A Study of the Structures and Reactions of Some Methyl Substituted Unsaturated C_{18} Ester Intermediates in the Synthesis of a Racemic Mixture of 10-Methyloctadecanoic Acid

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Methyl 10-undecenoate was hydrated to methyl 10-hydroxyundecanoate using mercury (II) acetate in aqueous tetrahydrofuran (THF). Chromic acid oxidation of methyl 10-hydroxyundecanoate gave methyl 10-oxoundecanoate, which was hydrolyzed to 10-oxoundecanoic acid. Reaction of n-octyl magnesium bromide complex in THF with 10-oxoundecanoic acid furnished 10-hydroxy-10-methyloctadecanoic acid after hydrolysis. The latter compound was esterified, and dehydration of methyl 10-hydroxy-10-methyloctadecanoate with *p*-toluenesulfonic acid in benzene gave a mixture of unsaturated branched fatty ester intermediates: *viz.* methyl 10-methyl-9-octadecenoate, 10-methyl-10-octadecenoate and 10-octyl-10-undecenoate. Treatment of the mixture of unsaturated branched fatty ester intermediates with mercury (II) acetate in methanol gave exclusively methyl 10-methoxy-10-methyloctadecanoate. Epoxidation of the same mixture of unsaturated fatty esters with *m*-chloroperbenzoic acid provided a mixture of epoxy derivatives: methyl 9,10-epoxy-10-methyloctadecanoate, 10,11-epoxy-10-methyloctadecanoate and 2-octyl-oxirane-nonanoate. Catalytic hydrogenation of the mixture of unsaturated fatty esters gave a racemic mixture of methyl 10-methyloctadecanoate, which was hydrolyzed to 10-methyloctadecanoic acid. The structures of the mixture of unsaturated branched fatty ester intermediates and their derivatives were characterized by chemical and spectroscopic analyses.
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Tuberculostearic acid, (*R*)-(−)-10-methyloctadecanoic acid, has been found in lipid extracts of various strains of bacteria, *viz. Mycobacterium* (1), *Nocardia* (2-4), *Corynebacterium* (5), *Saccharopolyspora* (6), *Streptomyces* and *Actinomadura* (7). The biochemical characteristics and fatty acid composition of some armadillo-derived mycobacteria have been studied recently (8). Many modern analytical techniques have been specially developed to detect the range of methyl substituted long chain fatty acids in bacterial lipid extracts, including tuberculostearic acid (9-11). Serodiagnostic tests for tuberculosis (12), and methods for detection of antibodies to phospholipids from *M. Tuberculosis* have been designed to assist in the clinical diagnosis of tuberculosis in humans (13). In our effort to conduct immunological studies involving tuberculostearic acid *in vivo* experiments, it was necessary to develop a facile synthesis method for the preparation of a racemic mixture of 10-methyloctadecanoic acid.

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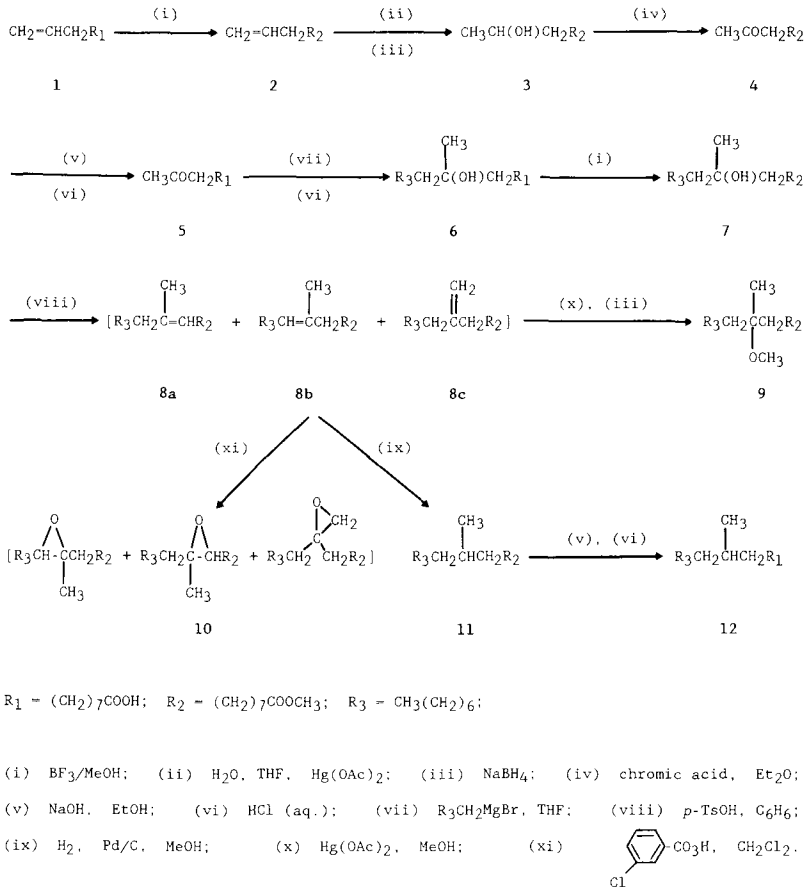
Abbreviations: APT, Attached Proton Test; ECL, equivalent chain length; GC, gas chromatography; GC-MS, gas chromatography-mass spectrometry; IR, infrared; NMR, nuclear magnetic resonance; THF, tetrahydrofuran; TLC, thin layer chromatography.

Various methods for the preparation of optically pure and racemic mixtures of 10-methyloctadecanoic acid have been reported (1). Cason and coworkers prepared optically pure 10-methyloctadecanoic acid in a 10-step sequence starting from enantiomeric 2-decanol (14). Both Ställberg-Stenhagen (15) and Weedon and coworkers (16) employed methyl hydrogen β-methylglutarate in a multistep chain elongation procedure or by anodic synthesis for the preparation of tuberculostearic acid. Another interesting method leading to 10-methyloctadecanoic acid involved chain extension via a thiophene nucleus (17). While these procedures emphasized the production of optically pure isomers of 10-methyloctadecanoic acid, the racemic mixture of this compound could also be obtained by the same procedures when the racemic substrate was employed.

In designing our synthesis route to obtain a racemic mixture of 10-methyloctadecanoic acid (Scheme 1), we realized that several interesting methyl substituted unsaturated C_{18} fatty ester intermediates would be obtained. As methyl substituted unsaturated C_{18} fatty acids are not common in seed oils, this project provided an opportunity to investigate the physical and chemical nature of such long chain fatty esters. Branched-chain saturated and unsaturated fatty acids are found in lipid extracts of insects, fungi, bacteria and in some lower forms of marine organisms (18-21). Patients suffering from Refsum's disease are reported to be unable to metabolize phytanic acid, 3,7,11,15-tetramethylhexadecanoic acid (22). Epoxidation of the C_{18} unsaturated fatty ester intermediates would also provide an opportunity to study the physical and biological properties of epoxy fatty acids containing a methyl group at an epoxy carbon position. Such methyl branched epoxy fatty acids are not found in nature, although numerous C_{18} and C_{20} unsaturated epoxy fatty acids have been reported (23-25).

MATERIALS AND METHODS

Thin layer chromatography (TLC) was performed on microscope glass plates coated with silica (about 0.1 mm thickness), and a mixture of n-hexane/diethyl ether in various concentrations was used as developer. Preparative $AgNO_3$ TLC was carried out on glass plates (20 × 20 cm) coated with silica GF_{254} (0.7 mm thickness) containing 15% $AgNO_3$ (w/w) and n-hexane/diethyl ether, 9:1, v/v, which was used as developer. Column chromatography was performed on silica using gradient elution of mixtures of n-hexane/diethyl ether. Collected fractions were checked by TLC before pooling. Gas chromatographic (GC) analysis was carried out on a Hewlett Packard 5970 GC fitted with a 10 m microbore glass column (0.53 mm diameter, 2.65 μm film thickness, SE-30 stationary phase), using nitrogen (20 ml/min) as the carrier gas under temperature programmed condition (initial temperature of 140°C, final temperature 250°C, rate 5°C/min) or isothermally (200°C), and a flame ionization



SCHEME 1. Synthesis of a racemic mixture of 10-methyloctadecanoic acid and reactions involving some unsaturated intermediates.

detector. External methyl ester standards (12:0, 14:0, 16:0, 18:0, 20:0 and 22:0) were used as reference compounds and the equivalent chain length (ECL) values calculated accordingly for each compound. Infrared spectra were obtained on a Perkin Elmer model 577 spectrophotometer and ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra on a JEOL FX90Q (90 MHz) instrument. Mass spectral analyses were conducted on a Hewlett Packard GC with a Mass Selective Detector™ fitted with a 12 m capillary glass column (0.2 mm internal diameter, 0.33 μm film thickness, cross-linked methyl silicone gum), and helium (ca. 2 ml/min) was used as the carrier gas under temperature programmed condition (initial temperature 140°C, final temperature 240°C, rate 5°C/min).

Methyl 10-undecenoate (2). A mixture of 10-undecenoic acid (1, 18.4 g, 0.1 mole), methanol (100 ml) and BF_3-methanol complex (5 ml, 15% w/w) was refluxed for 15 min. Water (100 ml) was added to the cooled reaction mixture and extracted with n-hexane (2 × 60 ml). The organic extract was washed with water (2 × 20 ml) and dried over anhydrous Na_2SO_4. Evaporation of the solvent under reduced pressure gave pure 2 (18 g, 91%). R_f = 0.8 (PE30) (PE30 = n-hexane/diethyl ether, 70:30, v/v, where the number denotes the amount [%] of diethyl ether) and ECL = 10.9 (SE-30); IR (NaCl) 1740 cm^{-1} (s, C=O, str.).

Methyl 10-hydroxyundecanoate (3). A mixture of 2 (50 ml) and tetrahydrofuran (THF, 50 ml) was stirred at room temperature for four days. Sodium borohydride (1.9 g) in sodium hydroxide (2 M, 50 ml) was added to the reaction mixture. Aqueous sodium chloride (10%, 50 ml) was added and the THF layer isolated. The aqueous layer was extracted with diethyl ether (2 × 100 ml) and the combined organic extract washed with water (50 ml) and dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure and the product (9.4 g) purified by silica column chromatography (200 g silica, gradient elution of mixtures of n-hexane/diethyl ether) to yield pure 3 (8.5 g, 79%). R_f = 0.3 (PE30) and ECL = 12.8 (SE-30); IR (NaCl) 3400 (s, br. OH str.), 1740 (s, C=O str.) cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.1 (*d*, 3H, C<u>H</u>$_3$) 1.2-1.8 (*m*, 14H, C<u>H</u>$_2$), 2.3 (*t*, 2H, <u>C</u>H$_2$COOCH$_3$), 2.2 (*s*, 1H, O<u>H</u>), 3.5-3.8 (*m*, 1H, C<u>H</u>OH), 3.6 (*s*, 3H, COOC<u>H</u>$_3$); ^{13}C NMR ($CDCl_3$) ppm: 23.5 (C-11), 25.0 (C-3), 29.2-29.7 (<u>C</u>H$_2$), 34.1 (C-2), 51.4 (COO<u>C</u>H$_3$), 67.8 (C-10), 174.3 (C-1).

Methyl 10-oxoundecanoate (4). Chromic acid (20 ml, prepared from 5 g $Na_2Cr_2O_7$, 7 g H_2SO_4 and 16 ml water) was added to a solution of 3 (3 g, 13.9 mmol) in diethyl ether (150 ml) contained in a 250 ml separatory funnel. The total mixture was carefully shaken for 5 min, releasing the pressure built up by the evaporating ether at intervals. The aqueous layer was removed and the organic layer was washed with water (50 ml). This process was repeated twice with chromic acid (20 ml). The ethereal

layer was isolated and dried over anhydrous Na_2SO_4. Silica column chromatographic purification gave pure **4** (2.2 g, 74%) R_f = 0.5 (PE30), ECL = 12.7 (SE-30); IR (NaCl) 1700 (s, C=O str.), 1740 (s, C=O, ester, str.) cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.2–1.8 (*m*, 12H, $C\underline{H}_2$), 2.1 (*s*, 3H, $C\underline{H}_3COCH_2$–), 2.3 (*t*, 2H, $C\underline{H}_2COOCH_3$), 2.4 (*t*, 2H, $CH_3COC\underline{H}_2$–), 3.65 (*s*, 3H, $COOC\underline{H}_3$) ppm; ^{13}C NMR ($CDCl_3$) ppm: 23.4 (C-8), 24.5 (C-3), 28.7 ($\underline{C}H_2$), 29.2 (C-11), 33.7 (C-2), 43.2 (C-9), 50.8 ($COO\underline{C}H_3$), 173.5 (C-1), 208.1 (C-10).

10-Oxoundecanoic acid (**5**). A mixture of **4** (2.2 g, 10.3 mmol), KOH (1 g), water (2 ml) and ethanol (30 ml) was refluxed for 30 min. Water (50 ml) was added and the mixture acidified with conc. HCl (10 ml). The cooled reaction mixture was extracted with diethyl ether (3 × 30 ml). The organic extract was successively washed with water (20 ml), $NaHCO_3$ (2 M, 20 ml), water (20 ml), then dried over anhydrous Na_2SO_4. Evaporation of the solvent under reduced pressure gave pure **5** (1.9 g, 95%). R_f = 0.2 (PE30); IR (NaCl) 3300 (br., OH str.), 1730, 1700 (s, C=O, str.) cm^{-1}.

Methyl 10-hydroxy-10-methyloctadecanoate (**7**). A solution of **5** (15 g, 75 mmol) in THF (50 ml) was added to a suspension of n-octyl magnesium bromide (prepared from 57 g n-bromooctane, 7.2 g magnesium, 100 ml THF) at 20°C. The mixture was stirred for 2 hr and then refluxed for 1 hr. Dilute HCl (2M, 100 ml) was carefully added to the cooled reaction mixture, and the THF layer was isolated. The aqueous layer was extracted with diethyl ether (2 × 50 ml) and the combined organic extract washed with water (2 × 50 ml) and dried. Crude **6** (23 g, 98%) was obtained after evaporation of the solvent under reduced pressure. The latter was dissolved in absolute methanol (150 ml) and refluxed for 10 min in the presence of BF_3-methanol (20 ml, 15% w/w). The reaction mixture was diluted with water (150 ml) and extracted with diethyl ether (3 × 50 ml). The organic extract was washed with water (50 ml) and dried. Silica column chromatographic purification gave **7** (16.7 g, 68%). R_f = 0.4 (PE30); ECL = 20.2 (SE-30); IR (NaCl) 3400 (s, br. OH str.), 1740 (s, C=O str.) cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.88 (*t*, 3H, terminal $C\underline{H}_3$), 1.1 (*s*, 3H, $C\underline{H}_3$), 1.2–1.6 (*m*, 28H, $C\underline{H}_2$), 2.3 (*t*, 2H, $C\underline{H}_2COOCH_3$), 2.5 (*s*, 1H, $O\underline{H}$), 3.6 (*s*, 3H, $COOC\underline{H}_3$) ppm. ^{13}C NMR ppm: 14.1 (C-18), 22.7 (C-17), 23.9, 24.0 (C-8, C-12), 25.0 (C-3), 27.0 ($\underline{C}H_3$ at C-10), 29.2–29.6 ($\underline{C}H_2$), 30.2, 30.3 (C-7, C-13), 31.9 (C-16), 34.1 (C-2), 41.9 (C-9, C-11), 51.4 ($COO\underline{C}H_3$), 174.3 (C-1).

Dehydration of **7**. A mixture **7** (13.2 g, 40 mmol), benzene (50 ml) and *p*-toluenesulfonic acid (0.5 g) was refluxed for 3 hr using a Dean-Stark water separator fitted with a water cooled condenser. Water (20 ml) was added to the cooled reaction mixture and the benzene layer isolated and dried over anhydrous Na_2SO_4. The solvent was evaporated under reduced pressure and separation by silica column chromatography gave a mixture of **8** consisting of methyl 10-methyl-9-octadecenoate (**8a**), 10-methyl-10-octadecenoate (**8b**) and 10-octyl-10-undecenoate (**8c**) (9.6 g, 77%). R_f = 0.7 (PE30), ECL = 18.0 (32%), 18.3 (68%) (SE-30); IR (NaCl) 1740 (s, C=O, str.), 1450 (m, CH_2, bend), 890 (m, CH of $>C=CH_2$, bend) cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.9 (*t*, 3H, $C\underline{H}_3$), 1.2–1.4 (*m*, ~26H, $C\underline{H}_2$), 1.51 (*s*, ~2H, $C\underline{H}_3C=C$), 2.3 (*t*, 2H, $C\underline{H}_2COOCH_3$), 3.6 (*s*, 3H, $COOC\underline{H}_3$), 4.68 (*s*, ~1H, $>C=C\underline{H}_2$), 5.1 (*t*, 1H, J = 8 Hz, $CH_3C=C\underline{H}$) ppm; ^{13}C NMR ($CDCl_3$) ppm: 14.1 (*q*), 15.9 (*q*), 22.7, 23.5 (*q*), 25.0, 27.9, 28.1, 29.2, 29.3, 29.6, 31.8, 31.9, 34.1, 36.1, 39.8, 51.4, 108.4 (*t*), 124.6 (*d*), 125.3 (*d*), 135.1 (*s*), 150.4 (*s*), 174.3 (*s*) ppm.

Separation of **8** *by silver ion TLC.* A mixture of **8** (300 mg) in n-hexane (4 ml) was streaked on three preparative TLC plates (20 × 20 cm, 1.0 mm thick, silica GF_{254}, 15% $AgNO_3$, w/w) using a TLC Sample streaker (Applied Science Laboratories, State College, PA) and developed with a mixture of n-hexane/diethyl ether, 9:1, v/v. Detection under ultraviolet light showed an intense band at R_f = 0.6–0.8 and a faint band at R_f = 0.5–0.6. The bands were scraped off and extracted with diethyl ether (5 × 10 ml), the less polar band gave fraction A (250 mg), while the more polar band furnished fraction B (22 mg). Fraction A consisted of a mixture of **8a** and **8b**. ECL = 18.0 (35%) and 18.3 (65%) on SE-30; ^1H NMR ($CDCl_3$) δ 0.9 (*t*, 3H, $C\underline{H}_3$), 1.2–1.4 (*m*, 22H, $C\underline{H}_2$), 1.5 (*s*, 3H, $C\underline{H}_3C=CH$) 1.6–1.8 (*m*, 4H, $C\underline{H}_2CH=CCH_3C\underline{H}_2$), 2.3 (*t*, 2H, $C\underline{H}_2COOCH_3$), 3.65 (*s*, 3H, $COOC\underline{H}_3$), 5.1 (*t*, 1H, $CH_3C=C\underline{H}$); ^{13}C NMR ($CDCl_3$) ppm: 14.1 (*q*), 15.9 (*q*), 22.7, 23.5, 25.0, 28.1, 29.2, 29.3, 29.5, 29.8, 31.8, 32.0, 34.2, 39.8, 51.4 (*q*), 124.6 (*d*), 125.3 (*d*), 135.1 (*s*), 174.3 (*s*) ppm.

Fraction B consisted of **8c**. ECL = 18.3 (SE-30); IR (NaCl) 1735 (s, C=O, str.), 1650 (m, C=C str.), 890 (s, C–H of $>C=CH_2$, bend) cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.9 (*t*, 3H, $C\underline{H}_3$), 1.2–1.4 (*m*, 22H, $C\underline{H}_2$), 1.6–2.0 (*m*, 4H, $C\underline{H}_2CH_2COOCH_3$, $CH_2=CC\underline{H}_2$), 2.3 (*t*, 2H, $C\underline{H}_2$-$COOCH_3$), 3.6 (*s*, 3H, $COOC\underline{H}_3$), 4.68 (*s*, 2H, $C\underline{H}_2=C<$) ppm; ^{13}C NMR ($CDCl_3$) ppm: 14.1 (C-18), 22.7 (C-17), 25.0 (C-3), 27.9 (C-8, C-12), 29.2–29.6 ($\underline{C}H_2$), 32.0 (C-16), 34.1 (C-2), 36.1 (C-9, C-11), 51.4 ($COO\underline{C}H_3$), 108.4 ($>C=\underline{C}H_2$), 150.4 ($>\underline{C}=CH_2$), 174.3 (C-1).

Methyl 10-methoxy-10-methyloctadecanoate (**9**). A mixture of **8(a, b** and **c)** (0.22 g), mercury (II) acetate (0.7 g, 2.3 mmol) and methanol (20 ml) was stirred for four days at room temperature. Sodium borohydride (1.2 g) in dilute NaOH (2M, 10 ml) was added and the total reaction mixture stirred for 2 hr. Saturated aqueous NaCl (30 ml) was added and the mixture extracted with diethyl ether (3 × 30 ml). Silica column chromatographic separation gave **9** (240 mg, 27%). R_f = 0.8 (PE30), ECL = 20.0 (SE-30); ^1H NMR ($CDCl_3$) δ 0.9 (*t*, 3H, terminal $C\underline{H}_3$), 1.07 (*s*, 3H, $C\underline{H}_3$), 1.2–1.4 (*m*, 24H, $C\underline{H}_2$), 1.4–1.8 [*m*, 4H, $C\underline{H}_2C$-$(OCH_3)CH_3$], 2.3 (*t*, 2H, $C\underline{H}_2COOCH_3$), 3.13 (*s*, 3H, $OC\underline{H}_3$), 3.66 (*s*, 3H, $COOC\underline{H}_3$) ppm. ^{13}C NMR ($CDCl_3$) ppm: 14.1 (C-18), 22.7 (C-17, $\underline{C}H_3$ at C-10), 23.5 (C-8, C-12), 25.0 (C-3), 29.2–29.6 (CH_2), 30.2, 30.3 (C-7, C-13), 31.9 (C-16), 34.1 (C-2), 37.6 (C-9, C-11), 48.7 ($O\underline{C}H_3$ at C-10), 51.4 ($COO\underline{C}H_3$), 75.6 (C-10), 174.3 (C-1). MS (ei) m/e (rel. int.): 279 (2), 230 (16), 229 (100), 197 (4), 172 (12), 171 (93), 125 (6), 97 (12).

Reaction of **8** *with m-chloroperbenzoic acid.* A mixture of **8 (a, b** and **c)** (0.7 g, 1.9 mmol), *m*-chloroperbenzoic acid (0.4 g, 2.4 mmol) and dichloromethane (20 ml) was stirred at room temperature for 48 hr. Water (50 ml) was added and the mixture extracted with dichloromethane (2 × 30 ml). The organic extract was successively washed with dilute Na_2SO_3 (10%, 20 ml), $NaHCO_3$ (10%, 20 ml) and dried over anhydrous Na_2SO_4. Evaporation of the solvent gave a mixture of epoxy derivatives (**10**, 0.5 g, 95%). IR (NaCl) 1740 (s, C=O str.), 1170 (m, C–O–C) cm^{-1}; ^1H NMR δ 0.9 (*t*, 3H, CH_3), 1.23 (*s*, 3H, $C\underline{H}_3$), 1.2–1.6 (*m*,

24H, $C\underline{H}_2$), 2.3 (*t*, 2H, $C\underline{H}_2COOCH_3$), 2.57 (*s*, 2H, $C\underline{H}_2$ of epoxy), 2.7 (*t*, 1H, $C\underline{H}$ of epoxy), 3.67 (*s*, 3H, $COOC\underline{H}_3$).

Oxidation cleavage of mixture of 8 (**a, b** and **c**). A mixture of 8 (62 mg), Lemieux-von Rudloff reagent (26) (63 ml, prepared from 0.9 g $KMnO_4$, 22.4 g $NaIO_4$, 1000 ml H_2O), aqueous K_2CO_3 (5 ml, 5%), tert. butanol (70 ml) and water (10 ml) was stirred at room temperature for 48 hr. Sulfur dioxide gas was passed through the reaction mixture until the solution turned light yellow in color. NaOH pellets (1.2 g) were added and the solvent evaporated under reduced pressure. Dil.HCl (2 M, 20 ml) was added and the mixture extracted with diethyl ether (2 × 20 ml). The diethyl ether extract was washed with saturated aqueous NaCl (10 ml). The solvent was distilled and the residue refluxed with BF_3-methanol complex (20 ml, 10%) for 15 min. Saturated aqueous NaCl (30 ml) was added and the product extracted with n-hexane (3 × 20 ml). The organic extract was washed with saturated NaCl solution (20 ml), dried over Na_2SO_4, and filtered. The product was analyzed by gas chromatography-mass spectrometry (GC-MS) and was shown to consist of the following compounds: $CH_3(CH_2)_6COOCH_3$, $CH_3CO(CH_2)_8COOCH_3$, $CH_3(CH_2)_7COCH_3$, $CH_3OOC(CH_2)_7COOCH_3$, $CH_3(CH_2)_7CO(CH_2)_8COOCH_3$.

Methyl 10-methyloctadecanoate (**11**). A mixture of 8 (**a, b** and **c**) (9.6 g, 31 mmol), methanol (50 ml) and palladium on charcoal (50 mg, 5%) was shaken in an atmosphere of hydrogen for 12 hr at 780 mm Hg pressure. The mixture was filtered and the methanol evaporated under reduced pressure. Column chromatographic separation on silica gave pure **11** (8.8 g, 91%). R_f = 0.8 (PE20), ECL = 18.3 (SE-30); IR (NaCl) 1740 (s, C=O str.), 1460 (m, CH_2 bend) cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.9 (*t*, 6H, $C\underline{H}_3$), 1.2–1.8 (*m*, 28H, $C\underline{H}_2$), 2.3 (*t*, 2H, $C\underline{H}_2COOCH_3$), 3.66 (*s*, 3H, $COOC\underline{H}_3$) ppm; ^{13}C NMR ($CDCl_3$) ppm: 14.1 (C-18), 19.8 ($\underline{C}H_3$ at C-10), 22.7 (C-17), 25.0 (C-3), 27.2 (C-8, C-12), 29.2–29.8 ($\underline{C}H_2$), 30.0, 30.2 (C-7, C-13), 32.0 (C-16), 32.9 (C-10), 34.2 (C-2), 37.2 (C-9, C-11), 51.3 ($COO\underline{C}H_3$), 174.0 (C-1).

10-Methyloctadecanoic acid (**12**). A mixture of **11** (1.8 g, 5.8 mmol), KOH (1 g), water (2 ml) and ethanol (30 ml) was refluxed for 30 min. Water (30 ml) was added and the reaction mixture acidified with conc. HCl (10 ml). The cooled reaction mixture was extracted with diethyl ether (2 × 30 ml). The extract was washed successively with water (10 ml), saturated aqueous NaCl (10 ml) and dried over anhydrous Na_2SO_4. Evaporation of the solvent gave **12** (1.6 g, 94%). M.p. 22–23°C; IR (NaCl) nujol 3300 (br, OH str.), 1700 (s, C=O str.); ^1H NMR ($CDCl_3$) δ 0.9 (*t*, 6H, $C\underline{H}_3$), 1.2–1.6 (*m*, 28H, $C\underline{H}_2$), 2.3 (*t*, 2H, $C\underline{H}_2COOH$), 9.7 (*s*, 1H, $COO\underline{H}$) ppm; ^{13}C NMR ($CDCl_3$) ppm: 14.1 (C-18), 19.8 ($\underline{C}H_3$ at C-10), 22.7 (C-17), 24.8 (C-3), 27.1 (C-8, C-12), 29.2–29.8 ($\underline{C}H_2$), 30.1 (C-7, C-13), 32.0 (C-16), 32.9 (C-10), 34.1 (C-2), 37.2 (C-9, C-11), 180.0 (C-1).

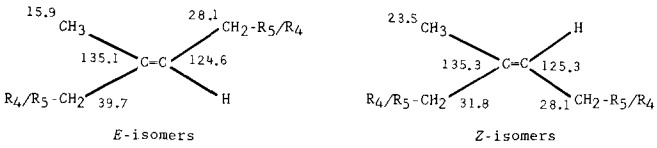
RESULTS AND DISCUSSION

Hydration of the ethylenic center of the methyl ester of commercially available 10-undecenoic acid (**1**) gave the methyl 10-hydroxyundecanoate (**3**) as described by Brown and Geoghegan (27) exclusively. Two-phase oxidation of the latter compound furnished methyl 10-oxoundecanoate (**4**) (28). 1-Bromooctane was reacted with magnesium in THF to give the corresponding Grignard reagent, which was allowed to react with 10-oxoundecanoic acid (**5**). The resulting 10-hydroxy-10-methyloctadecanoic acid (6) was esterified and *p*-toluenesulfonic acid catalyzed dehydration reaction of the ester gave a mixture of two positional methyl substituted unsaturated C_{18} ester isomers: methyl 10-methyl-9-octadecenoate (**8a**), 10-methyl-10-octadecenoate (**8b**) and a vinyl containing C_{18} ester derivative (methyl 10-octyl-10-undecanoate, **8c**). Hydrogenation of this mixture of unsaturated fatty ester intermediates (**8**) over palladium on charcoal gave methyl 10-methyloctadecanoate (**11**) exclusively, which yielded the required 10-methyloctadecanoic acid (**12**) in 23% yield based on 10-undecenoic acid on hydrolysis. As the hydrogenation process was nonstereospecific, a racemic mixture of 10-methyloctadecanoic aid was produced, one of the isomers being tuberculostearic acid (Scheme 1).

The presence of methyl 10-octyl-10-undecenoate (**8c**) as one of the products of the dehydration of methyl 10-hydroxy-10-methyloctadecanoate (**7**) was initially detected in the IR (890, 1650 cm^{-1}, vinyl group), ^1H NMR (4.68δ, *s*, $C\underline{H}_2$=C<), and ^{13}C NMR (108.4, 150.4 ppm, $\underline{C}H_2$=$\underline{C}$<, respectively) spectra of the reaction product. GC analysis of this mixture gave two peaks on SE-30 stationary phase with ECL = 18.0 (32%) and 18.3 (68%). Oxidative cleavage of the mixture of **8a, 8b** and **8c** gave the following oxidation products after methylation: $CH_3(CH_2)_6COOCH_3$, $CH_3CO(CH_2)_8COOCH_3$, $CH_3(CH_2)_7COCH_3$, $CH_3OOC(CH_2)_7COOCH_3$ and $CH_3(CH_2)_7CO(CH_2)_8COOCH_3$, as identified by GC-MS. From these results it could be confirmed that the dehydration of methyl 10-hydroxy-10-methyloctadecanoate furnished not only the methyl substituted unsaturated C_{18} ester intermediates, but also a significant amount of a vinyl-containing intermediate (**8c**) as indicated by the presence of methyl 10-oxooctadecanoate in the oxidation product mixture.

Also, two fractions were isolated when the mixture **8a, b** and **c** was chromatographed ($AgNO_3$ TLC). The slightly less polar fraction (A, 83% w/w) showed two peaks on GC (SE-30) with ECL = 18:0 (35%) and 18.3 (65%), and both peaks gave similar mass spectral fragmentation patterns. The ^1H NMR spectrum of fraction A confirmed the presence of the methyl substituted unsaturated C_{18} ester intermediates from the appearance of signals at 1.5 (*s*) and 5.1 (*t*) δ. Only a trace of **8c** was detected in fraction A from the very weak signal at 4.68 (*s*) δ for the protons of the vinyl system ($C\underline{H}_2$=C<). From these results it could be concluded that fraction A consisted wholly of a mixture of **8a** and **8b**. From a mechanistic consideration, the dehydration of **7** would most likely furnish the *E*-isomers as the major product. This assumption agrees with the GC results, as the major component (at ECL = 18.3, 65%) correspond to the *E*-isomers, as *E*-isomers of unsaturated long chain fatty ester are known to have slightly longer retention times than the corresponding *Z*-isomers on non-polar stationary phases when analyzed by GC (29). The ^{13}C NMR spectral analysis of fraction A provided further evidence of the presence of different geometric isomers with an *E/Z* ratio of about 2:1 as reflected by the intensities of the associated signals for the methyl substituted unsaturated system (CH_3C=CH–) in these molecules. The assignments of the signals for these carbon nuclei are shown

below and are supported by data presented by Stothers (30) for methyl substituted alkenes.



$R_4 = CH_3(CH_2)_{5/6}$; $R_5 = (CH_2)_{6/7}COOCH_3$

Fraction B gave a single peak by GC with an ECL = 18.3 on SE-30 stationary phase. The IR spectrum gave characteristic absorption bands at 1650 (C=C str.) and 890 (C-H of CH_2=C< bend) cm^{-1} for the vinyl system. The ^1H NMR and ^{13}C NMR spectra showed fraction B to be completely free from any methyl substituted unsaturated esters (**8a,8b**). The ^1H NMR spectrum gave the characteristic signal at 4.68δ for the protons of the $C\underline{H}_2$=C< system, while the ^{13}C NMR spectrum confirmed the structure of this molecule by the shifts of the vinyl carbons at 108.4 and 150.4 ppm. These results were in full agreement with data observed for methyl 12-hexyl-12-tridecenoate, an isomeric vinyl-containing long chain fatty ester derived from a different route (Lie Ken Jie, M.S.F., and Zheng, Y.F., unpublished data). The assignments of the chemical shifts of the various carbon atoms was based on data recorded for long chain fatty esters and derivatives (31-34) and methyl substituted alkanes (35).

In a further effort to study the chemical behavior of **8**, this mixture of compounds was treated with methanol in the presence of mercury (II) acetate. The nucleophilic attack of methanol on the mercurated complex of **8a, b** and **c** preferred the tertiary carbonium ion site at position C-10 of the alkyl chain, resulting in the exclusive production of methyl 10-methoxy-10-methyloctadecanoate (**9**). This derivative was readily identified by the characteristic ^1H NMR signals at 3.13 (*s*) and 1.07 (*s*) δ for the methoxy and methyl protons attached to C-10, respectively. The ^{13}C NMR spectral analysis gave signals at 48.7 and 22.7 ppm for the carbon nuclei of these same groups. The shift of the methyl carbon attached to C-10 coincided with the shift of the methylene carbon at C-16 (22.7 ppm), however, these signals were differentiated by application of the Attached Proton Test (APT) technique (36). The mass spectrum gave two very intense peaks at *m/z* 171 (93%) and 229 (100%, base peak), which permitted the unequivocal establishment of the position of the methyl and methoxy groups at C-10 of the alkyl chain of compound **9**.

Epoxidation of **8** gave a mixture of the corresponding epoxy derivatives **10**. The ^1H NMR spectral analysis displayed a distinct singlet at 2.57δ, due to the shift of the methylene protons of the 2,2-epoxide, and a distorted triplet at 2.7δ for the methine proton of the 2,3-epoxide system present in the product. These observations reconfirmed the structures of **8a, b** and **c** as products derived from the dehydration of compound **7**.

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Recovery of Fish Oil-Derived Fatty Acids in Lymph of Thoracic Duct-Cannulated Wistar Rats

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The absorption of equivalent doses of eicosapentaenoic and docosahexaenoic acids was compared in rats when administered as the ethyl ester concentrate, ethyl ester concentrate plus olive oil, free fatty acid or triacylglycerol (menhaden oil). Lymph was collected from a thoracic duct cannula for 24 hr after dosing via an indwelling duodenal catheter. After 24 hr, the absorption of eicosapentaenoic acid was greater for the free fatty acid and menhaden oil than for the ethyl ester form, but docosahexaenoic acid absorption was comparable for all forms. Other rats had greater plasma levels of eicosapentaenoic and docosahexaenoic acids 5 hr after oral gavage dosing with menhaden oil than did rats dosed with the ethyl ester form. ***Lipids 25,*** **6-10 (1990).**

Consumption of fish oils has been advocated for antiatherogenic effects attributed to constituent long-chain n-3 polyunsaturated fatty acids, particularly eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids. EPA and DHA may be ingested as dietary components of fish, or as a dietary supplement containing purified fish oil or fish oil-derived fatty acid ethyl esters.

The proposed advantage of the purified products is reduced levels of saturated fatty acids, cholesterol, calories, fat-soluble vitamins and environmental contaminants relative to the levels of the putative therapeutic components, EPA and DHA. The ethyl ester concentrate (EE) is a more concentrated delivery vehicle for EPA and DHA than the fish oil-containing capsule. However, there are questions regarding the absorbability, efficacy and safety of the EE since the ethyl ester form is not a natural component of the human diet, and its effects compared to those of triacylglycerol EPA and DHA have not been extensively studied. Several studies have compared the absorption of the EE and the triacylglycerol EPA and DHA (1-4) by using the incorporation of EPA and DHA into plasma phospholipids, cholesteryl esters or triacylglycerol fatty acids as an indicator of absorption. Less EPA and DHA was incorporated into human plasma triacylglycerol after a single oral dose of EPA and DHA as the EE than as the triacylglycerol (2-4). However, in longer-term animal studies, the EE and the triacylglycerol forms enriched plasma phospholipids with EPA to the same extent (1).

The purpose of the present study was to compare the absorption of EPA and DHA as the ethyl ester, triacylglycerol (menhaden oil, MO) or the free fatty acid (FFA) form by direct measurement of EPA and DHA in the lymph of thoracic duct-cannulated rats. Previous experience with the thoracic duct cannulation technique in the rat has shown this to be a useful model for the study of lipid absorption (5).

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Abbreviations: C, lipid-free emulsion medium; DHA, docosahexaenoic acid; EE, ethyl ester concentrate; EPA, eicosapentaenoic acid; FFA, free fatty acid; MO, menhaden oil; OO, olive oil.

MATERIALS AND METHODS

Male Wistar rats (Harlan Sprague-Dawley, Indianapolis, IN) were individually housed in suspended stainless steel mesh cages. Rats were fed a commercial diet (Purina Rodent Chow 5002, Purina Mills, Inc., St. Louis, MO) and distilled water *ad libitum* prior to surgical procedures and treatment with lipid emulsions. All rats were 6-7 weeks old and weighed approximately 250 g at the time of the experiments.

Under sodium pentobarbital anesthesia, a midline abdominal incision was made and the left thoracic lymph duct was cannulated according to the procedure of Bollman *et al.* (6). A cannula was placed into the duodenum at the pylorus. Cannulas were secured with cyanoacrylate, the incisions were sutured and the rats were placed in restraining cages. A glucose-saline solution (5% glucose, 0.9% NaCl, w/v) was continuously infused through the duodenal cannula (3 ml/hr), and the rats had free access to the same solution for drinking. After an overnight recovery period, the rats were dosed with 1.5 ml lipid emulsion via the duodenal cannula. The compositions of the lipid emulsions are shown in Table 1. The emulsions were prepared according to the methods of Vahouny *et al.* (7). To prepare the emulsions, a stock solution of bovine serum albumin (BSA) (25 g/l), sodium taurocholate (139 g/l) and NaCl (9 g/l) was prepared. On the day the emulsion was to be used, sufficient lipid for 15 ml of emulsion (i.e., 10 doses) was dissolved in 5 ml

TABLE 1

Lipid Emulsion Composition

Thoracic duct-cannulated rats

Component[a]/1.5 ml emulsion	Group EE	MO	FFA	EE + OO
Bovine serum albumin (mg)	25	25	25	25
Sodium taurocholate (mg)	139	139	139	139
Ethyl ester concentrate (mg)	54	0	0	54
Menhaden oil (mg)	0	170	0	0
Olive oil (mg)	0	0	0	116
EPA (mg)	0	0	23	0
DHA (mg)	0	0	15	0

Gavaged rats

Component/1.5 ml emulsion	Group EE	MO	Lipid-free control
Bovine serum albumin (mg)	25	25	25
Sodium taurocholate (mg)	139	139	139
Ethyl ester concentrate (mg)	60	0	0
Menhaden oil (mg)	0	175	0

[a]The EE, MO, FFA and EE + OO groups were administered equivalent amounts of EPA and DHA. In the first experiment: 23 mg EPA plus 15 mg DHA per 1.5 ml emulsion. In the second experiment: 26 mg EPA plus 17 mg DHA per 1.5 ml emulsion.

of ethyl ether; 10 ml of the BSA/taurocholate solution was added to the lipid suspension and the mixture was emulsified in a glass-Teflon homogenizer until milky. The ether was evaporated under N_2 at 37°C. The final volume was brought to 15 ml with saline. Emulsions were rehomogenized immediately before use. Lymph was collected on ice over a 24-hr period. Fatty acid composition of the collected lymph was determined by capillary gas chromatography (GC) by a modification of the procedure of Einig and Ackman (8). A 0.25-ml aliquot of lymph was used for lipid extraction by the Folch method (9). The lipid extracts with tricosanoic acid added as an internal standard were treated with methanolic sodium hydroxide at 100°C for 7 min. After cooling, methanolic BF_3 was added and the mixture was heated at 100°C for 5 min (10). The fatty acid methyl esters were extracted by the addition of isooctane and saturated NaCl solution, and were analyzed on a Varian 3700 gas chromatograph using a 30 m × 0.531 mm DB-wax megabore capillary column (J & W Scientific, Folsom, CA) with appropriate GC conditions. The fatty acid methyl esters were identified by their retention times with respect to fatty acid methyl ester standards (Sigma Chemical Co., St. Louis, MO). The EPA and DHA content of lymph collected from rats that were not given a lipid emulsion dose was also determined. This baseline value for endogenous fatty acids was subtracted from the recovery values obtained for the rats in the groups receiving lipid emulsions.

In a second experiment, male Wistar rats were dosed with lipid emulsions by intragastric gavage (Table 1). These rats were fasted for 16 hr prior to dosing. At 2.5 and 5 hr post-dosing, the animals were anesthetized and blood was obtained by cardiac puncture, then the animals were euthanized and the small intestine was excised. The small intestine was repeatedly rinsed with ice-cold 0.9% saline, and the intestinal mucosa was scraped, homogenized in 2× the volume with 0.9% saline, and centrifuged at 40,000 × *g* for 30 min. Lipid was extracted from plasma and the intestinal mucosa supernatant (9) and analyzed for fatty acid composition (8,10), as in the previous experiment.

MO and EE (80% EPA and DHA ethyl esters in a 1.6:1 ratio) were supplied by the National Marine Fisheries Service (Charleston, SC). EPA was provided as a gift by Ocean Organics (Peace Dale, RI); DHA and olive oil (OO) were obtained from Sigma Chemical Co. Tertiary butylhydroquinone and *α*- and *γ*-tocopherol were added to the MO and the EE as antioxidants.

RESULTS

The percentages of administered EPA and DHA recovered in lymph at 4-, 8- and 24-hr intervals after dosing are shown in Figures 1 and 2, respectively. At 4 hr, the recoveries of EPA and DHA in lymph were greatest when the lipid emulsion contained EPA and DHA as FFA. At 8 hr, recoveries of EPA and DHA in lymph were in the following order: FFA = MO > EE = (EE + OO). At 24 hr, lymph EPA in the groups receiving MO or FFA was still greater than in those receiving EE, while lymph DHA was not different among the groups. DHA was absorbed at a slower rate but with greater efficiency than was EPA when administered in all but the FFA form. The percentage of DHA recovered in 24 hr was significantly higher ($P < 0.05$) than that of EPA only when DHA was given as the EE (76.1% vs 60.6%). At no time was EPA or DHA absorption affected by supplementation of the EE with OO.

The EPA/DHA ratio of the lipid emulsion was 1.5. Table 2 presents the ratios of EPA/DHA found in the

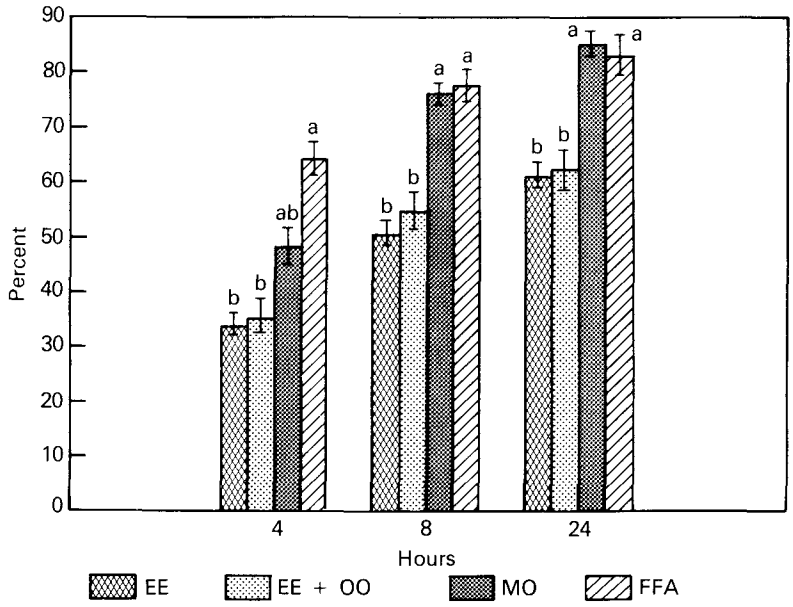

FIG. 1. Percent recovery of EPA in lymph at 4, 8 and 24 hr after dosing with lipid emulsions containing EPA/DHA ethyl ester concentrate (EE), menhaden oil (MO), free fatty acids (FFA) or EE plus olive oil (EE + OO). Values represent means ± SEM expressed as percentages of administered dose; n = 5–8 animals per group. Bars without common superscripts within the same time periods are significantly different ($P < 0.05$).

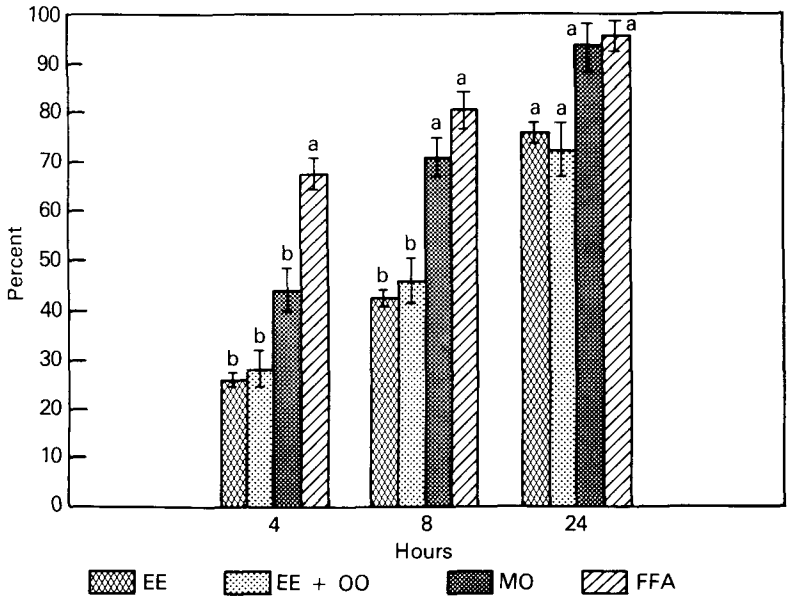

FIG. 2. Percent recovery of DHA in lymph at 4, 8 and 24 hr after dosing with lipid emulsions. Values represent means ± SEM; n = 5–8 animals per group. Bars without common superscripts within the same time periods are significantly different (P <0.05).

TABLE 2

Ratio of EPA/DHA in Lymph Lipids

Group	Hours after dosing 0–4	n	4–8	n	8–24	n
EE	2.75 ± 0.18^a	7	1.98 ± 0.28^a	7	0.55 ± 0.04^a	5
MO	2.08 ± 0.17^b	6	1.85 ± 0.15^a	6	0.90 ± 0.18^a	6
FFA	2.09 ± 0.12^b	8	1.79 ± 0.10^a	8	0.87 ± 0.18^a	7
EE + OO	2.80 ± 0.19^a	7	2.53 ± 0.36^a	7	0.67 ± 0.09^a	7

Each value is the mean ± SEM of n observations. Values without common superscripts are significantly different (P < 0.05). The ratio of EPA/DHA in the initial lipid emulsion was 1.5.

collected lymph. Over the initial 4 hr, the lymph EPA/DHA ratio of the EE-dosed groups was significantly greater than that of the MO- or FFA-dosed groups. The lymph ratio at 4 hr for all groups was well above that of the administered emulsion. Beyond 4 hr there were no significant differences between groups in EPA/DHA ratios. Beyond 8 hr, the lymph ratio decreased to below 1.0 for all groups.

The EPA and DHA concentrations in plasma or intestinal mucosa supernatant after EE or MO intragastric gavage are shown in Figures 3 and 4, respectively. At 5 hr post-gavage, plasma EPA was higher in the MO group than in the EE group. Plasma DHA was greater than the endogenous level only at 2.5 hr after gavage dosing with MO. EPA and DHA concentrations in intestinal tissue were not different at equivalent times for MO and EE groups.

No EPA or DHA ethyl esters were found by thin layer chromatography in 4-hr lymph samples collected from rats receiving EE. These results were confirmed by GC with a limit of determination of 1 ng fatty acid/0.25 ml lymph (data not shown).

DISCUSSION

In a recent review, Nelson and Ackman (11) questioned the suitability of the ethyl ester as a vehicle for the administration of EPA and DHA. They suggested that EPA from the ethyl ester can enter the circulation from two lipid pools, one in chylomicron form into lymph and one through the portal vein. It was also suggested that because the ethyl esters are poor substrates for pancreatic lipase and are poorly hydrolyzed in the intestinal lumen, they may (i) be carried farther in the intestinal tract to be hydrolyzed by intestinal flora or (ii) enter the enterocyte unhydrolyzed and then undergo hydrolysis or transesterification in the cell.

The results from the present study indicate that the EE form of EPA and DHA was not absorbed as well by rats as were the triacylglycerol and FFA forms. The 24-hr recoveries in lymph from the EE form were about 60% for EPA and 70% for DHA. These recoveries indicate that significant portions of the EPA and DHA ethyl esters were hydrolyzed and absorbed and that they entered the lymphatic system in chylomicron form. The present study did not consider the fate of the remaining 30–40% of the EPA/DHA ethyl ester dose, i.e., excretion or absorption of some portion via the portal blood. Plasma EPA and DHA levels obtained from the gavage experiment (Fig. 3) support the findings in lymph that the EPA and DHA were not absorbed as well from a single dose of the EE form as from triacylglycerol EPA and DHA.

Table 2 reflects differences in absorption kinetics between EPA and DHA. EPA was initially absorbed more rapidly than was DHA. Hence the lymph collected during

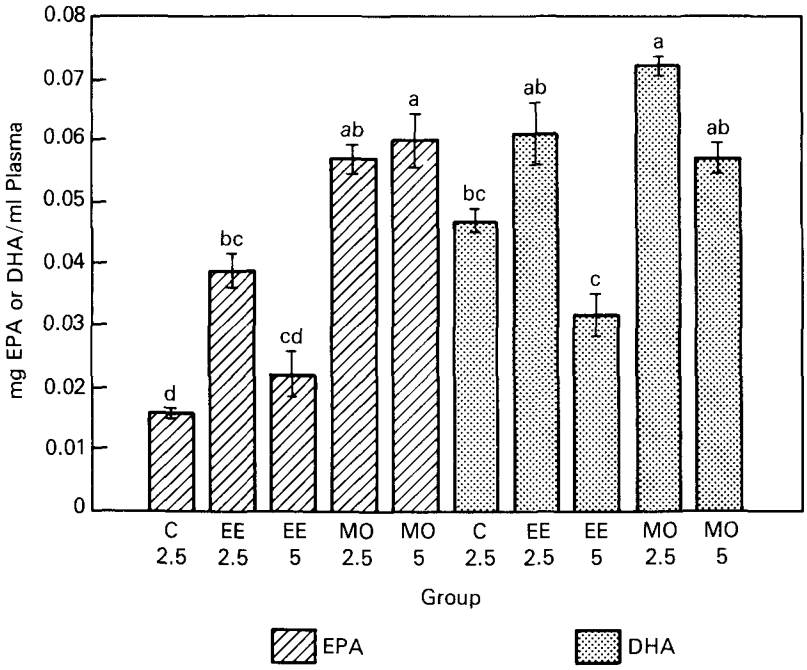

FIG. 3. Amount of EPA or DHA/ml plasma at 2.5 or 5 hr after dosing with lipid emulsions. Rats were gavaged with emulsions of ethyl ester concentrate (EE), menhaden oil (MO) or with lipid-free emulsion medium (C). Values represent means ± SEM; n = 4 animals per group. Bars without common superscripts for the same fatty acid are significantly different ($P < 0.05$).

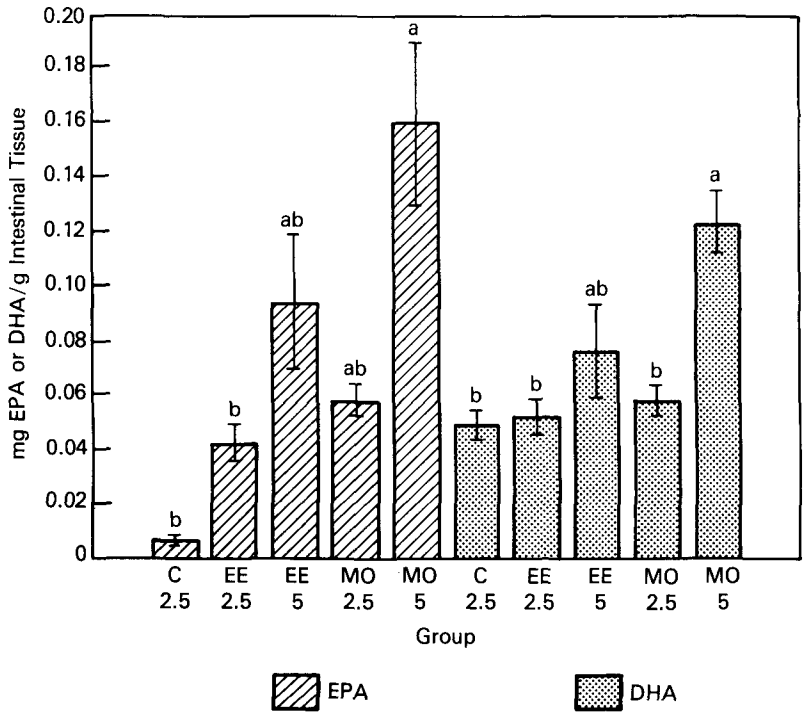

FIG. 4. Amount of EPA or DHA/g intestinal tissue at 2.5 or 5 hr after dosing with lipid emulsions. Values represent means ± SEM; n = 4 animals per group. Bars without common superscripts for the same fatty acid are significantly different ($P < 0.05$).

the first 4 hr was EPA-enriched relative to the dosing emulsions (lymph EPA/DHA ratios of 2.1-2.8 vs emulsion EPA/DHA ratio of 1.5). DHA was absorbed at a slower, but longer sustained rate. Beyond 8 hr, the rate of EPA transport into the lymph had fallen to below that of DHA (i.e., lymph EPA/DHA ratio of <1.0; Table 2). Although initial EPA transport exceeded DHA transport, at 24 hr post-dosing the portion of administered DHA recovered in the lymph exceeded that of administered EPA (Figs. 1 and 2).

Resistance of the fatty acid ethyl ester to lipolysis by pancreatic lipase has been implicated as the reason for lower absorption of the EPA and DHA ethyl esters compared to that of triacylglycerol EPA and DHA (2,3). EPA and DHA ethyl esters were not found in rat lymph in the present study (data not shown), and other studies have not found ethyl esters in human or rat circulation after EE administration (1,12,13). Since a significant portion of the EPA and DHA was recovered in the lymph of rats given the EE, it appears that resistance to pancreatic hydrolysis may not significantly impair absorption in rats.

Yang *et al.* (14) examined luminal digestion and fatty acid micellar incorporation of MO triglycerides and the corresponding fatty acid ethyl esters. Fatty acid composition of the micelles was reported to reflect the composition of the initial ethyl esters or of the terminal positions of the MO triglycerides. It was also shown that the ethyl esters were hydrolyzed four times more slowly than MO, which was fast enough to maintain a saturated micellar solution of fatty acids in the lumen of the jejunum during absorption.

The metabolic effects attributed to fish oil ingestion are also observed after ingestion of n-3 polyunsaturated fatty acid ethyl esters. Long-term EPA ethyl ester ingestion by humans resulted in decreased platelet aggregation (13,15), lowered serum cholesterol or triglycerides (16,17) and improved erythrocyte rheological properties (13). These reports show the efficiency of EPA intestinal absorption from EPA ethyl esters to be sufficient for therapeutic effectiveness. The differences in EPA absorption from MO (80% of dose) and from EE (60% of dose), which were observed in the present study, may not be significant in terms of therapeutic effectiveness.

Recently, it was reported that in humans the absorption of fatty acid ethyl esters is highly dependent on the amount of co-ingested lipid (4). In the present study addition of OO to the EE lipid emulsion administered to thoracic duct-cannulated rats did not influence EPA and DHA recoveries.

Additional studies are required to determine whether there is a dose-response relationship between the amounts of EPA and DHA administered as the EE and the percent recoveries in the lymph, and to determine the amounts of EPA and DHA required to produce the desired metabolic effects.

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Intestinal Absorption of Unconjugated Dihydroxy Bile Acids: Non-Mediation by the Carrier System Involved in Long Chain Fatty Acid Absorption

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Experiments were performed using isolated mucosal cells from the rat jejunum or using the perfused jejunum in the anesthetized rat to test whether lipophilic unconjugated dihydroxy bile acids are absorbed from the proximal small intestine via the same carrier mechanism involved in the uptake of long chain fatty acids. With isolated jejunal mucosal cells, the cellular uptake rate of deoxycholic acid or chenodeoxycholic acid increased linearly with time, showed no evidence of saturation, and was not decreased by the presence of a monospecific antibody to the membrane fatty acid binding protein. In contrast, oleate uptake was saturable, was inhibited by the same antibody, but was not affected by the presence of chenodeoxycholic acid or deoxycholic acid. Bile acid uptake by isolated enterocytes occurred at one-eighth the rate of fatty acid uptake if expressed in relation to total solute concentration; if expressed in relation to monomeric concentration, initial bile acid uptake was four orders of magnitude slower than fatty acid uptake. In the isolated perfused jejunal segment, chenodeoxycholic acid and deoxycholic acid uptake was not influenced by the presence of the antibody to membrane fatty acid binding protein, whereas absorption of oleate was inhibited by more than 70%. These experiments indicate that absorption of unconjugated lipophilic dihydroxy bile acids in the rodent jejunum does not involve the carrier mediated uptake mechanism involved in the absorption of long chain fatty acids—the mechanism is likely to be passive nonionic diffusion.

Lipids 25, 11-16 (1990).

The absorption of endogenous unconjugated bile acids from the distal small intestine is a normal physiologic event in humans (1), and absorption of unconjugated bile acids from anywhere in the small intestine may occur in pathological conditions causing increased bile acid deconjugation (2). Efficient absorption of exogenous unconjugated bile acids is desirable when they are administered for therapeutic purposes, such as cholesterol gallstone dissolution (3) or to correct inborn errors of bile acid biosynthesis, such as cerebrotendinous xanthomatosis (4).

Previous studies have shown that lipophilic dihydroxy bile acids, such as chenodeoxycholic acid (CDC) or deoxycholic acid (DC), are rapidly absorbed by the perfused jejunum, but that cholic acid, an unconjugated trihydroxy bile acid, is absorbed more slowly (5-9). Because the absorption rate of unconjugated bile acids is inversely proportional to their hydrophilicity, as assessed by octanol/water partition coefficients (10), it has generally been assumed that bile acids are absorbed passively by partitioning into the lipid domains followed by subsequent "flipflop" across the lipid bilayer. The rates at which unconjugated bile acids permeate artificial bilayers have recently been reported and have been shown to be inversely proportional to the hydrophilicity of the molecule (11).

Whether passive absorption is the sole mechanism of absorption of lipophilic unconjugated bile acids has not been examined experimentally. Recent studies on the mechanism of long chain fatty acid uptake by isolated intestinal cells have suggested that fatty acid absorption is, at least in part, a carrier mediated process. Two lines of evidence have supported this hypothesis. First, long chain fatty acid uptake has been shown to be saturable; second, fatty acid uptake can be inhibited by a monospecific antibody prepared against a membrane fatty acid binding protein (MFABP) (12,13). This antibody also has been shown to inhibit oleate uptake by isolated hepatocytes (14,15) and cardiomyocytes (16).

The chemical structure of unconjugated dihydroxy bile acids can be considered to be analogous to hydroxy fatty acids such as ricinoleic acid in that an acidic group is present at the end of a hydrophobic body with one or more hydroxyl groups. We hypothesized that the intestinal absorption of lipophilic unconjugated bile acids might also use the putative carrier system involved in long chain fatty acid absorption. If so, the absorption of such unconjugated bile acids should be saturable, should inhibit that of fatty acids, and should be inhibited by the monospecific antibody to MFABP. Two experimental approaches were used to test this hypothesis: The first measured the rate of uptake of solutes by isolated rat jejunal enterocytes; and the second measured the rate of uptake of solutes by a jejunal segment perfused in single pass fashion in the anesthetized rat. For both, the uptake rate of the unconjugated bile acids CDC and DC was compared with that of oleate. The effect of increasing bile acid concentration on uptake of oleate was also examined using isolated enterocytes. Finally, the effect of added monospecific antibody to MFABP on the uptake of either bile acids or oleate was defined using both systems.

MATERIALS AND METHODS

Materials. [24-^{14}C]Chenodeoxycholic acid (CDC) and [24-^{14}C]deoxycholic acid (DC) were prepared using a modification of the method of Tserng *et al.* (17). The final products, with a specific activity of 50 μCi/μmol, were purified by thin-layer chromatography (TLC). [9,10-^{3}H]-Oleic acid, L-[2,3-^{3}H]alanine, [1,2-^{14}C]- and [1,2-^{3}H]polyethylene glycol 4000 ([^{14}C]PEG, [^{3}H]PEG) and Aquasol

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Abbreviations: CDC, chenodeoxycholic acid; DC, deoxycholic acid; MFABP, membrane fatty acid binding protein; PEG, polyethylene glycol; LDH, lactate dehydrogenase; TLC, thin-layer chromatography; UDC, ursodeoxycholic acid.

were from New England Nuclear, Dreieich, West Germany. Collagenase (type I) was from Worthington, Freehold, NJ. Nonradioactive CDC was generously provided by Diamalt AG, Raubling, West Germany; and DC was purchased from Aldrich Chemical Co., Milwaukee, WI. Oleic acid, bovine serum albumin (fraction V, essentially fatty acid free), D-glucose, L-glutamine, L-alanine, insulin (bovine), PEG 4000, and HEPES were from Sigma Chemie GmbH, Deisenhofen, West Germany.

All reagents were of analytical grade; doubly distilled, deionized water was used in all experiments. Glassware was acid washed.

Wistar rats were obtained from the Zentralinstitut für Versuchstiere, Hannover, West Germany, and were fed a standard chow diet (Altromin 1314).

Preparation of enterocytes. Jejunal mucosal cells of male Wistar rats (200–250 g body weight) that were fasted overnight were prepared by a vascular collagenase perfusion technique (12). The final combined mucosal cell preparation was filtered through nylon gauze (70 μm pore size), centrifuged at 70 × *g* for 2 min, and washed twice using the incubation medium which contained 125 mM NaCl, 2.6 mM KCl, 5.7 mM Na_2HPO_4, 1.2 mM KH_2PO_4, 10 mM HEPES buffer and 5.5 mM glucose (pH 7.4). Thereafter the cells were diluted to 2×10^6 cells/ml in incubation medium (20°C). To maintain the physiological transcellular ion gradients of Na^+ and K^+, it was essential to use the cells within 2 hr after preparation and to prevent their exposure to temperatures below 20°C, as such is known to reduce Na^+/K^+-ATPase levels markedly (18). Viability and purity of the mucosal cell preparations were assessed by phase contrast microscopy and the ability to exclude trypan blue dye (16). Additional critiera of viability were based on the intracellular K^+-concentration determined by atomic absorption spectroscopy as well as the release of lactate dehydrogenase (LDH) (19,20).

The isolated enterocytes retained both morphological and functional integrity. Phase contrast microscopy of the isolated mucosal cell preparations demonstrated that 92 ± 4% (M ± SD) of the cells revealed the typical features of intestinal epithelium with oval or elongated cell bodies, basal nuclei, and prominent brush borders at the apical pole. Of these cells, 93 ± 5% excluded trypan blue; the intracellular K^+-concentration remained >85 mM; and loss of cellular LDH was less than 15% during 3 hr after preparation of the cells. As another criterion of their functional integrity, the uptake kinetics of L-[2,3-^{3}H]alanine, which is known to be actively transported, were examined as described (12). With increasing L-[^{3}H]alanine concentrations, the cellular influx showed saturation kinetics with a V_{max} of 17.0 nmol × min^{-1} per 10^6 mucosal cells and a K_m of 2.3 mM.

Cellular uptake studies. Isolated jejunal mucosal cells (125 μl, 2×10^6 cells/ml) were incubated with 368 μM CDC or 368 μM DC or with 173 μM [^{3}H]oleate bound to albumin in molar ratios of 1:1 and 2:1, prepared as previously described (12). The final incubation volume was 1 ml, the temperature 37°C. After certain incubation periods, 200 μl sample aliquots were pipetted into 3 ml of ice-cold incubation medium or in experiments with [^{3}H]oleate into 3 ml ice-cold 0.5% albumin in incubation medium (stop/chase-solution) (16). After vacuum filtration, the cell associated radioactivity was measured as described previously (12). Nonspecific association of radioactivity to filters and cells was determined in each experiment by adding the cold stop solution before the addition of corresponding aliquots of cells and radioactive solutes. This blank always constituted less than 2% of the incubated radioactivity and was subtracted from all determinations. All incubations were performed in triplicate and all observations were confirmed with at least three separate cell preparations. The fraction of [^{3}H]oleate incorporated into cellular lipids and the amount oxidized to CO_2 during the course of uptake was determined as previously described (21).

Antibody inhibition studies. To determine the effect of the antibody to the MFABP on cellular influx of CDC, DC and oleate, 2 ml of the cell suspension (2×10^6 cells/ml) were incubated for 30 min at room temperature in gently rotating polypropylene tubes with 100 μg of the IgG-fraction of the antiserum to the MFABP isolated from rat liver or of the preimmune serum as controls (16). After centrifugation and washing the cells three times in medium, the viability of the cells remained >90% as determined by trypan blue exclusion. The cells were then diluted to 2×10^6 cells/ml and solute uptake was examined as described above.

Absorption of solutes by in vivo *single pass perfused jejunal segments.* Male Wistar rats (200–250 g body weight) were fasted overnight and anesthetized with pentobarbital. Jejunal segments of 25 cm length were canulated beginning at the ligament of Treitz. The perfusate contained 85 mM NaH_2PO_4/45 mM NaH_2PO_4 (pH 7.4). PEG 4000 containing [^{14}C]PEG or [^{3}H]PEG was added to a concentration of 5 g/l (5 μCi/l) and used as a nonabsorbable marker of the perfusate. After flushing the intestinal lumen with 20 ml of this medium, perfusion was started at a flow rate of 2 ml/min. The abdominal cavity was closed and the body temperature of the anesthetized rat, as well as of the perfusion medium, were maintained at 37°C. After an equilibrium period of 20 min, the absorption rate of 500 μl bolus injections with increasing quantitites (37.5–375 nmol) of [^{14}C]CDC or [^{14}C]DC, as well as of 0.1–5 μmol [^{3}H]oleate in the presence of equimolar concentrations of Na taurocholate was examined. The effusate was collected over a period of 10 min, at which time no further radioactivity appeared in the effluent. For all perfusion studies it was shown that the recovery of PEG was between 98–100%. The fraction of solute absorbed was calculated from the difference of the total amount of radioactivity infused in relation to the amount of radioactivity that was recovered. Absorption rates (nmol solute/min/25 cm jejunal segment) were analyzed as a function of the logarithmic mean of the solute concentration in the perfused segment, using the assumption that the concentration of fatty acid at the beginning of the test segment was equal to that of the infused bolus (22). Clearance of solutes (ml/min) was defined as the rate of solute absorption divided by the logarithmic mean concentration of the solutes in the perfused segment (22). The clearance calculated in this manner is certainly a very crude measure of the permeation ratios, but does permit uptake rates of different ligands to be compared.

For determination of the effect of the anti-MFABP on the overall absorption process of bile acids and fatty acids, the gut lumen was perfused in a recirculating

system for 10 min with perfusion medium containing 5 mg/100 ml of the IgG-fraction of the antiserum; as control, 5 mg of the IgG-fraction of the preimmune serum was used. Thereafter, recirculation was discontinued and the lumen perfused in single pass fashion for 10 min (equilibrium period) with IgG-free medium. Then absorption of bolus injections (500 μl) containing 375 nmol [^{14}C]CDC or 375 nmol [^{14}C]DC or 2.5 μmol [^{3}H]oleate in the presence of 2.5 μmol Na taurocholate was determined (see above).

In all experiments, radioactivity was measured as described earlier (12). Quench correction was made by external standardization. Samples containing two isotopes were counted in two channels. Counts per minute were converted into disintegrations per minute for each isotope with a computer program, which corrected for quenching and spillover of ^{14}C into the ^{3}H channel (12). Spillover of ^{3}H into the ^{14}C channel was less than 1%. Since the recovery of PEG was between 98 and 100% (see Results), the absorption rate was determined from the difference between infused and recovered radioactivity.

RESULTS

Solute uptake by isolated jejunal mucosal cells. Uptake of bile acids or fatty acids. CDC and DC uptake from a 368 μM solution was linear over the entire observation period of 4 min (Fig. 1). The uptake rate corresponded to a "clearance" of 0.43 pl/min/cell and was similar for the two dihydroxy bile acids.

Initial uptake of oleate (representing cellular influx) from solutions having a monomeric concentration of 117 nM or 401 nM (14) was linear over the initial 30 sec incubation period. This initial rate of oleate uptake occurred about eight times as rapidly as bile acid uptake if related to total fatty acid concentration. When expressed in relation to the calculated monomeric concentration, the "clearance" of oleate was 12,000 times greater than that of bile acids. For both fatty acid and bile acid uptake, intracellular metabolism is unlikely to play a role, at least in the initial stages of uptake. For oleate uptake, 87 ± 9% (M ± SD) of the fatty acids were present in unmodified form after 30 seconds, and no oxidation was detectable; for bile acids, intestinal biotransformation is considered not to occur (7).

Effect of unconjugated bile acid on fatty acid uptake. To determine the effect of added DC or CDC on fatty acid uptake, the rate of oleate uptake was measured using a solution of albumin:oleate (2:1, albumin:oleate; total oleate concentration, 173 μM; monomeric concentration, 401 nM). The addition of CDC or DC to concentrations of 5, 20, or 100 μM had no effect on the rate of fatty acid uptake (Fig. 2). Higher concentrations of bile acids were not used because of the likelihood of nonspecific cytotoxic effects (23-25).

Effect of the monospecific antibody to MFABP on bile acid uptake. Experiments were performed to define the effect of the MFABP antibody on DC or CDC uptake (Fig. 3). Uptake of DC or CDC was uninfluenced by the addition of the antibody to MFABP. In contrast, uptake of oleate was 67% inhibited by the addition of this antibody; uptake was not inhibited by the IgG-fraction of preimmune serum.

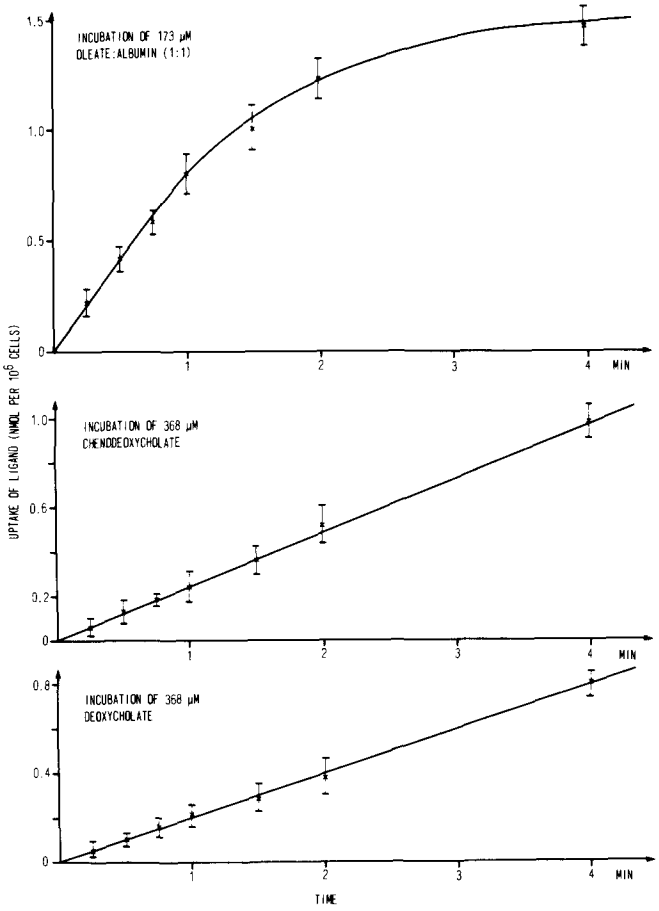

FIG. 1. Time course of solute uptake by isolated jejunal rat enterocytes. 173 μM [^{3}H]oleate/albumin (1:1), 368 μM DC or 368 μM CDC were incubated in 1 μl incubation medium at 37°C with 125 μl of isolated jejunal mucosal cells (2 × 10^{6} cells per ml). At the times indicated, uptake was terminated as described in Methods. Values are means ± SD of three replicate experiments.

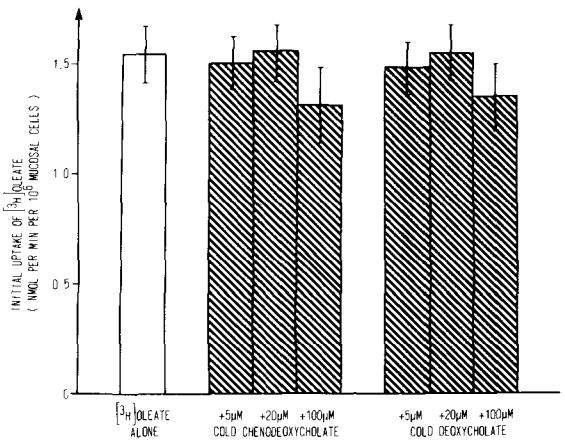

FIG. 2. Effect of increasing concentrations of unlabeled CDC or DC on initial uptake rate of [^{3}H]oleate by isolated jejunal rat enterocytes. 173 μM [^{3}H]oleate/albumin (2:1) were incubated either alone or in the presence of 5, 20 and 100 μM unlabeled CDC or DC with 125 μl of isolated jejunal mucosal cells (2 × 10^{6} cells per ml) at 37°C. The initial rate of uptake was determined as described in Methods. Values are means ± SD of three replicate experiments. The differences are not statistically significant.

Solute uptake by the perfused rat jejunum. Uptake of bile acids or fatty acids. Absorption of DC and CDC increased linearly with increasing perfusate concentrations (Fig. 4). From the slopes of the absorption curves, mean uptake rates of 0.544 and 0.608 nmol/min-cm rat jejunal segment were determined for a logarithmic mean segment concentration of 0.1 mM CDC and DC, respectively (Table 1 and Fig. 4). Under the conditions of our experiment (perfusion rate of 2 ml/min), this corresponds to a clearance of 57.3 ± 0.58 for CDC and 6.13 ± 0.78 for DC (μl/min-cm rat jejunal segment, M ± SD).

In contrast, after instillation of 0.1 to 5 μmol [^{3}H]-oleate, absorption of oleate was highly efficient and followed saturation kinetics with a maximal uptake rate of 55.52 nmol/min cm jejunal segment (half saturation at a logarithmic mean segment concentration of 0.7 mM).

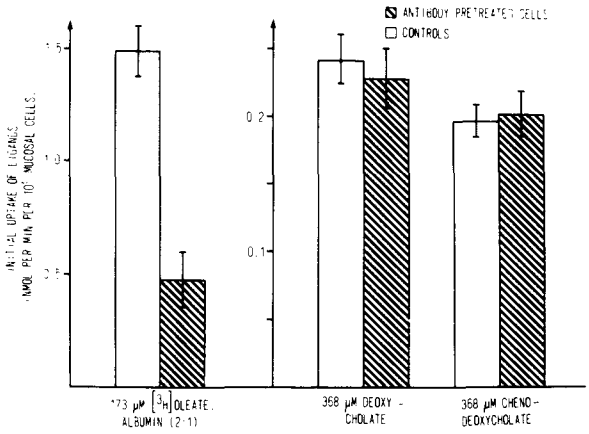

FIG. 3. Effect of anti-MFABP monoclonal antibody on initial uptake of solutes by isolated jejunal enterocytes. Cells that had been pretreated with 100 μg of the IgG-fraction of the antiserum were compared to control preparations pretreated with 100 μg of the IgG-fraction of the preimmune serum. 125 μl of such cell suspensions (2 × 10^6 cells/ml) were incubated with 173 μM [^{3}H]oleate/albumin (2:1), 368 μM DC or CDC. Initial rates of uptake were determined as described in Methods. Values are means ± SD of three replicate experiments.

Effect of the monospecific antibody to MFABP on bile acid uptake by the perfused rat jejunum. Uptake of CDC or DC was not influenced by the addition of the monospecific antibody to MFABP (Fig. 5). In contrast, pretreatment of the jejunal segments resulted in a marked decrease (71%) in fatty acid uptake. The addition of the IgG fraction of preimmune serum did not influence oleate uptake.

DISCUSSION

These experiments indicate that the jejunal uptake of CDC and DC, two common lipophilic unconjugated dihydroxy bile acids, is first order and is not influenced by the simultaneous uptake of oleate or the presence of an antibody to a brush border fatty acid binding protein which inhibits oleate absorption. Thus, uptake of these two bile acids does not involve the transport mechanism used by long chain fatty acids.

Previous studies using perfused jejunal segments of rodent (6,8,9) or humans (5,7) have shown rapid absorption

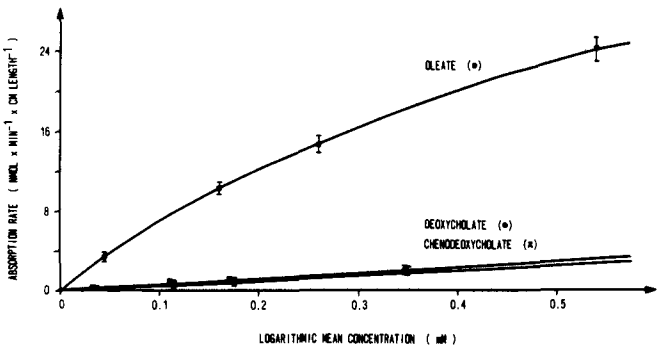

FIG. 4. Absorption rates of [^{3}H]oleate, [^{14}C]CDC, and [^{14}C]DC as a function of the logarithmic mean solute concentration in the perfusion medium. Jejunal segments (25 cm) were perfused at a flow rate of 2 ml/min and the fraction of absorbed solute was determined as described in Methods. Values are means ± SD of three replicate experiments.

TABLE 1

Uptake of Oleate, Chenodeoxycholate, or Deoxycholate from Perfused Rat Jejunal Segment[a]

	Mass of bolus (nmol)	Entry conc. (mM)	Exit conc. (mM)	Log mean conc. (mM)	Absorption rate (nmol/min-cm length)	Clearance (μl/min-cm length)[b]
Oleate	100	0.2	0.0125	0.045	3.40	75.6
	300	0.6	0.0435	0.160	10.24	64.0
	500	1.0	0.066	0.260	14.72	56.8
	1000	2.0	0.157	0.550	24.24	44.0
Chenodeoxycholate	37.5	0.075	0.0161	0.0345	0.21	6.16
	125	0.25	0.0536	0.114	0.71	6.24
	187.5	0.375	0.0829	0.175	0.87	5.00
	375	0.75	0.1635	0.350	1.92	5.52
Deoxycholate	37.5	0.075	0.0165	0.035	0.18	5.12
	125	0.25	0.053	0.110	0.76	6.88
	187.5	0.375	0.0797	0.170	1.12	6.60
	375	0.75	0.162	0.344	2.04	5.92

[a]Each value is the mean of three perfusions.

[b]The perfused segment was a 25 cm segment of jejunum; values obtained were divided by 25.

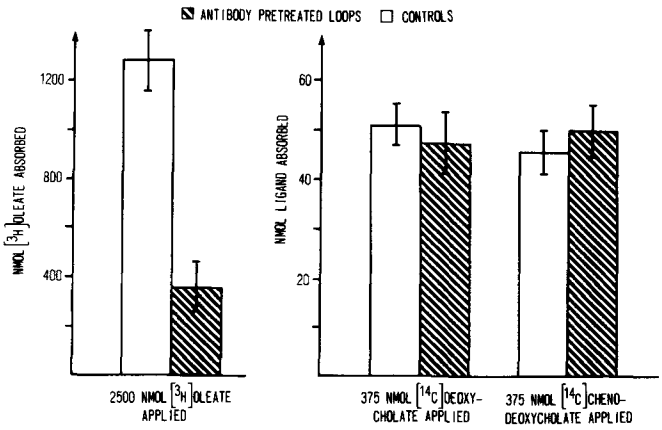

FIG. 5. Effect of the anti-MFABP on the overall absorption of solutes by an isolated perfused jejunal segment. The gut lumen was perfused in a recirculating system (10 min) with medium containing the IgG-fraction of the antiserum or, as control, the IgG-fraction of the preimmune serum; the IgG concentration was 5 mg per dl in both. Thereafter, recirculation was discontinued and the lumen was perfused in a single pass fashion for 10 min with IgG-free medium. The absorption of a 500 μl bolus injection of 2500 nmol [^{3}H]oleate, 375 nmol [^{14}C]DC or 375 nmol CDC was determined. Values are means ± SD of three replicate experiments.

of CDC. DC has similar physicochemical properties (10,26) and is also rapidly absorbed (8). Cholic acid, the most common trihydroxy bile acid, is absorbed more slowly (5,6,8,9). Extremely hydrophilic epimers of these three bile acids have been synthesized (27), and their absorption rate is likely to be far slower than that of CDC and DC (9). The present studies indicate that although unconjugated lipophilic bile acids are absorbed rapidly, their rate of absorption is far slower than that of long chain fatty acids.

Intestinal absorption of unconjugated lipophilic bile acids occurs only to a limited extent under physiological conditions since bile acids are secreted mostly in conjugated form and any lipophilic, unconjugated bile acid secreted into the canaliculus is likely to be absorbed in the biliary tract (28,29). In the distal small intestine, there is partial deconjugation during the normal enterohepatic cycling of bile acids, but the magnitude of this is small; and the majority of bile acids are absorbed by active transport from the terminal ileum without undergoing deconjugation (1). Unconjugated dihydroxy bile acids are used for dissolution of cholesterol gallstones, but the magnitude of the dose used (<15 mg/kg-day) is far less than daily fat intake (1000–2000 mg/kg-day). The absorption of orally administered CDC has been shown to be complete for commonly used doses (12–15 mg/kg-day) (30), but falls at higher doses (31,32). The absorption of ursodeoxycholic acid (UDC) appears to be incomplete (30). UDC is absorbed nearly as rapidly as CDC for a given monomeric activity (6), and its incomplete absorption (33) is likely to be explained by its insolubility at pH values prevailing in the small intestinal lumen (34).

We conclude that although the unconjugated lipophilic dihydroxy bile acids are absorbed rapidly from the jejunum, their absorption is far slower than that of fatty acid and, like the absorption of short and medium chain fatty acids in the jejunum, is not mediated by the fatty acid-Na^+ cotransporter. The mechanism is likely to be passive nonionic diffusion, that is, adsorption of the protonated monomer into lipid domains of the enterocyte followed by transbilayer movement (11). In hepatic sinusoidal membranes, an unconjugated bile acid/bicarbonate (hydroxide) exchange system has been described for cholate uptake (35), but it is not known whether such is present in the small intestine. In the hamster, cholate is absorbed more distally in the intestine than DC or C (9). Unconjugated bile acids, in contrast to long chain fatty acids, are not biotransformed to transport through the enterocyte. The mechanism of transport across the basolateral membrane is likely to involve an anion-exchange protein (36).

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The Uptake of (R,R,R)α-Tocopherol by Human Endothelial Cells in Culture

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Endothelial cells from human umbilical cord vein in culture incorporate physiological and pharmacological amounts of (R,R,R)α-tocopherol in a time-dependent and dose-dependent manner. Incorporated tocopherol was found to associate predominantly with membrane fractions of the cell. When expressed on the basis of organelle protein, the highest amount of tocopherol was found in plasma membrane, and decreasing amounts in mitochondria, endoplasmic reticulum and cytosol. With the relatively wide range of tocopherol concentrations used in these studies (23.2–92.8 μM), there was no apparent toxicity on the cells as judged by unaltered cell numbers and cell viability. When the cells were enriched with tocopherol and cell tocopherol levels were monitored in tocopherol-free medium, there was a rapid phase of tocopherol disappearance, which was followed by a slower phase. The half-time for the disappearance of incorporated tocopherol was found to be approximately 65 ± 8.6 hr (mean ± SD, n = 3). The results of this study clearly show that human endothelial cells in culture are a feasible model for the study of vitamin E uptake. The cell culture model could potentially be used to study other fat-soluble vitamins and essential nutrients.
Lipids 25, 17–21 (1990).

The vascular endothelial monolayer is in immediate contact with the circulation, and it forms a selective barrier for nutrient transport to various tissue compartments. The cells are under continuous physical and biochemical stress. Exposure to prooxidant generating components of the blood, such as platelets and neutrophils, necessitates an adequate antioxidant defense system in these cells in order to maintain endothelial integrity (1). In the hydrophilic compartment of the cell, the defense system chiefly consists of ascorbate, the glutathione-redox cycle, and an array of enzymic systems aimed at the removal of radicals and peroxides. In contrast, in the hydrophobic region, α-tocopherol remains the key membrane-bound antioxidant. Recent experimental evidence indicates that cultured endothelial cells possess a powerful system which is capable of oxidizing low-density lipoprotein. The resulting modified low-density lipoprotein has been implicated to play a significant role in atherogenesis (2–4). Therefore, the vitamin E status of the endothelium can potentially influence not only the generation of oxidized low-density lipoprotein, but it also has been documented by various laboratories to affect prostacyclin production in these cells (5–8). In order to better understand the turnover of tocopherol in the endothelium, this paper reports on a method we developed which enabled us to study the uptake and decay of tocopherol in cultured endothelial cells derived from human umbilical veins. By using this approach, it is feasible to enrich endothelial cells and study the effect of vitamin E-enrichment on the turnover of arachidonic acid (9,10).

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Abbreviations: DMSO, dimethylsulfoxide; EDTA, ethylenediaminetetraacetic acid; HBS, Hepes buffered saline; HPLC, high performance liquid chromatography; PBS, phosphate buffered saline.

MATERIALS AND METHODS

Materials. Medium 199 with Hanks' salt and L-glutamine, heat-inactivated fetal bovine serum, sodium penicillin G (10,000 units/ml), streptomycin sulfate (10,000 μg/ml), fungizone (250 μg Amphotericin B and 205 μg sodium deoxycholate/ml), trypsin-ethylenediaminetetracetic acid (EDTA) and culture dishes were from Gibco (Burlington, Ontario, Canada). Endothelial cell growth supplement was from Collaborative Research, Inc. (Bedford, MA). Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid], collagenase type IV, gentamicin sulfate and heparin were purchased from Sigma Chemical Co. (St. Louis, MO). (R,R,R)α-Tocopherol was a generous gift from the Vitamin E Research Information Service Henkel Co. (LaGrange, IL) and Eisai Co. Ltd. (Tokyo, Japan); *rac*-α-tocopherol acetate was from Sigma. High performance liquid chromatography (HPLC) grade solvents were purchased from BDH Chemicals, Inc. (Toronto, Ontario). All glassware was silanized prior to use.

The medium 199, pH 7.4, was supplemented with heparin (90 μg/ml), Hepes (25 mM), gentamicin sulfate (40 μg/ml), sodium penicillin G (200 U/ml), streptomycin sulfate (100 μg/ml), fungizone (2.5 μg Amphotericin B/ml) and 20% heat inactivated fetal bovine serum. The endothelial cell growth supplement (30 μg/ml) was added after each change of medium (11).

Culture of endothelial cells. Endothelial cells were derived from human umbilical cord veins by the method of Jaffe *et al.* (12). Cords were cannulated with tubing from butterfly needles and flushed with 50 ml of warm Hepes buffered saline (HBS; 10 mM Hepes, 0.14M NaCl, 4 mM KCl, 11 mM glucose, pH 7.4) to remove residual blood. The vein was then filled with 10 ml of 0.2% collagenase in HBS and incubated at 37°C in a bath of phosphate buffered saline (PBS) for 15 min. The collagenase/cell mixture was flushed with 40 ml HBS into a 50 ml plastic centrifuge tube containing 5 ml medium 199, and then centrifuged at 180 × *g* (or 1000 rpm) for 10 min. The cell pellet was resuspended in 8 ml of fresh medium 199 and cultured on a 2% gelatin-coated culture-dish (35 mm diameter). After 24 hr in a 5% CO_2:95% air incubator, the medium was replaced with fresh medium that was supplemented with endothelial cell growth supplement. Confluency was usually reached within 7–10 days, and cell medium was changed every three days. Cells were deteached by trypsinization and were subcultured in a 1:3 ratio. Cells used in these experiments were in passages 2–5. Endothelial cells were characterized by the presence of von Willebrand factor antigen using immunofluorescent microscopy (13).

Preparation of (R,R,R)α-tocopherol-containing medium. An appropriate amount of (R,R,R)α-tocopherol in ethanol

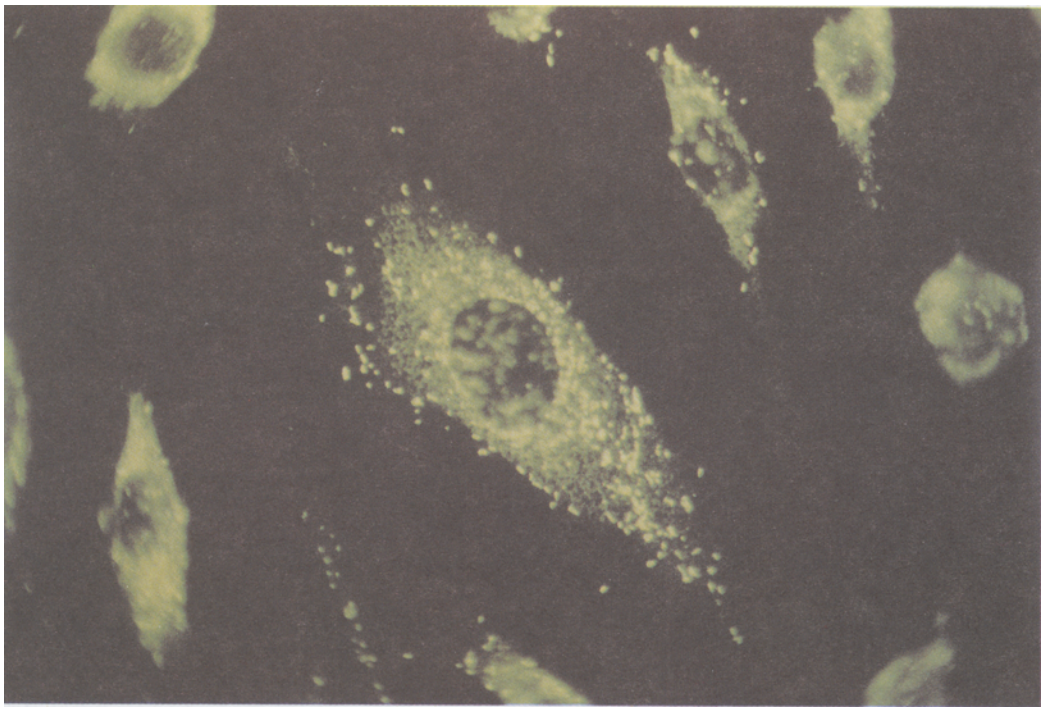

FIG. 1. Immunofluorescent microscopy of cells showing the presence of von Willebrand factor antigen, verifying that cells were of endothelial origin.

was added into a 50 ml conical centrifuge tube and the ethanol was evaporated under N_2. A microliter volume of dimethylsulfoxide (DMSO) was added to dissolve the tocopherol. The maximum amount of DMSO used never exceeded 0.4% of the final medium volume. Fetal calf serum, which reprsented 20% of the final medium volume, was prewarmed at 37°C and added to the tocopherol-DMSO mixture. This solution was vortexed and incubated at 37°C for 15–20 min, in the dark. Medium 199 and antibiotics were added last, vortexed, and the mixture was further incubated in 37°C for another 15–20 min. Tocopherol incorporated by this method was determined by HPLC; the recovery was >95%.

Tocopherol enrichment of cells and HPLC determination of (R,R,R)α-tocopherol. Confluent monolayers were used in all experiments. The tocopherol-enriched medium was added to the cell monolayer, and after various incubation times, the medium was removed and the monolayer was rinsed twice with HBS containing 0.25% bovine serum albumin. Cells were detached by trypsinization and sedimented by centrifugation (180 × *g*) for 10 min. The cell pellet was washed, resedimented, and resuspended in HBS. An aliquot was taken for cell number determination. Total lipid from the remaining cell suspension was extracted by the method of Bligh and Dyer (14), with the addition of *rac*-α-tocopherol acetate as internal standard. Tocopherol was quantitated by reversed phase HPLC using a UV detector (280 nm). The mobile phase contained methanol/water/trifluroacetic acid (99:1:0.1, v/v/v). Flow rate through a Bondpak C-18 column was adjusted to 2.5 ml/min, modified as described by Bieri *et al.* (15). Minimum detection of tocopherol was 40 pg.

Isolation of subcellular fractions. After incubation with (R,R,R)α-tocopherol-enriched medium (23.2 μM) for 21 hr, cells from eight dishes (58 cm^2) were harvested, washed, and homogenized in a tissue grinder homogenizer with 5 ml of Hepes buffer (10 mM Hepes, 0.25M sucrose, 0.2 mM EDTA, pH 7.5). An aliquot of the homogenate was taken for tocopherol determination. The homogenate was centrifuged at 1000 × *g* for 10 min at 4°C to remove nuclei and unbroken cells. The mitochondrial pellet was isolated by centrifugation of the supernatant at 10,000 × *g* for 30 min in a Beckman L8-M centrifuge using a 70-1 Ti rotor. The post-mitochondrial supernatant was centrifuged at 27,000 × *g* for 30 min to obtain the crude plasma membrane pellet, and finally the endoplasmic reticulum was isolated from this supernatant after 100,000 × *g* centrifugation for 1 hr. All subcellular pellets were resuspended in 1 ml of PBS, from which an aliquot was taken for protein determination (16), and the remainder was used for tocopherol determination as described above.

RESULTS

To verify that the cells isolated by collagenase digestion of umbilical cord veins were of endothelial origin, immunofluorescent microscopy against von Willebrand factor antigen was used on the primary cell cultures. Figure 1 clearly shows that intense fluorescent particles stained for von Willebrand factor were located throughout the cytoplasm of these cells, an observation which is in agreement with Jaffe (12), who demonstrated this unique characteristic of endothelial cells expressing this factor.

When incubated with a concentration similar to that of normal human plasma (R,R,R)α-tocopherol (10 μg/ml or 23.2 μM), confluent endothelial monolayer incorporated

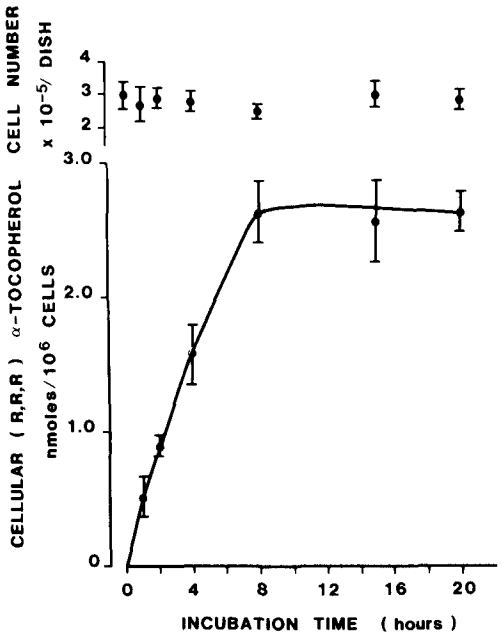

FIG. 2. Time course of incorporation of (R,R,R)α-tocopherol by human endothelial cells. Confluent monolayers from the third passage were incubated for the indicated time periods with 23.2 μM (10 μg/ml) of (R,R,R)α-tocopherol in complete culture medium. The cells were harvested as described in Methods, an aliquot was taken for cell number determination and the remainder was extracted for lipid analysis in the presence of *rac*-α-tocopherol acetate as internal standard. Tocopherol was determined by HPLC as described in Methods. Values are means ± S.D. from three dishes.

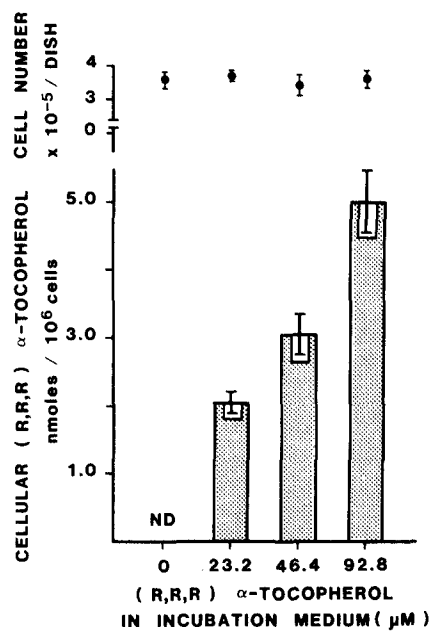

FIG. 3. Dose dependent uptake of (R,R,R)α-tocopherol by human endothelial cells. Confluent cells were incubated with different tocopherol concentrations. After 20 hr, cells were rinsed with Hepes buffered saline which contained 0.25% of essential fatty acid free bovine serum albumin to remove adhering tocopherol. Cells were detached by trypsinization for 1 min and immediately harvested by sedimentation. Cellular lipids were extracted and tocopherol was determined as described in Methods. Values are means ± S.D. from triplicate dishes.

the vitamin in a linear manner for up to 8 hr. Addition of tocopherol to the culture medium appeared to have no effect on the cell numbers of the endothelial monolayer (Fig. 2). The response of endothelial monolayers to different tocopherol concentrations (23.2-92.8 μM) after 20 hr of incubation showed a dose-dependent proportional uptake of tocopherol by these cells (Fig. 3). Again, no appreciable change in cell number was detected, even when these cells were grown at the highest tocopherol concentration.

In order to determine the distribution of incorporated tocopherol in these cells, subcellular fractions were isolated after cells were grown in tocopherol-enriched medium (23.2 μM) for 21 hr. The incorporated tocopherol was mainly associated with organelle membrane, and when tocopherol was expressed on the basis of membrane protein, the highest amount was found in plasma membrane > mitochondria > endoplasmic reticulum > cytosol (Table 1). We attempted to measure various marker enzymes of the respective organelle fractions, but despite repeated attempts we were unable to detect the presence of the mitochondrial enzymes (succinate dehydrogenase and cytochrome C oxidase) in the mitochondrial fraction. This appears to be largely due to the fact that expression of the two mitochondrial enzymes was greatly diminished upon subculturing of endothelial cells, which was necessary to obtain sufficient cell quantities for the fractionation experiments. The data presented herein indicate that the tocopherol incorporated is predominately associated with the membrane fractions of the cell.

TABLE 1

Subcellular Distribution of (R,R,R)α-Tocopherol in Human Endothelial Cells[a]

Fractions	Tocopherol (nmol)	Protein (mg)	Tocopherol/protein (nmol/mg)
Plasma membrane	1.10	0.06	18.3
Mitochondria	7.26	0.59	12.3
Endoplasmic reticulum	2.46	0.36	6.8
Cytosol	1.15	5.56	0.2
Total cell homogenate	19.53	9.22	2.12

[a]Cell monolayers were incubated with (R,R,R)α-tocopherol, 23.2 μM for 21 hr. Subcellular fractions were isolated as described in Materials and Methods.

The kinetics of tocopherol disappearance from these cells were examined next. After incubating confluent monolayers with 23.2 μM of (R,R,R)α-tocopherol for 4 hr, the tocopherol-enriched medium was replaced with fresh medium without tocopherol addition. Cellular tocopherol content was then monitored over the next 80 hr. Under these conditions (Fig. 4), the tocopherol content of the cells showed a rapid decline during the initial 15 hr (33.3 pmol/hr/10^6 cells), but thereafter the disappearance of tocopherol from the cells was much slower (6.7 pmol/hr/10^6 cells). The half-life of (R,R,R)α-tocopherol retained by the cells was estimated from the linear portion of the curve to be 55 hr (Fig. 4). To determine the half-time of

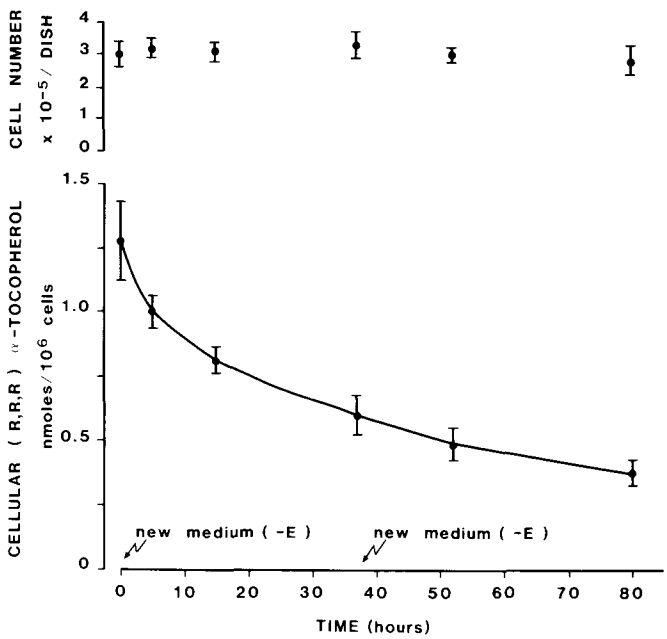

FIG. 4. Disappearance of (R,R,R)α-tocopherol in human endothelial cells. Endothelial cells were incubated with 23.2 μM of (R,R,R)α-tocopherol for 4 hr, after which the tocopherol-enriched medium was removed. The cells were gently rinsed with warm Hepes buffer saline containing 0.25% of essential fatty acid free bovine serum albumin and fresh culture medium without tocopherol (-E). At indicated time points, cells were harvested and cellular lipids were extracted to determine tocopherol as described in Methods. After 37 hr of incubation, medium was replaced in order to maintain cell viability (arrow). Values are means ± S.D. from three dishes.

TABLE 2

Half-Life of (R,R,R)α-Tocopherol Retained in Human Endothelial Cells[a]

Trial	Half-life (hr)	Cell passage
1	70	2
2	55	4
3	70	5
Mean ± SD	65 ± 8.6	

[a]Endothelial cells derived from different cords at different cell passages were incubated with 23.2 μM (R,R,R)α-tocopherol for 4 hr, at which time the tocopherol enriched medium was removed and replaced with fresh medium without tocopherol. Retention of tocopherol by cells was determined as described in Figure 4, and half-life was estimated from the extrapolated linear portion of the plot.

tocopherol disappearance from cells isolated from different umbilical cords, similar experiments were conducted and the results are summarized in Table 2. The mean half-time for (R,R,R)α-tocopherol retention by endothelial cells isolated from three cords was 65 ± 8.6 hr. Differences in the number of cell passages appeared to have no appreciable effect on the rate of tocopherol disappearance.

DISCUSSION

Using our method of incorporating tocopherol into medium at physiological temperatures, which combines the lipid solubilizing effects of DMSO and takes advantage of the lipoproteins present in fetal calf serum, the maximum medium concentration of tocopherol that can be attained is about 100 μM. This concentration coincides with the upper attainable limit of tocopherol in human plasma of individuals who take mega-vitamin E supplements. Results from these studies show that human endothelial cells take up tocopherol from the medium in a time-dependent and concentration-dependent manner. Judging from the successful passages of the cells grown in the relatively "tocopherol-deficient" medium, it is quite clear that the requirement of tocopherol for proper cell division and cell growth is indeed very small. Conversely, the toxicity levels for tocopherol in the endothelial cells appear to lie beyond the highest concentration (92.8 μM) used in our experiments as cell numbers and cell viability were not altered.

Apart from serving as a selective barrier for nutrient clearance and transport, the endothelial monolayer also synthesizes potent, hormone-like substances, such as the eicosanoids, platelet activating factor and endothelium-derived relaxing factor, and its role as an endocrine organ has recently been suggested (17). Indeed, tocopherol has been reported to affect the synthesis of these lipid mediators (5-7,10,17-20). The present study demonstrates that human endothelial cells readily accumulate tocopherol in their cellular membranes in a dose and time-dependent manner. Under our experimental conditions, the half-time of tocopherol disappearance from human cells is about 65 hr. Our results clearly show that human endothelial cells in culture are a useful model to study the effect of tocopherol on cell function. In view of the difficulties in extrapolating data obtained on animals to humans, this human cell culture model could potentially be used to also study other fat-soluble vitamins and essential nutrients.

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Identification of Vitamin E-Dependent Water Soluble Fluorescent Compounds in Mouse Tissues

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The present paper describes the identification of two vitamin E-dependent, water soluble fluorescent compounds in mouse tissues. Ultraviolet and fluorescent spectroscopy, derivatization with 1-dimethylamino-naphtalene-5-sulfonyl chloride (dansyl chloride) and cochromatography using high performance liquid chromatography (HPLC) were utilized for the identification of the unknown compounds. The water soluble fluorescent compounds in mouse tissues were identified as tyrosine and tryptophan. The compounds were previously found to increase significantly in vitamin E deficiency in various tissues.
***Lipids 25,* 22-26 (1990).**

Fluorescent pigments exist ubiquitously in various types of animal tissues (1,2), and they have been shown to increase in vitamin E deficiency and aging (3-9). Because of their histological staining properties, these lipofuscin pigments are generally assumed to be organic solvent soluble. They are thought to exist primarily as Schiff base type compounds which arise from lipid peroxidation and have a maximal fluorescence in the range of 430-470 nm (10-12). After further purification, the 435 nm emitting material was termed "organic solvent-soluble lipofuscin pigment" (OSLP) (13). However, OSPL was found to be significantly elevated in vitamin E deficiency only in the liver, not in other tissues (14). This was surprising because of the great increase of fluorescence that occurs in intact uteri of rats kept on a vitamin E deficient diet (8). It appeared that some of the fluorescence seen in the intact tissues could not be extracted with organic solvent.

Therefore, a systematic search for the presence of water-soluble fluorescent compounds in tissues was conducted in our laboratory. It was found that up to four fluorescent, vitamin E-dependent materials were present in the water extracts of various tissues (15). These materials were separated by Sephadex G-15 column chromatography. They had the following maximum excitation/emission wavelengths (in order of elution): i) high molecular wieght compound 275 nm/350 nm, and ii) low molecular weight compounds 270 nm/310 nm, 320 nm/380 nm and 275 nm/350 nm (15). Two of these low molecular weight compounds, the 270 nm/310 nm and the slower eluting 275 nm/350 nm materials (designated in the present paper as compounds A and B, respectively), were significantly increased in vitamin E deficiency, as well as upon ozone exposure (16). The identity of the higher molecular weight (275 nm/350 nm) material was recently reported in a separate paper (17). The purpose of the present study was to identify the two low molecular weight, water-soluble fluorescent compounds designated A and B.

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Abbreviations: Dansyl chloride, 1-dimethyl-naphtalene-5-sulfonyl chloride; HPLC, high performance liquid chromatography; OSLP, organic solvent-soluble lipofuscin pigment; TRP, DL-tryptophan; TYR, DL-tyrosine.

MATERIALS AND METHODS

Animals, diets and extraction. Female weanling mice (C57BL/6J, The Jackson Laboratories, Bar Harbor, ME) were fed a vitamin E deficient or supplemented diet (30 mg RRR-α-tocopheryl acetate/kg diet) as described previously (15). After 14 months, the mice were decapitated, the livers were removed, washed with cold saline-phosphate solution and blotted dry. The livers were frozen at $-70°C$ until they were homogenized with chloroform/methanol (2:1, v/v) and extracted with distilled water as reported previously (15). Extraction of the tissues was followed by the lyophilization of the aqueous phase, and then redissolved in distilled water. Aliquots were used for chromatographic separation of the low molecular weight, fluorescent water-soluble compounds using Sephadex G-15 or G-25 columns (Pharmacia Fine Chemicals, Piscataway, NJ) (Table 1). Initial fluorescence measurements were performed using an Aminco-Bowman Ratio Spectrophotofluorometer (American Instrument Co., Silver Spring, MD) standardized as previously described (15).

High performance liquid chromatography (HPLC) equipment. Instrumentation for HPLC consisted of a Model 210 Injection Valve (Beckman Instruments, Inc., Berkeley, CA), a model 110 A Solvent Metering Pump (Beckman Instruments, Inc.), a Perkin-Elmer Model 650-10S Fluorescence Spectrophotometer (Norwalk, CT) equipped with a horizontal, flat, 20 μl capacity, quartz flow cell, a Model SP8400 Variable Wavelength Ultraviolet/Visible Detector (Spectra Physics, Arlington, IL) equipped with a 10 μl quartz flow cell, and a Model SP4100 Computing Integrator (Spectra Physics).

The analytical columns included a 0.75 $\times$ 30 cm Micropak TSK G2000 SW column (Varian Associates, Inc., Walnut Creek, CA) and a 10 μm, 0.46 $\times$ 25 cm LiChrosorb RP-18 column (EM Reagents, Cincinnati, OH). Samples were injected with a 100 or a 200 μl blunt needle Hamilton Syringe (Rainin Instrument Co., Inc., Woburn, MA). Injection volumes were less than 200 μl.

Fluorescence and absorption spectra of low molecular weight compounds A and B. The compound A (270 nm/310 nm) and the later eluting compound B (275 nm/350 nm) (excitation wavelength/emission wavelength) were collected from the Sephadex G-15 column using distilled water for elution. Fluorescence spectra for these materials, DL-tyrosine (TYR) and DL-tryptophan (TRP) were obtained using the Perkin-Elmer fluorometer and recorded using a LKB Model 2210, two-channel recorder (Rockville, MD). Ultraviolet absorption spectra were measured and recorded using a Beckman DU-8 Wavelength Scanning System (Beckman Instruments, Inc.). Small quantities of hydrochloric acid or sodium hydroxide were added to the cuvette to produce pH < 2, pH $= 7$ or pH > 12 for the pH-dependent scans.

Cochromatography of compounds A and B with standards. Compounds A (270 nm/310 nm) and B (275 nm/350 nm) were collected as above and were cochromatographed with pure TYR and TRP (Sigma Chemical Co.,

TABLE 1

Identification of Vitamin E Related, Low Molecular Weight, Water-Soluble Fluorescent Compounds in Mouse Tissues

A. Fluorescence spectra for the 270/310 and 275/350 Compounds A and B agreed with TYR and TRP standards, respectively.

B. Cochromatography of the 270/310 Compound A with TYR and that of the 275/350 Compound B with TRP was accomplished under the following chromatographic conditions:

Column	Mobile phase	Elution volume (ml)	
		270/310 nm (A) with TYR	275 nm/350 nm (B) with TRP
G-15 (1.6 × 49 cm)	0.02% NaN_3	96.3	175.5
TSK G2000 SW	0.6 M phosphate buffer, 0.3 M NaCl, 0.02% NaN_3, pH 7.0	11.5	15.2
TSK G2000 SW	H_2O	8.5	12.3
LiChrosorb RP-18	Water/methanol/acetic acid (70:29:1, v/v/v)	4.1	10.0
LiChrosorb RP-18	30% methanol in water	3.8	7.7
LiChrosorb RP-18	20% methanol in water	—	14.9
LiChrosorb RP-18	10% methanol in water	5.4	—
LiChrosorb RP-18	100% water	9.9	—
LiChrosorb RP-18	10% acetonitrile in water	4.3	14.5
LiChrosorb RP-18	5% acetonitrile in water	6.7	—

C. Derivatization (dansylation by use of 1-dimethylamino-napthalene-5-sulfonyl chloride) of these compounds and stock solutions of TYR and TRP followed by appropriate cochromatography on LiChrosorb RP-18.

St. Louis, MO), respectively, on several different types of columns with different mobile phases (Table 1). Cochromatography was first performed on a 1.6 × 49 cm Sephadex G-15 column eluted with 0.02% sodium azide. Two-milliliter fractions were collected and their relative fluorescence was measured at their appropriate wavelengths using an Aminco-Bowman fluorometer. Cochromatography was also accomplished on a 0.75 × 30 cm TSK G2000 SW column eluting with either i) 0.066 M phosphate, 0.3 M sodium chloride, 0.02% sodium azide (pH 7.0) or ii) distilled water at 1 ml/min. The third type of cochromatography experiment was performed on a 0.46 × 25 cm LiChrosorb RP-18 column eluting with either: i) distilled water; ii) 10% methanol in water; iii) 20% methanol in water; iv) 30% methanol in water; v) 5% acetonitrile in water; vi) 10% acetonitrile in water; or vii) water/methanol/acetic acid (70:29:1, v/v/v) at 1 ml/min. Fluorescent peaks were detected with a Perkin-Elmer 650-10S fluorometer.

Derivatization of compounds A and B. Dansylation and subsequent cochromatography of compound A and TYR and compound B and TRP were done by a modification of the method of O'Keefe *et al.* (18). Ten μl 0.01 N NaOH, 100 μl 0.1 M borate buffer (pH 9.0) and 100 μl 1 mM 1-dimethylamino-napthalene-5-sulfonyl chloride (dansyl chloride; Sigma Chemical Co.) in acetonitrile were added to a 100 μl sample. This mixture was stirred in a Vortex mixer, enclosed, and placed in a 40°C water bath for 45 min. The sample was cooled to room temperature and 75 μl aliquots were injected onto a 0.46 × 25 cm LiChrosorb RP-18 column. A model 421 CRT Microprocessor-Controller (Beckman Instruments, Inc.) was used to control two HPLC pumps at a total flow rate of 3 ml/min. A linear gradient with solvent A and B was used. Solvent A consisted of nine parts of 0.01 M phosphate buffer (pH 7.0) and one part of acetonitrile. Solvent B consisted of one part each of 0.1 M phosphate buffer (pH 7.0) and acetonitrile. The 20 minute HPLC analysis involved a 10 minute linear gradient from 25% B to 70% B, followed by a 10 minute hold at 70% B. The column was made ready for the next injection by running a 0.1 min gradient from 70% B to 25% B, and holding at 25% B at least 10 minutes. Detection of the separated materials was accomplished using the Perkin-Elmer 650-10S fluorometer (330 nm excitation/565 nm emission) and the Spectra Physics SP8440 UV monitor (254 nm) in series. Standards for cochromatography were *N,O*-didansyl 1-TYR in two parts of 0.1 M borate buffer, pH 9.0 and one part of acetonitrile and dansylated DL-TRP (synthesized as described above).

Aeration experiment. A homogenate (60 mg wet liver/ml water) from the liver of a ten-month-old, freshly killed, normal mouse was aerated by bubbling ambient air through the homogenate at 150 cc/min for up to 138 hr at room temperature. Samples were collected during and at the end of the aeration period and extracted by vigorously mixing 1 ml of the aerated sample with 1 ml of chloroform:methanol (2:1, v/v). The samples were centrifuged at 3000 × *g* for 10 min, and the aqueous layer was applied to a 1.5 × 35 cm Sephadex G-15 column and chromatographed as described previously (15).

RESULTS

Fluorescence spectra of the isolated low molecular weight compounds A and B were compared to those of known standards with similar fluorescent characteristics. It was found that compound A (270 nm/310 nm) and the later eluting compound B (275 nm/350 nm) were similar to TYR and TRP, respectively. These spectra were obtained using a Perkin-Elmer fluorometer, while the one used in the previously described vitamin E response study was an

Aminco-Bowman Spectrofluorometer (15). Slight wavelength differences were found between the two instruments using the same materials. The observed excitation and emission maxima were 282 nm/307 nm for both compound A and TYR standard (Fig. 1) and 285 nm/359 nm for both compound B and the TRP standard (Fig. 2).

The ultraviolet absorption spectra for compounds A and B were measured at pH < 2, pH = 7 and pH > 12 and compared to those for standard TYR and TRP. The ultraviolet spectrum of compound B and that of TRP agreed well at each pH (Fig. 3), indicating the presence of the same chromophore in each compound. Compound A could not be compared to TYR due to the presence of a contaminating material with a maximum absorbance at 250 nm.

A summary of several columns and mobile phases used to determine whether the unknown compounds would comigrate with the standards is shown in Table 1. The unknown was applied to the column first. This was followed by the appropriate standard (TYR for Compound A and TRP for Compound B). Finally, a mixture of equal quantities by fluorescence of the standard and the unknown was applied to the column. The elution profiles of the sample peak, the standard peak and the mixture peak were identical for Compound A and standard TYR and for Compound B and standard TRP (Fig. 4 and 5, respectively).

Final identification of the two fluorescent unknown compounds was by derivatization. Dansylated TYR, dansylated Compound A and a mixture of these two with equal fluorescence were injected onto the LiChrosorb RP-18 column. All amples eluted at 8.24 ± 0.05 min with no recognizable shoulders (Fig. 6). The peak height ratio of the maximum fluorescence at 330 nm/565 nm to ultraviolet absorbance at 254 nm was 10.8 ± 0.2 (arbitrary units) for each sample injected. Elution profiles for dansylated TRP, dansylated Compound B and a mixture of these two is shown in Figure 7 with a retention time of 11.9 ± 0.1 min. The shape of the peak is the same for each sample and the peak height ratio of fluorescence at 330 nm/565 nm to ultraviolet absorbance at 554 nm was constant at 6.5 ± 0.2 (arbitrary units). Chromatograms were compared to a suitable blank treated exactly

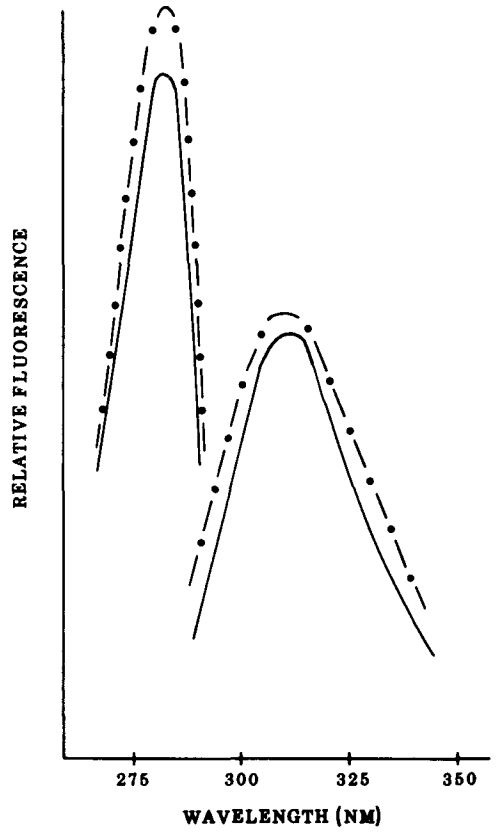

FIG. 1. Excitation and emission spectra of the "270 nm/310 nm compound" (——) and tyrosine standard (—●—●—).

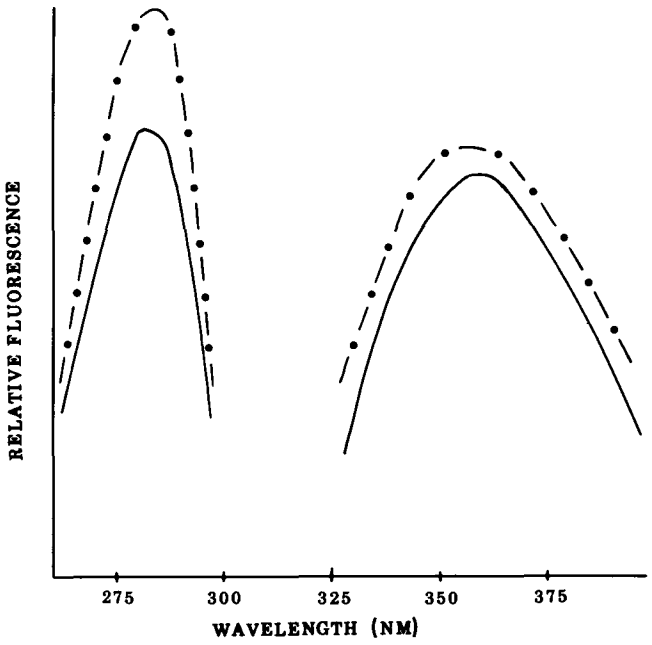

FIG. 2. Excitation and emission spectra of the "275 nm/350 nm compound" (——) and tryptophan standard (—●—●—).

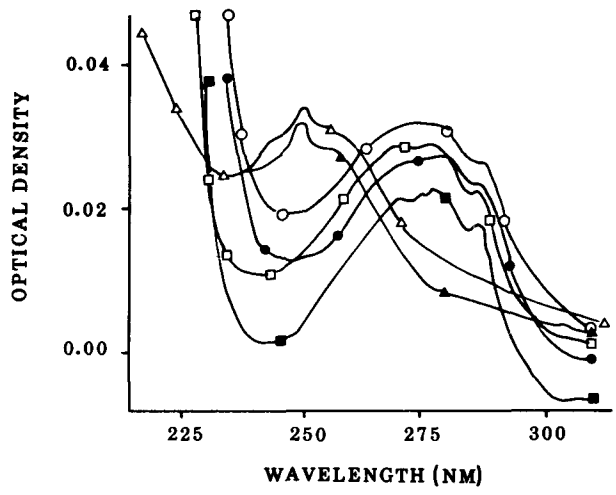

FIG. 3. Ultraviolet absorption spectra of the "275 nm/350 nm compound" at pH <2 (△—△), at pH 7 (□—□), at pH >12 (○—○); and tryptophan at pH <2 (▲—▲), at pH 7 (■—■), at pH >12 (●—●).

as the standards, except that 100 μl water was used instead of 100 μl TYR or TRP solution for the dansylation reaction. Other than the dansylated amino acid, no new peak of any appreciable size was observed.

The third low molecular weight, water-soluble fluorescent compound that was previously found to be related to dietary vitamin E in mouse liver was the "320 nm/380 nm compound" (15). The identification of this compound was not carried out because it appeared that it was not produced *in vivo* by the mouse or by man. Livers from mouse, rat, pig, and human were analyzed for the presence of the "320 nm/380 nm compound," and there was no sizeable quantity found in fresh livers from the following animals: i) a six-month-old pig, ii) vitamin E deficient Sprague-Dawley rats, iii) vitamin E sufficient 6-month, 13-month, and 49-month-old mice (strain C-57BL/6J), or iv) a 64-year-old male human. The only livers that contained any sizeable concentration of the "320 nm/380 nm compound" were i) 14-month-old C-57BL/6J strain mice whose livers had been stored for more than three months at −70°C, and ii) 6-month-old pork livers stored for more than two years at −70°C. The mouse livers analyzed were from vitamin E deficient and supplemented animals, and the pig was fed a cracked corn and soybean oil diet. These

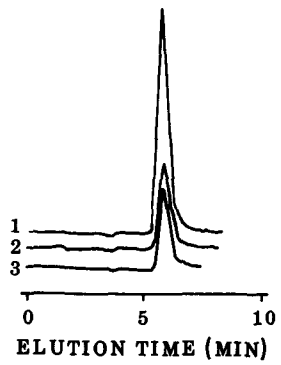

FIG. 4. Cochromatography of tyrosine and the "270 nm/310 nm compound" on LiChrosorb RP-18 column, eluted with 10% methanol in water. (1) Mixture, (2) "270 nm/310 nm compound," and (3) tyrosine.

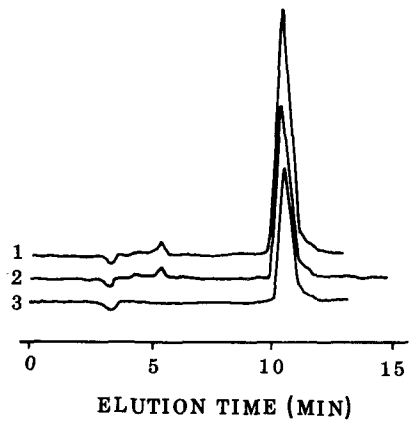

FIG. 5. Cochromatography of tryptophan and the "275 nm/350 nm compound" on LiChrosorb RP-18 column, eluted with water/methanol/acetic acid (70:29:1, v/v/v). (1) Mixture, (2) "275 nm/350 nm compound," and (3) tryptophan.

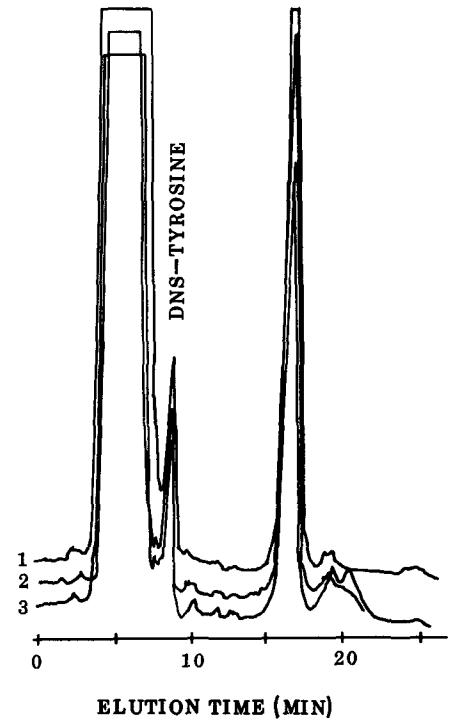

FIG. 6. Cochromatography of dansylated tyrosine standard and dansylated "270 nm/310 nm compound." (1) Dansylated tyrosine, (2) mixture, and (3) dansylated "270 nm/310 nm compound."

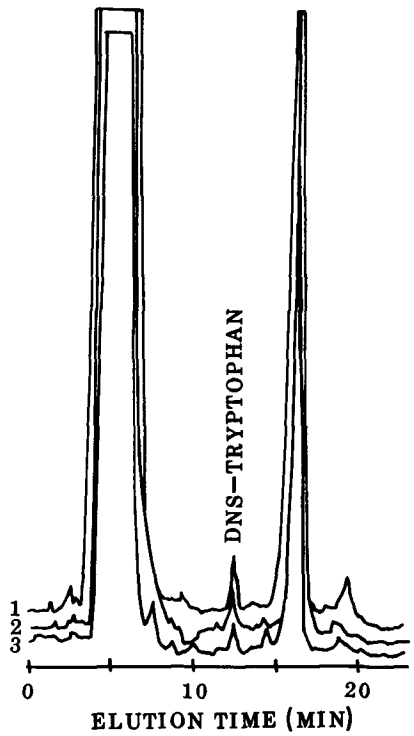

FIG. 7. Cochromatography of dansylated tryptophan standard and dansylated "275 nm/350 nm compound." (1) Dansylated tryptophan, (2) mixture, and (3) dansylated "275 nm/350 compound."

results suggest that the "320 nm/380 nm compound" is not produced *in vivo*.

An aeration experiment was conducted to see if oxidation was the cause of the production of the "320 nm/380 nm compound." After about 100 hr of aeration, the "320 nm/380 nm compound" was seen to increase quite dramatically to a maximum concentration. The produced compound eluted at the same position off the Sephadex G-15 column as did the "320 nm/380 nm compound" originally found in the tissues. It was concluded that the "320 nm/380 nm compound" was an artifact caused by oxidation.

DISCUSSION

Several cochromatography experiments were performed in which several physical parameters such as size (Sephadex and HPLC size exclusion columns), polarity, and solubility (HPLC reversed phase column) were varied. By varying the ionic strength, polarity, buffering capacity and mobile phases, up to a three-fold change in the elution time of the compound could be obtained. The sample peaks always had the same elution time and peak shape as did the TYR or TRP standard. Cochromatography of the dansyl derivative of the unknown compounds with those obtained from the standard TYR or TRP solutions confirmed the identity of Compounds A (270/310 nm) and B (275/350 nm) as TYR and TRP, respectively.

The variation in fluorescence excitation and emission wavelengths for TYR and TRP using different fluorometers are within the expected range. Spectra of the same material may differ by as much as 10–15 nm on two different fluorometers (18,19). Teale and Weber (20) reported the wavelengths for TYR and TRP to be 275 nm/303 nm and 287 nm/348 nm, respectively. Duggan *et al.* (21) reported them to be 275 nm/310 nm (TYR) and 285 nm/359 nm (TRP). In the present study, the excitation and emission wavelengths of compound A and TYR were both found to be 282 nm/307 nm and those for compound B and TRP were both found to be 285 nm/359 nm. These variatios between differing instruments are well within the expected range.

Previous experiments in this laboratory have shown a significant rise in the concentration of both of these water-soluble fluorescent compounds in mouse liver due to vitamin E deficiency or ozone, following whole animal exposure (15,16). Vitamin E deficiency and ozone exposure similarly seem to lead to the increased oxidation of lipid membranes, including lysosomal membranes. Injury to membranes results in release of lytic enzymes, increased hydrolysis of proteins and a concomitant rise in the level of free amino acids. Alterations in free amino acid concentration have been reported due to vitamin E deficiency in rabbits and chicks (22,23). Electrophoretic changes in serum proteins of rabbits and chicks are detected at early stages of vitamin E deficiency (24). The muscle proteins, myosin and actomyosin, are decreased in advanced stages of skeletal muscular dystrophy and free amino acids concentrations are increased in muscle extracts and urine (24). Since TYR and TRP are the main amino acids that fluoresce (phenylalanine is only slightly fluorescent), fluorescence analysis of the water-soluble fraction of tissue extracts should be expected to show a relative increase of both TYR and TRP. The observed increase in the concentration of water-soluble fluorescent compounds due to vitamin E deficiency or ozone exposure (15) indicates that the rise in TYR and TRP concentration is a secondary effect of lipid peroxidation.

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Effects of Dietary Fish Oil on Biliary Phospholipids and Prostaglandin Synthesis in the Cholesterol-fed Prairie Dog

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Cholesterol gallstone formation in the prairie dog is accompanied by an increase in the percentage of biliary phospholipids containing arachidonic acid, and an increase in gallbladder prostaglandin (PG) synthesis, but the pathogenetic significance of these changes is unclear. Dietary supplementation with eicosapentaenoic acid (EPA), an omega-3 fatty acid which is commonly found in fish oil, decreases prostaglandin synthesis in some tissues by replacing arachidonic acid, and by competitively inhibiting prostaglandin synthesis. We studied the effect of dietary fish oil on gallbladder PG synthesis, and the relative abundance of various molecular species of phosphatidylcholines and phosphatidylethanolamines in bile and gallbladder epithelium in the cholesterol-fed prairie dog. Prairie dogs were maintained for 4 weeks on one of four diets: i) control, ii) cholesterol-supplemented (0.34%), iii) menhaden oil (50 g/kg chow), or iv) cholesterol plus menhaden oil. Supplementation with menhaden oil resulted in a replacement of arachidonic and linoleic acids with EPA and docosahexaenoic acids in the phospholipids of bile and gallbladder mucosa. In cholesterol-fed animals, supplementation with menhaden oil prevented increased gallbladder PG synthesis. Menhaden oil also reduced the incidence of cholesterol monohydrate crystals among cholesterol-fed animals (9/20 with cholesterol plus menhaden oil vs 21/22 with cholesterol alone), but the improvement could not clearly be attributed to decreased PG synthesis since supplementation with menhaden oil also increased the total phospholipid concentration in bile, and decreased the degree of cholesterol saturation. These results demonstrate that dietary supplementation with omega-3 fatty acids significantly influences biliary phospholipids, and decreases the incidence of cholesterol monohydrate crystal formation in this animal model.
***Lipids* 25, 27–32 (1990).**

Alterations in the relative amounts of various phospholipid molecular species in the biliary tract may be an important factor in the pathogenesis of cholesterol gallstones. The bile of human cholesterol gallstone formers has been reported to have an increased percentage of phospholipids containing arachidonic acid (1), and a decreased percentage of phospholipids containing linoleic acid (2). Recent studies in our laboratory have demonstrated similar changes in the prairie dog model for cholesterol cholelithiasis, and we have also shown that these changes are accompanied by an increase in the arachidonic acid content of phosphatidylcholines and phosphatidylethanolamines in the gallbladder epithelium (3). Arachidonic acid is a precursor for prostaglandin (PG) synthesis, and increased availability of arachidonic acid in gallbladder phospholipids might account for the increase in gallbladder PG synthesis which occurs during gallstone formation in the prairie dog model (4).

These alterations in biliary phospholipid metabolism have the potential to contribute to cholesterol gallstone pathogenesis. Prostaglandins are known to have potent effects on biliary smooth muscle, and may be responsible for the increased cystic duct resistance and impaired gallbladder emptying which occur during cholelithiasis in the prairie dog (5). In addition, PGs are known to stimulate mucin secretion from gastric epithelium (6), and increases in gallbladder PG synthesis could be responsible for the hypersecretion of gallbladder mucin which also occurs during cholesterol gallstone formation in the prairie dog (7). Gallbladder stasis and hypersecretion of gallbladder mucin are believed to contribute to cholesterol gallstone formation by promoting the nucleation of cholesterol crystals in cholesterol-supersaturated bile (8–10).

The availability of free arachidonic acid in tissues is an important factor regulating the rate of PG synthesis (11). It has been shown, for example, that dietary supplementation with linoleic acid, the precursor of arachidonic acid, produces an increase in PG synthesis in gastric mucosa (12). Eicosapentaenoic acid (20:5) and docosahexaenoic acid (22:6) are fatty acids of the omega-3 series which, when provided in the diet, tend to replace arachidonate in tissue PL's (13–15). In some tissues, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DCHA) decrease PG synthesis by acting as competitive inhibitors of cyclooxygenase (16,17). The present study was conducted to determine whether dietary supplementation with menhaden oil, a fish oil relatively rich in EPA and DCHA, could prevent the cholesterol-induced increase in gallbladder arachidonyl phospholipids, and the associated increase in PG synthesis.

METHODS

Experimental design. Animal studies were reviewed and approved by the Institutional Animal Care and Use Committee at Boston University Medical School. Adult female prairie dogs *(Cynomys ludovicianus)* weighing 0.75–1.0 kg were purchased form Otto M. Locke, New Braunfels, TX. All animals were fed a cholesterol-free control diet (TD 73176, Teklad, Madison, WI) for at least 2 weeks prior to study, after which animals either continued the cholesterol-free control diet, or were switched to an experimental diet for an additional 4 weeks. The cholesterol-supplemented diet contained 0.34% cholesterol by weight (TD 83484, Teklad), and was identical to the control diet except that 3.4 g of sucrose per kilogram of chow was replaced with cholesterol. Two additional experimental diets were prepared by coating the control and cholesterol-supplemented diets with 50 g of menhaden oil (Zapata Haynie Corp., Reedville, VA) per kg of chow.

After an overnight fast, anesthesia was induced by intramuscular injection of ketamine HCl 100 mg (Ketalar,

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Abbreviations: DCHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; HPLC, high performance liquid chromatography; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PL, phospholipid; PG, prostaglandin.

Parke-Davis, Morris Plains, NJ) and acepromazine maleate 2 mg (PromeAce, Fort Dodge Laboratories, Fort Dodge, Iowa). A laparotomy was performed, and the gallbladder was excised promptly.

Measurement of cholesterol, bile salt, and total phospholipid concentrations in bile. The effect of dietary treatment on cholesterol saturation and cholesterol monohydrate crystal formation was determined in a total of 73 animals (as will later be summarized in Table 8). Fresh samples of gallbladder bile were examined for crystals by direct microscopy. Cholesterol monohydrate crystals were identified by their birefringence and typical notched rhomboidal shape as described by Juniper and Burson (18). Unspun samples of bile were analyzed for total bile salts by the hydroxysteroid dehydrogenase method of Talalay (19) as modified by Admirand and Small (20). Aliquots of bile were extracted in chloroform and methanol, and analyzed for total cholesterol content by the method of Rudell and Morris (21). Total phospholipids in bile were measured by the method of Bartlett (22) after extraction of bile with chloroform/methanol (2:1, v/v). The cholesterol saturation index (CSI) was calculated as described by Kuroki *et al.* (23).

Analysis of phospholipid molecular species in bile and gallbladder epithelium. Because of the detailed nature of the analysis of phospholipid molecular species, these analyses were conducted only in 6–8 subjects within each dietary group (as will be summarized in Tables 1–6). For these studies the gallbladder was rinsed with normal saline, and samples of gallbladder epithelium were removed by gentle scraping and extracted by the method of Folch *et al.* (24). Folch extraction was also performed on aliquots of gallbladder and hepatic bile immediately after their collection. Samples of hepatic bile were collected for 1 hr after cannulating the common hepatic duct with polyethylene tubing (PE-10) ligated in place.

Phospholipid high performance liquid chromatography (HPLC). HPLC was performed with a Shimadzu (Columbia, MD) system consisting of an LC-6A solvent delivery system, an SPD-6A variable wavelength detector, and a C-R3A Chromatopac integrator.

Phospholipid classes in bile and gallbladder mucosa were separated by HPLC by the method of Patton *et al.* (25). Lipid extracts were evaporated under nitrogen and redissolved in hexane/isopropanol/water(40:54:6, v/v/v) prior to injection. A 250 × 4.6 mm Hibar column packed with 10 μ LiChrospher Si-100 (Alltech Associates, Deerfield, IL) was used for separation of PL classes with detection at 205 nm. The elution solvent (isopropanol/hexane/25 mM phosphate buffer, pH 7.0/ethanol/acetic acid [490:367:62:100:1.25, by vol.]) was delivered at 0.5 ml/min through the elution of phosphatidylserine, and then increased to 1.5 ml/min.

Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) fractions were perbenzoylated by the method of Ullman *et al.* (26) in order to obtain quantitative detection at 230 nm during separation into subclasses and molecular species. The derivatized diacyl PC and PE fractions were obtained by a modification of the HPLC method of Nakagawa *et al.* (27). Samples were evaporated and redissolved in hexane prior to chromatography on a 250 × 4.6 mm Hibar column packed with 10 μ LiChrospher Si-100 (Alltech). The elution solvent consisted of cyclohexane/hexane/methyl-*t*-butyl ether/acetic acid (375:125:10:0.1, by vol delivered at a flow rate of 1.0 ml/min.

Diacyl molecular species of perbenzoylated PC and PE were separated and quantitated by reverse phase HPLC according to Patton *et al.* (28) in which the fatty acyl moieties were identified by gas-liquid chromatography of their methyl esters. The HPLC separations utilized a 250 × 4.6 mm Ultrasphere ODS column (Beckman, San Ramon, CA), and the solvent system consisted of methanol/water/acetonitrile (950:35:15, v/v/v delivered at a rate of 2.0 ml/min.

Measurement of prostaglandin synthesis. Gallbladder strips were incubated *in vitro* to determine rates of PG synthesis as previously described (4). Prostanoids were extracted with ethyl acetate, purified by silicic acid column chromatography, and measured by radioimmunoassay as previously described by us (4).

Data analysis. Results for groups of animals are reported as mean with correction for multiple comparisons. Differences in the incidence of cholesterol monohydrate crystals were tested for significance by chi-square analysis.

RESULTS

Chow consumption among different dietary groups was comparable, with no weight loss or illness occurring in any group. At laparotomy, all livers were grossly normal.

Effects of diet on phospholipids in bile. As previously reported by this laboratory (3) phosphatidylcholine (PC) and phosphatidylethanolamine (PE) accounted for approximately 95% and 4%, respectively, of the phospholipids in bile, regardless of dietary treatment. Approximately 30 molecular species were measured during each reverse phase elution. However, the major changes occurred in those species containing linoleic acid, arachidonic acid, or one of the omega-3 fatty acids. Consequently, for clarity of presentation, Tables 1–6 focus on these species.

Table 1 shows the effect of the diets on the molecular species of PC in hepatic bile. Animals supplemented with cholesterol alone had significant increases in species containing arachidonic acid (20:4) when compared to controls. Addition of fish oil to the control diet decreased the percentage of phospholipid species containing the omega-6 fatty acids linoleate (18:2) and arachidonate (20:4), while species containing the omega-3 fatty acids (20:5, 22:5, and 22:6) were increased. The same effects were noted when fish oil was added to the cholesterol-supplemented diet.

As shown in Table 2 similar changes were found in gallbladder bile. Cholesterol-supplementation again increased the percentage of PC species containing arachidonic acid by a small, but significant amount. Addition of fish oil to either the control or cholesterol-supplemented diets decreased the percentage of phospholipid species containing linoleate and arachidonate, and both groups receiving fish oil had increases in species containing omega-3 fatty acids.

The effect of dietary manipulation on PE species in hepatic bile is shown in Table 3. Cholesterol-supplementation had little effect, except for a decrease in the 18:0–18:2 species. Addition of fish oil to either the control or cholesterol-supplemented diets reduced linoleoyl species even further, and produced significant decreases

TABLE 1

Effect of Dietary Cholesterol and Menhaden Oil on Molecular Species of Phosphatidylcholines in Hepatic Bile (% ± SD)

Species	Control (N = 6)	Cholesterol (N = 6)	Fish oil (N = 8)	Cholesterol + fish oil (N = 8)
Linoleoyl species				
16:0-18:2	21.9 ± 3.2	19.7 ± 6.0	20.3 ± 5.4	26.5 ± 5.5
18:0-18:2	13.5 ± 1.9	11.7 ± 2.5	7.6 ± 1.9[a]	10.4 ± 2.7[a,c]
18:1-18:2	11.2 ± .7	13.1 ± 2.4[a]	3.0 ± .6[a,b]	5.7 ± 1.2[a,b,c]
Arachidonyl species				
16:0-20:4	4.7 ± .6	5.9 ± 1.1[a]	4.8 ± .9[b]	4.9 ± .5[b]
18:0-20:4	5.8 ± 1.1	7.3 ± 2.5	2.4 ± 1.2[a,b]	3.2 ± .4[a,b]
18:2-20:4 and 16:1-18:2	2.3 ± .3	3.5 ± .8[a]	1.2 ± .3[a,b]	1.9 ± .4[b]
Omega-3 species				
16:0-20:5 and 14:0-22:5	.8 ± .2	.1 ± .2	6.1 ± 2.7[a,b]	3.9 ± 1.6[a,b,c]
16:0-22:5	1.2 ± .3	1.2 ± .1	2.2 ± .6[a,b]	2.2 ± .8[a,b]
16:0-22:6 and 14:0-22:4	.9 ± .1	1.0 ± .2	8.7 ± 2.4[a,b]	4.8 ± 1.4[a,b,c]

[a]Differs from control ($p < .05$). [b]Differs from cholesterol ($p < .05$). [c]Differs from fish oil alone ($p < .05$).

TABLE 2

Effect of Dietary Cholesterol and Menhaden Oil on Molecular Species of Phosphatidylcholines in Gallbladder Bile (% ± SD)

Species	Control (N = 6)	Cholesterol (N = 6)	Fish oil (N = 8)	Cholesterol + fish oil (N = 8)
Linoleoyl species				
16:0-18:2	18.1 ± 3.6	15.8 ± 5.9	16.3 ± 6.2	19.5 ± 7.7
18:0-18:2	10.5 ± 2.3	8.4 ± 3.2	5.7 ± 2.1[a,b]	6.9 ± 2.3[a]
18:1-18:2	9.7 ± 1.2	9.3 ± 1.3	2.1 ± .7[a,b]	3.9 ± 1.2[a,b,c]
Arachidonyl species				
16:0-20:4	4.4 ± .6	6.0 ± 1.3[a]	4.0 ± .9[b]	5.1 ± .9[c]
18:0-20:4	6.7 ± 1.4	8.8 ± 3.3	2.1 ± .9[a,b]	2.9 ± .5[a,b]
18:2-20:4 and 16:1-18:2	1.9 ± .3	2.5 ± .5[a]	.9 ± .3[a,b]	1.7 ± .3[b,c]
Omega-3 species				
16:0-20:5 and 14:0-22:5	.1 ± .2	.0 ± .0	8.6 ± 2.6[a,b]	6.4 ± 1.9[a,b,c]
16:0-22:5	1.4 ± .3	1.4 ± .2	2.9 ± .8[a,b]	2.6 ± .4[a,b]
16:0-22:6 and 14:0-22:4	.7 ± .1	.8 ± .3	8.5 ± 2.1[a,b]	5.2 ± 1.7[a,b,c]

[a]Differs from control ($p < .05$). [b]Differs from cholesterol ($p < .05$). [c]Differs from fish oil alone ($p < .05$).

TABLE 3

Effect of Dietary Cholesterol and Menhaden Oil on Molecular Species of Phosphatidylethanolamines in Hepatic Bile (% ± SD)

Species	Control (N = 6)	Cholesterol (N = 6)	Fish oil (N = 8)	Cholesterol + fish oil (N = 8)
Linoleoyl species				
16:0-18:2	18.1 ± 2.7	16.0 ± 3.4	11.5 ± 1.3[a,b]	14.7 ± 1.5[a,c]
18:0-18:2	29.0 ± 1.5	24.9 ± 4.6[a]	11.1 ± 1.6[a,b]	15.9 ± 4.0[a,b,c]
18:1-18:2	17.6 ± 2.5	17.4 ± 4.4	2.1 ± 1.2[a,b]	5.7 ± 1.8[a,b,c]
Arachidonyl species				
16:0-20:4	4.2 ± .5	4.8 ± 1.1	2.8 ± 1.3[a,b]	4.3 ± .5[c]
18:0-20:4	12.5 ± 1.2	12.1 ± 2.1	6.9 ± 1.4[a,b]	9.8 ± 2.2[a,b,c]
18:2-20:4 and 16:1-18:2	.4 ± .2	.5 ± .3	.1 ± .1[a,b]	.4 ± .2[c]
Omega-3 species				
16:0-20:5 and 14:0-22:5	.2 ± .2	.4 ± .3	5.8 ± 2.4[a,b]	4.0 ± 2.0[a,b,c]
16:0-22:5	1.0 ± .5	.7 ± .5	10.9 ± 4.0[a,b]	8.3 ± 3.6[a,b]
16:0-22:6 and 14:0-22:4	1.6 ± .42	1.8 ± .5	24.6 ± 3.6[a,b]	14.8 ± 4.0[a,b,c]

[a]Differs from control ($p < .05$). [b]Differs from cholesterol ($p < .05$). [c]$p < .05$).

TABLE 4

Effect of Dietary Cholesterol and Menhaden Oil on Molecular Species of Phosphatidylethanolamines in Gallbladder Bile (% ± SD)

Species	Control (N = 6)	Cholesterol (N = 6)	Fish oil (N = 8)	Cholesterol + fish oil (N = 8)
Linoleoyl species				
16:0-18:2	20.6 ± 3.4	18.7 ± 3.7	10.3 ± 1.1[a,b]	12.8 ± 1.5[a,b]
18:0-18:2	27.7 ± 1.8	24.7 ± 3.6[a]	10.2 ± 2.1[a,b]	11.2 ± 2.4[a,b]
18:1-18:2	14.8 ± 2.4	15.6 ± 4.1	1.8 ± .5[a,b]	4.3 ± 1.6[a,b]
Arachidonyl species				
16:0-20:4	5.1 ± .9	5.7 ± 1.2	3.2 ± .4[a,b]	4.6 ± .5[b,c]
18:0-20:4	11.3 ± .7	11.3 ± .6	5.5 ± 1.4[a,b]	7.6 ± 2.0[a,b,c]
18:2-20:4 and 16:1-18:2	.5 ± .1	.8 ± .3[a]	.1 ± .1[a,b]	.5 ± .1[b,c]
Omega-3 species				
16:0-20:5 and 14:0-22:5	.3 ± .1	.5 ± .1	6.9 ± 2.3[a,b]	5.6 ± 2.3[a,b]
16:0-22:5	1.2 ± .2	1.1 ± .2	12.8 ± 3.6[a,b]	9.7 ± 2.9[a,b,c]
16:0-22:6 and 14:0-22:4	1.7 ± .5	1.7 ± .6	22.0 ± 2.5[a,b]	16.2 ± 3.6[a,b,c]

[a]Differs from control ($p < .05$). [b]Differs from cholesterol ($p < .05$). [c]Differs from fish oil alone ($p < .05$).

TABLE 5

Effect of Dietary Cholesterol and Menhaden Oil on Molecular Species of Phosphatidylcholines in Gallbladder Mucosa (% ± SD)

Species	Control (N = 6)	Cholesterol (N = 6)	Fish oil (N = 8)	Cholesterol + fish oil (N = 8)
Linoleoyl species				
16:0-18:2	23.9 ± 3.3	24.1 ± 3.7	26.6 ± 2.9	26.2 ± 5.7
18:0-18:2	7.7 ± 2.9	9.3 ± 2.5	7.7 ± 1.4	9.1 ± 1.7
18:1-18:2	12.1 ± 1.2	11.2 ± 2.2	6.3 ± .7[a,b]	7.6 ± 1.8[a,b]
Arachidonyl species				
16:0-20:4	6.5 ± 1.4	8.3 ± 2.1[a]	6.8 ± .8[b]	7.3 ± 1.6
18:0-20:4	8.5 ± 1.4	8.3 ± 2.1	5.0 ± .8[a,b]	6.2 ± 1.1[a,b]
18:2-20:4 and 16:1-18:2	.8 ± .2	1.3 ± .4[a]	.7 ± .1[b]	.9 ± .2[b]
Omega-3 species				
16:0-20:5 and 14:0-22:5	.7 ± .2	.6 ± .3	7.0 ± 2.5[a,b]	4.5 ± 1.8[a,b,c]
16:0-22:5	.8 ± .3	.7 ± .2	2.8 ± .7[a,b]	2.6 ± 1.1[a,b]
16:0-22:6 and 14:0-22:4	.4 ± .1	.5 ± .3	1.8 ± .9[a,b]	1.9 ± 1.3[a,b]

[a]Differs from control ($p < .05$). [b]Differs from cholesterol ($p < .05$). [c]Differs from fish oil alone ($p < .05$).

TABLE 6

Effect of Dietary Cholesterol and Menhaden Oil on Molecular Species of Phosphatidylethanolamines in Gallbladder Mucosa (% ± SD)

Species	Control (N = 6)	Cholesterol (N = 6)	Fish oil (N = 8)	Cholesterol + fish oil (N = 8)
Linoleoyl species				
16:0-18:2	6.1 ± 1.1	5.6 ± 1.3	7.4 ± 1.3[a,b]	6.1 ± 1.3[c]
18:0-18:2	10.0 ± 1.7	10.0 ± 2.6	10.0 ± 2.27	9.25 ± 2.5
18:1-18:2	12.3 ± 1.9	10.0 ± 2.2[a]	6.8 ± .8[a,b]	6.6 ± 3.2[a,b]
Arachidonyl species				
16:0-20:4	8.5 ± 1.0	10.5 ± 2.1[a]	7.6 ± 1.2[b]	9.8 ± 3.0[c]
18:0-20:4	31.4 ± 2.5	31.9 ± 6.2	21.4 ± 3.2[a,b]	26.6 ± 4.3[a,b,c]
18:2-20:4 and 16:1-18:2	.1 ± .2	.5 ± .3[a]	.1 ± .1[b]	.1 ± .1[b]
Omega-3 species				
16:0-20:5 and 14:0-22:5	.4 ± .2	.3 ± .4	4.8 ± 2.3[a,b]	3.7 ± 1.5[a,b]
16:0-22:5	.5 ± .4	.6 ± .4	10.3 ± 3.4[a,b]	7.6 ± 2.7[a,b,c]
16:0-22:6 and 14:0-22:4	.2 ± .2	.8 ± .7	2.0 ± 1.7	4.3 ± 3.7[a,b,c]

[a]Differs from control ($p < .05$). [b]Differs from cholesterol ($p < .05$). [c]Differs from fish oil alone ($p < .05$).

TABLE 7

Effects of Dietary Cholesterol and Menhaden Oil on Gallbladder Prostaglandin Synthesis (ng/mg tissue protein/30 min ± SD)

	Control	Cholesterol	Fish oil	Fish oil + cholesterol
PGE	2.81 ± 1.51	7.42 ± 2.53[a]	3.92 ± 1.98	4.46 ± 1.97[b]
PGF_{2a}	3.71 ± 2.28	7.21 ± 3.43[a]	2.47 ± 1.25	3.23 ± 1.74
6-Keto-PGF_{1a}	1.98 ± .84	3.53 ± 2.48[a]	1.94 ± .58	2.04 ± .66
Thromboxane B_2	2.24 ± 1.38	3.51 ± 2.08[a]	1.07 ± .85	1.34 ± .92

[a]Differs from all other dietary groups ($p < .05$).

[b]Differs from control ($p < .05$).

TABLE 8

Effects of Dietary Cholesterol and Menhaden Oil on Biliary Lipid Concentrations, Cholesterol Saturation, and Cholesterol Monohydrate Crystal Formation in Gallbladder Bile

	Control	Cholesterol	Fish oil	Fish oil + cholesterol
Bile salts (mmol/l)	143.0 ± 38.9	146.9 ± 48.4	132.8 ± 20.2	154.2 ± 45.4
Phospholipids (mmol/l)	15.7 ± 6.8	21.6 ± 11.0[a]	18.7 ± 4.4	31.9 ± 12.5[a,b,c]
Cholesterol (mmol/l)	4.2 ± 2.6	13.6 ± 10.4[a]	4.1 ± .8[b]	11.2 ± 6.4[a,c]
Chol. saturation index	.7 ± .3	1.6 ± .9[a]	.6 ± .2[b]	1.1 ± .7[a,b]
Cholesterol crystals	0/23	21/22	0/8	9/20

[a]Differs from control ($p < .05$).

[b]Differs from cholesterol-fed ($p < 0.05$).

[c]Differs from fish oil alone ($p < .05$).

in arachidonyl species as well. These decreases in omega-6 fatty acids were compensated by increases in species containing omega-3 fatty acids.

The effect of the diets on PE in gallbladder bile is shown in Table 4. In the group supplemented with cholesterol alone, there were relatively minor changes with an isolated decrease in the percentage of 18:0-18:2 and a small increase in the 18:2-20:4 species. Addition of fish oil to either the control or cholesterol-supplemented diet resulted in marked decreases in the concentrations of species containing linoleate and arachidonate, with corresponding increases in those with omega-3 fatty acids.

Effects of diet on phospholipids in gallbladder mucosa. Table 5 shows the effect of the various diets on the molecular species of PC in gallbladder mucosa. Cholesterol-fed animals showed small, but significant, increases in two of the arachidonate-containing species. Fish oil-supplementation of both the control and cholesterol diets resulted in significant decreases in linoleoyl and arachidonyl species, while species with the omega-3 fatty acids (20:5, 22:5, and 22:6) were increased.

The dietary manipulations produced a similar pattern of change in the epithelial PE species (Table 6). Cholesterol supplementation increased the relative concentration of 16:0-20:4, while the concentration of 18:1-18:2 was decreased. In the two groups supplemented with fish oil, there were even greater decreases in 18:1-18:2, as well as small, but significant, decreases in species containing 20:4. Conversely, the percentage of species with omega-3 fatty acids was increased.

Effects of diet on gallbladder prostaglandin synthesis. Table 7 shows the effects of dietary cholesterol and menhaden oil on PG synthesis by the gallbladder. The group supplemented with cholesterol alone showed significant increases in all four prostanoids measured. These increases were largely eliminated when fish oil was fed concurrently with cholesterol, with an exception—PGE synthesis was still significantly increased above control levels.

Effects of diet on cholesterol saturation and cholesterol monohydrate crystal formation. Effects of dietary cholesterol and menhaden oil on the lipid concentrations and cholesterol saturation index of gallbladder bile are shown in Table 8. The gallbladder bile of both cholesterol-fed groups was supersaturated with cholesterol. Total phospholipid concentrations increased in the cholesterol-fed groups as well, but, to a greater extent, in the cholesterol/menhaden oil group. As a result, the increase in cholesterol saturation index in this group was not as great as in the group receiving cholesterol alone. The incidence of cholesterol monohydrate crystals in the cholesterol/fish oil group (9/20) was significantly less than that in the group receiving cholesterol alone (21/22, $p < .05$), but the difference was consistent with the incidence of cholesterol supersaturation in each group.

DISCUSSION

As in our earlier studies (3), cholesterol feeding produced small, but significant, increases in the percentage of phosphatidylcholines and phosphatidylethanolamines containing arachidonic acid, both in bile and in gallbladder mucosa. The availability of arachidonic acid in tissues is an important factor in the regulation of PG synthesis,

and it is possible that these small changes were responsible for producing the associated increases in gallbladder prostaglandin synthesis. Dietary supplementation with fish oil produced a consistent replacement of arachidonic acid and its precursor, linoleic acid, with the omega-3 fatty acids found in fish oil (i.e., 20:5, 22:5, and 22:6). The increases in gallbladder PG synthesis resulting from cholesterol-supplementation were also prevented when menhaden oil was added to the diet, with an exception—PGE remained somewhat elevated above control levels.

A potential role for prostaglandins in cholesterol gallstone formation was initially suggested by the studies of Lee *et al.* who showed that high doses of aspirin, an inhibitor of PG synthesis, prevented gallbladder mucin hypersecretion and gallstone formation in the cholesterol-fed prairie dog despite the continued presence of cholesterol-supersaturated bile (29). LaMont *et al.* subsequently reported that exogenous arachidonic acid stimulated gallbladder mucin secretion in organ culture, while indomethacin caused a dose-dependent inhibition (30). Our earlier studies demonstrated that gallstone formation in the cholesterol-fed prairie dog is accompanied by increases in the arachidonic acid content of phosphatidylcholines and phosphatidylethanolamines in bile and gallbladder mucosa (3), and that these changes are associated with increased gallbladder PG synthesis (4).

Previous studies have shown that omega-3 fatty acids such as EPA and DCHA compete with arachidonate for esterification into tissue phospholipids (31-33). In addition to replacing arachidonate, EPA and DCHA can decrease PG synthesis by competitively inhibiting cyclooxygenase (16,17). In the present study, dietary fish oil consistently increased species of PC and PE containing the omega-3 fatty acids, EPA and DCHA, at the expense of arachidonic acid and its precursor, linoleic acid. Furthermore, these changes appeared to prevent most of the increase in gallbladder PG synthesis usually seen with cholesterol feeding.

Supplementation with menhaden oil also reduced the incidence of cholesterol monohydrate crystals, but it is not clear that this was due to the associated reduction in gallbladder PG synthesis. Besides increasing the percentage of phospholipids containing omega-3 fatty acids, the addition of menhaden oil to the diet of cholesterol-fed animals also resulted in a substantial increase in the total phospholipid concentration in gallbladder bile with a corresponding decline in cholesterol saturation which, by itself, could have been responsible for the lower incidence of crystallization. Furthermore, the mechanism by which gallbladder PG synthesis is stimulated during cholelithiasis is unclear, but it is likely to be related in some way to the presence of cholesterol-supersaturated bile. If so, it is possible that menhaden oil prevented increased PG synthesis by decreasing cholesterol saturation, rather than by replacing arachidonic acid from tissue phospholipids. Finally, neither mucin secretion rates nor biliary motility was studied in these animals, and the effect of menhaden oil on these parameters still needs to be established. Nevertheless, regardless of the mechanism involved, a 50% reduction in the incidence of crystallization is substantial and warrants further investigation.

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Autoxidation of Polyunsaturated Triacylglycerols. I. Trilinoleoylglycerol[1]

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The hydroperoxides and secondary products formed from trilinoleoylglycerol autoxidized at 40°C were isolated and characterized to clarify their contribution to oxidative deterioration of vegetable oils. The products were purified by high performance liquid chromatography (HPLC) and identified, as intact triacylglycerols, by ultraviolet, infrared, ^{1}H NMR and ^{13}C NMR analyses, and after derivatization by lipolysis, gas chromatography, and gas chromatography-mass spectrometry. The main, primary products included mono-, *bis*- and tris-9-hydroperoxy-*trans*-10,*cis*-12-; 9-hydroperoxy-*trans*-10,*trans*-12; 13-hydroperoxy-*cis*-9,*trans*-11; and 13-hydroperoxy-*trans*-9,*trans*-11-linolenoyl glycerols. The structures of the minor secondary products analyzed after derivatization were consistent with known oxidative degradation products of linoleate hydroperoxides. HPLC analyses showed that the *bis*- and tris-hydroperoxides were formed from the mono-hydroperoxides during autoxidation at peroxide values above 18 and 28 meq/kg. Studies on the further oxidation of the mono-hydroperoxides support a mechanism for the consecutive formation of *bis*- and tris-hydroperoxides from the monohydroperoxides. HPLC analyses showed that no preferential oxidation occurred between the 1(3)- and 2-triglyceride positions. Hydroperoxides of linoleate triacylglycerols may be important precursors of volatile compounds contributing to off-flavors of vegetable oils.

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Although considerable research has been published on the oxidation of pure fatty acids and esters (1-6), there is relatively little information on the oxidation of mixed fatty acids, their interactions and how their relative positions in the triacylglycerol molecule influence flavor and oxidative stability. Some structural studies have been published on the oxidation products of simple unsaturated triacylglycerols and vegetable oils. Triacylglycerol mono-hydroperoxides were prepared by column chromatography and thin-layer chromatography (TLC) from oxidized safflower oil (7). Mono-, *bis*- and tris-hydroperoxides were isolated by gel permeation chromatography from trilinoleoylglycerol subjected to oxidation photosensitized with methylene blue (8). These hydroperoxides were characterized by peroxide value (PV), ultraviolet (UV) and infrared (IR) analyses. The isomeric composition of mono-hydroperoxides from photooxidized and autoxidized trioleylglycerol, trilinoleoylglycerol and trilinolenoylglycerol were later shown to be the same as those of the corresponding methyl esters by gas chromatography-mass spectrometry (GC-MS) after hydrogenation and methanolysis (9,10). In vegetable oils, the proportion of linoleic and linolenic hydroperoxides changed with the level of oxidation. Linolenic hydroperoxides in soybean oil and linoleic hydroperoxides in olive oil decreased significantly at high levels of oxidation (10). The respective 1(3)- and 2-positional isomers, 1-(hydroperoxy)-linoleoyl-2,3-dilinoleoylglycerol and 2-(hydroperoxy)-linoleoyl-1,3-dilinoleoyl glycerol, from trilinoleoylglycerol autoxidized in the dark, were partially separated by normal phase high performance liquid chromatography (HPLC) and characterized by partial lipase hydrolysis (11). The 2-positional isomers of 9- and 13-*cis,trans*-monohydroperoxides were isolated as the only products formed from 2-linoleoyl-1,3-dipalmitoylglycerol in the presence of 5% tocopherol (12). In contrast to linoleic acid and simple esters, trilinoleoylglycerol did not follow classical autoxidation kinetics (13). The rate was not a simple, direct function of substrate concentration, but was also affected by lipid aggregation at low concentration of trilinoleoylglycerol (13).

To elucidate the effect of triacylglycerol structures on the susceptibility of vegetable oils to autoxidation, a basic study was undertaken with both simple and mixed polyunsaturated triacylglycerols. This paper presents a structural characterization of the autoxidation products of trilinoleoylglycerol as a model for further studies of synthetic polyunsaturated triacylglycerols. Structural analysis techniques used included ^{1}H and ^{13}C NMR, capillary gas chromatography (GC) of derivatives after hydrolysis by pancreatic lipase, and reversed and normal phase HPLC, to determine the relative 1(3)- and 2-positions of hydroperoxide isomers on the triacylglycerol molecules. In subsequent papers of this series we characterize the autoxidation products of trilinolenoylglycerol and of synthetic triacylglycerols containing linoleic and linolenic acids in different known positions, and the volatile decomposition products formed from these autoxidized triacylglycerols.

MATERIALS AND METHODS

All solvents were HPLC grade or analyzed reagent grade. Trilinoleoyl glycerol (99+% purity) was purchased from NuCheck Prep, Inc. (Elysian, MN). Sodium cholate and pancreatic lipase were purchased from Sigma Chemical Co. (St. Louis, MO). Before autoxidation, trilinoleoylglycerol was purified by passing 200 mg through a short commercial silica column ("Bond-Elut," Analytichem International, Harbor City, CA), by eluting with a mixture of diethyl ether/hexane (5:95, v/v), and verifying a PV of 0 by the ferric thiocyanate method (14). Trilinoleoylglycerol (1.0 g) was autoxidized at 40°C to predetermined levels with pure oxygen in a 1 × 15 cm test tube. Oxidation was monitored by analyzing 10 mg samples for PV and by reversed phase TLC and HPLC.

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Abbreviations: *bis*-OOH, *bis*-hydroperoxides; GC, gas chromatography; GC-MS, gas chromatography-mass spectrometry; HPLC, high performance liquid chromatography; IR, infrared; mono-OOH, monohydroperoxides; NMR, nuclear magnetic resonance; OTMS, trimethylsilyl; PV, peroxide value; RI, refractice index; TLC, thin-layer chromatography; tris-OOH, tris-hydroperoxides; UV, ultraviolet.

HPLC separations and analyses. The mono-hydroperoxides from autoxidized trilinoleoylglycerol were separated from unoxidized triglyceride and other oxidation products by preparative reversed phase HPLC, using a mixture of methylene chloride/acetonitrile (30:70, v/v), a flow rate of 5.0 ml/min, a column of 5 micron C-18 (25.0 × 2.14 cm, Dynamax, Rainin Instrument Co., Inc., Woburn, MA), sample loads of 30–50 mg, and a UV detector set at 235 nm in series with a refractive index (RI) detector. The mono-hydroperoxides of trilinoleoylglycerol were further resolved into isomeric components by preparative normal phase HPLC, using a mixture of 2-propanol/hexane (1:99, v/v), a column of 5 micron silica (25.0 × 2.14 cm, Dynamax), and sample loads of 10–50 mg. The relative concentrations of mono-, *bis*-, and tris-hydroperoxides in autoxidized trilinoleoylglycerol were determined by analytical reversed phase HPLC, with the same methylene chloride/acetonitrile mixture (30:70, v/v) as the preparative system, a flow rate of 1.0 ml/min, using either a 5 micron C-18 column (25 × 0.49 cm, Zorbax, Du Pont Instrument Division, Wilmington, DE), or a radial compression 5 micron C-18 column (1 × 10 cm, Waters Associates, Milford, MA), and UV and RI detectors. The triacyl glycerol positions of various mono-hydroperoxide isomers were determined by analytical normal phase HPLC, with a 2-propanal/hexane (0.5:99.5, v/v) mixture, a flow rate of 0.3 ml/min, using two 5 micron silica Zorbax columns (25 × 0.49 cm) in series.

Characterization. Oxidation products isolated by HPLC were analyzed quantitatively by UV (Carey 219 spectrophotometer, Varian Associates, Walnut Creek, CA), and qualitatively by TLC with either normal phase silica plates (Merck, Darmstadt, Germany), developed with a diethyl ether/hexane mixture (40:60, v/v), or reversed phase C-18 plates (Whatman, Maid Store, England), developed with a methylene chloride/acetonitrile mixture (30:70, v/v). Separated products were examined with a UV light to detect conjugated dienes and with a KI spray to detect hydroperoxide components (15), followed by charring with chromic-sulfuric acid. Oxidized products were further characterized by Fourier transform IR spectrometry (Model 1750, Perkin Elmer, Norwalk, CN) in CS_2, and by ^1H and ^{13}C NMR in $CDCl_3$ (16–18).

The oxidation products of trilinoleoylglycerol were further characterized by GC and GC-MS analyses of the trimethylsilyl (OTMS) ethers and compared with reference compounds (19). These derivatives were prepared by either reducing with sodium borohydride or hydrogenating with PtO_2 catalyst in methanol (20), transmethylating in 0.5 N KOH methanol at room temperature, and then silylating with *bis*(trimethylsilyl) trifluoroacetamide (Supelco, Inc., Bellefonte, PA). The same GC procedure was used as described previously (20), except that a 30-min hold at 250°C was used to allow the elution of di- and tri-OTMS stearates. The triacylglycerol positions of mono-hydroperoxides were determined by lipolysis by the method of Yoshida (21), modified by using 20 mg samples, 20–25 mg pancreatic lipase incubated for 8–16 min at 37°C in aqueous "Tris" buffer (pH 8.1) containing sodium cholate and calcium chloride. The lipolysis products extracted with ether were hydrogenated with PtO_2 in methanol, methylated with diazomethane and silylated before capillary GC analyses.

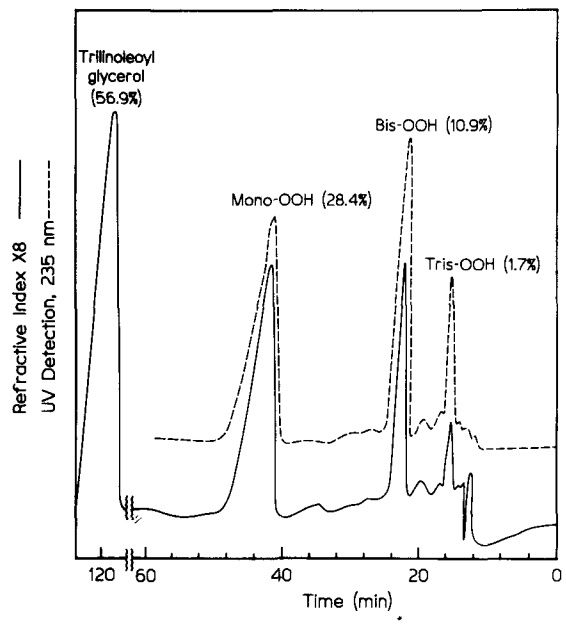

FIG. 1. Preparative reversed phase HPLC of trilinoleoylglycerol autoxidized at 40°C to a peroxide value of 2950 me/kg. Solid line, refractive index detection; broken line, ultraviolet detection. Mono-OOH, mono-hydroperoxides; *Bis*-OOH, *bis*-hydroperoxides; Tris-OOH, tris-hydroperoxides.

RESULTS AND DISCUSSION

Trilinoleoylglycerol was autoxidized at 40°C to a PV of 125 for kinetic studies and to a PV of 2950 for structural studies. Autoxidized trilinoleoylglycerol was separated by reversed-phase HPLC into mono-, *bis*-, and tris-hydroperoxides containing one, two and three hydroperoxy functions per triacylglycerol molecule, respectively (Fig. 1). These primary products decreased in concentrations from the mono-hydroperoxides (28.4%), to the *bis*-hydroperoxides (10.9%) and tris-hydroperoxides (1.7%) at a PV of 2950. The number of hydroperoxide moieties per glycerol unit in autoxidized trilinoleoylglycerol was determined quantitatively by GC analysis of the OTMS ether derivatives. The ratio of OTMS ether/unoxidized linoleic acid was 0.51 for the mono-hydroperoxide, and 2.22 for the *bis*-hydroperoxide. The theoretical value is 0.50 for mono-hydroperoxides (1 OTMS/2 unoxidized linoleic acid) and 2.0 for the *bis*-hydroperoxide (2 OTMS/1 unoxidized linoleic acid). As expected, the tris-hydroperoxide produced only OTMS linoleate after $NaBH_4$ reduction and OTMS stearate after hydrogenation.

The 9- and 13-hydroperoxide isomers of trilinoleoylglycerol were found to occur in the same 1:1 ratio as autoxidized methyl linoleate (19), as determined by GC and GC-MS analysis of the OTMS ethers prepared after transmethylation and hydrogenation. The ratios of 9- + 13-*cis,trans* to *trans,trans* isomers of the hydroperoxides were estimated by GC of the OTMS ethers prepared after transmethylation and sodium borohydride reduction (20), as follows: 59.1:40.9% for the mono-hydroperoxides, 63.8:36.2% for the *bis*-hydroperoxides, and 79.5:20.5% for the tris-hydroperoxides. Molecular absorptivities at 232–233 nm in methanol from conjugated dienes increased from mono- (E_M 24,554) to *bis*- (E_M 51,657) and

to tris-hydroperoxides (E_M 63,080). Similarly, IR analyses show absorptions corresponding to mono-, *bis*- and tris-hydroperoxides: 3446 (free C-OOH), 3550, 3200 (bonded C-OOH), 3007 (olefinic-H), 1746 (carbonyl) and 987, 950 cm^{-1} (conjugated *cis,trans* and *trans,trans* unsaturation).

Reversed phase HPLC analyses of trilinoleoylglycerol autoxidized at 40°C at different PV's show that the mono-hydroperoxides are the only products formed initially (Fig. 2). The formation of mono-hydroperoxides is followed by the formation of *bis*-hydroperoxides at PV's above 18 and tris-hydroperoxides at PV's above 28. To clarify the mechanism of *bis*- and tris-hydroperoxide formation, the mono-hydroperoxides of trilinoleoyl glycerol, isolated by reversed phase HPLC, were further oxidized at 40°C. The kinetic curves in Figure 3 show that the disappearance of mono-hydroperoxides correspond to the appearance of *bis*-hydroperoxides as major products and tris-hydroperoxides as minor products. These products were also accompanied by more polar minor products observed by HPLC and characterized below. These results support a sequential mechanism for the autoxidation of trilinoleoylglycerol (Fig. 4). Mono-hydroperoxides formed initially in either the 1(3)- or 2-triacylglycerol position undergo further oxidation to the 1,3- and 1,2-*bis*-hydroperoxides, which are, in turn, oxidized to the tris-hydroperoxides. The proportion of *cis,trans* to *trans,trans* dienes was shown above to be smaller for the mono-hydroperoxides than for the *bis*- and tris-hydroperoxides of trilinoleoyl glycerol. Isomerization of *cis,trans* to *trans,trans* dienes becomes, therefore, more important in the mono-hydroperoxides because their further oxidation favors the initial formation of the *cis,trans* diene configuration in the *bis*- and tris-hydroperoxides, which are formed consecutively from the mono-hydroperoxides (Fig. 4). These results also show that at the low PV's, which are relevant to flavor deterioration in polyunsaturated vegetable oils (1), the mono-hydroperoxides of trilinoleoylglycerol are clearly the most important precursors of oxidation volatile flavor compounds.

The secondary oxidation products of trilinoleoylglycerol were identified by GC-MS of derivatives obtained after reduction with sodium borohydride or after hydrogenation (20). After hydrogenation, silylation and transmethylation, the three secondary products formed, respectively: methyl 9,13-diOTMS stearate, 9/13-mono-OTMS stearate, and stearate in a ratio of 1:1:1; methyl 9,13-diOTMS stearate, epoxystearate, and stearate in a ratio of 1:1:1; and methyl 9,13-diOTMS stearate and stearate in a ratio of 2:1. This evidence indicates that the three secondary oxidation products were: *bis*-(dioxygenated linoleoyl)(mono-oxygenated linoleoyl) monolinoleoylglycerol; *bis*-(di-oxygenated linoleoyl)-(mono-epoxyene linoleoyl)-monolinoleoylglycerol; and *bis*-(dioxygenated linoleoyl)-monolinoleoylglycerol, respectively. The di-oxygenated product is the same as previously identified from oxidized methyl linoleate (19,20), and may be formed by selective hydrogenolysis of epoxyhydroxy (or hydroperoxy)-octadecenoate (22). The mono-oxygenated product comes from the mixture of 9- and 13-mono-hydroperoxides of linoleate (19,20). Other minor secondary products, identified by TLC and GC after sodium borohydride

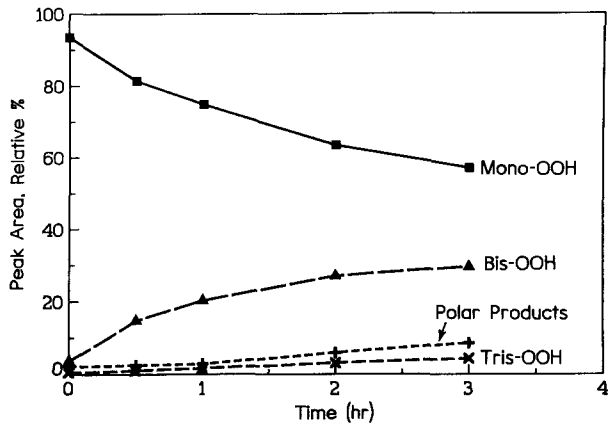

FIG. 3. Further oxidation of trilinoleoylglycerol mono-hydroperoxides at 40°C. Analysis by reversed phase HPLC. Conditions the same as indicated for Figure 2.

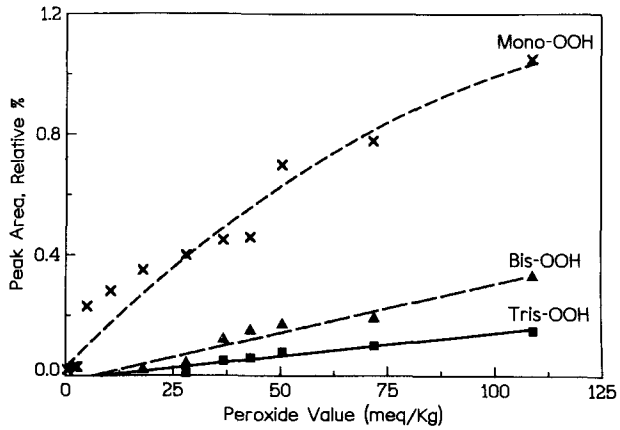

FIG. 2. Autoxidation of trilinoleoylglycerol at 40°C. Analyses by reversed phase HPLC. Conditions: RCM C-18 column; flow rate: 1.0 ml/min eluting solvent: CH_2Cl_2/CH_3CN (30:70, v/v); sample load: 15–25 μl; area % based on RI detection. Mono-OOH, mono-hydroperoxides; *Bis*-OOH, *bis*-hydroperoxides; Tris-OOH, tris-hydroperoxides.

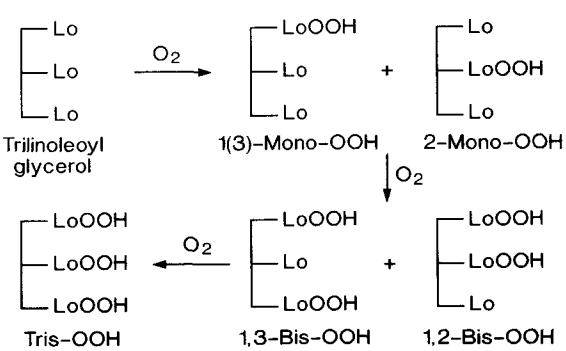

FIG. 4. Mechanism of autoxidation of trilinoleoylglycerol. 1(3)-Mono-OOH, 1(3)-mono-hydroperoxides of trilinoleoylglycerol; 2-Mono-OOH, 2-mono-hydroperoxides of trilinoleoylglycerol; 1,3-*Bis*-OOH, 1,3-*bis*-hydroperoxides of trilinoleoylglycerol; 1,2-*Bis*-OOH, 1,2-*bis*-hydroperoxides of trilinoleoylglycerol; Tris-OOH, tris-hydroperoxide of trilinoleoylglycerol.

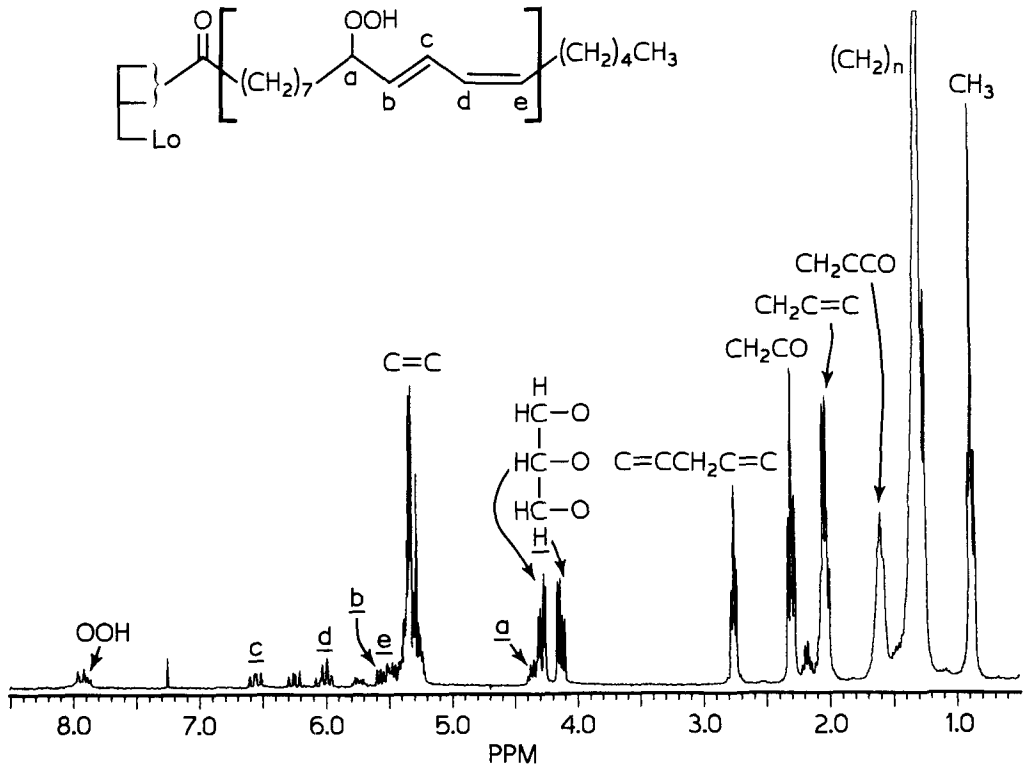

FIG. 5. ^{1}H NMR spectrum of mono-hydroperoxides of trilinoleoyl glycerol.

reduction, silylation and transmethylation, included fatty acids containing mono-keto diene, monoepoxyhydroperoxyene, and 9,13-dihydroxy-epoxyene (20). There was no evidence of dimer or polymer formation in either highly oxidized samples of trilinolein (PV 2950) or further oxidized mono-hydroperoxides of trilinoleoylglycerol.

The ^{1}H NMR spectrum of the mono-hydroperoxides of trilinoleoyl glycerol shows proton signals for the hydroperoxy and linoleoyl residues (Fig. 5). Characteristic signals for the hydroperoxides include 7.92 for OOH; 6.56, 6.0, 5.58, and 5.45 for protons c, d, b, and e of the conjugated diene system of *cis,trans*-9- and 13-hydroperoxides of linoleate (23,24); and 4.35 ppm for the methine proton a of the hydroperoxide bearing carbon. Signals at 6.25 and 5.75 ppm for protons of conjugated diene systems are apparently due to the corresponding *trans,trans*-9- and 13-hydroperoxide isomers (23). Signals characteristic of the linoleoyl groups include 5.38 for protons of carbon-carbon unsaturation, 4.30 and 4.13 for protons on 2- and 1(3)-carbons of glycerol; 2.76 for methylene protons between two double bonds, 2.26 for methylene protons alpha to ester carbonyl; 2.18, 2.06 for methylene protons adjacent to double bonds; 1.25 and 1.65 for chain methylene protons, and 0.90 ppm for terminal methylene protons.

Quantitative ^{1}H NMR analyses showed one, two, and three protons corresponding to the respective number of hydroperoxy groups (7.91–7.99 ppm); methine proton of the hydroperoxide bearing carbon (4.34 ppm); and four, eight and twelve protons for the conjugated diene systems (5.3–6.58 ppm) of the mono-, *bis*- and tris-hydroperoxides of trilinoleoylglycerol. Signals were also observed at 6.25, 6.00 and 5.75 ppm for protons of the conjugated diene systems (Fig. 5) of mixtures of mono-, *bis*-, and tris-hydroperoxides. Signals for methylenes between two double bonds (2.76 ppm) corresponded to four protons for the mono-hydroperoxide, two protons for the *bis*-hydroperoxide, and was absent in the tris-hydroperoxide. The protons of a methylene group adjacent to a diene system (2.1–2.2 ppm) decreased from 10 for the mono- to eight for the *bis*- and to six for the tris-hydroperoxides, respectively.

^{13}C NMR data showed the linoleoyl carbonyl (173.2, 172.8 ppm) in a 2:1 ratio for 1(3)- to 2-glycerol substitution in mono-, *bis*- and tris-hydroperoxides of trilinoleoylglycerol (Table 1). Signals were observed for the carbons of the conjugated diene system of linoleate hydroperoxides (127.4–136.3 ppm), for the hydroperoxide bearing carbons (86.4 ppm), for the carbons adjacent to *cis,trans* and *trans,trans* conjugated diene systems (32.4 ppm) and for the carbons of the methylene interrupted diene system of unoxidized linoleate in the mono- and *bis*-hydroperoxides, but not in the tris-hydroperoxide. Assignments were based on published data for carbons in unsaturated and oxygenated long chain fatty acids (25–29). The 1(3)- and 2-carbons of the substituted glycerol moiety were also observed in relative intensity of 2:1.

The different positional isomers of the mono-hydroperoxides on trilinoleoylglycerol were separated by analytical normal phase HPLC. Eight peaks were resolved (Fig. 6) and identified by capillary GC of derivatives prepared after pancreatic lipase hydrolysis, followed by hydrogenation and methylation. The scheme in Figure 7

TABLE 1

^{13}C NMR of Trilinoleoylglycerol Hydroperoxides

HPLC fractions[a]	Shifts, ppm (assignments)[b]
Mono-OOH	173.2, 172.8 (C=O), 136.3, 136.0, 134.7, 133.7, 133.4, 131.4, 129.5, 129.2, 129.1, 129.0, 127.6, 127.4 (C=C-C=C, *cis,trans/trans,trans*), 86.4 (C-C-OOH), 34.0 (C-OOH), 32.4 (*trans* C-C=C), 27.1 (*cis* C-C=C), 25.0 (C-C-OOH)
Bis-OOH	173.2, 172.8 (C=O), 136.33, 136.06, 134.74, 134.68, 133.66, 133.37, 133.34, 131.46, 131.44, 131.26, 129.56, 129.49, 129.25, 129.23, 129.21, 129.18, 129.10, 129.00, 127.93, 127.41 (C=C-C=C, *cis,trans/trans,trans*), 86.4 (C-C-OOH), 34.0 (C-OOH), 33.9 (C-2, linoleate), 32.4 (*trans* C-C=C), 27.1 (*cis* C-C=C), 25.0 (C-C-OOH)
Tris-OOH	173.2, 172.8 (C=O), 136.84, 136.52, 135.19, 134.15, 133.78, 129.97, 129.29, 129.11 (C=C-C=C, *cis,trans/trans,trans*), 86.6 (C-C-OOH), 34.1 (C-OOH), 32.56 (*trans* C-C=C), 27.2 (*cis* C-C=C), 25.0 (C-C-OOH)

[a]See Figure 1. Mono-OOH, mono-hydroperoxides, *Bis*-OOH, *bis*-hydroperoxides; Tris-OOH, tris-hydroperoxides.

[b]See Figure 5 for structure of mono-hydroperoxides.

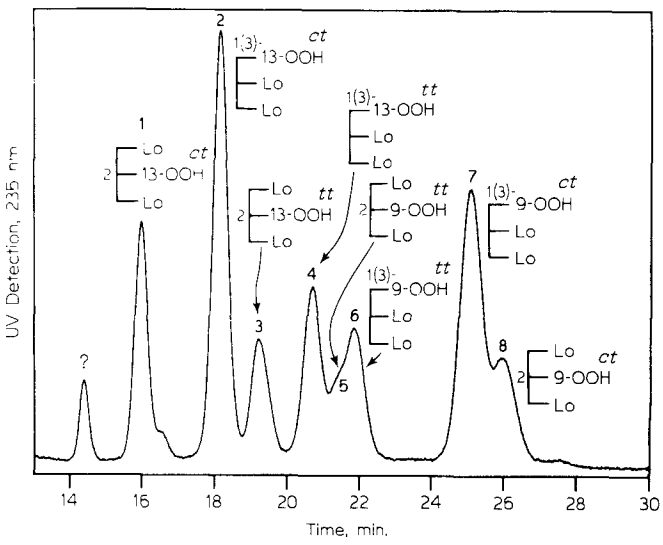

FIG. 6. Analytical normal phase HPLC of mono-hydroperoxide mixture from trilinoleoylglycerol autoxidized to PV 43 meq/kg. Lo, linoleoyl residue; *ct* 13-OOH, *cis,trans* linoleic 13-hydroperoxide; *tt* 13-OOH, *trans,trans* linoleic 13-hydroperoxide; *ct* 9-OOH, *cis,trans* linoleic 9-hydroperoxide; and *tt* 9-OOH, *trans,trans* linoleic 9-hydroperoxide.

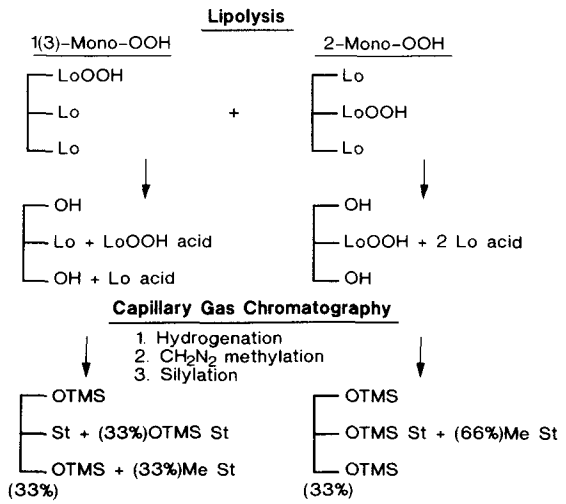

FIG. 7. Analytical scheme for pancreatic lipase products of 1(3)- and 2-monohydroperoxides of trilinoleoylglycerol. Lo, linoleoyl residue; Lo acid, linoleic acid; LoOOH, linoleic hydroperoxide residue (9- or 13-hydroperoxide isomers); LoOOH acid, linoleic acid hydroperoxides.

is based on the work of Terao and Matsushita (9) who identified the lipase hydrolysis products by GC-MS. According to this scheme, 1(3)-mono-hydroperoxides of trilinoleoylglycerol produce linoleic acid hydroperoxides, monolinoleoylglycerol, and linoleic acid. The corresponding products obtained after hydrogenation, methylation and silylation included methyl OTMS stearate, monostearoylglycerol (OTMS derivative), and methyl stearate. 2-Mono-hydroperoxides of trilinoleoylglycerol produce linoleic acid and mono-hydroperoxy linoleoylglycerol. The corresponding products obtained after hydrogenation, methylation and silylation included methyl stearate and mono-hydroxystearoylglycerol (OTMS derivative). On the basis of this scheme, capillary GC analyses indicate that peaks 1, 3, 5 and 8 correspond to 2-mono-hydroperoxides of trilinoleoylglycerol, and peaks 2, 4, 6 and 7 correspond to 1(3)-mono-hydroperoxides of trilinoleoylglycerol (Table 2). Moreover, peaks 1, 2, 3 and 4 correspond to the 13-hydroperoxide isomer, and peaks 5, 6, 7 and 8 correspond to the 9-hydroperoxide isomer.

Further analyses by IR and NMR showed that the hydroperoxides eluted in peaks 1, 2, 7 and 8 had the *cis,trans* configuration, and the hydroperoxides eluted in peaks 3, 4, 5 and 6 had the *trans,trans* configuration. ^1H

TABLE 2

Capillary GC Analysis of Lipolysis Products of Trilinoleoylglycerol Mono-Hydroperoxide Isomers Separated by HPLC[a,b]

Product[c]	HPLC fraction[a]							
	1	2	3	4	5	6	7	8
Methyl stearate	65.6	35.5	67.0	34.7	64.2	33.5	31.2	66.6
9-OTMS stearate	—	—	—	—	—	31.9	35.6	—
13-OTMS stearate	—	30.3	—	30.0	—	—	—	—
2-Monostearin	—	34.2	—	35.3	—	34.6	33.2	—
2-Mono-OH stearin-9-OTMS stearoyl	—	—	—	—	35.8	—	—	33.4
13-OTMS stearoyl	34.4	—	33.0	—	—	—	—	—

[a] See Figure 6 for HPLC separation.

[b] See Figure 7 for analytical scheme of lipolysis products of mono-hydroperoxides of trilinoleoylglycerol.

[c] Monostearin (monostearoylglycerol) and mono-OTMS stearin products are analyzed as the OTMS ethers of glycerol (see Fig. 7). Products were identified by comparing GC retention with authentic compounds. 2-Mono-9(13)-hydroxystearoylglycerol was identified as the OTMS derivative from 2-mono-9(13)-hydroxylinoleoylglycerol after hydrogenation and transmethylation producing methyl 9- and 13-hydroxystearate (OTMS derivative).

TABLE 3

HPLC Analysis of 1(3)-:2-Isomers of Mono-Hydroperoxides of Trilinoleoylglycerol[a]

Peroxide values	Hydroperoxide isomer (area %)[b]		Ratio 1(3)-:2-
	1(3)-mono	2-mono	
cis,trans-13-hydroperoxides			
5	24.1	16.6	1.5
19	22.6	9.1	2.1
72	17.0	9.0	1.9
109	13.4	8.6	2.1
1989	13.7	7.3	1.9
Average	16.7 ± 0.8	8.5 ± 4.4	1.9 ± 0.2
cis,trans-9-hydroperoxides			
5.2	18.7	10.5	1.8
15.0	18.9	9.2	2.1
Average	18.8 ± 0.1	9.9 ± 0.9	2.0 ± 0.2

[a] See Figure 6 for HPLC separation.

[b] Analyses for 1(3)-mono-hydroperoxides were based on areas of peaks 2 and 7 for 13-OOH and 9-OOH isomers respectively; analyses for 2-mono-hydroperoxides were based on areas of peaks 1 and 8 for 9-OOH isomers respectively (Fig. 6).

and ^{13}C NMR showed no distinguishing signals between 1(3)- and 2-mono-hydroperoxides of trilinoleoylglycerol. The proton of the hydroperoxy group gave a signal at 9.32 ppm. The olefinic protons of the *cis,trans* isomers gave signals at 6.56, 6.00 and 2.18 ppm (CH_2-C=C-C-C) (Fig. 4). The olefinic protons of the *trans,trans* isomer gave signals at 6.25 and 5.75 ppm, and the protons of a methylene carbon adjacent to conjugated diene gave a signal at 2.09 ppm. The rest of the ^1H NMR spectrum of each isomeric hydroperoxide was the same as that of the trilinoleoyl-glycerol mono-hydroperoxide mixture (Fig. 4). ^{13}C NMR spectra of the same mono-hydroperoxide isomers showed no difference between 1(3)- and 2-mono-hydroperoxides of trilinoleoylglycerol. The *cis,trans* isomers gave signals at 131.31 (C-b), 129.94 (C-c), 127.59 (C-d) and 133.76 ppm (C-e), and the *trans,trans* isomers at 129.23 (C-c), 129.03 (C-d), 135.18 (C-b) and 136.48 ppm (C-e) (Table 1). The rest of the ^{13}C NMR spectrum of each isomeric hydroperoxide was the same as that of the trilinoleoylglycerol mono-hydroperoxide mixture (Table 1).

The ratios of 1(3)- to 2-mono-hydroperoxide isomers of trilinoleoylglycerol were estimated by normal phase HPLC of trilinoleoylglycerol oxidized at different peroxide values. The ratios of peak areas of *cis,trans* triacylglycerol isomers, which were more completely resolved than the *trans,trans* isomers (Fig. 6) gave a 1(3):2-ratio averaging 2.0 for the 9-hydroperoxide isomers between PV's of 5 and 1989, and averaging 1.9 for the 13-hydroperoxide isomers between PV's of 19 and 1989 (Table 3). While the concentration of 1(3)-glycerol isomers

of mono-hydroperoxides decreased linearly between PV's 5 and 109, the concentration of the 2-glycerol isomers of mono-hydroperoxides decreased at a greater rate between PV's of 5 and 19 and remained fairly constant at higher PV's. At low PV's some selectivity appears to exist for oxidation of the 2-triacylglycerol position. However, considering probabilities for oxidation, the average 1(3)-glycerol isomers of mono-hydroperoxides of 8.35% (16.7%/2) is slightly less than the average 2-glycerol isomers of mono-hydroperoxides of 8.5%, but this difference is within experimental error. Oxidation of trilinoleoylglycerol is, therefore, not selective toward either the 1(3)- or 2-triacylglycerol position. These results agree with those of Park *et al.* (11), who found equal amounts of triacylglycerols with the same isomeric mono-hydroperoxide (9- or 13-*cis,trans* isomer) in either the 1(3)- or 2-position. By separating positional isomers of mono-hydroperoxides of trilinoleoylglycerol, this HPLC method provides a more reliable quantitative technique than lipase hydrolysis, which may be affected by unusual fatty acid structures.

The *bis*-hydroperoxides of trilinoleoylglycerol produced the same lipolysis derivatives as the corresponding 1(3)- and 2-mono-hydroperoxides of trilinoleoylglycerol analyzed by capillary GC (Table 2). These results indicate that the *bis*-hydroperoxides have two linoleoyl mono-hydroperoxides in both the 1,3- and 1,2-triacylglycerol positions. The tris-hydroperoxide of trilinoleoylglycerol produced by lipolysis only have linoleic acid mono-hydroperoxides and 2-monolinoleoyl-hydroperoxide.

The HPLC procedures developed in this study permit, for the first time, the complete identification of all eight positional and geometric monohydroperoxides isomers expected from trilinoleoylglycerol, and the kinetic investigations supporting a consecutive mechanism for the formation of mono-, *bis*-, and tris-hydroperoxides. Moreover, ^{1}H and ^{13}C NMR, and capillary GC of lipolysis product derivatives permitted a more complete characterization of the mono-, *bis*- and tris-hydroperoxides of trilinoleoylglycerol than previously reported (8). These analytical tools proved valuable in the structural studies of oxidation products of trilinolenoylglycerol and synthetic polyunsaturated triacylglycerols presented in the accompanying papers of this series. On further oxidation, methyl linoleate and its hydroperoxides form significant amounts of dimers with relative ease by intermolecular condensation of peroxyl radicals (30–32). In contrast, in the present study we found no evidence for dimer formation in either highly oxidized trilinoleoylglycerol or further oxidized trilinoleoylglycerol mono-hydroperoxides. Dimerization is evidently only significant in the methyl esters of unsaturated fatty acids, because intermolecular condensations of peroxyl radicals are favored. In contrast, during autoxidation of trilinoleoylglycerol, further oxidation of the mono-hydroperoxides to *bis*- and tris-hydroperoxides is apparently favored because hydrogen abstraction from the linoleoyl residues can occur intramolecularly more favorably than intermolecular condensation of the peroxyl radicals to form dimers. At the levels of oxidation studied in this work we found no evidence for dimerization of tris-hydroperoxides. Therefore, this work demonstrates that simple esters of unsaturated fatty acids do not necessarily provide valid models for the autoxidation of unsaturated triacylglycerols.

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Autoxidation of Polyunsaturated Triacylglycerols. II. Trilinolenoylglycerol[1]

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The hydroperoxides and secondary products formed from trilinolenoylglycerol autoxidized at 40°C were isolated and characterized to clarify the mechanism of oxidative deterioration of polyunsaturated vegetable oils. The products were purified by high performance liquid chromatography (HPLC) and identified either as intact triacylglycerols spectrophotometrically, or after lipolysis (pancreatic lipase) and capillary gas chromatography and gas chromatography-mass spectrometry. The products included 9-, 12-, 13-, and 16-mono-, *bis*-, tris-hydroperoxy, 9- and 16-hydroperoxy epidioxy, 9- and 16-hydroperoxy bicycloendoperoxy and 9,12-, 13,16-, and 9,16-dihydroperoxy linolenoylglycerols. The mono-hydroperoxides and hydroperoxy epidioxides were the only main products initially formed at peroxide values (PV) below 30. *Bis*- and tris-hydroperoxides were formed consecutively as minor products from the mono-hydroperoxides at PV's between 31 and 47. Hydroperoxy bicycloendoperoxides and monodihydroperoxides were also formed as minor secondary products at PV's above 75. HPLC analyses show a small preference for the formation of 16-hydroperoxides on the 1(3)-position over the 2-position of trilinolenoylglycerol. However, there was no selectivity for the formation of the 9-, 12- and 13-hydroperoxides and for the hydroperoxy epidioxides between the 1(3)- and the 2-positions in trilinolenoylglycerol. Mono-hydroperoxides and hydroperoxy epidioxides of linolenate triacylglycerols may be important precursors of volatile compounds contributing to oxidative deterioration of vegetable oils.
***Lipids* 25, 40-47 (1990).**

In the preceding paper (1) autoxidation products of trilinoleoylglycerol separated by reversed phase and normal phase high performance liquid chromatography (HPLC) were characterized both spectrally and by pancreatic lipase hydrolysis. In addition to mono-, *bis*- and tris-hydroperoxides, all eight positional and geometric monohydroperoxide isomers expected from trilinoleoylglycerol were identified. ^{1}H and ^{13}C NMR, and capillary GC of lipolysis product derivatives proved to be valuable analytical tools for the structural studies of oxidation products of trilinoleoylglycerol.

Few structural studies have been reported on the oxidation products of trilinolenoylglycerol. The mono-hydroperoxide mixture produced by photosensitized oxidation of trilinolenoylglycerol was partially characterized, while *bis*- and tris-hydroperoxides were suggested to be analogous to those produced during photosensitized oxidation of trilinoleoylglycerol (2). An attempt to separate the individual 1(3)- and 2-mono-hydroperoxide isomers of trilinolenoylglycerol was unsuccessful (3). The isomeric composition of mono-hydroperoxides of trilinolenoyl glycerol was reported to be constant with oxidation level (4), and the same as reported for methyl linolenate (5).

This paper presents a structural characterization of the autoxidation products of trilinolenoylglycerol, which is used as a model for further studies of synthetic polyunsaturated triacylglycerols. The same structural techniques applied to trilinoleoylglycerol (1) were used to determine the relative 1(3)- and 2-positions of hydroperoxide isomers of the trilinolenoylglycerol molecules. Novel triacylglycerol products identified include mono-hydroperoxy epidioxides, mono-dihydroperoxides and monohydroperoxy bicycloendoperoxides.

MATERIALS AND METHODS

Trilinolenoylglycerol (99+% purity) was purchased from NuChek Prep, Inc. (Elysian, MN). Procedures for autoxidation and analytical methods were as described in the preceding paper (1). Mono-hydroperoxides and monohydroperoxy epidioxides were first separated by reversed phase HPLC, and then were further resolved into isomeric components by normal phase HPLC by the same procedures as the mono-hydroperoxides of trilinoleoylglycerol (1).

RESULTS AND DISCUSSION

Trilinolenoylglycerol was autoxidized at 40°C to a PV of 2157 for structural studies, and to PV's below 125 for analytical and rate studies. Autoxidized trilinolenoylglycerol was separated by preparative reversed phase HPLC into mono-hydroperoxides (22.1%) and monohydroperoxy epidioxides (8.8%), as major products (Fig. 1). Minor oxidation products include tris-hydroperoxides (0.9%), *bis*-hydroperoxides (1.7%), mono-9,12-, 13,16- and 9,16-dihydroperoxides (1.2%) and mono-9- and 16-hydroperoxy endoperoxides (1.6%) and unidentified compounds (0.7%). These oxidation products were identified by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) of suitable derivatives by comparison with reference compounds that were previously identified in autoxidized methyl linolenate (5-8). The number of hydroperoxide moieties per glycerol unit in autoxidized trilinolenoylglycerol was determined quantitatively by GC analysis of the trimethylsilyl (OTMS) ether derivatives. The ratio of OTMS ethers/unoxidized linolenic acid was 0.52 for the mono-hydroperoxide and 2.12 for the *bis*-hydroperoxide. The theoretical value is 0.50 for mono-hydroperoxides (1 OTMS/2 unoxidized linoleic acid) and 2.0 for the *bis*-hydroperoxides (2 OTMS/1 unoxidized linoleic acid). As expected, the tris-

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Abbreviations: *Bis*-OOH, *bis*-hydroperoxides; GC, gas chromatography; GC-MS, gas chromatography-mass spectrometry; HPLC, high performance liquid chromatography; IR, infrared; mono-OOH, monohydroperoxides; NMR, nuclear magnetic resonance; OTMS, trimethylsilyl; PV, peroxide values,; TLC, thin-layer chromatography; tris-OOH, tris-hydroperoxides; UV, ultraviolet.

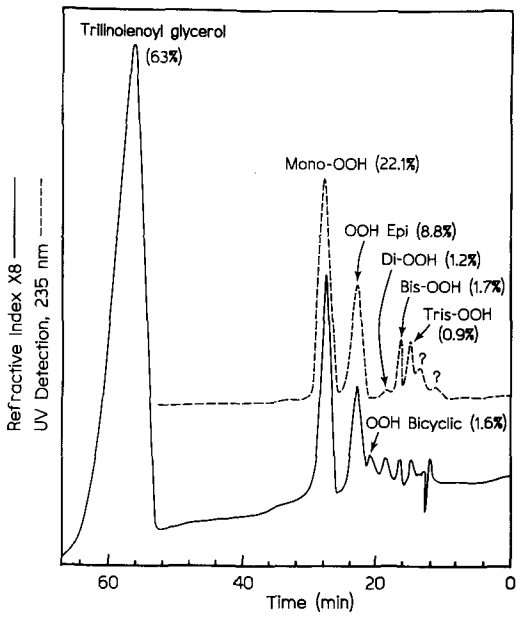

FIG. 1. Preparative reversed phase HPLC of trilinolenoylglycerol autoxidized at 40°C to a peroxide value of 2157 me/kg. Solid line, refractive index detection; broken line, ultraviolet detection. Mono-OOH, *mono-hydroperoxides*; OOH Epi, *hydroperoxy epidioxides*; Di-OOH, dihydroperoxides; *Bis*-OOH, *bis*-hydroperoxides; Tris-OOH, tris-hydroperoxides; OOH Bicyclic, hydroperoxy bicycloendoperoxides.

hydroperoxide produced only OTMS linolenate after $NaBH_4$ reduction and OTMS stearate after hydrogenation. The ratio of OTMS ether/unoxidized linolenic acid was 0.57 for both the mono-hydroperoxy epidioxides and mono-hydroperoxy bicyclo endoperoxides, and 0.55 for the mono-dihydroperoxides. The theoretical value is 0.50 (1 OTMS/2 unoxidized linoleic acid).

GC-MS analyses showed that the mono-, *bis*- and tris-hydroperoxides contain a mixture of one, two and three 9-, 12-, 13-, and 16-hydroperoxide isomers. The relative proportions of 9-, 12-, 13- and 16-hydroperoxide isomers were similar to the corresponding hydroperoxides of methyl linolenate (5), as follows (in respective order): 34.5, 9.3, 10.9 and 45.4% for the monohydroperoxides; 33.3, 6.8, 8.1 and 51.8% for the *bis*-hydroperoxides; 33.2, 14.6, 14.8 and 37.5% for the tris-hydroperoxides. Capillary GC analyses, after $NaBH_4$ reduction, showed that about 92% of the mono-, *bis*- and tris-hydroperoxides were in the *cis,cis,trans* configuration.

After hydrogenation, transmethylation and silylation, GC and GC-MS of the mono-hydroperoxy epidioxides showed the presence of one hydroperoxy epidioxy group per trilinolenoylglycerol molecule, identified as a mixture of methyl 9,10,12- and 13,15,16-triOTMS stearate derivatives corresponding to the 9-hydroperoxy-10,12- and 16-hydroperoxy-13,15-epidioxides in autoxidized methyl linolenate (7). GC-MS also confirmed the structures of 9- and 16-hydroperoxy bicyclo endoperoxide derivatives and those of the di-hydroperoxides, which gave the same mass fragmentation as the corresponding methyl ester derivatives in autoxidized and photooxidized methyl linolenate (8,9). Capillary GC analyses of the mono-dihydroperoxide fractions of autoxidized trilinolenoylglycerol showed 43.8% 9,16-, 28.7% 9,12- and 27.5% 13,16-isomers, respectively.

Ultraviolet (UV) analyses for conjugated diene gave a molar absorptivity of 21,400 at 232 nm for the mono-, 42,900 at 234 nm for the *bis*-, and 72,200 at 235 nm for the tris-hydroperoxides, and 22,700 at 236 nm for the hydroperoxy epidioxides of trilinolenoylglycerol. These results are in agreement with previous analyses of hydroperoxides of methyl linolenate (7). A mixture of mono 9,16-, 9,12- and 13,16-dihydroperoxides isolated by reversed phase HPLC of autoxidized trilinolenoylglycerol showed absorption maxima for conjugated diene at 234 nm and for conjugated triene at 268 nm with apparent molar absorptivities of 8700 and 8500, respectively. Infrared (IR) analyses showed typical absorptions for mono-, *bis*-, and tris-hydroperoxides: 3431 (free C-OOH), 3550-3200 (bonded C-OOH), 3007 (olefinic-H), 1746 (carbonyl) and 987, 950 cm^{-1} (conjugated *cis,trans* unsaturation). The hydroperoxy epidioxides absorbed at 3421 (free C-OOH), 3600-3200 (bonded C-OOH), 3010 (olefinic C-H), 1745 (carbonyl), and 990-950 cm^{-1} (conjugated diene unsaturation). The hydroperoxy bicyclo endoperoxide had absorption at 967 cm^{-1} for isolated *trans* double bond adjacent to the hydroperoxy-bearing carbon. The dihydroperoxide showed IR absorptions for *cis,trans* conjugated diene at 980 and 950 cm^{-1}, and *trans,trans,trans* conjugated triene at 980, and major absorption for the *trans,cis,trans* conjugated triene at 960 cm^{-1} (7).

Analyses by reversed phase HPLC of trilinolenoylglycerol autoxidized at 40°C to different levels showed that mono-hydroperoxides and hydroperoxy epidioxides are the only products formed initially and they become major products at PV's above 30 (Fig. 2). *Bis*- and tris-hydroperoxides were detected in small amounts at PV's of 31 and 47, respectively. Hydroperoxy bicyclo endoperoxides and mono-dihydroperoxides were also formed as minor secondary products at PV's above 75. These results support a mechanism in which mono-hydroperoxides and mono-hydroperoxy epidioxides are formed

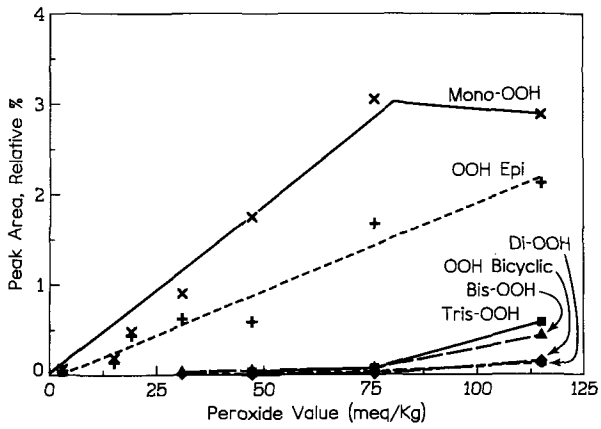

FIG. 2. Autoxidation of trilinolenoylglycerol at 40°C. Analyses by reversed phase HPLC. Conditions: RCM C-18 column, 1.0 ml/min mobile $CH_2,CL_2/CH_3CN$ (30:70, v/v), 15-25 μl sample, area % RI detection calibrated against mixtures of known compounds. Mono-OOH, mono-hydroperoxides; OOH Epi, hydroperoxy epidioxides; Di-OOH, dihydroperoxides; *Bis*-OOH, *bis*-hydroperoxides; Tris-OOH, tris-hydroperoxides; OOH Bicyclic, hydroperoxy bicycloendoperoxides.

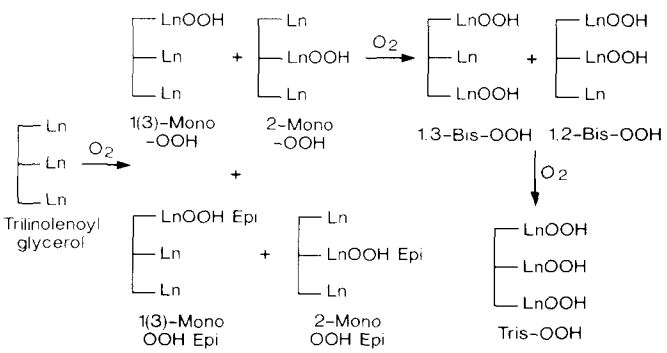

FIG. 3. Mechanism of autoxidation of trilinolenoylglycerol. 1(3)-Mono-OOH, 1(3)-mono-hydroperoxides of trilinolenoylglycerol; 2-Mono-OOH, 2-mono-hydroperoxides of trilinolenoylglycerol; 1(3)-mono epi, 1(3)-mono hydroperoxy epidioxides; 2-mono epi, 2-mono hydroperoxy epidioxides; 1,3-*Bis*-OOH, 1,3-*bis*-hydroperoxides; 1,2-*Bis*-OOH, 1,2-*bis*-hydroperoxides; Tris-OOH, tris-hydroperoxides.

rapidly as initial products (Fig. 3), by the same process recognized for the autoxidation of methyl linolenate (6,7, 10). The mono-hydroperoxides undergo consecutive oxidation to *bis*-hydroperoxides, which are in turn oxidized to the tris-hydroperoxides. The relative concentrations of *bis*- and tris-hydroperoxides are much smaller in autoxidized trilinolenoylglycerol than in trilinoleoylglycerol (1). This difference may be attributed to cyclization of the 12- and 13-hydroperoxide isomers of trilinolenoylglycerol leading to the hydroperoxy epidioxides, which is competitive with the formation of *bis*- and tris-hydroperoxidation in trilinolenoylglycerol but not in trilinoleoylglycerol (1).

The ^{1}H nuclear magnetic resonance (NMR) spectrum of the mono-hydroperoxides of trilinolenoylglycerol shows proton signals for the hydroperoxy and linolenoyl residues (Fig. 4A). Signals characteristic of the trilinolenoyl monohydroperoxide include 8.40 for C-OOH; 6.55, 6.00, 5.56, and 5.42 for protons c, d, b, e of the *cis,trans* conjugated diene systems; 5.38 for protons g and h of the isolated double bond; 4.35 for the methine proton a of the hydroperoxide bearing carbon; and 2.88 ppm for the double allylic proton f. Signals characteristic of the linolenoyl groups include 5.38 for protons of carbon-carbon unsaturation; 4.28 and 4.11 for protons on 2- and 1(3)-carbons of glycerol; 2.76 for methylene protons between two double bonds; 2.26 for methylene protons α to ester carbonyl; 2.10 for methylene protons adjacent to double bonds; 1.62 for methylene protons β to ester carbonyl; 1.30 for chain methylene protons; and 0.95 ppm for terminal methyl protons (Fig. 4A).

The ^{1}H-NMR spectrum of the mono-hydroperoxy epidioxides of trilinolenoylglycerol agreed with those reported for the hydroperoxy epidioxides of methyl linolenate (6,7). Figure 4B shows characteristic signals at 4.75, 4.45 ppm for protons b, d, and 2.44 and 2.65 ppm for protons c of the methine and methylene protons of the epidioxide ring; at 6.64, 6.00, 5.62, and 5.40 ppm for protons f, g, e and h of the *cis,trans* conjugated diene systems; and at 4.30 ppm for the methine proton a of the hydroperoxy-bearing carbon. Signals for the linolenoyl group and the glycerol moiety are the same as those for monohydroperoxides and support a triacylglycerol structure containing one hydroperoxy epidioxide and two linolenic acid units. The ^{1}H NMR spectra of the di-hydroperoxide and hydroperoxy bicyclo endoperoxide are consistent with the corresponding products previously identified in autoxidized and photooxidized methyl linolenate (8,9).

^{13}C NMR analyses of the mono-, *bis*- and tris-hydroperoxides show signals for the carbons of the conjugated diene system of linolenoyl hydroperoxides, for the carbons of the isolated olefinic substituents, for the hydroperoxy-bearing carbons, and for carbons adjacent to *cis,trans* conjugated diene systems (Table 1). The hydroperoxy epidioxide of trilinolenoylglycerol showed the same signals for the hydroperoxy-bearing carbon, conjugated diene system, and for the methine and methylene carbons of the epidioxide group, as previously reported for the hydroperoxy epidioxide of methyl linolenate (7,8). The methylene carbon of the epidioxide ring had two signals at 41.5 and 42.0 ppm, consistent with two diastereoisomers (8).

The triacylglycerol position of the mono-hydroperoxides of trilinolenoylglycerol was determined by analytical normal phase HPLC (Fig. 5). Six peaks were resolved and identified according to the same scheme used for the mono-hydroperoxides of trilinoleoylglycerol, by pancreatic lipase hydrolysis and capillary GC after hydrogenation and methylation (1). 2-Mono-hydroperoxylinolenoylglycerols eluted in peaks 1, 2 and 5, and produced methyl stearate and 2-mono-OTMS stearoylglycerol (as OTMS ethers) in a 2:1 ratio (Table 2). Peak 5 corresponded to the 9-hydroperoxide isomer, peak 1 to a mixture of 12- and 13-hydroperoxide isomers, and peak 2 to the 16-hydroperoxide isomer. 1(3)-Mono-hydroperoxylinolenoylglycerols eluted in peaks 4 and 6, produced methyl stearate, methyl OTMS stearate and 2-monostearoylglycerol (OTMS ether) in a 1:1:1 ratio. Peak 6 corresponded to the 9-hydroperoxide, and peak 4 to the 16-hydroperoxide isomer. Partially resolved peak 3 was assumed to be due to a mixture of 1(3)-glycerol isomers of 12- and 13-hydroperoxides on the basis of its area relative to that of peak 1 corresponding to the 2-glycerol isomers of 12- and 13-hydroperoxides. The structures of mono-hydroperoxide isomers separated by normal phase HPLC were further confirmed by ^{1}H and ^{13}C NMR, showing the same signals as the trilinolenoylglycerol mono-hydroperoxide mixture (Fig. 4A and Table 1). Moreover, IR analyses showed the expected *cis,trans* configuration for the conjugated diene system allylic to the hydroperoxy-bearing carbon.

Analytical normal phase HPLC separation of the monohydroperoxy epidioxides of trilinolenoylglycerol showed eight peaks, which were identified by the same scheme as the mono-hydroperoxides (Fig. 6). 2-Glycerol isomers of mono-hydroperoxy epidioxides eluted in peaks 7, 8, 11 and 13, produced methyl stearate and 2-mono-9,10,12-(13,15,16)tri-OTMS stearoylglycerol (as TMS ethers) in a 2:1 ratio (Table 3). 1(3)-Glycerol isomers of monohydroperoxy epidioxides eluted in peaks 9, 10, 12 and 14, produced methyl stearate, methyl 9,10,12- or 13,15,16-tri-OTMS stearates and monostearoylglycerol (TMS ethers) in a 1:1:1 ratio. ^{1}H-NMR confirmed the structures of the mono-hydroperoxy epidioxides and further established that peaks 7, 8, 9 and 10 corresponded to the *cis,trans* 9-hydroperoxy-10,12-epidioxide isomers, peaks 11 and 12 to the *cis,trans* 16-hydroperoxy-13,15-epidioxide isomers, and peaks 13 and 14 to a mixture of *trans,trans* 9- and 16-hydroperoxy epidioxide isomers. Examination of

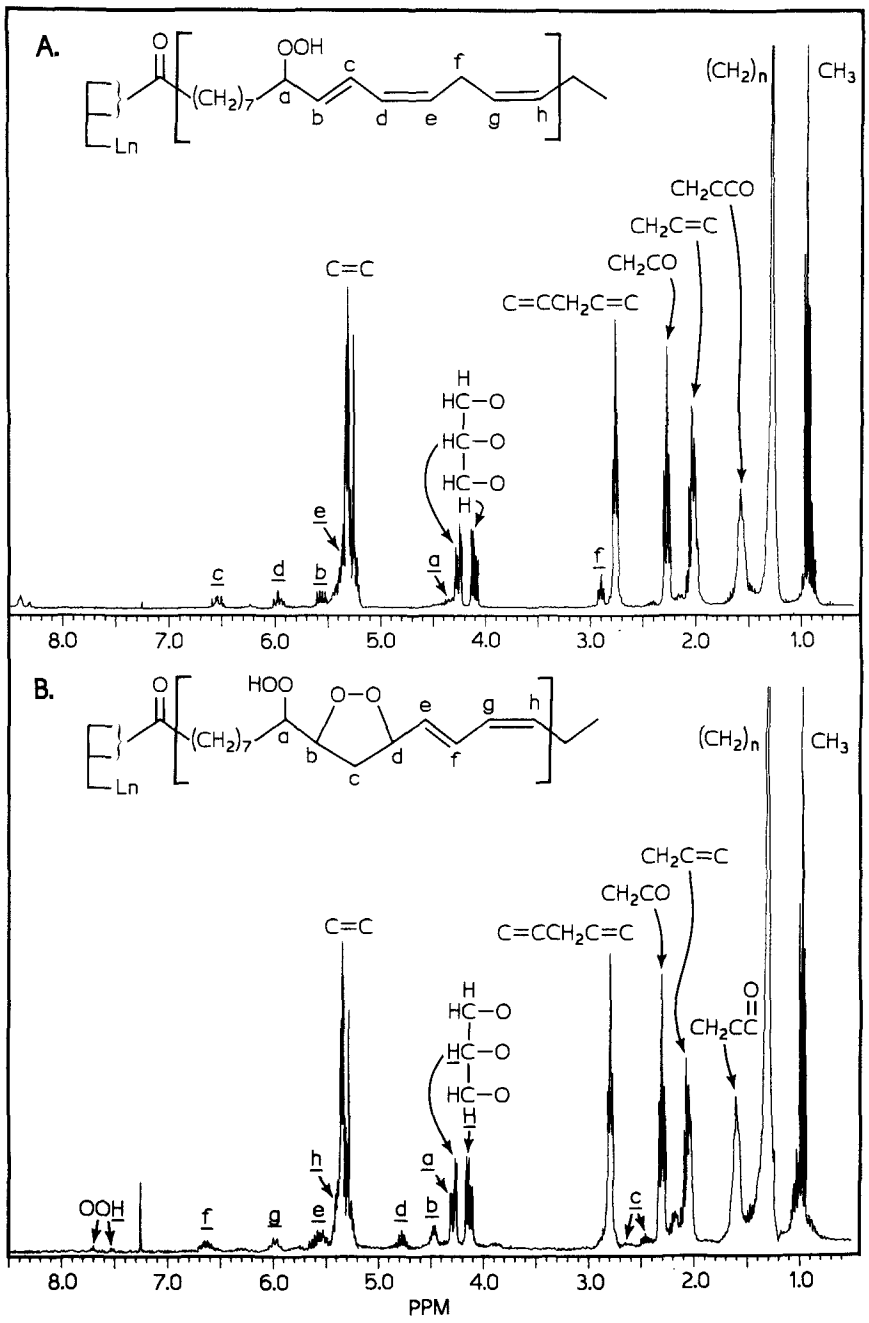

FIG. 4. (A) ^{1}H NMR spectrum of mono-hydroperoxides of trilinolenoylglycerol. General structure shown for 9- or 16-linolenic hydroperoxides. (B) ^{1}H NMR spectrum of mono-hydroperoxy epidioxides of trilinolenoylglycerol. General structure shown for 9- or 16-hydroperoxy epidioxides.

chemical shifts for methine proton of the hydroperoxy-bearing carbon and α proton of the methylene carbon of the epidioxide ring showed that the components eluted in peaks 7 and 8, and peaks 9 and 10 are two diastereomeric pairs, but the diastereomers did not separate in peaks 11, 12, 13, 14.

The ratio of 1(3)- to 2-glycerol isomers of trilinolenoylglycerol mono-hydroperoxides were determined by normal phase HPLC of trilinolenoyl glycerol autoxidized at various PV's (Table 4). On the one hand, the ratio of peak areas of *trans,cis,cis*-9-hydroperoxy and *cis,trans,cis*-12(13)-hydroperoxy isomers (Fig. 5) gave a 1(3):2-ratio averaging 2.0. On the other hand, the ratio of peak areas of *cis,cis,trans* 16-hydroperoxy isomers gave a 1(3)-:2-ratio averaging 2.7, which was significantly higher than 2.0. Therefore, these results indicate that the formation of 9-, 12- and 13-hydroperoxides is not selective toward either the 1(3)- or 2-triacylglycerol position, but the formation

TABLE 1

^{13}C NMR of Trilinolenoylglycerol Hydroperoxides and Hydroperoxy Epidioxides

HPLC fractions[a]	Shifts, ppm (assignments)[b]
Mono OOH	172.88, 172.50 (C=O), 132.42, 132.00, 131.72, 131.46, 131.33, 130.72, 130.70, 130.65, 129.73, 129.63, 129.32, 129.23, 127.61, 127.58, 126.98, 126.94 (C=C-C=C-C-C=C, *cis,trans/trans,trans* 1(3)- and 2-isomers), 86.7 (C-OOH), 34.10 (C-COOH), 34.17 (*trans* C-C=C), 27.67 (*cis* C-C=C), 24.84 (C-C-OOH)
Bis OOH	173.25, 172.8 (C=O), 132.49, 131.71, 131.54, 130.90, 130.87, 130.80, 129.51, 128.82, 127.55, 127.51, 126.95 (C=C-C=C-C-C=C, *cis,trans/trans,trans* 1(3)- and 2-isomers), 88.0-87.0 (C-COOH), 34.15 (*trans* C-C=C), 27.20 (*cis* C-C=C), 25.51 (C-C-OOH)
Tris OOH	173.2, 172.8 (C=O), 131.71, 131.54, 130.90, 130.87, 128.82, 127.55, 127.51 (C=C-C=C-C-C=C, *cis,trans/trans,trans* 1(3)- and 2-isomers), 88.0-87.0 (C-OOH), 34.0 (C-COOH), 34.13 (*trans* C-C=C), 27.17 (*cis* C-C=C), 25.58 (C-C-OOH)
OOH Epi	173.2, 172.8 (C=O), 132.49, 131.71, 131.54, 130.90, 130.87, 130.80, 127.0, 126.6 (C=C-C=C, *cis,trans/trans,trans* 1(3)- and 2-isomers), 83.50, 82.99 (methine C's of epidioxide ring), 87.40, 87.2 (C-OOH), 42.0, 41.5 (CH_2 of epidioxide ring), 34.17 (C-COOH), 32.4 (*trans* C-C=C) 27.78 (*cis* C-C=C), 24.90 (C-C-OOH)

[a] See Figure 1. Mono-OOH, mono-hydroperoxides; *Bis*-OOH, *bis*-hydroperoxides; Tris-OOH, tris-hydroperoxides; OOH Epi, hydroperoxy epidioxides.

[b] See Figure 4A and B for structures of mono-hydroperoxides and hydroperoxy epidioxides.

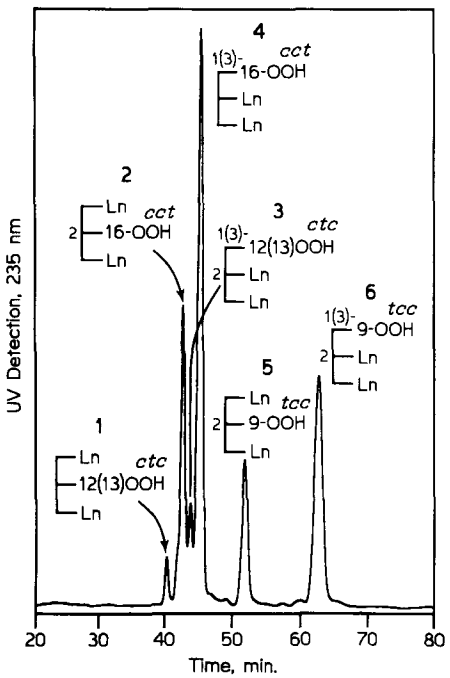

FIG. 5. Analytical normal phase HPLC of mono-hydroperoxide mixture from trilinolenoylglycerol autoxidized to PV 115 meq/kg. Ln, linolenic acid residue; *ctc* 12(13)OOH, *cis,trans,cis* linolenic 12 + 13-hydroperoxides; *cct* 16-OOH, *cis,cis,trans* linolenic 16-hydroperoxide; *tcc* 9-OOH, *trans,cis,cis* linolenic 9-hydroperoxide.

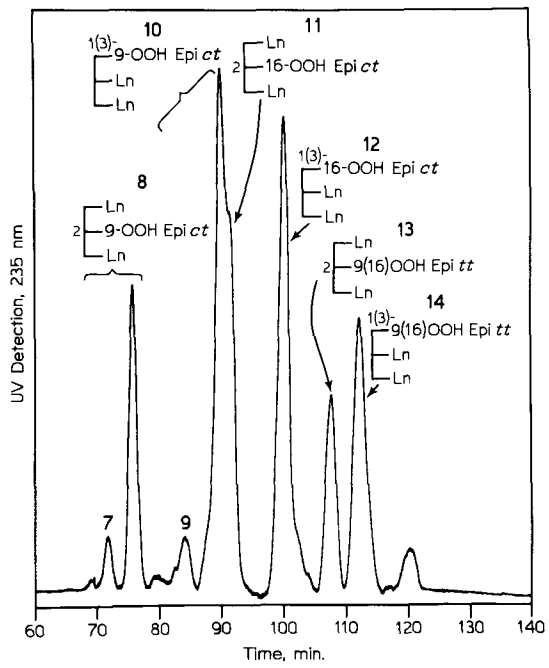

FIG. 6. Analytical normal phase HPLC of mono-hydroperoxy epidioxide mixture from trilinolenoylglycerol autoxidized to PV 115 meq/kg. Ln, linolenic acid residue; *ct* 9-OOH Epi, *cis,trans* 9-hydroperoxy epidioxide; *ct* 16-OOH Epi, *cis,trans* 16-hydroperoxy epidioxide; *tt* 9 + 16-OOH Epi, *trans,trans* 9 + 16-hydroperoxy epidioxides.

TABLE 2

Capillary GC Analysis of Lipolysis Products of Trilinolenoylglycerol Mono-Hydroperoxide Isomers Separated by HPLC[a,b]

	HPLC fraction[a]				
Product[c]	1	2	4	5	6
Methyl stearate	64.2	68.5	34.6	66.8	31.8
9-OTMS stearate	—	—	—	—	33.0
16-OTMS stearate	—	—	35.1	—	—
2-Monostearin	—	—	30.9	—	35.2
2-Mono-OH stearin-9-OTMS stearoyl	—	—	—	33.2	—
12-OTMS stearoyl	16.4	—	—	—	—
13-OTMS stearoyl	19.4	—	—	—	—
16-OTMS stearoyl	—	31.5	—	—	—

[a] See Figure 5 for HPLC separation.

[b] See Figure 7 in reference (1) for analytical scheme of lipolysis products of mono-hydroperoxides of trilinoleoylglycerol.

[c] Monostearin (monostearoylglycerol) and mono-OTMS stearin products are analyzed as the OTMS ethers of glycerol. Products were identified by comparing GC retention with authentic compounds. 2-Mono-9(12,13,16)-hydroxystearoylglycerol was identified as the OTMS derivative from 2-mono-9(12,13,16)-hydroxylinolenoylglycerol after hydrogenation and transmethylation producing methyl 9-, 12-, 13- and 16-hydroxystearate (OTMS derivative).

TABLE 3

Capillary GC Analysis of Lipolysis Products of Trilinolenoylglycerol Mono-Hydroperoxy Epidioxides Separated by HPLC[a,b]

	HPLC fraction[a]							
Product[c]	7	8	9	10	11	12	13	14
Methyl stearate	65.3	62.3	57.3	37.8	66.5	36.0	63.8	34.7
Tri-OTMS stearate								
9,10,12-	—	—	20.4	33.8	—	—	—	19.3
13,15,16-	—	—	—	—	—	34.2	—	13.4
2-Monostearin	—	—	22.3	28.4	—	29.8	—	—
2-Mono-triOH stearin Tri-OTMS stearoyl								
9,10,12-	34.7	37.8	—	—	—	—	21.1	18.4
13,15,16-	—	—	—	—	33.5	—	15.1	14.2

[a] See Figure 6 for HPLC separation.

[b] See Figure 7 in reference (1) for analytical scheme of lipolysis products of mono-hydroperoxides of trilinoleoylglycerol. 2-Mono-hydroperoxy epidioxides produce methyl stearate and a mixture of tri-OTMS stearate; 1(3)-mono-hydroperoxy epidioxides produce methyl stearate, a mixture of tri-OTMS stearate and 2-monostearoylglycerol.

[c] Monostearin (monostearoylglycerol) and mono-triOTMS stearin products are analyzed as the OTMS ethers of glycerol. Products were identified by comparing GC retention with authentic compounds. 2-Mono-tri-hydroxy stearoylglycerol was identified as the OTMS derivative after hydrogenation and transmethylation producing methyl 9,10,12- and 13,15,16-trihydroxystearate (OTMS derivative).

of 16-hydroperoxides has a small but significant preference for the 1(3)-triacylglycerol position.

The ratio of 1(3)-:2-isomers of trilinolenoylglycerol monohydroperoxy epidioxides were also determined by normal phase HPLC of trilinolenoylglycerol autoxidized at various PV's (Table 5). The 1(3)-:2-ratios of *cis,trans* 9-hydroperoxy-10,12-epidioxides, *cis,trans*-16-hydroperoxy 13,15-epidioxides and *trans,trans*-9 + 16-hydroperoxy epidioxides averaged 1.8–1.9, and were not significantly different. The cyclization leading to the formation of hydroperoxy epidioxides is, therefore, not selective toward either the 1(3)- or 2-triacylglycerol position. Furthermore, the observation that each hydroperoxy epidioxide remained fairly constant relative to peroxide values support the previous conclusion that cyclization is competitive with formation of *bis*- and tris-hydroperoxides.

Our study shows that oxidation of the n-3 double bond of linolenic acid forming the 16-hydroperoxide is slightly selective toward the 1(3)-position of trilinolenoylglycerol. Hydroperoxidation of the central double bonds of

TABLE 4

HPLC Analysis of 1(3)-:2-Glycerol Isomers of Mono-Hydroperoxides of Trilinolenoylglycerol[a]

Peroxide values	Hydroperoxide isomer (area %)								
	t,c,c-9			*c,t,c*-12+13			*c,c,t*-16		
	1(3)-	2-	1(3)-:2-	1(3)-	2-	1(3)-:2-	1(3)-	2-	1(3)-:2-[b]
6	21.5	10.3	2.09	9.4	4.4	2.14	39.9	14.6	2.73
9	21.2	10.5	2.02	7.9	3.9	2.03	40.6	15.6	2.60
15	22.6	11.5	1.97	7.9	3.7	2.14	39.9	15.0	2.66
18	21.7	10.8	2.01	7.8	3.9	2.00	41.1	14.9	2.76
28	20.7	11.3	1.83	7.3	3.8	1.92	42.2	14.7	2.87
35	21.5	11.2	1.92	7.6	4.1	1.85	41.3	15.4	2.68
41	21.5	11.1	1.92	7.5	4.2	1.79	40.9	14.8	2.76
50	25.3	11.4	2.22	7.2	3.9	1.85	38.8	13.5	2.87
150	19.6	10.3	1.90	7.3	3.6	2.03	43.3	16.8	2.58
842	20.7	11.8	1.75	7.2	3.1	2.32	41.3	15.9	2.60
1110	22.3	11.1	2.01	7.3	3.8	1.92	37.9	17.6	2.15
Average	21.7	11.0	1.97	7.7	3.9	2.00	40.7	15.4	2.66
S.D.	±1.5	±0.5	±0.1	±0.6	±0.4	±0.1	±1.5	±1.1	±0.2

[a] See Figure 5 for HPLC separation. Analyses were based on areas of peaks 5 and 6 for *trans,cis,cis*-9-hydroperoxides (*t,c,c*-9), peaks 1 and 3 for *cis,trans,cis*-12 + 13-hydroperoxides (*c,t,c*-12+13), and peaks 2 and 4 *cis,cis,trans*-16-hydroperoxides (*c,c,t*-16) (Fig. 5).

[b] 1(3)-:2-ratio of 2.66 was significantly higher ($p < 0.01$ by *t*-test) than 2.00. S.D. = standard deviation.

TABLE 5

HPLC Analysis of 1(3)- and 2-Glycerol Isomers of Trilinolenoylglycerol Mono-Hydroperoxy Epidioxides[a]

Peroxide values	Hydroperoxy epidioxides (area %)								
	c,t-9-OOH-10,12-epi			*c,t*-16-OOH-13,15-epi			*t,t*-9+16-OOH-epi		
	1(3)-	2-	1(3)-:2-	1(3)-	2-	1(3)-:2-	1(3)-	2-	1(3)-:2-[b]
6	30.8	15.2	2.03	21.8	12.8	1.70	12.2	7.3	1.67
9	26.8	14.3	1.87	22.0	13.4	1.64	15.2	8.3	1.83
15	26.3	16.4	1.60	22.3	12.5	1.78	14.6	7.9	1.85
18	26.9	12.8	2.10	22.8	12.3	1.85	15.9	9.3	1.71
28	26.4	15.0	1.76	22.6	10.8	2.09	14.8	7.4	2.00
35	25.8	14.8	1.74	22.9	12.5	1.83	15.8	8.2	1.93
41	24.3	12.3	1.98	23.9	15.2	1.57	15.3	9.0	1.70
50	25.6	14.4	1.78	23.4	13.4	1.75	15.5	7.7	2.01
150	25.5	13.7	1.86	25.2	15.6	1.62	12.5	7.5	1.67
842	26.2	13.5	1.98	22.2	11.0	2.02	16.7	10.4	1.61
1110	25.7	14.3	1.80	22.3	11.1	2.01	17.2	10.5	1.69
Average	26.4	14.2	1.86	22.9	12.8	1.81	15.0	8.5	1.79
S.D.	±1.6	±1.2	±0.1	±1.0	±1.6	±0.2	±1.5	±1.2	±0.2

[a] See Figure 6 for HPLC separation. Analyses were based on areas of peaks 7, 8, 9 and 10 for *cis,trans*-9-hydroperoxy-10,12-epidioxides (*c,t*-9-OOH-10,12-epi), peaks 11 and 12 for *cis,trans*-16-hydroperoxy-13,15-epidioxides (*c,t*-16-OOH-13,15-epi), and peaks 13 and 14 for *trans,trans*-9-+16-hydroperoxyepidioxides (*t,t*-9+16-OOH-epi) (Fig. 6).

[b] 1(3)-:2 ratios were not significantly different with a $p = 0.18$ by *t*-test. S.D. = standard deviation.

linolenic acid leading to the 9-, 12- and 13-hydroperoxide isomers is not selective toward either the 1(3)- or 2-triacylglycerol positions. Because the formation of the 12- and 13-hydroperoxide isomers is not selective, their cyclization leading to the hydroperoxy epidioxides is also not selective. The greater susceptibility to oxidation of the n-3 double bond of methyl linolenate and isomeric octadecadienoates is well established (5,10,11). Our present results suggest that the n-3 double bond of linolenic acid may be more sterically available to oxygen in the 1(3)- than the 2-positions of trilinolenoylglycerol. Decomposition of the main 16-hydroperoxide formed may be expected to contribute larger amounts of n-3 volatiles, including propanal, propenal and ethane (12). Our kinetic study indicates further that the mono-hydroperoxides and mono-hydroperoxy epidioxides may be regarded as

the most important precursors of volatile products contributing to the oxidative deterioration of linolenate-containing triacylglycerols.

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Autoxidation of Polyunsaturated Triacylglycerols. III. Synthetic Triacylglycerols Containing Linoleate and Linolenate[1]

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Four triacylglycerols containing linoleate (L) and linolenate (Ln) in specific positions were synthesized to determine the effect of fatty acid position on their relative rates and products of autoxidation. Analyses by reversed-phase high performance liquid chromatography (HPLC) showed that autoxidation of L- and Ln-containing triacylglycerols form monohydroperoxides and hydroperoxy epidioxides as the main products. The peroxyl radicals of internal 12- and 13-mono-hydroperoxides of Ln triacylglycerol components cyclized rapidly and their relative triacylglycerol position had no influence on their rates of cyclization. A good linear relation was obtained between total HPLC peak areas (detected at 235 nm) of the main oxidation products and peroxide values. Reversed phase HPLC analyses thus provide a useful method to estimate oxidation of polyunsaturated triacylglycerols. The ratios of Ln to L mono-hydroperoxides were twice the ratios of Ln to L in the triacylglycerol substrates. Ln triacylglycerol components, therefore, oxidized twice as much as the L components. At 40°C, LnLnL oxidized slightly faster than LnLLn with respective induction periods of 45 and 47 hr. LLnL oxidized faster than LLLn with respective induction periods of 56 and 60 hr. Dilinolenoyl-linoleoylglycerols are, therefore, slightly less stable to oxidation when Ln is in the 1,2- than the 1,3-triacylglycerol position. Dilinoleoyl-linolenoylglycerols are less stable when L is in the 1,3- than the 1,2-triacylglycerol position.
Lipids 25, 48–53 (1990).

Although many papers have been published on the products of autoxidation of simple esters of unsaturated fatty acids (1–4), only a few reports have appeared on the structure of primary and secondary oxidation products of unsaturated triacylglycerols (5,6). Relatively scant attention has been given to the effects of mixed fatty acids and how their relative positions in the triacylglycerol molecule influence oxidative and flavor stability.

Studies to determine if any preferential oxidation of the unsaturated acids occurs in different triacyglycerol positions are controversial. Raghuveer and Hammond (7) reported that the stability of several fats when oxidized at 37 and 50°C decreased after randomization by *trans* esterification. They suggested that concentration of unsaturated fatty acids in the 2-position of triacylglycerols increases stability. Similarly, Lau *et al.* (8) reported that randomized corn oil oxidizes at 28°C three or four times faster than natural corn oil, but concluded that the oxidation attack is random. In contrast, Zalewski and Gaddis (9) reported that randomization did not alter oxidative stability when measured at 60°C. Hoffmann *et al.* (10) found that randomization had a drastic effect in lowering the stability of palm oil, but not that of lard. Their results were attributed to a larger change of SSO (where S = saturates, O = oleate) content in palm oil (from 7 to 20%) than in lard (from 25 to 19%). The marked instability of SSO might explain the discrepancy of different reports in the literature. These authors also found that 1,3-dioleyl-2-linoleoylglycerol (OLO) (where L = linoleate) is much more stable to oxidation at 85°C than 1,2-dioleyl-3-linoleoyl-glycerol (OOL), and that SOS was much more stable than SSO, but LOL was only slightly more stable than OLL. These authors concluded that 1,3-triglycerides tend to be more stable to oxidation than 1,2 isomers, but this difference was not observed in oleyldilinoleoylglycerols.

Wada *et al.* (11) reported that randomized mixtures prepared from tripalmitoylglycerol, tristearoylglycerol, trioleylglycerol, and trilinoleoylglycerol (LLL) were more oxidatively stable than the equivalent mixtures of the same triacylglycerols before transesterification. Their evidence supported the conclusion of Raghuveer and Hammond (7), that triacylglycerols having unsaturated fatty acids in the 2-position are more oxidatively stable than triacylglycerols with these acids in the 1(3)-positions. In another study, Park *et al.* (12) reported that if tocopherols are carefully removed from soybean oil triacylglycerols, by charcoal treatment, randomization had no significant effect on oxidative stability measured at 37 and 50°C on the basis of peroxide values. They attributed the effect of transesterification in early studies to the loss of tocopherol. In another paper (13), these authors reported no difference in rate of oxidation between PLnP and PPLn, and between PLP and PPL (where P = palmitate, Ln = linolenate).

To better understand the interrelationship of unsaturated fatty acids on the oxidative stability of polyunsaturated fats, we evaluated a number of model synthetic triacylglycerols containing L and Ln in known positions. We previously described the syntheses and characterizations of all possible diacid triacylglycerols containing L and Ln for oxidation studies (14). We also separated and characterized the major autoxidation products of LLL and trilinolenoylglycerol (LnLnLn), and determined their relative triacylglycerol position by high performance liquid chromatography (HPLC) and by lipolysis (15,16). In the preceeding paper of this series, we established that hydroperoxy epidioxides are formed as important initial secondary products of the autoxidation of LnLnLn (16).

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Abbreviations: HPLC, high performance liquid chromatography; TLC, thin layer chromatography; GC, gas chromatography; GC-MS, gas chromatography-mass spectrometry; NMR, nuclear magnetic resonance; UV, ultraviolet; FI, flame ionization; TMS, trimethylsilyl; O, oleate; S, saturated compounds; U, unsaturated compounds; P, palmitate; L, linoleate; Ln, linolenate; LLL, trilinoleoylglycerol; LnLnLn, trilinolenoylglycerol; LnLL, 1-linolenoyl-2,3-dilinoleoylglycerol; LLnL, 2-linolenoyl-1,3-dilinoleoylglycerol; LLnLn, 1-linoleoyl-2,3-dilinolenoylglycerol; LnLLn, 2-linoleoyl-1,3-dilinolenoylglycerol.

In this paper, we oxidized four synthetic polyunsaturated triacylglycerols to determine the effect of triacylglycerol structures on their relative rates of oxidation, and on the types and amounts of products, to clarify their contribution to oxidative deterioration of vegetable oils. Reversed phase HPLC techniques were developed to analyze quantitatively the relative amounts of monohydroperoxides and hydroperoxy epidioxides derived from the L and Ln components of mixed triacylglycerols at peroxide values below 50.

MATERIALS AND METHODS

Preparations. All solvents were HPLC grade or analyzed reagent grade. LLL and LnLnLn (99+% purity) were purchased from NuChek Prep, Inc. (Elysian, MN). 1-Linolenoyl-2,3-dilinoleoylglycerol (LnLL), 2-linolenoyl-1,3-dilinoleoylglycerol (LLnL), 1-linoleoyl-2,3-dilinolenoylglycerol (LLnLn), and 2-linoleoyl-1,3-dilinolenoylglycerol (LnLLn) were synthesized and purified by dry column chromatography as described previously (14). The functional purities of these synthetic triacylglycerols ranged between 97.8 and 99.7% by gas chromatography (GC), and their isomeric purities between 97.4 and 99.0% (by lipolysis). The syntheses of these triacylglycerols were replicated two or three times and their analyses were replicated with standard deviations averaging 0.39% (14). Before oxidation, all triacylglycerols were further purified by preparative reversed phase HPLC (15) and by silicic acid column chromatography to obtain a peroxide value of zero by the ferric thiocyanate method (17).

Autoxidation. The chromatographed triacylglycerols (500 to 600 mg) were autoxidized to different levels by bubbling pure oxygen at 40°C in a 1 × 15 cm test tube. Aliquot samples were taken for peroxide value determination and for analytical reversed phase HPLC. The isomeric purity of each synthetic triacylglycerol was checked by lipolysis after autoxidation at 40°C and found to be the same as before autoxidation. There is therefore no acyl migration under our conditions of autoxidation.

HPLC analyses. Mono-hydroperoxides and hydroperoxy epidioxides were determined by analytical reversed phase HPLC with a solvent mixture of acetonitrile/methylene chloride/methanol (85:15:1, v/v/v), a flow rate of 1.5 ml/min, in a 5 micron C-18 column (25 × 0.46 cm, Zorbax, Du Pont Instrument Division, Wilmington, DE). The effluent was monitored with a variable ultraviolet (UV) detector (Model 770, Schoeffel Instruments, Westwood, NJ) set at 235 nm, and with a flame ionization (FI) detector (Model 945, Tractor, Inc., Austin, TX).

Capillary analyses. The mono-hydroperoxides and hydroperoxy epidioxides separated by HPLC were identified by capillary GC and GC-MS of the trimethylsilyl (TMS) ethers prepared after catalytic hydrogenation and transmethylation (15). These oxidation products were also analyzed by capillary GC of the TMS ethers prepared after reduction with $NaBH_4$ followed by transmethylation (18,19). A capillary column (DB-225, 30 m × 0.32 mm, 1 micron film, J & W Scientific Co., Folsom, CA) was used with the temperature programmed from 160 to 200°C at 4°C per min.

RESULTS AND DISCUSSION

The relative oxidative stabilities at 40°C were determined by following the increase in peroxide values. The synthetic triacylglycerols LnLLn, LLnLn, LLnL and LnLL were compared with LnLnLn, LLL, and 2:1 or 1:2 mixtures of LnLnLn:LLL. As expected, the oxidative stabilities of triacylglycerols decreased with increasing total unsaturation (Fig. 1). A large difference in oxidative stability was observed between LLL and LnLnLn with respective induction periods of 24 and 160 hr. However, small but significant differences were found between triacylglycerols having the same degrees of unsaturation. LnLnL was more susceptible to oxidation than LnLLn, with respective induction periods of 45 and 47 hr. The oxidation rate of a 2:1 LnLnLn:LLL mixture was intermediate between LnLnL and LnLLn, with an induction period of 46 hr (data not shown). LLnL oxidized faster than the corresponding LLLn, with respective induction periods of 56 and 60 hr. The corresponding 1:2 LnLnLn:LLL mixture oxidized at about the same rate as LLnL (data not shown). These results show that the dilinolenoyl-linoleoylglycerols are more easily oxidized when Ln is in the 1,2- than the 1,3-triacylglycerol position, but the difference in oxidative stability is small. The dilinoleoyl-linolenoylglycerols are less oxidatively stable when L is in the 1,3- than the 1,2-triacylglycerol position.

In the previous papers of this series, we developed reversed phase HPLC systems to separate the autoxidation products of LLL and LnLnLn (15,16). These HPLC systems were further refined to separate and analyze quantitatively the oxidation products of synthetic triacylglycerols (Fig. 2). The L and Ln mono-hydroperoxides (peaks B and C) were only partially separated in autoxidized LnLnL, LLnL and LLLn. This separation was easier with LnLLn than with LnLnL. The Ln mono-hydroperoxides were also partially separated into two components in LnLLn and LLLn.

The components of each HPLC peak were identified by capillary GC after derivatization and by ^1H and ^{13}C NMR (15). The hydrogenated and silylated derivatives from components eluted in HPLC peak B included an equal mixture of 9- and 13-OTMS stearates and were thus attributed to L mono-hydroperoxides. The corresponding derivatives from components eluted in HPLC peak C included a mixture of 9-, 12-, 13- and 16-OTMS stearates (35, 10, 13 and 42%, respectively), and were attributed to LN mono-hydroperoxides. Quantitative GC analyses

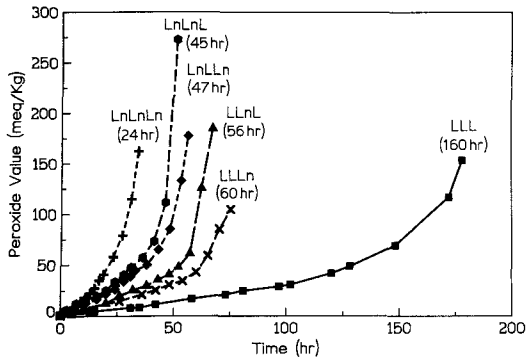

FIG. 1. Peroxide development during autoxidation of synthetic triglycerides at 40°C. Oxidation runs and peroxide value determinations were made in duplicate and the relative standard deviations averaged ±5%.

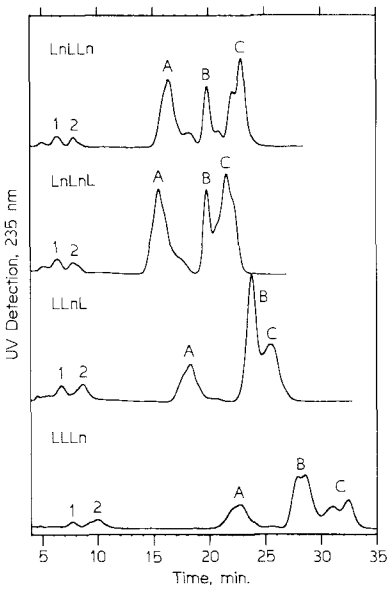

FIG. 2. Reversed phase HPLC analyses of synthetic triglycerides autoxidized at 40°C. Peak 1, tris-hydroperoxides; peak 2, *bis*-hydroperoxides; peak A, linolenate hydroperoxy epidioxides; peak B, linoleate mono-hydroperoxides; peak C, linolenate mono-hydroperoxides.

gave ratios of 2:1 for methyl stearate to methyl OTMS-stearate, supporting monoacyl hydroperoxide glycerol structures. Capillary GC analyses of the $NaBH_4$-reduced and silylated derivatives showed the expected mixture of 9- and 13-dienols (as TMS ethers) from the L mono-hydroperoxide triacylglycerol components, and the mixture of 9-, 12-, 13- and 16-trienols (as TMS ethers) from the corresponding Ln mono-hydroperoxides.

In addition to mono-hydroperoxides, another important component found in HPLC peak A (Fig. 2) corresponds to hydroperoxy epidioxides derived from the Ln components. This was identified by capillary GC, which showed an equal mixture of 9,10,12-triOTMS and 13,15,16-triOTMS stearates. This cyclic peroxide is readily separated by HPLC from the mono-hydroperoxides and represents a major product of Ln-containing triacylglycerols. Quantitative capillary GC analyses gave ratios of 2:1 for methyl stearate to methyl triOTMS-stearate, supporting monoacyl hydroperoxy epidioxide glycerol structures.

Minor secondary oxidation products in HPLC peaks 1 and 2 (Fig. 2) were identified by capillary GC after hydrogenation, silylation and transmethylation. Components from HPLC peak 1 produced a mixture of mono OTMS stearates and no methyl stearate, and were attributed to tris-hydroperoxides (15,16). Components from HPLC peak 2 produced methyl stearate and mono OTMS stearate in a ratio of 1:2 and were attributed to *bis*-hydroperoxides.

The ^1H NMR of mono-hydroperoxide components in HPLC peaks B and C showed broad signal for the hydroperoxide proton (7.90 ppm), conjugated diene protons (5.45-6.56 ppm), and for the methine proton of the carbon bearing the hydroperoxide group (4.35-4.45 ppm). The spectrum for the Ln hydroperoxide in HPLC peak C showed a unique signal at 2.95 ppm attributed to the methylene between an isolated double bond and the carbon bearing the hydroperoxide group [C=CC$\underline{H}_2$C(COOH)C=CC=C]. ^1H NMR of components in HPLC peak A confirmed the structures of hydroperoxy epidioxides (16), with signals for the methine protons (5.55 and 5.30 ppm) and methylene proton of the epidioxide ring (2.44 and 2.16 ppm), hydroperoxy protons (7.70 and 7.50 ppm), protons of the conjugated diene system (5.55-6.64), and methine proton of the carbon bearing the hydroperoxide group (3.82-3.95 ppm).

Mono-hydroperoxides and hydroperoxy epidioxides of polyunsaturated triacylglycerols have only one conjugated diene system (15,16). Thus, reversed phase HPLC with a UV detector was used to analyze oxidation products from synthetic triacylglycerols. The ratios of Ln to L mono-hydroperoxides (HPLC peaks C/B) for autoxidized LLnL (0.55 to 0.69) were similar to those of LLLn (0.59 to 0.64) (Table 1). These ratios, on the other hand, were lower for autoxidized LnLLn (2.10 to 2.24) than those for LnLnL (2.43 to 2.51). These results show that in the dilinoleoylglycerols, the position of the polyunsaturated fatty acids has no effect on the relative ratios of hydroperoxides. However, in the dilinolenoylglycerols, Ln in the 1,2-positions seems to affect the relative ratios of hydroperoxide components more than in the 1,3-positions, and a larger proportion of Ln is oxidized in LnLnL than in LnLLn.

The ratio of total Ln (mono-hydroperoxides plus hydroperoxy epidioxides) to L products (HPLC peaks A+C/B) did not change significantly during autoxidation at low peroxide values and ranged from 0.94 to 1.16 for autoxidized LLnL, from 0.96 to 1.14 for autoxidized LLLn, from 3.67 to 3.99 for autoxidized LnLLn, and from 4.21 to 4.70 for autoxidized LnLnL (Table 1). These Ln to L oxidation product ratios are about twice as high as the corresponding Ln to L ratios in the starting triacylglycerols. Analyses by capillary GC of the $NaBH_4$-reduced derivatives after silylation confirmed the HPLC analyses in showing that the ratio of Ln to L mono-hydroperoxides did not change significantly at peroxide values below 50 and was about twice as high as the substrate ratio of Ln to L. Therefore, the extent of Ln oxidation was twice as high as that of L oxidation in these synthetic polyunsaturated triglycerides at 40°C at peroxide values between 3 and 48 (Table 1). These results are in agreement with previous kinetic studies (20,21) showing that an increase in the number of methylene-interrupted double bonds in a fatty acid or ester increases the rate of oxidation by a factor of two.

The ratios of hydroperoxy epidioxides to Ln mono-hydroperoxides (ratios of HPLC peaks A/C) ranged between 0.62 to 0.90 (Table 1). These ratios are slightly lower than those reported for autoxidized methyl linolenate (22,23), ranging between 0.92 and 1.20, and suggest that cyclization of the peroxyl radicals of the 12- and 13-mono-hydroperoxides is more difficult in the triacylglycerols than in the methyl esters. There was also little change in these hydroperoxy epidioxides/monohydroperoxides ratios during autoxidation of the synthetic polyunsaturated triacylglycerols (Table 1). These results show that cyclization of the peroxyl radicals of 12- and 13-hydroperoxides of the Ln components occurred at about the same rate in the 1(3)-position of LLLn as in the 2-position of LLnL, and in the 1,3-positions of LnLLn as

TABLE 1

Reversed Phase HPLC Analyses of Synthetic Triacylglycerols Autoxidized at 40°C (Peak Integration Units × 10000)[a]

Peroxide values	Hydroperoxides L	Hydroperoxides Ln	OOH Epi	Ratios			Total
	B	C	A	C/B	(A+C)/B	A/C	A+B+C
LLnL							
6.4	3.42	1.90	1.30	0.55	0.94	0.68	6.62
12.4	5.95	3.63	2.64	0.61	1.05	0.73	12.2
20.0	9.45	5.85	3.96	0.62	1.04	0.68	19.3
25.4	11.3	7.36	5.16	0.65	1.11	0.70	23.8
29.9	14.2	9.66	6.28	0.68	1.12	0.65	30.1
35.7	15.4	10.6	7.21	0.69	1.16	0.68	33.2
48.2	21.4	14.3	10.2	0.67	1.14	0.71	45.9
LLLn							
2.9	1.62	0.96	0.60	0.59	0.96	0.62	3.19
7.7	3.98	2.35	1.63	0.59	1.00	0.69	7.97
14.7	6.35	3.89	2.73	0.61	1.67	0.70	13.0
21.2	9.72	6.18	4.22	0.64	1.07	0.68	20.1
25.6	11.7	7.54	5.36	0.64	1.10	0.71	24.6
34.5	13.9	8.59	7.24	0.62	1.14	0.84	29.8
43.2	19.7	12.1	9.04	0.61	1.07	0.75	40.8
LnLLn							
5.1	1.28	2.87	1.83	2.24	3.67	0.64	5.98
10.4	2.08	4.54	3.42	2.18	3.83	0.75	10.0
12.8	2.55	5.55	4.50	2.18	3.94	0.81	12.6
17.1	3.48	7.38	6.03	2.12	3.85	0.82	16.9
21.8	4.72	9.90	7.73	2.10	3.70	0.78	22.3
33.9	6.39	13.4	12.1	2.10	3.99	0.90	31.9
44.7	8.51	18.2	15.7	2.14	3.98	0.86	42.4
LnLnL							
5.7	1.16	2.91	1.97	2.51	4.21	0.68	6.04
12.6	2.05	5.12	4.00	2.50	4.45	0.78	11.2
16.2	3.04	7.46	6.03	2.45	4.44	0.81	16.5
24.9	4.17	10.3	8.71	2.47	4.56	0.84	23.2
33.8	5.58	14.0	11.6	2.51	4.59	0.82	31.2
40.5	7.24	18.2	15.8	2.51	4.70	0.87	41.3
48.0	9.21	22.4	18.0	2.43	4.39	0.81	49.6

[a]See Figure 2 for HPLC chromatograms; L, linoleate; Ln, linolenate; OOH Epi, hydroperoxy epidioxides; LLnL, 1,3-linoleoyl-2-linolenoylglycerol; LLLn, 1,2-linoleoyl-3-linolenoylglycerol; LnLLn, 1,3-linolenoyl-2-linoleoylglycerol; LnLnL, 1,2-linolenoyl-3-linoleoylglycerol.

in the 1,2-positions of LnLnL. Therefore, there is no influence of the triacylglycerol positions on the relative rates of cyclization of the internal peroxyl Ln components.

A plot of the total oxidation products (HPLC peaks A+B+C) vs oxidation times shows that the relative rates of oxidation decrease in the order of LLnLn, LnLLn, LLnL, and LLLn (Fig. 3). These HPLC analyses are in agreement with the oxidative stabilities based on increases of peroxide values with time (Fig. 1), and indicate that HPLC analyses correspond to peroxide value determinations. This correspondence is demonstrated by the good linear relationship obtained between integrations of total HPLC peak areas of oxidation products and peroxide values. Correlation coefficients between HPLC peak areas and peroxide values were calculated to be 0.9964, 0.9984, 0.9957, and 0.9990 for LLLn, LLnL, LnLnL, and LnLLn, respectively.

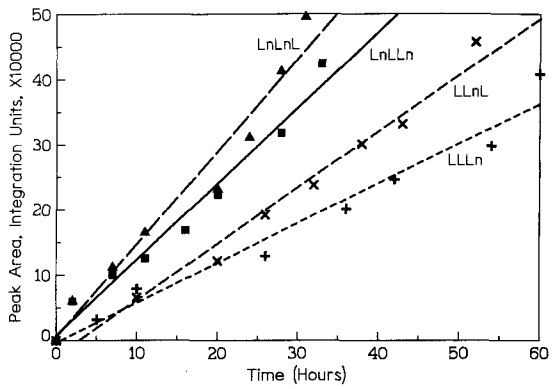

FIG. 3. Total oxidation products by HPLC (total peak areas A+B+C, Fig. 2) vs autoxidation time at 40°C. HPLC analyses were made in duplicate and the relative standard deviations average ±5%.

The usefulness of analytical reversed phase HPLC was further checked by using an FI detector, which can detect both conjugated and nonconjugated products of autoxidation. Although chromatograms obtained by HPLC with both detectors were in good agreement, the sensitivity of the HPLC method with a UV detector was 13 to 18 times greater than that with the FI detector. These results are in agreement with those of Hara *et al.* (24) who reported that normal phase HPLC with a UV detector was useful for the determination of oxidative rancidity in vegetable oils at low levels of oxidation. The formation of hydroperoxy epidioxides in these oxidized vegetable oils was, however, not considered in their HPLC analyses. In the present work, we found reversed phase HPLC of autoxidized triacylglycerols to be quantitatively more reliable than normal phase HPLC with a 5 micron silica column. With this normal phase HPLC system, we obtained significantly lower recovery; 67% of the injected autoxidized LLLn (peroxide value of 53), compared to 95% by reversed phase HPLC.

The results of this work are difficult to compare with the published literature because of the complexity of interrelationships between triglycerides of different unsaturated fatty acid substrates, oxidized under different conditions, and analyzed by different methods. The present study shows that the rates of oxidation and amounts of oxidation products are affected by the relative 1,2- and 1,3-triacylglycerol positions of Ln in dilinolenoyl-linoleoylglycerols. The relative triacylglycerol positions of L in dilinoleoyl-linoleoylglycerols affect the rates of oxidation but have no effect on the relative amounts of oxidation products. Our results on relative rates of oxidation do not agree with those of Hoffmann *et al.* (10) who compared the oxidative stability of OLO with OOL, and concluded that 1,3-equiacyl triglycerides tend to be more stable than 1,2-equiacyl triglycerides. Clearly, this conclusion does not apply to the polyunsaturated triglycerides compared in the present study, because LnLLn was more stable than LnLnL, but LLnL was less stable than LLLn (Fig. 1). However, not only the interactions of oleate and linoleate may be different than those of linoleate and linolenate, but Hoffman *et al.* (10) based their stabilities on oxygen absorption and oxidized their substrates at 85°C, whereas we used peroxide values, and our oxidation temperature was 40°C.

The studies of Raghuveer and Hammond (7) and of Wada *et al.* (11), suggesting that the unsaturated fatty acids in the 2-triacylglycerol position is more important that the 1(3)-positions in increasing the stabilities of triacylglycerols, are also not comparable to the present work. Their triacylglycerols contained significant amounts of saturated fatty acids and the greater stability of SUS than SSU (U = unsaturates) is well established (10,11).

In the present study, the competitive interactions between L and Ln were found to be important factors affecting oxidation. These results suggest that the lower oxidative stabilities of LnLnL than LnLLn may be due to the higher interactions between the two linolenoyl residues than between linolenoyl and linoleoyl residues. On the other hand, the lower oxidative stabilities of LLnL than LLLn may be due to the higher interactions between linolenoyl and linoleoyl residues than between the two linoleoyl residues. We previously found that the formation of *bis*- and tris-hydroperoxides is greater in LLL than in LnLnLn (16), because cyclization of the peroxyl radicals of the internal 12- and 13-hydroperoxides of linolenate competed with further hydroperoxidation of mono-hydroperoxides in triacylglycerols. To eliminate the interactions between linoleoyl and linolenoylglycerol components, it may be interesting in future studies, to compare the relative oxidative stabilities of SLL, LSL, and of SLnLn, LnSLn. These interactions are, however, expected to play a role in the oxidative stability of polyunsaturated triacylglycerols present in vegetable oils. Low erucic acid rapeseed oil is reported to contain 2.5% LnLnO and 11.8% OOLn (25) whereas soybean oil has no LnLnO and 0-0.5% OOLn (25,26). The interactions and triacylglycerol positions of O and Ln may thus be important in clarifying the difference in oxidative stabilities found between these oils (27). In comparing different polyunsaturated vegetable oils, we also found marked differences in the oxidative and flavor stabilities depending on storage conditions and types of analyses used to measure oxidative deterioration (27). In future studies of oxidative stabilities of unsaturated triglycerides, it is therefore imperative that comparisons be made under the same conditions of oxidation and with the same methods of analyses.

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Incorporation of Linoleic Acid and Its Conversion to γ-Linolenic Acid in Fungi

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The incorporation of [1-^{14}C]linoleic acid (LA) into lipids of *Mortierella ramanniana* var. *angulispora* was studied to determine which lipid classes participated in the Δ6-desaturation of [1-^{14}C]LA. [1-^{14}C]LA was rapidly taken up into fungal cells and esterified into various lipids. Comparison of the profile of [1-^{14}C]LA incorporation between fungal cells at the exponential growth phase and the stationary growth phase showed that [1-^{14}C]LA incorporation into most lipids—except for triacylglycerol (TG) and phosphatidylcholine (PC)—were greatly reduced at the stationary growth phase. Desaturation of [1-^{14}C]LA into γ-linolenic acid (GLA) readily occurred at the exponential growth phase, but was greatly decreased at the stationary growth phase. Moreover, pulse-chase experiments revealed that the radiolabel incorporated into phosphatidylserine (PS) and PC rapidly turned over, while that in TG and diacylglycerol (DG) accumulated after the 4 hr chase. In addition to the change of the radiolabel in individual lipids, the content of radiolabeled GLA converted from [1-^{14}C]LA varied with individual lipids. In phospholipids such as PC, phosphatidylethanolamine (PE) and PS, radiolabeled GLA rapidly increased after 1 hr and then decreased after 4 hr. On the other hand, a gradual increase in radiolabeled GLA until 4 hr was observed in TG. These results suggest that LA, which has been esterified into phospholipids such as PC, PE and PS, is readily desaturated to GLA, which is then transferred to TG. These differences in the fate of GLA derived from LA between phospholipids and neutral lipids may be reflected in the GLA content in the individual lipids.
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Though fatty acid desaturation systems in eucaryotic microorganisms such as yeasts and fungi have been studied for a long time (1), the biosynthesis of polyunsaturated fatty acids is poorly understood. One of the fatty acid desaturases participating in the biosynthesis of polyunsaturated fatty acids, Δ6-desaturase, was purified to homogeneity from rat liver microsomes and it was shown that the purified Δ6-desaturase acted on the CoA derivative of linoleic acid (LA) to form γ-linolenic acid (GLA) (2). On the other hand, the desaturation of phospholipid-linked oleic acid has been reported to occur in yeasts (3,4), fungi (5), algae (6) and higher plants (7). Moreover, it has been suggested that glycolipid-linked LA is a substrate of Δ15-desaturase, which produces α-linolenic acid in plants (8,9). Thus, the desaturation systems which produce polyunsaturated fatty acids have been shown to use several types of fatty acid derivatives as substrates depending on the organism. Recently, we found that the filamentous fungus, *Mortierella* genus, proliferated well under high glucose concentrations and efficiently produced GLA (10,11). Thus, we tried to examine the synthesis of GLA from LA and the regulation of GLA synthesis. Since little is known about the Δ6-desaturation of LA to GLA in fungi, the comparison with Δ6-desaturase previously described in mammals is interesting.

In a previous paper (12), we described the effects of metal ions in culture media and of temperature on the GLA content in *Mortierella ramanniana* var. *angulispora*. The results suggested that these factors had different effects on the GLA content of polar lipids and neutral lipids in this fungus. However, the content of GLA was affected not only by the Δ6-desaturation, but also by the acylation or degradation of GLA. In the present paper we examine the incorporation of [1-^{14}C]LA into individual lipids and its conversion into GLA. The process of Δ6-desaturation is discussed from the distribution of ^{14}C-labeled GLA among various lipid classes.

MATERIALS AND METHODS

Materials. [1-^{14}C]stearic acid (59 mCi/mmol), [1-^{14}C]oleic acid (59 mCi/mmol) and [1-^{14}C]LA (59 mCi/mmol) were obtained from New England Nuclear Corporation (Boston, MA). Unlabeled stearic acid, oleic acid, LA and GLA were purchased from Sigma Chemical Co. (St Louis, MO). Silica gel G thin-layer chromatography (TLC) plates were obtained from Merck (Darmstadt, Federal Republic of Germany), and KC18 (reversed phase) TLC plates were acquired from Whatman (Maidstone, U.K.). All solvents were of reagent grade.

Microorganisms and culture conditions. Mortierella ramanniana var. *angulispora* (IFO 8187) was obtained from the culture collection of the Institute of Fermentation (Osaka, Japan). The fungi were maintained on a yeast-extract, malt-extract agar medium. The liquid medium contained glucose, inorganic salts and vitamins as described previously (12).

Incorporation of ^{14}C-labeled compounds into fungal lipids. One ml of fungal cell culture grown in rotary shakers (180 rpm) at 30°C for one day, when cells were at the exponential growth phase, were incubated with 3.4 μM (0.2 μCi/ml) [1-^{14}C]fatty acids at 30°C for 1–6 hr. In some experiments, fungal cells which were at the stationary growth phase and precultured for eight days, were incubated with ^{14}C-labeled compounds. After incubation, the fungal cells were cooled on ice and washed with 1 ml of 0.1 M phosphate buffer (pH 6.0) by centrifugation (1000 *g*, 5 min) to remove ^{14}C-labeled com-

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Abbreviations: DG, diacylglycerol; MG, monoacylglycerol; FFA, free fatty acid; GL, glycolipid; GLA, γ-linolenic acid; LA, linoleic acid; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SE, sterol ester; TG, triacylglycerol; TLC, thin-layer chromatography.

pounds not taken up into the fungal cells. For the pulse-chase experiments, fungal cells were incubated with [1-^{14}C]LA for 10 or 60 min, and then washed twice with a liquid medium containing 3.4 μM unlabeled LA. After the washing, these cells were suspended in the same medium and chased with 3.4 μM unlabeled LA, which was the same amount as labeled LA.

Extraction and analysis of lipids. Lipids were extracted from 1 ml of fungal cell suspension with 3 ml of chloroform/methanol (1:2,v/v). After 1 hr, 1 ml of chloroform and 1 ml of 0.1 M phosphate buffer were added. The upper aqueous layer was washed twice with 1 ml of chloroform, and the lower chloroform layers were collected and evaporated to dryness under N_2. The extracted lipids were analyzed for the distribution of radioactivity in individual fatty acids and individual neutral lipid classes and polar lipid classes. For fatty acid analysis, extracted lipids were transmethylated and the resultant fatty acid methyl esters were separated by reversed phase TLC on KC18 plates with acetonitrile/acetic acid (200:1, v/v). Neutral lipid classes were separated by TLC on Silica gel 60 plates with benzene/diethyl ether/ethanol/NH_3 (50:40:2:0.5, by vol) as the first solvent, and hexane/dimethyl ether (94:6, by vol) as the second solvent. Polar lipid classes were separated by TLC on Silica gel 60 plates with chloroform/acetone/methanol/acetic acid/H_2O (50:20:10:10:5, by vol). When necessary, two-dimensional TLC was performed for checking ^{14}C-labeled TLC fractions of polar lipids. Chloroform/methanol/H_2O (65:25:4, v/v/v) was used as the developing solvent in the second dimension. ^{14}C-Labeled spots were detected by autoradiography and scraped into scintillation vials. Radioactivity was determined with a Beckman liquid scintillation system (model LS1701) with automatic quenching correction. In some experiments, ^{14}C-labeled fractions of neutral or polar lipids detected by autoradiography were scraped off and extracted with chloroform/methanol (2:1, by vol). Then, the extracted neutral and polar lipid classes were transmethylated for analysis of their fatty acids. The mass fatty acid composition of various lipid classes was analyzed by gas liquid chromatography as described previously (12). For quantifying the amounts of the fatty acids in individual lipid classes, heptadecanoic acid was added as the internal standard. To confirm the identity of ^{14}C-labeled incubation products such as GLA, the position of the first double bond from the carboxyl end in 1-^{14}C-labeled products was determined using the permanganate-periodate procedure (13). The resultant ^{14}C-labeled dibasic acids were separated by TLC on Silica gel 60 plates with xylene/phenol/n-butanol/formic acid/water (70:30:10:8:2, by vol) (14). Since adipic acid which contained 6 carbon atoms, was obtained from a ^{14}C-labeled incubation product corresponding to GLA, GLA was shown to be synthesized from [1-^{14}C]LA and [1-^{14}C]oleic acid.

Other methods. The dry cell weight and total lipid content were measured by weight as described previously (12).

RESULTS

Incorporation of [1-^{14}C]*LA into fungal lipids.* [1-^{14}C]LA was incorporated into fungal lipids as shown in Figure 1. The incorporation profile of [1-^{14}C]LA was changed when fungal cells at different phases of growth were incubated with [1-^{14}C]LA. In fungal cells at the exponential growth phase, [1-^{14}C]LA was taken up into fungal cells so rapidly that the radioactivity of the free fatty acid (FFA) fraction was very high at zero time, and gradually decreased afterward. Corresponding to the decrease in [^{14}C]FFA, an increase in ^{14}C-incorporation into triacylglycerol (TG) occurred. On the other hand, ^{14}C-incorporation into phosphatidylcholine (PC) or phosphatidylethanolamine (PE) reached a plateau early in the incubation period and decreased at later incubation times. Incorporation of radioactivity into phosphatidylserine (PS) was the most transient, and it reached a plateau after 1 hr.

In fungal cells, at the stationary growth phase, the incorporation of [1-^{14}C]LA into most lipid classes was reduced in comparison with fungal cells at the exponential growth phase. However, the degree of reduction was different for each lipid class. Incorporation of radioactivity into TG or PC was not affected by the growth phase, though a difference in ^{14}C-incorporation into TG was found at longer incubation times.

In the same experiment, the distribution of [^{14}C]fatty acids in the total lipids is shown in Figure 2. In fungal cells, at the exponential growth phase, amounts of [^{14}C]GLA formed increased linearly for the first 4 hr, and reached a plateau after a 6-hr incubation period, whereas amounts of [1-^{14}C]LA reached a maximum after 2 hr, and gradually decreased afterward. The ratio of [^{14}C]GLA to the total ^{14}C-label taken up into the fungal cells reached 15–20% after 6 hr. In fungal cells at the stationary growth phase, the synthesis of radiolabeled GLA was greatly reduced (5% of total ^{14}C-label after 6 hr), even though the reduction of [^{14}C]LA was taken into consideration. Thus, it seemed that differences in ^{14}C-incorporation patterns between the fungal cells at two growth phases were correlated with the conversion of [1-^{14}C]LA into [1-^{14}C]GLA.

To further examine the pathway of [1-^{14}C]LA incorporation and its conversion, we performed a pulse-chase experiment in fungal cells at the exponential phase of growth. Figure 3 shows the ^{14}C-incorporation into various lipid classes after a 10-min or 60-min pulse. The amounts of radiolabel in TG, diacylglycerol (DG) and glycolipid (GL) increased after the chase, which meant that [^{14}C]LA or [^{14}C]GLA of these lipids had a tendency to be accumulated. On the other hand, radiolabels in phospholipids were more exchangeable. The amount of radiolabel in PS decreased more rapidly than that in other phospholipids, and this was similar to the decrease of radiolabel in FFA. The results obtained from the 10-min and 60-min pulse experiments showed a similar tendency, though the 60-min pulse made the amounts of individual lipid classes less interchangeable after the chase. The distribution of [^{14}C]fatty acids in the total lipids after the chase is shown in Figure 4. The amounts of radiolabel in GLA were increased greatly after the chase, while the amounts of [^{14}C]LA decreased after the chase. These results suggested that GLA produced from LA was likely to be stored and not further metabolized into other fatty acid derivatives.

Fatty acid composition of various lipid classes in

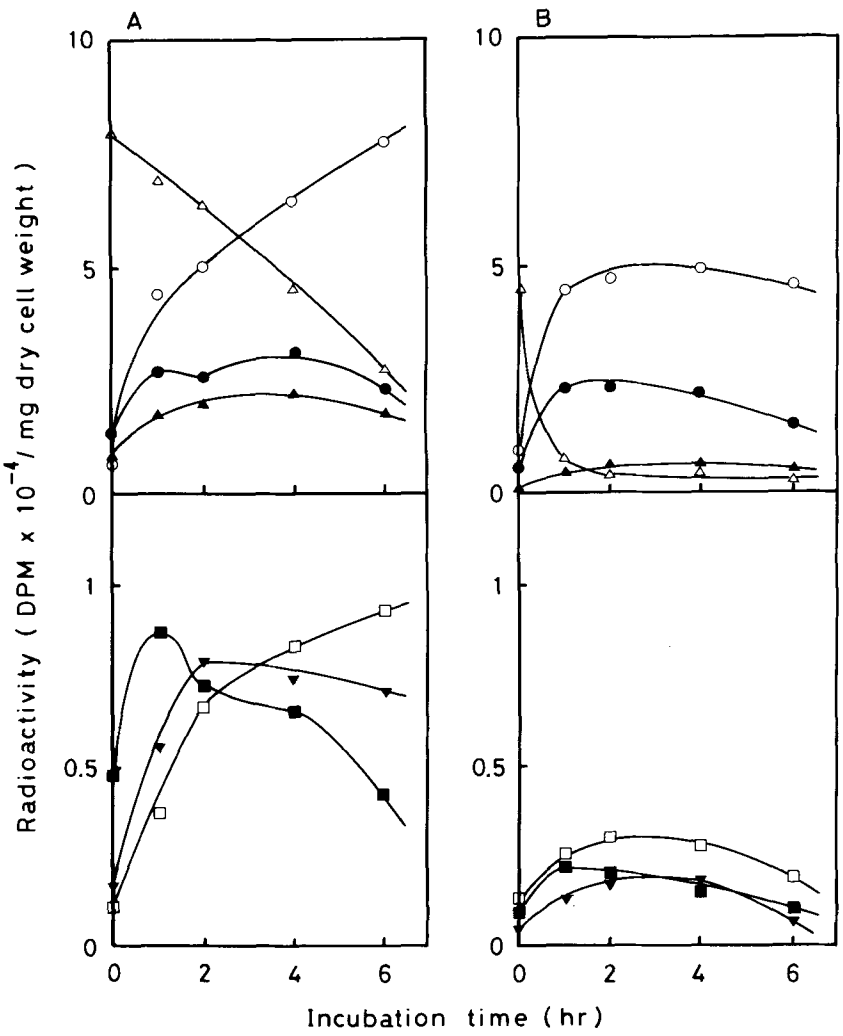

FIG. 1. Incorporation of [1-^{14}C]LA into fungal lipids. [1-^{14}C]LA was incubated with fungal cells at the exponential growth phase (A) [total lipids/dry cell weight (w/w) = 15%], or at the stationary growth phase (B) [total lipids/dry cell weight (w/w) = 50%]. [1-^{14}C]LA was incorporated into TG (○), FFA (△), DG (□), PC (●), PE (▲), PS (■) and GL (▼). The GL fraction was composed of a major unidentified glycolipid and several minor fractions. Radiolabeled PI, PA, SE and MG were also detected separately, but at lower levels. The incorporation of [1-^{14}C]LA at zero time may occur while the fungal cells were washed according to "Materials and Methods". When lipids were rapidly extracted by chloroform/methanol solution without washing at zero time, no [1-^{14}C]LA incorporation into individual lipids was observed. Values are means of duplicates for a typical experiment of several independent ones.

fungal cells at various growth phases. To determine the difference in the [1-^{14}C]LA incorporation into the fungal lipids at different growth phases, the fatty acid composition of various lipid classes in the fungal cells was analyzed. As shown in Table 1, some similarities in fatty acid compositions among the various lipid classes were observed. TG was the major fungal lipid, especially at longer culture time, and its fatty acid composition, which was similar to that of DG, was mostly unchanged during culture. FFA contained more saturated fatty acids, probably reflecting *de novo* fatty acid synthesis. Polar lipids were divided into two groups from the point of the fatty acid composition. One included PC and PE, which contained smaller amounts of palmitic acid and larger amounts of LA and GLA. The other included PS, PI and GL, which contained larger amounts of palmitic acid and relatively smaller amounts of LA and GLA. Though PS contained large amounts of GLA only in the one day culture, a decrease in the GLA content in PS was seen at longer culture time.

Incorporation of [1-^{14}C]stearic acid or [1-^{14}C]oleic acid into fungal lipids. The incorporation of fatty acids other than LA was also examined. Table 2 shows the conversions of incorporated [1-^{14}C]stearic acid, [1-^{14}C]oleic acid and [1-^{14}C]LA. Compared with stearic acid, which was mostly unchanged, oleic acid was actively converted to other fatty acids. It was notable

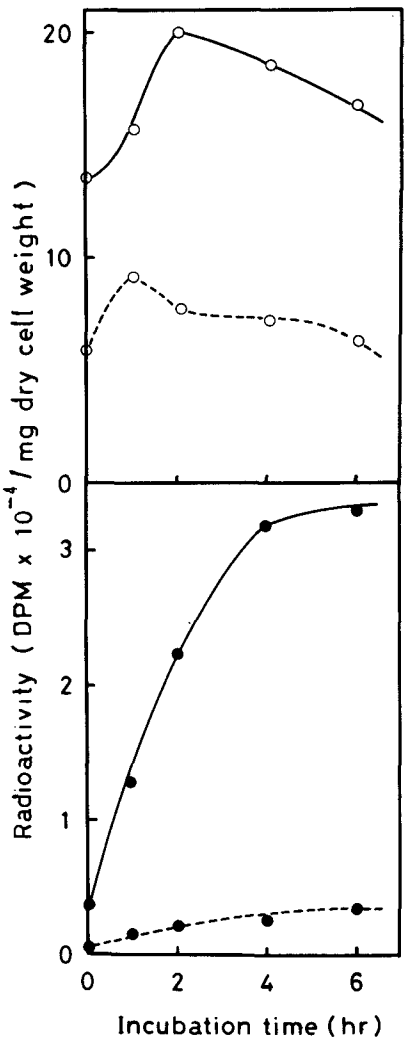

FIG. 2. Conversion of [1-^{14}C]LA into [1-^{14}C]GLA. After the incubation of [1-^{14}C]LA with fungal cells at the exponential phase of growth (——) or at the stationary phase of growth (----), the extracted lipids were transmethylated, and the amounts of radioactive LA (O) and GLA (●) were determined. Values are means of duplicates for a typical experiment of several independent ones.

that the desaturation of [1-^{14}C]oleic acid was sequential, that is, [^{14}C]GLA rather than [^{14}C]LA was accumulated after the [1-^{14}C]oleic acid incorporation.

Conversion of [1-^{14}C]LA to [1-^{14}C]GLA in fungal cells. To elucidate the role of substrates used in the conversion of LA to GLA, the distribution of [^{14}C]fatty acids in various lipid classes after the incorporation of [1-^{14}C]LA was examined. As shown in the lower panel of Figure 5, [^{14}C]GLA was present in the polar lipid fractions at the early incubation time. Moreover, the upper panel of Figure 5 shows that the ratio of [^{14}C]GLA to the incorporated radioactivity was high in the polar lipid fractions from the beginning of the incubation. Among the polar lipids, the content of [^{14}C]GLA in PS reached 60%. However, the amounts of [^{14}C]GLA in the polar lipid fractions, especially in PC and PS, decreased after 4 hr, when the amounts of [^{14}C]GLA in TG rapidly increased. Since the content of [^{14}C]GLA in TG also increased after 4 hr, the increase in [^{14}C]GLA in TG was likely to reflect transfer from the polar lipids, especially PC and PS. On the other hand, the content of [^{14}C]GLA in FFA was very low, and the amount of [^{14}C]GLA also remained unchanged. Thus, the desaturation process from LA to GLA probably involved fatty acid esterification into certain phospholipids.

DISCUSSION

The process of aerobic desaturation has been well analyzed, mainly through the studies of oleic acid desaturation (15). However, knowledge about the desaturation of polyunsaturated fatty acids still remains limited with respect to the desaturation mechanisms involved. Though the Δ6-desaturase, which catalyzed the synthesis of GLA from LA, was purified to homogeneity in mammalian cells, little was known about this enzyme in microorganisms or plants. Recently, the synthesis of GLA was examined in microsomes of plant seeds (16,17) and the desaturation process from LA to GLA in plants was found to be different from that of mammalian cells, i.e., the Δ6-desaturase purified from rat liver used linoleoyl-CoA as a substrate, while the plant Δ6-desaturase used as a substrate the linoleoyl moiety linked to phospholipid.

In the present study, we studied the incorporation of [1-^{14}C]LA into lipids of *Mortierella* genus to examine which lipid classes participated in Δ6-desaturation. First, we did a comparison of LA metabolism between fungal cells at the exponential growth phase and the stationary growth phase. In fungal cells at the stationary growth phase, the conversion of [1-^{14}C]LA into GLA was reduced compared with fungal cells at the exponential growth phase (Figure 2). The decrease of the conversion of [1-^{14}C]LA into GLA was accompanied by decreases in ^{14}C-incorporation into most lipid classes, but ^{14}C-incorporation into PC or TG was unchanged regardless of growth phase (Fig. 1). In pulse-chase experiments, radiolabels derived from [1-^{14}C]LA turned over at different rates depending on the lipid class (Fig. 3). The radioactive fatty acids in phospholipids turned over fast, whereas those in neutral lipids had a tendency to be more stable. Furthermore, Figure 5 shows that the radioactive GLA formed from LA first recurred in phospholipids, such as PC, PE and PS, and later accumulated in TG as it decreased in phospholipids.

In view of the results mentioned above, we postulated that the synthesis of radioactive GLA first occurred in the course of [1-^{14}C]LA incorporation into certain phospholipids, and that the synthesized radioactive GLA was then transferred into neutral lipids, mainly TG. As shown in Table 1, the mass content of GLA in phospholipids such as PC, PE and PS was high and varied during cell growth, while it was low in neutral lipids and remained almost constant. Thus, the difference in the metabolism of LA in phospholipids and neutral lipids may be reflected in the mass contents of GLA. These differences might also provide the clue to explain the different effects of metal ions and temperature on the GLA content of polar and neutral lipids as described previously (12).

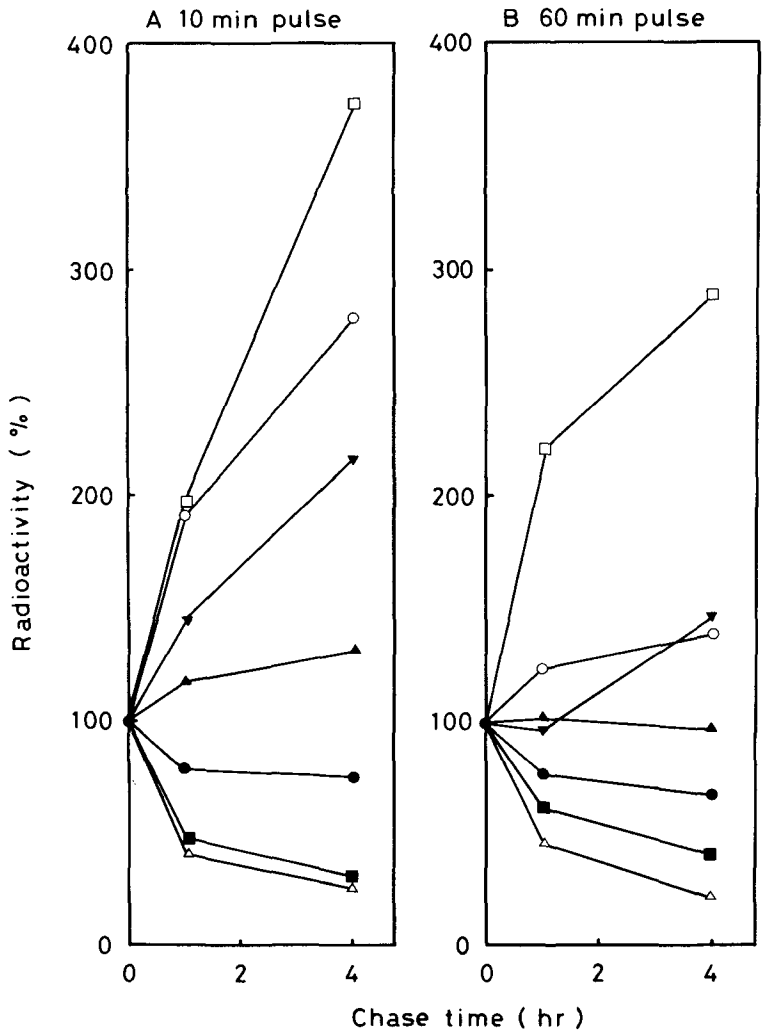

FIG. 3. Distribution of radioactivity in fungal lipids after pulse-chase with [1-^{14}C]LA. After 10-min pulse (A) or 60 min pulse (B) with [1-^{14}C]LA, incorporation of radioactivity into TG (○), FFA (△), DG (□), PC (●), PE (▲), PS (■) and GL (▼) was chased with unlabeled LA. Values are expressed as percentages of radioactivities incorporated into individual lipids during pulse (means of duplicates). The absolute values during the 10-min and 60-min pulse are in DPM × 10^{-3} of the fatty acid incorporated/mg dry cell weight, 10-min pulse: TG, 24.0; FFA, 78.8; DG, 1.0; PC, 30.0; PE, 11.8; PS, 10.1; GL, 1.4; and 60-min pulse: TG, 42.0; FFA, 41.2; DG, 1.4; PC, 29.9; PE, 15.2; PS, 6.3; and GL, 2.4.

Though formed GLA is found to exist preferentially in phospholipids in the early incubation time when GLA is actively formed by the Δ6-desaturase, it is still unclear which derivative is a substrate of the Δ6-desaturase, i.e., whether GLA formed as thioester derivative (either CoA or acyl carrier protein derivative) and is preferentially incorporated into phospholipids, or whether the Δ6-desaturase directly acts on phospholipid-linked LA to form GLA. The latter case has been reported for the Δ9-desaturase in some microorganisms and higher plants (3-7). The former case involves the regulatory systems which allow the desaturation products to be transacylated to specific lipids. In mammalian cells, the selective incorporation of specific fatty acids, such as eicosanoid precursor fatty acids into specific lipids, has been reported (18,19). Thus, it is possible that the specific acylation systems are involved in the LA and GLA incorporation into the specific lipids in this fungus. These fatty acids may be incorporated into phospholipids either by the deacylation-reacylation pathway (20) or by *de novo* synthesis, which would first form PA by acylation of glycerol-3-phosphate or 1-acyl-glycerol-3-phosphate (8). Since these acylation processes have been thought to be catalyzed by separate acyltransferases (21), the cooperation of these acyltransferases with the Δ6-desaturase may be responsible for the distribution of GLA among individual lipids.

Besides the LA incorporation, oleic acid seemed to be sequentially desaturated into GLA, which agrees with the results obtained from the plant seeds (16). Since the amounts of GLA formed from oleic acid were

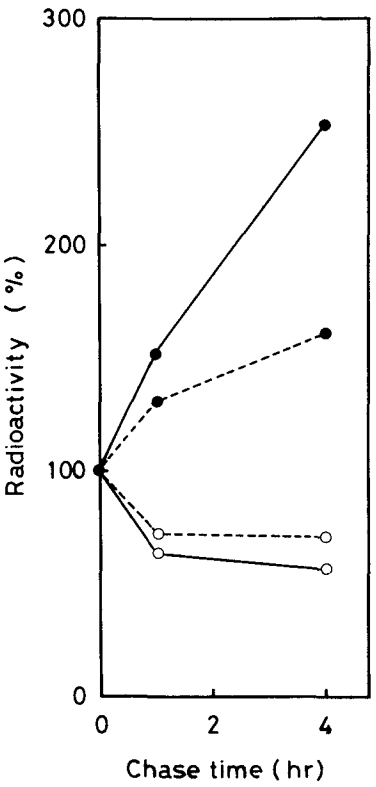

FIG. 4. Distribution of radioactivity in fatty acids after pulse-chase with [1-^{14}C]LA. After 10-min pulse (——) or 60-min pulse (----) with [1-^{14}C]LA, distribution of radioactivity in LA (○) or GLA (●) was chased with unlabeled LA. Values are expressed as percentages of radioactivities distributed after pulse (means of duplicates). The absolute values incorporated during the 10-min and 60-min pulse are in DPM × 10^{-3} of the fatty acid incorporated/mg dry cell weight, 10-min pulse: LA, 151.1; GLA, 12.4; and 60-min pulse: LA, 86.6; GLA, 20.5.

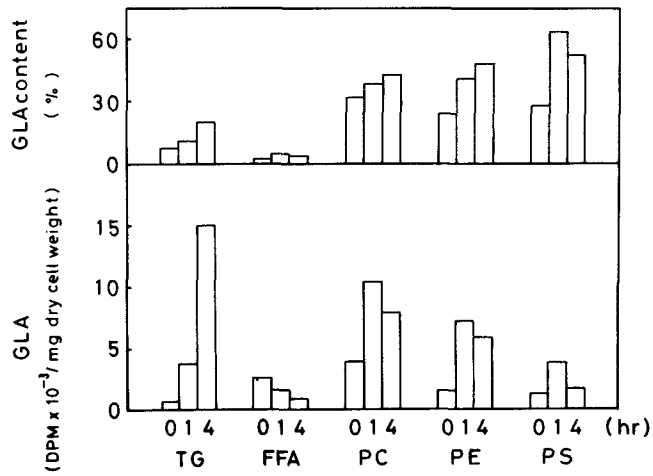

FIG. 5. Distribution of [^{14}C]GLA derived from [1-^{14}C]LA in major lipid classes. The distribution of [^{14}C]GLA at zero time was considered to be due to the same reason as mentioned in Figure 1. GLA content in upper column represents [^{14}C]GLA/[^{14}C]LA + [^{14}C]GLA. Values are means of triplicates.

almost equal to the amounts of GLA formed from LA, the possibility that LA formed from oleic acid enter pools different from those of LA taken up from culture must be considered.

Among filamentous fungi, several studies on the desaturation of unsaturated fatty acids have been reported in *Neurospora crassa* (22, 23), although the molecular nature of the desaturases still remains unclear.

TABLE 1

Fatty Acid Composition of Major Lipid Classes in *Mortierella ramanniana* var. *angulispora*[a]

Culture time (day)	Lipids	Acyl content (nmol/mg dry cell weight)	Fatty acid composition (%)				
			16:0	18:0	18:1	18:2	18:3[b]
1	TG	251.2	30.4	8.6	46.3	7.1	7.6
	DG	11.4	34.0	9.9	41.7	8.9	5.6
	SE	8.1	20.6	1.7	42.9	10.2	12.6
	FFA	4.3	42.1	17.1	23.0	1.9	4.4
	PC	26.5	19.9	2.6	17.0	27.1	27.6
	PE	37.1	16.7	1.8	32.9	18.3	27.7
	PS	9.3	43.7	2.9	15.3	11.5	24.1
	GL	10.1	34.5	2.5	30.6	19.6	6.4
	PI	4.4	47.0	2.0	17.6	21.1	7.5
8	TG	1034.3	27.9	4.8	51.7	9.2	6.0
	DG	62.3	30.4	6.2	49.4	9.1	5.3
	SE	13.0	15.4	2.6	39.5	22.8	10.7
	FFA	4.8	28.4	20.7	30.3	3.9	5.7
	PC	13.6	6.1	0.9	51.0	26.3	11.1
	PE	14.9	11.2	1.0	52.4	14.8	18.1
	PS	3.5	37.2	4.0	40.6	5.0	5.3
	GL	6.9	38.4	1.6	41.8	9.2	1.3
	PI	5.6	40.5	2.6	39.7	8.9	3.4

[a]Values are means of triplicates.
[b]GLA.

TABLE 2

Incorporation and Conversion of Other [1-^{14}C]Fatty Acids[a]

Incorporated fatty acid	Time (hr)	[^{14}C]fatty acid (%)				Total [^{14}C] (DPM × 10^{-4})
		18:0	18:1	18:2	18:3[b]	
[1-^{14}C]stearic acid	0	97.0	1.5	1.0	0.5	18.0
	1	94.7	2.8	1.5	0.7	23.5
	4	87.9	6.8	3.1	2.2	23.6
[1-^{14}C]oleic acid	0	2.5	89.7	1.3	6.5	8.8
	1	3.8	78.7	3.2	14.2	22.1
	4	3.5	72.7	7.9	15.9	23.5
[1-^{14}C]linoleic acid	0	0.2	1.0	96.3	2.6	14.1
	1	0.4	1.4	90.8	7.4	17.3
	4	1.0	2.0	82.7	14.3	22.3

[a]Values are means of duplicates. [1-^{14}C]fatty acids were incubated with fungal cells at the exponential growth phase.
[b]GLA.

Mortierella genus, which synthesizes large amounts of GLA instead of α-linolenic acid found in *Neurospora crassa* can be expected to have an active Δ6-desaturase system, although no biochemical or genetic insights have been gained so far. In addition to GLA, some species of the *Mortierella* genus produced large amounts of arachidonic acid (24,25) and eicosapentaenoic acid (26). Thus, the desaturation systems for producing polyunsaturated fatty acids in the *Mortierella* genus are of great interest. The results obtained in *Mortierella* genus suggest that LA, which is incorporated into some phospholipids, such as PS and PE, has a tendency to be desaturated into GLA, and the GLA produced is gradually accumulated mainly in TG from phospholipids. Since this fungus is used in the production of lipids (10), these findings on the role of individual lipids in the Δ6-desaturation may provide a strategy to improve the fatty acid composition of this fungus.

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The Distribution and Phylogenetic Significance of Desmethylsterols in *Chenopodium* and *Atriplex*: Coexistence of Δ^7 - and Δ^5 -Sterols

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Twenty-one species in the Chenopodiaceae were analyzed for sterol composition. In ten of eleven species of *Chenopodium*, the major desmethylsterols were Δ^7-sterols accompanied by lower proportions of Δ^5-sterols. In *C. fremontii* this pattern was reversed. The sterol profiles of five species of *Atriplex* were characterized by the coexistence of Δ^7- and Δ^5-sterols in ratios of 0.3:1 to 0.4:1. Male *Atriplex* plants contained higher proportions of Δ^5-sterols than female *Atriplex* plants. One *Ceratoides* and two *Salicornia* species contained Δ^5-sterols as their predominant sterols.
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Sterol distribution and biogenesis are chemotaxonomically significant in the algae (1) and fungi (2), as well as in lower tracheophytes (3). Two aspects of sterol structure have received particular attention among chemotaxonomists. First, the alkyl group usually present at C-24 may be α or β oriented. There are also three common types of unsaturation in ring B of the sterol nucleus. Most higher plants investigated contain 24α-alkyl sterols with 5(6) -unsaturation (4) as the dominant sterol, but there is increasing evidence that some angiosperms, including species in the Theaceae (5), Cucurbitaceae (6) and Sapotaceae (7) contain spinasterol, a 24 α-ethyl sterol with 7(8)-unsaturation, as the dominant sterol. The common occurrence of Δ^7-sterols in these three families suggests that sterol composition is chemotaxonomically significant at the family level. Δ^7-Sterols have also been reported in *Medicago sativa* (Leguminosae) (8), *Aesculus hippocastanum* (Hippocastanaceae) (9), *Lophocereus schottii* (Cactaceae) (10) and *Hacquetia epipactis* (Apiaceae) (11). Overall, these plants are considered relatively distant phylogenetically.

Since 1980, extensive sterol compositional data have been compiled for members of the Caryophyllales. Spinasterol and 7-stigmastenol have been identified as major sterols in *Phytolacca esculenta* (12), *Beta vulgaris* (13) and *Amaranthus* (14). Salt and Adler recently found that many of 13 Chenopodiaceae (15) and 13 Caryophyllaceae (16) are plants producing Δ^7-sterols as their dominant sterols. In previous work from this laboratory (17), sterol compositions of 40 species in the Caryophyllidae were determined. Nine out of twelve families in the order Caryophyllales included species accumulating Δ^7-sterols. However, most Cactaceae and some Portulacaceae lacked Δ^7-sterols, and only Δ^5-sterols were detected in the Polygonaceae (18). Further investigations are required to determine to what extent sterol composition varies within the families of the Caryophyllales.

In this study, a number of species belonging to the Chenopodiaceae are examined for sterol composition. The distribution of sterols in *Chenopodium* and *Atriplex* are examined in particular depth, as possible models of sterol distribution at the genus level in higher plants.

EXPERIMENTAL PROCEDURES

Atriplex hymenelytra (Torr.) Wats., *A. canescens* (Pursh) Nutt. and *A. polycarpa* (Torr.) Wats. were field collected by Duffie Clemons in San Diego Co., CA in the fall of 1986 and provided to the authors by Jack L. Reveal, San Diego, CA. *Atriplex semibaccata* R. Br., *A. patula* L., *A. lentiformis* Hall and Clements, *A. watsonii* Nelson, *Salicornia subterminalis* Parish, *S. virginica* L. and *Ceratoides lanata* (Pursh) J.T. Howell were field collected by Reveal in the fall of 1986 in southern California and generously donated to the authors. The remaining 12 *Chenopodium* species, *Chenopodium berlandieri* ssp. *nuttalliae* (Safford) Wilson and Heiser, *C. berlandieri* ssp. *berlandieri* Aellen, *C. quinoa* ssp. *quinoa*, *C. petiolare* H.B.K., *C. fremontii* S. Wats., *C. standleyanum* Aellen, *C. album* L., *C. gigantospermum* Aellen, *C. ambrosioides* L., *C. oahuense* Aellen, *C. graveolens* Lag. and Rodr. and *C. sanctae-clare* Johow. were herbarium specimens acquired through the generosity of Dr. Hugh D. Wilson, Department of Biology, Texas A & M Universtiy, College Station, TX. The voucher specimens were deposited in the herbarium of that institution.

Freshly collected plant material (mature photosynthetic tissue) was washed with tap water, cut into pieces and oven-dried for 48 hr at 85°C. Dry material was ground to pass a 20-mesh screen in a knife mill.

Dried samples (20 g fresh plant, 0.3 g herbarium specimen) were extracted with $CHCl_3$/MeOH (2:1, v/v) using a Soxhlet apparatus. The crude lipid extract was saponified with 20% KOH in 60% EtOH and the unsaponifiable lipids were partitioned into ether using a liquid-liquid extraction apparatus.

The free sterols were purified by alumina column chromatography (19). Separation of 4-desmethylsterols from 4-dimethylsterols was achieved on aluminum plates coated with 0.2 mm Silica Gel 60F in a solvent system of hexane/Et_2O/HOAc (60:39:1). The plate was air dried and sprayed with Rhodamine 6-G. Sterols were visualized under UV radiation, and recovered with Et_2O/MeOH (9:1).

Sterol identification. Sterols were identified and quantitated by gas-liquid chronatography (GLC) equipped with an FID detector and an integrating data system. The GLC analysis was performed on both capillary SP-2330 and SPB-1 columns. For a few samples, GLC capillary SE-30 and packed SE-30 columns were also utilized. The behavior of these TMSi-sterols on GLC capillary SE-30 column were reported previously (14). For preparation of TMSi-sterol derivatives, 25 μl pyridine, 10 μl Sylon BFT (Supelco, Bellefonte, PA) and 10 μg sterols were combined in a microreaction vessel for 30 min at 70°C. 100 μl Petroleum ether was added to the vessel and the solvent

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was evaporated under N_2. The samples were redissolved in iso-octane for GLC.

GLC/Mass spectral analyses were conducted on representative samples using a Finnigan-MAT Model 4500 spectrometer equipped with an Incos Data System and a DB-1 capillary GLC column.

RESULTS AND DISCUSSION

The trivial sterol names used in this paper (sitosterol, campesterol, etc.) imply a certain configuration at C-24. Specific methods to determine configuration at C-24 were not performed in this work, although the great majority of higher plant sterols are 24α-alkyl. It would be reasonable to expect the 24α-alkyl configuration in all sterols found here except brassicasterol, in which case the trivial names used would be correct. Ergosta-5,22-dienol, when found in higher plants, has the 24β-alkyl configuration, however, and is called brassicasterol. The isolated sterols were identified by their gas chromatographic characteristics on SPB-1, SP2330 and DB-1 capillary columns as well as by their mass spectral characteristics, which were in agreement with previously published values (14). Analyses of 7 *Atriplex* spp., 11 *Chenopodium* spp., 2 *Salicornia* spp. and *Ceratoides lanata* revealed the presence of at least 16 different sterols, most of which contained the Δ^5 or Δ^7 double bond (Table 1). From these data, four groups could be established based on the ratio of Δ^5-sterols to Δ^7-sterols in the individual sterol pofiles (Table 2). While many of the species also contained stanols from trace amounts (<1.0%) to 8% of the desmethylsterols, *Salicornia virginica* contained 36% stigmastanol and 4% 22-stigmastenol (Table 1).

Group 1: Δ^5 Species: ($\Delta^5 \geq 80\%$). Two *Salicornia* species (*S. virginica* and *S. subterminalis*) and *Ceratoides lanata* belong to this group. Exclusive of the high stanol content in *S. virginica* (Table 2), the proportion of Δ^5-sterols was greater than 90% of the combined Δ^5- and Δ^7-sterols. The major sterols produced by species in this group were sitosterol, stigmasterol and campesterol. All three species accumulated stanols, but the two *Salicornia* differed appreciably in the quantity of stanol. *S. vir-*

TABLE 1

Desmethylsterol Composition of Chenopodiaceae spp. (Numbers Represent Percent of Total Desmethylsterols)

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Salicornia virginica	—	—	—	—	21	4	35	—	36	—	—	—	—	4	—	—
S. subterminalis	2	—	—	2	24	—	58	—	7	—	—	—	—	6	—	1
Ceratoides lanata	6	12	7	14	10	—	38	3	—	2	—	3	2	1	—	2
Atriplex hymenelytra	—	—	—	—	5	2	2	—	—	—	—	3	45	27	12	4
A. semibaccata	—	—	—	t	5	1	2	—	t	—	—	1	11	44	34	1
A. watsonii (Female)	1	—	25	1	7	—	22	—	—	—	—	1	27	2	12	2
A. watsonii (male)	2	—	20	2	13	—	33	—	—	—	—	t	16	1	12	—
A. canescens	8	—	8	2	10	—	40	—	—	—	-	3	4	4	21	—
A. polycarpa (female)	6	—	3	6	10	1	43	—	—	—	—	2	1	4	24	—
A. polycarpa (male)	7	—	4	8	19	—	44	—	—	—	—	—	—	4	14	—
A. patula	2	—	6	—	11	—	46	—	—	—	—	—	9	7	19	—
A lentiformis	1	—	6	t	7	t	43	—	—	—	—	1	4	2	34	1
Genus: *Chenopodium*																
Section: *Chenopodia*																
C. berlandieri ssp. *nuttalliae*	1	t	—	3	14	—	16	2	3	4	1	2	t	23	29	1
C. quinoa ssp. *quinoa*	1	—	—	2	8	—	14	—	—	7	1	3	3	19	39	3
C. berlandieri ssp. *berlandieri*	2	—	—	2	12	—	24	—	4	3	—	1	3	15	34	—
C. petiolare	3	—	—	2	9	—	26	—	—	3	—	2	—	28	27	—
C. fremontii	8	1	—	3	8	—	42	1	7	2	t	2	1	4	19	1
C. standleyanum	5	—	—	4	11	—	21	—	7	6	—	3	—	12	29	2
C. album	5	—	—	3	10	—	20	—	3	12	1	3	3	18	21	1
C. oahuense	3	—	—	3	15	—	28	1	1	7	1	—	4	20	17	—
G. gigantospermum	6	—	—	4	13	—	16	—	—	4	—	3	—	35	19	—
Section: *Ambrina*																
C. ambrosioides	2	—	—	—	3	—	10	1	3	3	2	—	10	55	11	—
Section: *Botryoides*																
C. graveolens	1	—	—	1	5	—	6	1	2	—	—	1	t	54	26	2
Section: *Scottsbergia*																
C. sanctae-clare	4	—	—	—	8	—	13	—	—	1	—	—	8	24	42	—

1: cholesterol.
2: brassicasterol.
3: isofucosterol.
4: campesterol.
5: stigmasterol
6: 22-stigmastenol.
7: sitosterol.
8: campestanol.
9: stigmastanol.
10: cholest-7-enol.
11: 7,24-ergostadienol.
12: 7-ergostenol.
13: 7,24(28)-stigmastadienol
14: 7,22-stigmastadienol (spinasterol).
15: 7-stigmastenol.
16: 7,25-stigmastadienol.
t = trace amount (<%).

TABLE 2

Sterol Profiles of 22 Chenopodiaceae Species with Respect to the Ratio of Δ^7 to Δ^5 Double Bonds

Species	Ratio of Δ^7 : Δ^5	
Group 1: (Δ^5>80%)		
Salicornia virginica	7	93
S. subterminalis	8	92
Ceratoides lanata	10	90
Group 2: (80%>Δ^5>50%)		
Atriplex watsonii (female)	44	56
A. watsonii (male)	29	70
A. canescens	32	68
A. polycarpa (female)	31	69
A. polycarpa (male)	18	82
A. patula	35	65
A. lentiformis	42	57
Chenopodium fremontii	32	68
Group 3: (80%>Δ^7>50%)		
Chenopodium berlandieri ssp. *nuttalliae*	64	36
C. berlandieri ssp. *berlandieri*	58	42
C. quinoa ssp. *quinoa*	75	25
C. petiolare	60	40
C. standleyanum	56	44
C. album	61	39
C. oahuense	50	50
C. gigantospermum	61	39
C. sanctae-clare	75	25
Group 4: (Δ^7>80%)		
Atriplex hymenelytra	93	7
A. semibaccata	93	7
Chenopodium ambrosioides	84	16
C. graveolens	86	14

ginica contained stigmastanol (36% of the total desmethylsterol) while *S. subterminalis* accumulated only 7% stigmastanol (Table 1). *Ceratoides lanata* contained a substantial amount of brassicasterol (12%) and was the only plant to contain over 1% brassicasterol. It would be of interest to know if this elevated brassicasterol content were characteristic of the genus.

Group 2: $\Delta^{5/7}$ Species: 80%>Δ^5>50%. Plants in this category also produced sterol mixtures with higher proportions of Δ^5-sterols than Δ^7-sterols. However, in these cases the proportion of Δ^7-sterols exceed 18% of the combined Δ^5- and Δ^7-sterols. In addition to 7-stigmastenol and 7,24(28)-stigmastadienol, plants in this group contained spinasterol, the Δ^7 isomer of stigmasterol. Of the nine *Atriplex* samples tested, seven belonged to Group 2. In male *A. polycarpa*, Δ^5-sterols made up 82% of the total desmethylsterol. In the other six *Atriplex* samples — *A. watsonii* (male and female), *A. canescens*, *A. polycarpa* (female), *A. patula* and *A. lentiformis* — the proportions of Δ^5-sterols ranged from 56% to 70%. They were accompanied by proportions of Δ^7-sterols from 29% to 44%. The ratios of Δ^7-sterols to Δ^5-sterols varied from 0.3 to 1 to 0.4 to 1. Among Δ^5-sterols, cholesterol (1-8%), stigmasterol (7-9%), sitosterol (22-46%) and isofucosterol (3-25%) were identified in all seven samples. Spinasterol (1-7%) and 7-stigmastenol (12-34%) were the two Δ^7-sterols present in all seven samples tested. *Atriplex watsonii* differs from other *Atriplex* spp. by accumulating a very high proportion of isofucosterol (20-25%), and its Δ^7 analog, 7,24(28)-stigmastadienol (16-27%). In the other species, the proportions of these two sterols averaged 5% and 4%, respectively.

Some *Atriplex* species are monoecious, while others are dioecious. In order to determine how this influences the overall sterol profile, separate female and male samples were collected for *A. watsonii* and *A. polycarpa* (Table 2). In both cases, male plants produced higher proportions of Δ^5-sterols than female plants (70% vs. 56%, 82% vs. 68%). The possibility that Δ^7-sterols and Δ^5-sterols have different reproductive roles is worth further investigation. Finally, only one *Chenopodium* species examined, *C. fremontii*, produced Δ^5-sterols in larger quantities than Δ^7-sterols. This sterol profile was confirmed in two additional samples of *C. fremontii*. The major sterols identified in *C. fremontii* were sitosterol (42%), 7-stigmastenol (19%), cholesterol (8%), stigmasterol (8%), and stigmastanol (7%).

Group 3: Δ^7/Δ^5 species (80%>Δ^7>50%.) This group of plants had sterol profiles similar to those of Group 2, but accumulated a higher proportion of Δ^7-sterols (>50%) than Δ^5-sterols (<50%). In some species, the amounts of Δ^7-sterols and Δ^5-sterols were nearly equal.

Nine *Chenopodium* species (*C. berlandieri* ssp. *nuttalliae*, *C. quinoa* ssp. *quinoa*, *C. berlandieri* ssp. *berlandieri*, *C. petiolare*, *C. standleyanum*, *C. album*, *C. oahuense*, *C. gigantospermum* and *C. sanctae-clare*) are included in Group 3.

Group 4: Δ^7 species (Δ^7>80%). Both *A. hymenelytra* and *A. semibaccata* accumulated Δ^7-sterols (91%) as their predominant sterols and were assigned to Group 4. Spinasterol (27%, 44%), 7-stigmastenol (12%, 34%), and 7,24(28)-stigmastadienol (45%, 11%), were among Δ^7-sterols identified. The percentage of Δ^5-sterol was 7% in both species. Although each species contained a Δ^7-sterol as its major sterol, *A. semibaccata* contained a much higher proportion of 7-stigmastenol (34%) and a lower proportion of 7,24(28)-stigmastadienol (11%) than *A. hymenelytra* (12%, 45%). Spinasterol (55%) was present in *C. ambrosioides* in the highest proportion found in the genus *Chenopodium*, followed by 7-stigmastenol (11%), sitosterol (10%), 7,24(28)-stigmastadienol (10%) and few other minor sterols.

In Table 3, the *Chenopodium* species are arranged in sections following the scheme of Aellen and Just (22). The sterol compositions obtained from this work are incorporated along with data reported by several investigators (13,15,21,23) on other Chenopodiaceae. All species of *Chenopodium* tested produce Δ^7- and Δ^5-sterol mixtures. Some species contained very high proportions of Δ^7-sterols. The sole exception is based on a report by Mata *et al.* (21), that *C. graveolens*, which belongs to section *Botryoides* according to Aellen and Just (22), contains stigmasterol (0.003% of dry wt.), 22-stigmastenol (0.0025%) and sitosterol-glucoside (0.0036%). These identifications were based mainly on melting points and unspecified methods. In this work the sterol composition of *C. graveolens* was reinvestigated and the major sterols were clearly spinasterol and 7-stigmastenol with small amounts of other Δ^5- and Δ^7-sterols.

The family Chenopodiaceae is a highly diversified family. Δ^5 and Δ^7 mixtures seem almost universal, but

TABLE 3

Comparison of Sterol Profiles of Chenopodiaceae with Cronquist's (20) and Aellen and Just's Schemes (21)

Chenopodiaceae	Sterol profile[a]		Ref.
Subfamily: Chenopodioideae			
Genus: *Chenopodium* Section: *Chenopodia*			
Subsection: *Cellulata*			
Chenopodium berlandieri ssp. *berlandieri*	7/5	(56%/40%)	
Chenopodium berlandieri ssp. *nuttalliae*	7/5	(60%/34%)	
Chenopodium quinoa ssp. *quinoa*	7/5	(75%/25%)	
Subsection: *Leiosperma*			
Chenopodium petiolare	7/5	(60%/40%)	
Chenopodium fremontii	5/7	(68%/32%)	
Chenopodium standleyanum	7/5	(56%/44%)	
Chenopodium album	7/5	(61%/39%)	
Chenopodium album	7/5	(53%/45%)	15
Chenopodium album	7/5	(60%/40%)	17
Chenopodium oahuense	7/5	(50%/50%)	
Subsection: *Grossefoveata*			
Chenopodium gigantospermum	7/5	(61%/39%)	
Section: *Scottsbergia*			
Chenopodium sanctae-clare	7/5	(75%/25%)	
Section: *Ambrina*			
Chenopodium ambrosioides	7		15
Chenopodium ambrosioides	7		21
Section: *Botryoides* Subsection: *Botrys*			
Chenopodium graveolens	7	(86%/14%)	
Spinacea oleracea	7		17
Beta vulgaris	7/5	(73%/27%)	17
Beta vulgaris	7/5	(75%/25%)	13
Salicornia virginica	5		
Salicornia subterminalis	5		
Salicornia europea	7/5	(56%/44%)	15
Salicornia bigelovii	5/7	(51%/41%)	15
Bassia hirsuta	5		15
Kochia scoparia	5		15
Atriplex hymenelytra	7		
Atriplex semibaccata	7		
Atriplex watsonii (F)	5/7	(56%/43%)	
Atriplex watsonii (M)	5/7	(70%/30%)	
Atriplex canescens	5/7	(69%/31%)	
Atriplex polycarpa (F)	5/7	(69%/30%)	
Atriplex polycarpa (M)	5/7	(82%/18%)	
Atriplex patula	5/7	(66%/34%)	
Atriplex lentiformis	5/7	(59%/41%)	
Subfamily: Salisoloideae			
Ceratoides lanata	5		15
Salsola kali	5		15
Suaeda linearis	5		15

[a]7 = 80%<Δ^7; 7/5 = 50%<Δ^7<80%; 5/7 = 50%<Δ^5<80%; 5 = 80%<Δ^5.

proportions of the two sterol types vary widely. For example, all three species of Salisoloideae studied contain Δ^5-sterols almost exclusively (15). Subfamily Chenopodioideae also includes two Δ^5 species, *Bassia hirsuta* and *Kochia scoparia* (15), in addition to those examined here. At the opposite end of the spectrum, an early investigator (23) reported that *Spinacea oleracea* produces Δ^7-sterols almost exclusively, although several laboratories have recently shown it does contain small amounts of Δ^5-sterols (17, 24). In this study, we have confirmed the wide variation of sterol profiles in the Chenopodiaceae. Large differences may occur even between specifies of the same genus. For example, two *Salicornia* species tested in this work were primarily Δ^5 species, while two other species examined by Salt and Adler (15) contained nearly equal proportions of Δ^5- and Δ^7-sterols. The genus *Atriplex* generally accumulated a mixture of Δ^5- and Δ^7-sterols, with a higher proportion of Δ^5-sterols. *Chenopodium* species typically contained more Δ^7- than Δ^5-sterols.

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COMMUNICATIONS

Binding of Galactosylsphingosine (Psychosine) by Albumin

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On applying [^{3}H]galactosylsphingosine (psychosine) and albumin to a gel filtration column, either as a pre-incubated mixture or as separate solutions, the lipid and albumin co-eluted. When albumin and [^{3}H]galactosylsphingosine were incubated with Sephadex G-50, the concentration of galactosylsphingosine outside the gel increased as the concentration of albumin increased. These observations indicate that albumin binds galactosylsphingosine. By using an equilibrium gel procedure, it was estimated that one molecule of albumin has 7.4 binding sites for galactosylsphingosine, and that the dissociation constant is 3.9×10^{-6} M. These values appear compatible with the potency of albumin to suppress galactosylsphingosine-induced hemolysis.
Lipids 25, 65–68 (1990).

Galactosylsphingosine (psychosine) is enzymatically synthesized from UDP-galactose and sphingosine (1), and degradated by galactosylceramidase (2). In Krabbe disease (globoid cell leukodystrophy), galactosylceramidase is genetically deficient (3,4), and galactosylsphingosine accumulates in tissues, especially in the nervous system (5,6).

Since galactosylsphingosine is highly cytotoxic, the effects of the accumulated lipid are considered the basis for the devastating pathology in Krabbe disease (3,5,6). *In vitro,* galactosylsphingosine has a powerful inhibitory effect on cytochrome c oxidase in mitochondria (7), and it also causes hemolysis of washed erythrocytes (8,9). However, while γ-globulin has no such effect, albumin can completely abolish either the inhibition of cytochrome c oxidase or the hemolysis (9), thus implying a strong association between albumin and galactosylsphingosine. Furthermore, our ultrafiltration study suggested that albumin might bind galactosylsphingosine (9). We therefore examined further the interaction of albumin with galactosylsphingosine.

METHODS

Materials. Unlabelled galactosylsphingosine, galactosylceramide (for preparation of labelled galactosylsphingosine), albumin (from human serum, essentially fatty acid free) and γ-globulin (from human serum) were obtained from Sigma (St. Louis, MO). Pre-packed gel filtration column (Econo-Pac 10DG) and the dye for protein measurement were purchased from Nippon Bio-Rad (Tokyo, Japan). Sephadex G-50 (coarse) was from Pharmacia KK (Tokyo, Japan). Sodium [^{3}H]borohydride and galactose oxidase were from NEN Research Products (Boston, MA) and from Worthington (Freehold, NJ), respectively. [^{3}H]Galactosylsphingosine was prepared as described (2). Scintillation mixture (Scintisol EX-H) was purchased from Dojin (Kumamoto, Japan).

Gel-filtration. The pre-packed column (Econo-Pac 10DG) was equilibriated with 20 ml of 30 mM Tris-HCl (pH 7.4) containing 120 mM NaCl, 5 mM KCl and 2 mM $MgCl_2$. Elution was done with the same buffer, and fractions (1.0 ml) were collected. Radioactivity was measured in a Beckman LS-7000 liquid scintillation counter after 12.5 ml of the scintillation mixture was added to 0.4 ml of the fraction. Protein was determined by the Bradford method (10); after 0.02 or 0.01 ml of the fraction was mixed with 2 ml of water and 0.5 ml of the dye, the absorbance at 595 nm was measured.

Equilibrium gel procedure. Sephadex G-50 was washed and dried as described (11). To 0.1 g of the gel in a glass test tube (13 × 100 mm), 0.8–0.95 ml of the buffer was added and left overnight. An appropriate amount of the buffer containing [^{3}H]galactosylsphingosine and the buffer with or without protein (albumin or γ-globulin) were added making the total volume 1.1 ml. The tubes were incubated for 60 min with or without gentle shaking. During the incubation, each tube was agitated once by a vortex mixer. The mixture outside the gel (0.01–0.075 ml) was used for protein and radioactivity measurements, which were done as described above. Protein was measured in duplicate, and the radioactivity in triplicate. Bound and free ligands were calculated as described (11), and the number of binding sites and dissociation constant were obtained from Klotz plots (12).

Galactosylsphingosine-induced hemolysis and effects of albumin. Heparinized blood was obtained from a healthy male. After plasma and buffy coat were removed, the erythrocytes were washed 5 times with 30 mM Tris-HCl (pH 7.4) containing 120 mM NaCl, 5 mM KCl and 2 mM $MgCl_2$ (13). The erythrocytes were suspended in the buffer to make hematocrit 5%. The hemolytic concentration of galactosylsphingosine depends on the hematocrit (Igisu, unpublished). All other procedures were done as described previously (9).

RESULTS

Gel-filtration. When the mixture of [^{3}H]galactosylsphingosine and γ-globulin or albumin was incubated for 60 min and applied to the gel-filtration column, the peaks of galactosylsphingosine and γ-globulin were clearly separated (Fig. 1A) but the lipid and albumin co-eluted (Fig. 1B). On the other hand, when a large amount of galactosylsphingosine was applied to the column, a broad elution profile of the lipid was obtained. Addition of the buffer (without protein or with γ-globulin) to the column

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Abbreviations: Da, Dalton; KCl, potassium chloride; $MgCl_2$, magnesium chloride; NaCl, sodium chloride; Tris-HCl, tris(hydroxymethyl) aminomethane hydrochloric acid; UDP, uridine diphosphate.

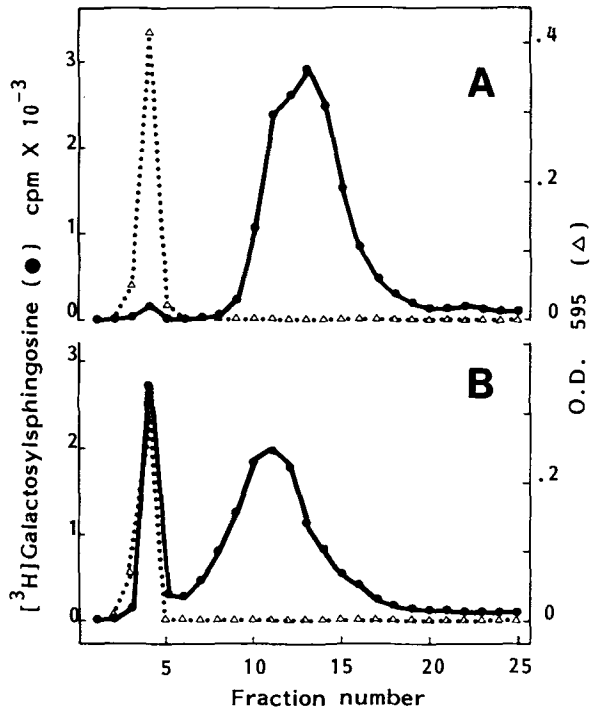

FIG. 1. Gel-filtration profiles for [^{3}H]galactosylsphingosine (●) and protein (△) after the mixture of galactosylsphingosine and protein [γ-globulin (A) or albumin (B)] was incubated. [^{3}H]Galactosylsphingosine (154 nmol, 520 cpm/nmol) was dissolved in 0.6 ml of the buffer [30 mM Tris-HCl (pH 7.4) with 120 mM NaCl, 5 mM KCl and 2 mM $MgCl_2$] and mixed with an equal amount of the buffer containing 2.82 mg of γ-globulin or 1.2 mg of albumin. After the mixture was incubated at 25°C for 60 min with gentle shaking, an aliquot (1.0 ml) was applied to the gel-filtration column (10-DG). The column was eluted with 27 ml of the buffer, and fractions (1.0 ml) were collected. For radioactivity and protein measurement, 0.4 and 0.02 ml of the fraction was used, respectively. It should be noted that the protein assay method used in the prsent experiments is more sensitive against albumin than γ-globulin.

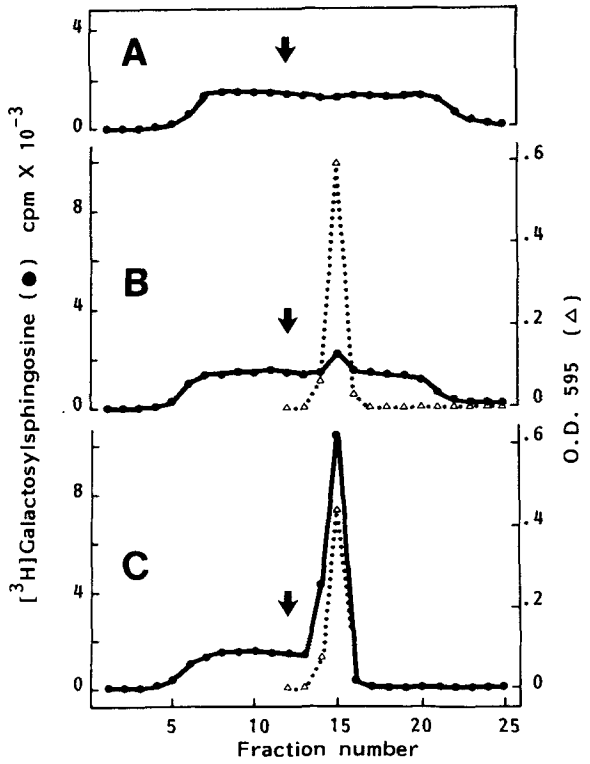

FIG. 2. Gel-filtration profiles for [^{3}H]galactosylsphingosine (●) and protein (△). In this experiment, 480 nmol of [^{3}H]galactosylsphingosine (170 cpm/nmol) dissolved in 1 ml of the buffer [30 mM Tris-HCl (pH 7.4) with 120 mM NaCl, 5 mM KCl and 2 mM $MgCl_2$] was first applied to the gel-filtration column (10 DG), and washed with 10 ml of the buffer; then, 1 ml of the buffer containing no protein (A), 7.04 mg of γ-globulin (B), or 3 mg of albumin (C), was applied (indicated by an arrow) and the elution was continued by applying 20 ml of the buffer. Fractions (1.0 ml) were collected, and 0.4 ml was used for radioactivity and 0.01 ml for protein determination.

had little effect on the filtration of galactosylsphingosine (Fig. 2A,B). In contrast, an addition of the buffer containing albumin caused marked elution of galactosylsphingosine with the protein (Fig. 2C).

Equilibrium gel procedure. Presence of γ-globulin caused only a slight increase of [^{3}H]galactosylsphingosine outside the gel. However, when albumin was added to the mixture, galactosylsphingosine increased outside the gel with the increase of the protein (Fig. 3). Changes of incubation temperature (18–37°C) or pH (5.4–8.6) of the buffer did not cause clear alteration of [^{3}H]galactosylsphingosine outside the gel in the presence of albumin.

Quantitation of the potency of albumin to bind galactosylsphingosine. The amount of [^{3}H]galactosylsphingosine was varied in the absence or presence of a constant concentration of albumin. The concentration of galactosylsphingosine was always higher outside the gel in the presence of albumin (Fig. 4A). The free and bound galactosylsphingosine were calculated following Hirose and Kano (11). From the Klotz plot (Fig. 4B), it was estimated that one molecule of albumin had 7.4 binding sites for galactosylsphingosine, and the dissociation constant was 3.9×10^{-6} M.

Effects of albumin on galactosylsphingosine-induced hemolysis. Based on the number of the binding sites and the dissociation constant obtained in the preceding experiment, concentration of free galactosylsphingosine was calculated in the presence of various amounts of albumin and 80 μM galactosylsphingosine. From this result and the galactosylsphingosine-induced hemolysis without albumin (shown in the inset in Fig. 5), expected hemolysis was calculated. In this estimation, it was assumed that bound galactosylsphingosine did not attack erythrocytes, and that no galactosylsphingosine was transferred between albumin and erythrocytes. Results obtained by this estimation and those obtained experimentally agreed reasonably well (Fig. 5).

DISCUSSION

It has been known that albumin binds various substances, either natural or synthetic, such as long-chain fatty acids, hormones, bilirubin, dyes and drugs (14). Present results obtained by gel-filtration on columns and by the equilibrium gel procedure all indicate that albumin can bind galactosylsphingosine also. These results are consistent

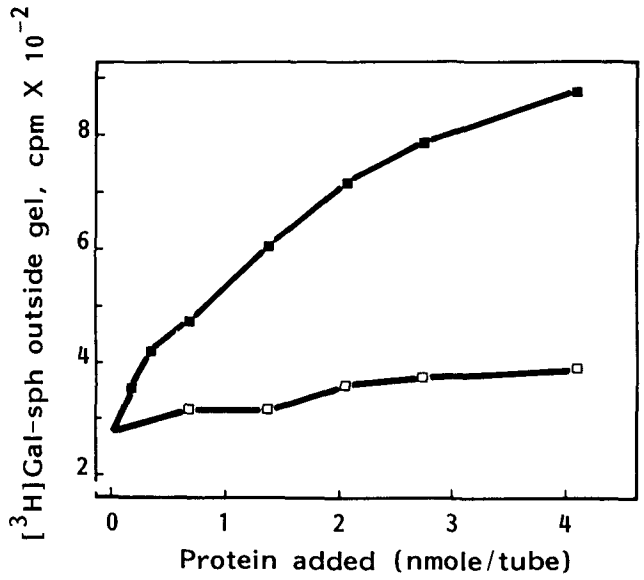

FIG. 3. Galactosylsphingosine (Gal-sph) outside the gel in equilibrium gel procedure. Each tube contained 0.1 g of Sephadex G-50 and 1.1 ml of the buffer [30 mM Tris-HCl (pH 7.4) with 120 mM NaCl, 5 mM KCl and 2 mM $MgCl_2$] with 3.6 nmol of [^{3}H]galactosylsphingosine (4400 cpm/nmol). The amount of γ-globulin (□) and albumin (■) was varied. The tubes were incubated at 37°C for 60 min with gentle shaking, and agitated once by a vortex mixer during the incubation. After the incubation, 0.05 ml of the mixture outside the gel was taken for radioactivity determination.

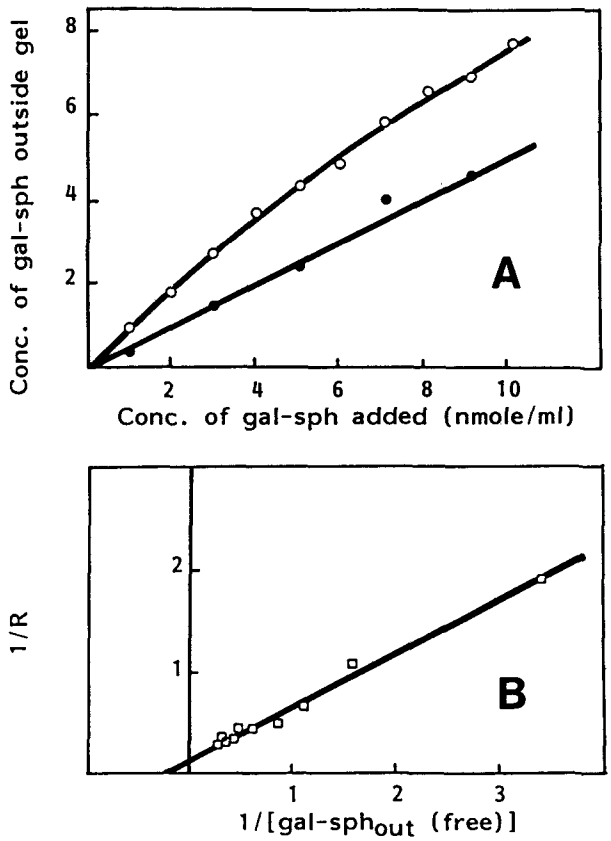

FIG. 4. Changes of the concentration of galactosylsphingosine (gal-sph) (nmol/ml) outside the gel in equilibrium gel procedure. The amount of [^{3}H]galactosylsphingosine (4100 cpm/nmol) was varied in the presence of 0.06 mg of albumin (○) or without it (●) (A). Bound and free galactosylsphingosine were calculated and plotted according to Klotz *et al.* (B). From the intercept of the line on the ordinate and the abscissa, the number of the binding sites (7.4) and the dissociation constant (3.9×10^{-6} M) were obtained.

with our previous findings obtained using ultrafiltration (9). Since albumin abolishes the effects of glucosylsphingosine and sphingosine as well as those of galactosylsphingosine (9), it is likely that lysosphingolipids other than galactosylsphingosine are also bound by albumin.

To quantitate the potency of albumin to bind galactosylsphingosine, we used the equilibrium gel procedure (11). The advantage of this procedure is that it permits determination of galactosylsphingosine binding to albumin under equilibrium and that it does not require a very large amount of galactosylsphingosine. In addition, the temperature can easily be controlled by this method. The number of binding sites and the dissociation constant obtained by this method appear compatible with the potency of albumin to suppress galactosylsphingosine-induced hemolysis.

The number of the binding sites per one molecule of albumin is apparently small when the difference of the mass of galactosylsphingosine (460 Da) and that of albumin (66000 Da) is considered. This suggests that albumin binds galactosylsphingosine at restricted region(s) of the molecule.

While the number of the binding sites is limited, the dissociation constant is small, indicating that the overall capacity of albumin to bind galactosylsphingosine is not small. Biological significance of the interaction of albumin with galactosylsphingosine is not clear. However, the strong association between these two substances observed in the present experiments may have practical significance. For instance, when the effects of galactosylsphingosine are examined, as in the loading test, attention should be paid to the possible presence of albumin.

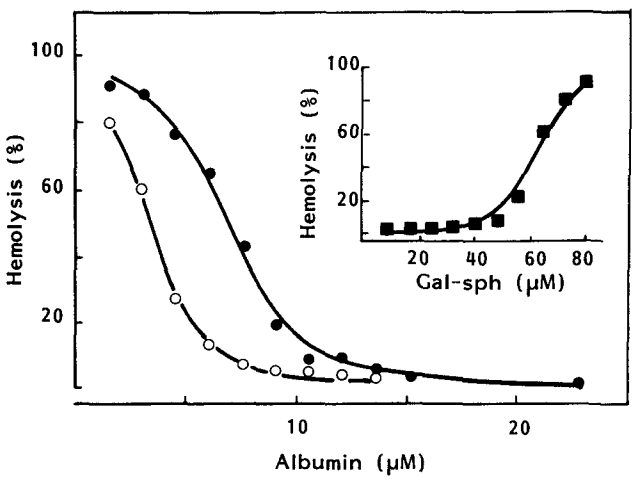

FIG. 5. Galactosylsphingosine-induced hemolysis and effect of albumin. Hemolysis caused by galactosylsphingosine (Gal-sph) without albumin is shown in the inset. Effect of albumin was experimentally determined in the presence of 80 μM galactosylsphingosine (●). From the data shown in the inset and the number of the binding sites and the dissociation constant obtained from the data shown in Fig. 4, the expected percentage of hemolysis was calculated (○). In this calculation, it was assumed that only free galactosylsphingosine could induce hemolysis, and that there was no transference of galactosylsphingosine between albumin and erythrocytes.

Furthermore, if albumin works as an acceptor of galactosylsphingosine produced within the cell, as in the case of lysophosphatidylcholine (15), albumin may be used (e.g., plasmapheresis) to remove this toxic lipid from the body.

ACKNOWLEDGMENT

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Identification of the New 23-Methyl-5,9-pentacosadienoic Acid in the Sponge *Cribrochalina vasculum*

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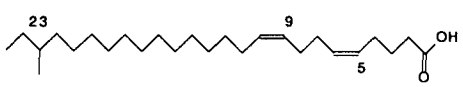
The phospholipid fatty acids from the sponge *Cribrochalina vasculum* were studied, revealing the presence of the new 23-methyl-5,9-pentacosadienoic acid (26:2) which completes the *iso-anteiso* 26:2 (Δ5,9) series. Other phospholipid fatty acids isolated include 26-methyl-5,9-heptacosadienoic (28:2) and 25- methyl-5,9-heptacosadienoic (28:2), as well as the branched acids 8-methylhexadecanoic and 11-methyloctadecanoic. The fatty acids described in this work were found in phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol. The sterol composition of *C. vasculum* consisted of petrosterol, an interesting cyclopropane containing sterol, and the more common sitosterol. The phospholipid fatty acid composition of the demosponge *Ircinia strobilina* is also reported upon. *Lipids 25,* 69-71 (1990).

It is now well established that the phospholipids of marine sponges are a rich source of structurally unusual and interesting fatty acids. Common phospholipid fatty acids from marine sponges include 5,9-hexacosadienoic (26:2), which occurs in most known sponges as described originally by Litchfield and coworkers (1), 5,9-heptacosadienoic (27:2) and 5,9-octacosadienoic (28:2). Interestingly enough, branched fatty acids of longer than usual chain-length have recently been encountered in several sponges. For example work by Ayanoglu *et al.* (2,3) with the sponges *Petrosia ficiformis* and *Jaspis stellifera* has revealed the presence of the unusual phospholipid fatty acids 25-methyl-5,9- hexacosadienoic and 24-methyl-5,9-hexacosadienoic, interesting cases of *iso* and *anteiso* terminal methyl branching in these so called "demospongic" acids. Just recently we reported that the sponge *Ectyoplasia ferox* also contains the novel 28:2 *iso* and *anteiso* series, i.e., the very long chain phospholipid fatty acids 25-methyl-5,9-heptacosadienoic and 26-methyl-5,9-heptacosadienoic, these being the longest set of Δ5,9 *iso* and *anteiso* phospholipid fatty acids from any marine sponge (4). However, much to our surprise, we have noticed that the 26:2 *iso* and *anteiso* series is incomplete, since in the sponges analyzed to date only the *iso* 26:2 acid has been reported before in nature, while the hitherto undescribed fatty acid 23-methyl-5,9-pentacosadienoic (1) has not been reported to occur in these invertebrates. In our search for novel acids in Caribbean sponges around Puerto Rico we have found that the sponge *Cribrochalina vasculum* (order Haplosclerida, family Niphatidae) contains the complete *iso* and *anteiso* 26:2, 27:2 and 28:2 series, including the undescribed 23-methyl-5,9- pentacosadienoic acid (**1**). The results of our investigation follows.



(1)

*To whom correspondence should be addressed

EXPERIMENTAL PROCEDURES

Cribrochalina vasculum was collected December 13, 1988 near the shelf edge of La Parguera, Puerto Rico, at a depth of 60 ft. The sponge (560 g) was washed in sea water, carefully cleaned of all nonsponge debris and cut into small pieces. Immediate extraction with 800 mL of chloroform/methanol (1:1, v/v) yielded the total lipids. The neutral lipids, glycolipids and phospholipids (113 mg) were separated by column chromatography on silca gel (60-200 mesh) using a similar procedure as that of Privett *et al.* (5). The phospholipid classes were investigated 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 (6), followed by purification on column chromatography eluting with hexane/ diethyl ether (9:1, v/v). The resulting methyl esters were analyzed by gas chromatography-mass spectrometry using a Hewlett Packard 5995 A gas chromatograph-mass spectrometer equipped with a 30 m × 0.25 mm nonpolar fused silica column coated with DB-1. For the location of double bonds, *N*-acylpyrrolidide derivatives were prepared by direct treatment of the methyl esters with pyrrolidine/acetic acid (10:1, v/v) in a capped vial (2 hr at 100°C), followed by ethereal extraction from the acidified solution and purification by preparative TLC. Hydrogenations were carried out in 10 mL of absolute methanol and catalytic amounts of platinum oxide (PtO_2). Mass spectral data for the key fatty acid in this discussion is presented below.

23-Methyl-5,9-pentacosadienoic acid methyl ester. MS *m/z* (rel intensity) 406(M^+,3), 396(0.1), 375(1.2), 357(1.6), 332(1.6), 292(2), 291(2.5), 290(1.5), 279(2.3), 264(5.7), 222(2), 196(3.7), 195(4.8), 182(8.9), 181(10), 168(9), 166(4), 164(13), 154(8), 151(11), 150(36), 149(17), 141(36), 140(19), 137(17), 136(29), 135(19), 124(14), 123(16), 121(17), 110(32), 109(68), 108(27), 107(13), 99(16), 97(34), 96(39), 95(35), 94(26), 83(38), 82(52), 81(100), 79(27), 74(26), 71(19), 69(40), 68(27), 67(65), 57(43).

23-Methylpentacosanoic acid methyl ester. MS *m/z* (rel intensity) 410(M^+,6), 368(0.9), 367(2.9), 325(1.0), 312(2.6), 298(1), 269(1.4), 255(3.3), 241(2.5), 213(2.7), 199(12.3), 185(7.6), 171(3.2), 157(5.7), 153(3), 149(4.5), 144(5.3), 143(38), 130(5.9), 129(14), 125(6.9), 115(5), 111(10), 101(12), 97(20), 87(11), 83(18), 75(42), 74(100), 71(18), 69(27), 68(5), 67(9), 57(45).

RESULTS

The phospholipid fatty acids isolated from *C. vasculum* are presented in Table 1. The fatty acid composition was interesting since ca. 60% of all of the fatty acids present were branched (the short ones probably arising from bacterial origin). Most of the branched acids were *iso* and *anteiso* isomers, i.e., with the methyl branch on the penul-

timate and antepenultimate carbon atoms, respectively. These acids were readily characterized by their typical ECL values, for example, the *iso* compound elutes first with typical fractional chain lengths (FCL) of 0.60-0.65, followed by the *anteiso* compound with values of 0.70-0.75. Mass spectrometry results confirmed our assignments.

Of particular interest to our work was the presence in this sponge of a complete series (14% of the total phospholipid fatty acid composition) of *iso* and *anteiso*, very long chain fatty acids with the typical Δ5,9 unsaturation pattern of "demospongic" acids. The Δ5,9 unsaturation pattern was readily recognized from the characteristic base peak at m/z=81 of the methyl esters and the allylic cleavage at m/z=180 of their corresponding pyrrolidides (7). Three rather interesting series of *iso* and *anteiso* fatty acids were recognized when a plot of retention time vs. number of carbon atoms was done (Fig. 1) for the hydrogenated mixture of the very long chain fatty acids from *C. vasculum*. Of particular significance was the finding of the *anteiso*-26:2 acid, i.e., 23-methyl-5,9-pentacosadienoic acid (1) which, to the best of our knowledge, is new in nature. The hydrogenated methyl ester of this acid presented an ECL value of 25.74, typical of *anteiso* acids, and mass spectrometry of the original acid confirmed the structure. The spectrum of the original fatty acid methyl ester included a molecular ion at m/z 406, consistent with the formula $C_{27}H_{50}O_2$, a base peak at m/z 81, typical of the Δ5,9 unsaturation, and characteristic peaks at m/z 74 and m/z 375 (M-31). In fact, the complete 26-28 series of *anteiso* and *iso* acids were readily recognized in the phospholipid fatty acid mixture and characterized in a similar way.

TABLE 1

The Phospholipid Fatty Acids from *Cribrochalina vasculum* and *Ircinia Strobilina*.

Fatty Acid	Abundance (%) *C. vasculum*	*I. Strobilina*
Tridecanoic (13:0)	0.3	—
Tetradecanoic (14:0)	1.7	8.9
4,8,12-Trimethyltridecanoic (16:0)	4.8	—
13-Methyltetradecanoic (*i*-15:0)	7.4	17.6
12-Methyltetradecanoic (*a*-15:0)	1.5	12.3
Pentadecanoic (15:0)	1.8	—
14-Methylpentadecanoic (*i*-16:0)	2.1	9.9
Hexadecanoic (16:0)	13.0	2.1
8-Methylhexadecanoic (17:0)	5.5	9.8
15-Methylhexadecanoic (*i*-17:0)	5.8	—
14-Methylhexadecanoic (*a*-17:0)	0.8	—
Heptadecanoic (17:0)	1.5	12.0
Octadecanoic (18:0)	9.1	1.0
11-Methyloctadecanoic (19:0)	12.7	7.2
Eicosanoic (20:0)	1.9	1.3
Heneicosanoic (21:0)	0.7	—
Docosanoic (22:0)	2.9	6.2
20-Methyldocosanoic (23:0)	0.6	—
Tricosanoic (23:0)	0.7	—
22-Methyltricosanoic (*i*-24:0)	0.4	—
5,9-Tetracosadienoic (24:2)	—	8.9
Tetracosanoic (24:0)	1.7	—
23-Methyltetracosanoic (*i*-25:0)	3.1	—
22-Methyltetracosanoic (*a*-25:0)	0.4	—
5,9-Pentacosadienoic (25:2)	—	1.3
Pentacosanoic (25:0)	0.5	—
24-Methyl-5,9-pentacosadienoic (*i*-26:2)	0.4	—
23-Methyl-5,9-pentacosadienoic (*a*-26:2)	2.0	—
5,9-Hexacosadienoic (26:2)	3.9	—
25-Methyl-5,9-hexacosadienoic (*i*-27:2)	2.1	—
24-Methyl-5,9-hexacosadienoic (*a*-27:2)	6.8	—
26-Methyl-5,9-heptacosadienoic (*i*-28:2)	0.8	—
25-Methyl-5,9-heptacosadienoic (*a*-28:2)	1.8	—
5,9-Octacosadienoic (28:2)	1.1	—

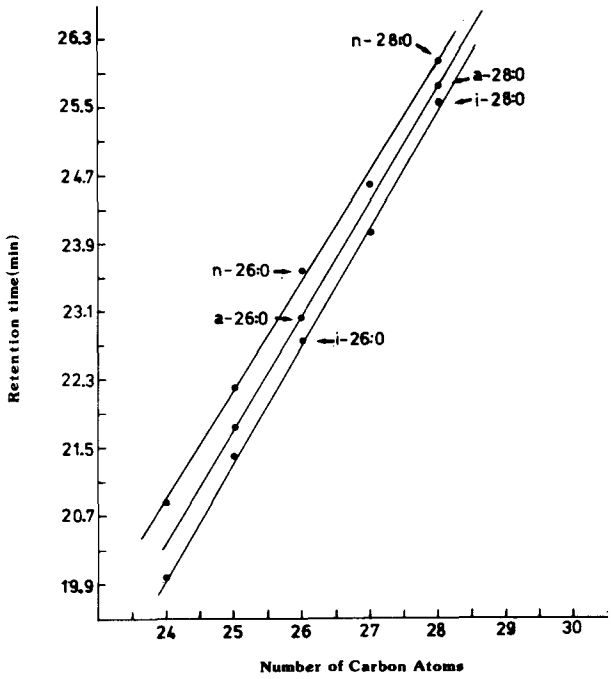


FIG. 1. Plot of retention time vs number of carbon atoms for the hydrogenated mixture of the very long chain fatty acids form *C. vasculum*.

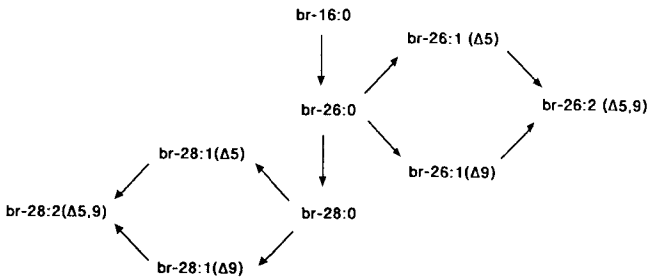
Also interesting was the finding of *two* branched-chain fatty acids with methyl branches in the center of the chain since they had lower retention times than those with methyl branches remote from the carboxyl group, *e.g.*, the *iso* and *anteiso* compounds. These acids were recognized to be 8-methylhexadecanoic and 11-methyloctadecanoic by their typical mass spectra, ECL values, and comparison with authentic samples isolated by us from other sponges (4). These acids have been shown to be present in several sponges, especially the 11-methyloctadecanoic, and the latter seems to be unique for sponges. As a mode of comparison, we have included the fatty acid composition of the demosponge *Ircinia stroblina* in Table 1 since it also contains these two branched fatty acids. The fatty acid composition of *I. strobilina* has not been reported before.

The phospholipid composition of *C. vasculum* was analyzed with the help of TLC and it was found that the principal phospholipids in this sponge were phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol. Much to our surprise, we detected only traces of phosphatidylcholine. The acids reported in this work were evenly distributed in these phospholipids. In this context we also studied the sterol composition of this sponge and found that it consisted mainly of the cyclo-

propane containing sterol petrosterol. ^{1}H-NMR (300 MHz) was key to its characterization. The typical C-18 and C-19 methyl groups were observed at 0.68 and 1.03 ppm, respectively, indicating a typical Δ5 nucleus as confirmed by the vinylic hydrogen at 5.36 ppm. The C-26 and C-27 hydrogens were observed at 0.92 ppm, while the C-29 hydrogens showed up at 1.01 ppm. The cyclopropyl group was confirmed by the absorptions at 0.06-0.16 ppm. A literature comparison of the NMR data confirmed the petrosterol structure as well as a HPLC retention time comparison with authentic petrosterol (8).

DISCUSSION

C. vasculum is an interesting sponge since it contains the complete series of *iso-anteiso* 26:2-28:2 fatty acids, adding a new dimension to the possible Δ5,9 branched very long chain "demospongic" acids. Our report establishes the presence of even- branched "demospongic" phospholipid fatty acids in sponges. In fact, *C. vasculum* is the second sponge from which the 28:2 acids have been isolated, the first sponge being *Ectyoplasia ferox*, and our present findings corroborate the fact that these acids are true sponge metabolites (4). Even more interesting is the finding of the *anteiso*-26:2 acid, which now completes the 26:2 series, opening up a more complete biosynthetic scheme for these "demospongic" acids. On the basis of previous biosynthetic experiments with the sponges *Microciona prolifera* and *Jaspis stellifera* (3), we can postulate the biosynthetic sequence depicted in Scheme 1. Either the *iso* or *anteiso* acids, denoted by "br" in the Scheme, could arise by chain elongation of br-16:0 to a br-26:0, which could give rise to the 26:2 series by introducing either the Δ5 or Δ9 double bond first, followed by Δ9 or Δ5 desaturation, respectively (9). In the same way, the br-28:0 could be obtained by a two carbon elogation of the br-26:0 series and subsequent double bond introduction in the order specified above. Much biosynthetic work has to be performed in order to put these biosynthetic routes on firm ground.



SCHEME 1. Possible biosynthetic routes for the very long-chain, even Δ5,9 acids isolated from *C. vasculum*.

ACKNOWLEDGMENTS

Dr. Vance Vicente from La Parguera, Puerto Rico, collected and classified the sponge. Dr. O. Rosario and Isaura Diaz assisted in the mass spectrometry. This work was supported by the National Science Foundation under Grant No. CHE-8715649 and the National Institute of Health under Grant No. S06 RR08102-17. We thank Loyda Lozada Rios for technical assistance with the *Ircinia strobilina*.

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ERRATUM

"Synthesis and Characterization of Triacylglycerols Containing Linoleate and Linolenate" by R.A. Awl, E.N. Frankel, and D. Weisleder, *Lipids 24*, 866–872, 1989. Under *Methods* in the EXPERIMENTAL section, the reference for ^{1}H and ^{13}C nuclear magnetic resonance spectra should be (13) not (7).

STATEMENT OF OWNERSHIP, MANAGEMENT AND CIRCULATION (Required by 39 U.S.C. 3685). 1. Title of publication: *Lipids*. 1a. Publication no.: 314700. 2. Date of filing: 12-13-89. 3. Frequency of issue: Monthly. 3a. No. of issues published annually: 12. 3b. Annual subscription price: $125. 4. Complete mailing address of known office of publication: 1608 Broadmoor Drive, P.O. Box 3489, Champaign County, Champaign, Illinois 61821-3489. 5. Complete mailing address of the headquarters of general business offices of the publishers: 1608 Broadmoor Drive, P.O. Box 3489, Champaign County, Champaign, Illinois 61821-3489. 6. Full names and complete mailing address of publisher, editor, and managing editor: Publisher—American Oil Chemists' Society, 1608 Broadmoor Drive, P.O. Box 3489, Champaign, Illinois 61821-0489; Editor—W. J. Baumann, Hormel Institute, 801 16th Avenue N.E., Austin, MN 55912; Managing Editor—Paul O'Dea, American Oil Chemists' Society, 1608 Broadmoor Drive, P.O. Box 3489, Champaign, Illinois 61821-3489. 7. Owner: American Oil Chemists' Society, 1608 Broadmoor Drive, P.O. Box 3489, Champaign, Illinois 61821-0489. 8. Known bondholders, mortgagees, and other security holders owning or holding 1 percent or more of total amount of bonds, mortgages or other securities: None. 9. For completion by nonprofit organizations authorized to mail at special rates (Section 423.12, DMM only): The purpose, function, and nonprofit status of this organization and the exempt status for Federal income tax purposes has not changed during preceding 12 months. 10A. Total no. copies (net press run): Average no. copies each issue during preceding 12 months—2,261; Actual no. copies of single issue published nearest to filing date—2,100. 10B. Paid circulation. 1. Sales through dealers and carriers, street vendors and counter sales: Average no. copies each issue during preceding 12 months—None; Actual no. copies of single issue published nearest to filing date—None. 2. Mail subscription: Average no. copies each issue during preceding 12 months—1,805; Actual no. copies of single issue published nearest to filing date—1947. 10C. Total paid circulation: Average no. copies each issue during preceding 12 months—1,805; Actual no. copies of single issue published nearest to filing date—1947. 10D. Free distribution by mail, carrier or other means, samples, complimentary, and other free copies: Average no. copies each issue during preceding 12 months—62; Actual no. copies of single issue published nearest to filing date—68. 10E. Total distribution: Average no. copies each issue during preceding 12 months—1,867; Actual no. copies of single issue published nearest to filing date—2,015. 10F. Copies not distributed. 1. Office use, left over, unaccounted, spoiled after printing: Average no. copies each issue during preceding 12 months—394; Actual no. copies of single issue published nearest to filing date—85. 2. Return from news agents: Average no. copies each issue during preceding 12 months—None; Actual no. copies of single issue published nearest to filing date—None. 10G. Total: Average no. copies each issue during preceding 12 months—2,261; Actual no. copies of single issue published nearest to filing date—2,100. 11. I certify that the statements made by me above are correct and complete. (Signed) Gloria B. Cook, Manager of Data Services, American Oil Chemists' Society.

420-23

Lipids

Notices
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Fourteenth Annual Meeting of The American Society of Preventive Oncology (ASPO), Hyatt Regency-Bethesda, Bethesda, MD, March 19-21, 1990. The meeting is intended for professionals in clinical, educational and research disciplines which contribute to comprehensive approaches to cancer prevention. Topics: Hormones and Cancer, Squamous Cell Cancers: Similarities and Contrasts of Etiology and Prevention. For further information contact: Richard R. Love, M.D., American Society of Preventive Oncology, 1300 University Avenue - 7C, Madison, WI 53706. Telephone: (608) 263-6919.

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The Na^+K^+ ATPase Activity in Cultured Human Fibroblasts with an Elevated Phospholipid Triene:Tetraene Ratio

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Human skin fibroblasts were cultured at low density for 11 days in MCDB 110, 0.4% fetal bovine serum, a mitogen mixture, and were supplemented with 18:2n-6 or 18:1n-9 as a fatty acid-albumin complex. The cells cultured with the 18:2n-6 supplement had a 20:3n-9/20:4n-6 ratio of 0.29 ± 0.07; the 18:1n-9 supplemented cells had a ratio of 1.51 ± 0.27. There was less than 4% difference in total growth of the cell population under the two culture conditions. The cells supplemented with 18:2n-9 had similar levels of protein/cell, K^+/mg cell protein and functional Na^+K^+ATPase activity.
Lipids 25, 73–77 (1990).

When cultured at low density in growth-promoting medium containing low levels of fetal bovine serum, human skin fibroblasts become depleted of n-6 polyunsaturated fatty acids (n-6 PUFA) (1). This fact is evident from a decrease in the total mole % of n-6 PUFA in cell phospholipids and by an increase in the 20:3n-9/20:4n-6 (triene:tetraene) ratio in cell phospholipids under these culture conditions (1). The culture system is of interest for two reasons: first, it can be utilized to study whether there is an n-6 PUFA requirement for fibroblasts in culture (2–4) and second, it can be used to examine the function of polyunsaturated fatty acids and their metabolites in cells (5–7). The biochemical adaptations necessary to permit rapid multiplication, under culture conditions which cause phospholipid triene:tetraene ratios to vary over a wide range (1), are undefined.

The Na^+K^+ATPase (EC 3.6.1.37) is an electrogenic pump located in the plasma membrane of mammalian cells. By exerting a direct effect on intracellular concentrations of Na^+ and K^+, this enzyme is a major factor in controlling membrane potential and cell volume (8). One of the reported effects of an n-6 PUFA deficiency in mammals is an elevated cell plasma membrane Na^+K^+ATPase activity (9–11). An elevated Na^+K^+ATPase activity may be part of an adaptive response of cells to the altered physiochemical properties of the cell plasma membrane under conditions of severe n-6 PUFA deficiency (9,12,13). As components of membrane phospholipid, n-6 PUFA have been postulated to have a controlling influence on membrane transport processes involving a variety of small molecules and water (14). The elevated Na^+K^+ATPase activity associated with n-6 PUFA deficiency may contribute to the increased basal metabolic rate characteristic of EFA-deficient animals (15–17).

These experiments were designed to determine whether an elevated cell phospholipid triene to tetraene ratio in a population of rapidly dividing human fibroblasts is associated with alterations in cell Na^+K^+ATPase activity and intracellular K^+ and protein concentrations.

EXPERIMENTAL

Cells and culture. The neonatal human skin fibroblasts (HF-1) were prepared by primary culture in our laboratory and stored at low population doubling number (1). Medium MCDB 110 (18) was prepared in our laboratory according to published procedures (19). The cells were cultured in MCDB 110 containing 0.4% fetal bovine serum (FBS) and supplemented with: 5 μg/ml human transferrin (Sigma), 4.5×10^{-8} M selenium (as Na_2HSeO_3), 0.95 μg/ml insulin (Sigma), 0.2 μg/ml dexamethasone and 0.03 μg/ml epidermal growth factor (Collaborative Research, culture grade). This supplement mixture is a modification of that used by Bettger *et al.* (18) to support clonal growth of human diploid fibroblasts under serum-free conditions. The lot of FBS used in these studies (Flow, lot #2916105) contained 17.2 mole % 18:1n-9, 5.8% 18:2n-6 and 9.9% 20:4n-6. All cell culture assays were performed in 5 ml total volume in 60 mm polystyrene plates (Falcon) coated with poly-D-lysine (Sigma, No. 7886) (1,20). All culture media were preincubated in the assay plates for 1 hr in the incubator prior to the inoculation of cells.

The procedure for the weaning and subsequent mild trypsinization of the cells prior to an assay has been previously described (1). The cells were counted by hemocytometer and introduced in a volume of 0.05 ml in solution A (30 mM HEPES [pH 7.6], 10 mM glucose, 130 mM NaCl, 1.0 mM Na_2HPO_4 and 3 mM KCl). The experiments were performed at low density, 400 cells per plate (20 cells/cm^2), with an 11 day incubation period at 37°C, 2% CO_2 in air and 100% humidity.

Fatty acid supplementation. The fatty acids (Sigma or Serdary) were purchased at a nominal purity of 99% or better. However, all fatty acids were evaluated for the presence of peroxides by thin layer chromatography (21). If peroxides were present, the preparation was purified according to the method of Marshall *et al.* (22), or by TLC on a plate previously run in a phospholipid resolving solvent (1); after the TLC plate was reactivated for at least 2 hr at 110°C, the fatty acid preparation was applied to the plate and was resolved in heptane/isopropyl ether/acetic acid (50:50:0.8, v/v/v). The fatty acid was visualized with 0.1% ANS (23), scraped into a tube and eluted by the method of Bligh and Dyer (24). This procedure resulted in fatty acid purity of 98.3% or better. The purified fatty acid was stored in toluene (scintillation grade, Fisher) under oxygen-free N_2 at −20°C. On the day of the assay, the fatty acids were dried under nitrogen and dissolved in the appropriate volume of 100% ethanol (Consolidated Alcohols Ltd., Toronto, Ont.). The fatty acid was added in 20 μl of 100% ethanol to 1.0 ml

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Abbreviations: ANS, 8-Anilino-1-naphthalene sulfonate; Ci, curie; EFA, essential fatty acid; FAMEs, fatty acid methyl esters; FBS, fetal bovine serum; GLC, gas-liquid chromatography; HEPES, *N*-2(-hydroxyethyl)piperazine-*N*-2-ethanesulfonic acid; MECL, modified equivalent chain length; Na^+K^+ATPase, sodium ion activated ATPase (adenosinetriphosphatase); PUFA, polyunsaturated fatty acid; TLC, thin-layer chromatography; UV, ultraviolet.

of 10 mg/ml of fatty acid-free albumin (Sigma #AO281) in solution A at room temperature. The freshly prepared fatty acid-free albumin complex was added at 0.05 ml to the medium,resulting in 100 μg/ml albumin, 0.02% ethanol and the final concentration of oleic and linoleic acid of 13.3×10^{-7} M and 5.3×10^{-7} M, respectively.

TABLE 1

Fatty Acid Composition of the Total Phospholipids of HF-1 Fibroblasts Supplemented with Oleic and Linoleic Acids

Phospholipid fatty acid	Fatty acid supplement[a,b]	
	Oleic	Linoleic
	mole %	
14:0	1.9 ± 0.5	2.5 ± 0.3
16:0	18.6 ± 1.3	22.3 ± 1.4
16:1n-9[c]	4.6 ± 0.3	4.5 ± 0.2
16:1n-7	4.4 ± 0.7	5.5 ± 0.4
18:0	8.4 ± 0.1*	10.5 ± 0.2
18:1n-9	27.1 ± 1.3*	18.3 ± 0.5
18:1n-7	4.6 ± 0.3*	5.5 ± 0.2
18:2n-9[c]	0.9 ± 0.1	0.8 ± 0.1
18:2n-7[c]	1.1 ± 0.2	1.1 ± 0.1
18:2n-6	0.4 ± 0.1*	2.3 ± 0.3
20:0	0.2 ± 0.1	0.2 ± 0.1
20:1n-9	0.6 ± 0.1*	0.1 ± 0.1
20:2n-9[c]	0.4 ± 0.1	0.4 ± 0.1
20:2n-7[c]	1.2 ± 0.1*	0.7 ± 0.1
20:3n-9[c]	3.9 ± 0.4*	1.6 ± 0.4
20:3n-7[c]	0.4 ± 0.1	0.5 ± 0.3
20:3n-6	0.4 ± 0.1*	1.1 ± 0.2
20:4n-6	2.7 ± 0.2*	6.3 ± 1.1
22:0	0.8 ± 0.1	1.7 ± 1.1
22:1n-9	0.3 ± 0.1	0.3 ± 0.3
22:3n-9[c]	2.2 ± 0.2*	0.8 ± 0.2
22:4n-6	1.3 ± 0.4	2.2 ± 0.3
22:5n-3[c]	1.0 ± 0.3	0.6 ± 0.1
22:6n-3	1.3 ± 0.4	0.9 ± 0.1
24:0	1.2 ± 0.1	1.3 ± 0.2
24:1n-9[d]	4.7 ± 1.4*	1.5 ± 0.2
24:1n-7[c]	0.3 ± 0.1	0.2 ± 0.1
% Unidentified	5.0 ± 1.3	5.7 ± 0.8
20:3n-9/20:4n-6	1.51 ± 0.27*	0.29 ± 0.07
22:3n-9/22:4n-6	1.95 ± 0.44*	0.37 ± 0.07
Fatty acid class		
Saturated	31.1 ± 1.6*	37.7 ± 1.4
Monounsaturated	46.8 ± 1.3*	35.7 ± 0.9
Polyunsaturated	18.0 ± 1.5*	22.0 ± 1.1
Double bond index (DBI)[e]	1.04 ± 0.06	1.07 ± 0.2
Unsaturation index[f]	3.4 ± 0.3	2.9 ± 0.2

[a]Cells were cultured in MCDB 110 plus 0.4% FBS, supplement MS and supplemented with either 13.3×10^{-7} M 18:1n-9 or 5.3×10^{-7} M 18:2n-6 as the albumin complex.

[b]Values represent mean ± SEM. Each value for 18:1n-9 supplemented cells is the mean of 4 independent experiments. Values having an (*) are significantly different by the Student's t-test.

[c]Tentatively identified by MECL analysis and argentation chromatography (1). Biological standards were also used to support the identification of 20:3n-9 and 22:3n-9 (livers from EFA-deficient rats) and 22:5n-3 (menhaden oil).

[d]Data were log transformed prior to statistical analysis.

[e]Average number of double bonds per fatty acid in the sample.

[f]Double bond index × 100/mole % of saturated fatty acids.

The concentration of unesterified fatty acids in the medium due to 0.4% FBS was $2.99 \pm 0.08 \times 10^{-7}$ M by analysis. The final concentrations of unesterified, oleic and linoleic acids due to the serum were $0.56 \pm 0.02 \times 10^{-7}$ M and $0.15 \pm 0.04 \times 10^{-7}$ M, respectively.

Lipid analysis. The methods used to extract the lipid from cells and FBS were outlined previously (1). The total phospholipids were isolated from neutral lipids on TLC (Merck) plates run twice in a resolving system containing heptane/isopropyl ether/acetic acid (60:4:3, v/v/v) (25). The lipid spots were visualized by U.V. after spraying with 0.1% ANS (23). The silica gel was scraped and the lipid transmethylated as previously described (1).

The resulting fatty acid methyl esters (FAMEs) were separated on a Megabore DB-225 column (J & W) by GLC analysis utilizing a temperature program: 140°C to 165°C at 1° per min to 175°C then held for 10 min, 1° per min to 210°C, end of program. The stationary phase was 25% cyanopropyl, 25% phenyl and 50% methyl polysiloxane (1). The FAMEs were identified by a comparison with known standards (NuChek Prep), by the use of argentation TLC, modified equivalent chain length (MECL) analysis and biological standards as previously described (1).

$^{86}Rb^+$ uptake. After the 11-day assay period, the plates were removed from the incubator. The complete growth medium was momentarily removed from the plates. To 3.6 ml of the growth medium was added either 0.4 ml of fresh medium containing ouabain (final concentration 10^{-3} M) or 0.4 ml of fresh medium without ouabain. The medium was returned to the plates and the plates preincubated for 10 min in the incubator. The medium was again removed from the plates and 2.3 ml of the medium was spiked with a $^{86}Rb^+$ tracer. The cells were then incubated at 37°C with 0.5 μCi/ml ^{86}RbCl (New England Nuclear; 4.89 Ci/mg) in 2.0 ml of the appropriate medium. After a 2.5 min incubation period, the labelled medium was discarded and the plates rapidly washed by immersing in 3 separate 2 L beakers containing ice-cold 0.1 M $MgCl_2$ (26). Washing was completed within 10 sec. After the last wash, 2.0 ml of 0.1 N NaOH was added and the cells scraped with a rubber policeman. The contents were transferred to a 7 ml plastic scintillation vial (Beckman) and the $^{86}Rb^+$ counted by Cherenkov radiation in a Beckman LS 7800 liquid scintillation counter. After analysis of radioactivity, the contents of the vials were processed for the determination of the protein using the method of Peterson (27); bovine serum albumin was used as the standard.

Determination of K^+. The plates after the 11-day incubation were washed as described for the $^{86}Rb^+$ uptake procedure, except, after the last wash, 1 to 2 ml of 0.1 M $MgCl_2$ was placed over the cells prior to scraping with a rubber policeman. The contents of the plates were transferred to plastic tubes, sonicated (Branson Sonic Power Co., Danburg, CT) at room temperature until a homogenous solution was produced and aliquots were taken for protein (27) and K^+ determination. The aliquot for K^+ determination was transferred to a Pyrex glass tube and wet ashed with nitric acid (28). The K^+ concentration was determined by atomic absorption spectroscopy (29). Analysis of National Bureau of Standards bovine liver sample 1577a by this method yielded 94.9 ± 0.8% of the certified potassium value.

Cell counting. The cells were rinsed with cold solution A prior to the addition of 1.0 ml of 0.01% trypsin in solution A (Sigma, salt free). After 3–4 min, when the cells

were beginning to round up and detach, 0.5 ml of 0.03% trypsin inhibitor (Sigma) was added. The cells were agitated with a Pasteur pipette, and the cell suspension was transferred to a 50 ml centrifuge tube after multiple washings of the plate. The cells were centrifuged at 500 × *g* for 5 min at 5°C. The supernate was discarded, and the cells were resuspended in a small volume of solution A. Aliquots were taken for protein and for cell counts by a Coulter counter (model Zm, Coulter Electronics Ltd.).

RESULTS

The fatty acid composition of the phospholipids of the human fibroblasts resulting from the supplementation of oleic (18:1n-9) and linoleic (18:2n-6) acids is displayed in Table 1. There is a significant ($P<0.05$) reduction in the accumulation of 18:0, 18:1n-7, 18:2n-6, 20:3n-6, 20:4n-6, total saturated and total polyunsaturated fatty acids in the cells supplemented with 18:1n-9 compared to the cells supplemented with 18:2n-6; there is a significant increase in 18:1n-9, 20:1n-9, 20:2n-7, 20:3n-9, 22:3n-9, 24:1n-9 and total monounsaturated fatty acids. The triene to tetraene ratio (20:3n-9/20:4n-6), an index of essential fatty acid deficiency, and an analogous ratio 22:3n-9/22:4n-6, were significantly greater for those cells supplemented with 18:1n-9 (1.51 ± 0.24 and 1.95 ± 0.44, respectively), than for those cells supplemented with 18:2n-6 (0.29 ± 0.07 and 0.37 ± 0.07, respectively).

Na^+K^+ATPase activity, cell K^+ and protein concentrations and cell growth are reported in Table 2. There is no significant difference in the total, ouabain insensitive or ouabain sensitive uptake of K^+ equivalents (Na^+K^+ATPase activity) between the cells cultured in the two media. Similarly, cell K^+ per mg protein and protein per cell was not affected. The 18:1n-9 supplemented cells grew significantly more over the assay period as assessed by final cell number after the 11-day culture period. Data on cell growth are expressed as the number of population doublings/11 days.

TABLE 2

Na^+K^+ATPase Activity and Protein and Potassium Concentrations in HF-1 Fibroblasts Supplemented with Oleic and Linoleic Acids

Parameter measured	Fatty acid supplement[a,b] 18:1n-9	18:2n-6
Na^+K^+ATPase activity		
Total uptake (n=6) (nmol K-equivalents/mg protein-min)	49.4 ± 12.8	52.7 ± 14.2
Ouabain-insensitive uptake (n=6) (nmol K-equivalents/mg protein-min)	36.5 ± 11.3	35.9 ± 10.8
(% of total uptake)	68.4 ± 9.1	63.9 ± 8.2
Ouabain-sensitive uptake[c] (n=6) (nmol K-equivalents/mg protein-min)	12.9 ± 3.8	16.9 ± 5.7
(% of total uptake)	31.6 ± 9.1	36.1 ± 8.2
Cell K^+ (μmol/mg protein) (n=7)	2.1 ± 0.1	2.3 ± 0.1
Cell protein (pg/cell) (n=5)	372 ± 56	396 ± 43
Cell growth[d] (number of population doublings/11 days) (n = 15)	8.2 ± 0.1*	7.9 ± 0.1
Phospholipid triene:tetraene ratio[e] (n=4) (n=6)	1.51± 0.27*	0.29 ± 0.07

[a]All values represent mean ± SEM. Number of independent experiments are listed for each parameter measured. Values having an asterisk (*) are significantly different ($P<0.05$) by the Student's t-test.

[b]The potassium concentration of the complete medium was 5.3 ± 0.1 mM upon analysis by atomic absorption spectrophotometry.

[c]Defined as Na^+K^+ATPase activity.

[d]These values are calculated assuming a plating efficiency of 100%.

[e]The ratio (mole%) of 20:3n-9/20:4n-6.

DISCUSSION

The culture system employed in this experiment was designed to elevate significantly the phospholipid triene:tetraene ratio in a population of fibroblasts while having a minimal effect on the growth and morphology of the cells. The culture system was developed by keeping culture conditions constant (MCDB 110, 0.4% FBS, supplement MS, 100 μg/ml fatty acid-free albumin), titrating supplemental 18:1n-9 and 18:2n-6 (albumin complex) and measuring the effect on growth and cell phospholipid triene:tetraene ratio in a 12-day culture assay. For the study of cellular Na^+K^+ATPase activity, an 11-day assay was employed to ensure that not only would growth of cells supplemented with 18:1n-9 or 18:2n-6 be nearly identical at the end of the assay period, but there would be some confidence that growth would continue in the next 24 hr period at a nearly identical rate. The results in Tables 1 and 2 suggest that the above objectives were accomplished. The total cell growth of 18:1n-9 supplemented cells at the end of the 11-day assay was only slightly (3.8%) greater than cells supplemented with 18:2n-6 while the cells had phospholipid triene:tetraene ratios of 1.51 and 0.29, respectively. A triene:tetraene ratio of >0.4 in cell lipids is the original biochemical index of EFA deficiency in experimental animals; by this standard, the cells supplemented with 18:1n-9 are n-6 PUFA-deficient while cells supplemented with 18:2n-6 are not. The nature of the culture system is of particular importance because of the sensitivity of cell Na^+K^+ATPase activity to a multitude of environmental and physiological parameters. Na^+K^+ATPase activity has been shown to be altered by the stage of the cell cycle (30), by log phase growth rate (31,32), by changes in cell shape/mechanical damage (33,34), by cell passage number (35) and by cell transformation (32,36). In addition, the enzyme activity can be affected by extracellular serum/mitogen (26,37), calcium (38), amino acid (39), hydrogen ion (40), lysophospholipid (41) and free fatty acid concentrations (41–43). In this culture system the test culture media were identical except that 18:1n-9-supplemented cultures had 2.5 times the supplemental free fatty acid concentration of the 18:2n-6-supplemented cultures (see footnote, Table 1). This inequity was necessary in order to achieve similar growth rates but very different triene: tetraene ratios in the cells.

In this experiment, Na^+K^+ATPase activity was analyzed by using a tracer dose of $^{86}Rb^+$ in the complete growth medium containing 5.3 mM K^+, and by defining Na^+K^+ATPase activity as ouabain-sensitive uptake of K^+-equivalents. The $^{86}Rb^+$ tracer was added to the

culture medium that was supporting cell growth over an 11-day period, i.e., the cells were not "refed," in an attempt to minimally perturb the population of dividing fibroblasts attached to the culture plate. This exacting procedure was an attempt to measure "functional" Na^+K^+ATPase activity in these cells (30). In this context, functional activity is defined as the Na^+K^+ATPase activity that actually occurs during culture to support the membrane potential, volume and intracellular Na^+ and K^+ concentrations necessary to sustain the growth of the fibroblast populations. The analysis of functional Na^+K^+ATPase activity has been performed previously on cells in culture (30,37,40) but not on cells cultured at low density, and growing in low serum, mitogen-supplemented media. The activity of Na^+K^+ATPase found in these experiments is within the range of the activities found in other cell types in a variety of culture systems but is slightly higher than the values reported in most studies [12.9–16.9 vs 7–38 nmol K^+-equivalents/mg protein-min] (26,38,39,44). Similarly, cell K^+ concentrations described in this study are slightly higher than those reported for other cell types cultured under a variety of conditions [2.1–2.3 vs 0.75–1.7 μmol/mg protein] (26,45–47). However, in the present studies, total cell K^+ (after wet ashing), as opposed to extractable cell K^+, has been analyzed.

It is clear from the results of these experiments that a significant (five-fold) elevation in fibroblast triene:tetraene ratio does not lead to an increase in Na^+K^+ATPase activity or an alteration in cell K^+ or protein concentrations. Though "functional" Na^+K^+ATPase activity is not altered in the cells with an elevated triene:tetraene ratio, the effect of an elevated triene:tetraene ratio on "optimal" Na^+K^+ATPase activity (30), total pump number per cell (48), regional distribution of pumps on the cell surface (49), enzyme temperature dependence (50) or enzyme stoichiometry (8) is unknown. Solomonson *et al.* (50) have demonstrated that Ehrlich ascites cell plasma membranes that have a phospholipid triene:tetraene ratio of 1.3 have a lower transition temperature for Na^+K^+ATPase activity than cells with a triene:tetraene ratio of 0.2. Though the cells with the high triene:tetraene ratio have a significantly higher energy of activation for Na^+K^+ATPase below the transition temperature, compared to low triene:tetraene containing controls, they have similar activation energies at 37°C. In addition, Blog *et al.* (51) have demonstrated in rat erythrocytes that the Hill coefficient (n) for the inhibition of Na^+K^+ATPase by F^- shows a significant negative linear correlation with the unsaturation index, but does not correlate with total n-9 or n-6 unsaturated fatty acids. It is also important to note that previous studies on the effect of n-6 PUFA deficiency demonstrate increased "optimal" Na^+K^+ATPase activity; "functional" Na^+K^+ATPase activity was not measure (9–11).

In summary, under these culture conditions, an elevated cellular phospholipid triene:tetraene ratio is not associated with altered growth rate, functional Na^+K^+ATPase activity, or cell K^+ and protein concentrations in human skin fibroblasts.

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Fatty Acid Hydroxylase System in the Japanese Harvest Mouse, *Micromys minutus*

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Liver microsomes of the Japanese harvest mouse (*Micromys minutus*), which is the smallest known mammal among rodents, catalyze the hydroxylation of various fatty acids (C_8 to C_{18}) to the corresponding ω-hydroxy and (ω-1)-hydroxy derivatives. Although laurate is most effectively hydroxylated among saturated fatty acids by liver microsomes of other species, harvest mouse liver microsomes most effectively catalyze the hydroxylation of decanoate. From inhibitor and cofactor studies, and from the substrate specificity for hydroxylation, it was concluded that ω- and (ω-1)-hydroxylation of fatty acids are catalyzed by different cytochrome P-450 species in the liver microsomes of the harvest mouse.

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Mammals other than rat, mouse, or rabbit have been used as laboratory animals (1-3) in a variety of studies (4-6). Nevertheless, there are numerous species of mammals in nature that, for a variety of specific research needs, may be more suitable as research subjects. In previous papers (7,8), we described the substrate specificity and other properties of the fatty acid hydroxylase system in liver microsomes of the musk shrew, *Suncus murinus*, and the Mongolian gerbil, *Meriones unguiculatus*. Both species have received attention as novel laboratory species because of such desirable characteristics as small size and rapid breeding. It was demonstrated that a cytochrome P-450 monooxygenase system in liver microsomes of the musk shrew and the Monogolian gerbil specifically and effectively catalyzed ω- and (ω-1)-hydroxylation of fatty acids and alcohols as compared to the monooxygenase systems in other species. Harvest mouse (*Micromys minutus*) is the smallest known mammal among rodents. It has also received attention as a possible laboratory species in recent years (9-11). However, little information is available concerning the fatty acid hydroxylase system in the harvest mouse liver. In this paper, various properties of a cytochrome P-450-dependent fatty acid hydroxylase system in the harvest mouse are described.

MATERIALS AND METHODS

Chemicals. ^{14}C-Labeled fatty acids (sp. act. 19–58 mCi/mmol) were purchased from Amersham International (Amersham, U.K.). The ω-hydroxy and (ω-1)-hydroxy derivatives of saturated fatty acids were synthesized chemically (12). *p*-Chloromercuribenzoate (PCMB), KCN, NaN_3, and menadione were purchased from Tokyo Kasei Chemical Co. (Tokyo). NADPH and NADH were from Oriental Yeast Co. (Tokyo). Cytochrome *c* (horse heart) was from Sigma Chemical Co. (St. Louis, MO). SKF 525-A was a gift from Professor M. Kusunose, Osaka City University Medical School. The organic solvents were reagent grade and freshly distilled before use.

Preparation of harvest mouse liver microsomes. The harvest mice used in this study were obtained from our breeding colonies. Twenty-four male harvest mice (7 weeks) weighing 7–12 g were used. The preparation of harvest mouse liver microsomes was carried out in the same manner as described for the preparation of frog liver microsomes (13). The protein concentration of the microsomal suspension was determined by the method of Lowry *et al.* (14). The cytochrome P-450 and cytochrome b_5 contents of the microsomal suspension were determined by the methods of Omura and Sato (15) and Omura and Takesue (16), respectively. NADPH-cytochrome *c* reductase activity was determined as described by Omura and Takesue (16).

Assays for hydroxylation of fatty acids. The standard incubations for the hydroxylation assay of fatty acids contained microsomes (110–150 μ g protein), 100 μ mol potassium phosphate (pH 7.5), 0.25 μ mol NADPH and 30 nmol ($4.7–5.3 \times 10^4$ cpm) of the appropriate ^{14}C-labeled fatty acids (added as the potassium salt). The final volume was 0.5 ml and the incubation was 10 min at 37°C. The hydroxylase activity of fatty acids was assayed by the same method as described previously (17, 18). All experiments were done in duplicate.

Isolation and determination of ω- and (ω-1)-hydroxy fatty acids. The several procedures used for isolation and identification of reaction products [ω- and (ω-1)-hydroxy fatty acids] have been described in detail previously (17, 18). The procedures used for radio-gas chromatographic analyses of the *O*-acetyl derivatives of hydroxy fatty acids have been decribed (7,18). All experiments were done in duplicte.

RESULTS

Electron transport components and hydroxylase activity of the harvest mouse. Body weight, liver weight and microsomal protein of the harvest mice used are shown in Table 1. The body weight of harvest mice is only ca. 9 g. Since no information is available on hepatic microsomal enzyme activities in the harvest mouse, cytochrome P-450 and cytochrome b_5 contents, NADPH-cytochrome *c* reductase activity and decanoate hydroxylase activity were measured (Table 1).

Effect of substrate chain length on the rate and positions (ω and ω-1) of hydroxylation. After it was found that decanoic acid could be hydroxylated by harvest mouse liver microsomes, other fatty acids were tested as substrates, and the polar products formed enzymatically were analyzed and identified. Table 2 shows the effect of substrate chain length on hydroxylation and distribution of ω- and (ω-1)-hydroxy products of fatty acids. Decanoate was the most active substrate. It is noteworthy that

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Abbreviations: NADH, dinucleotide phosphate; NADPH, reduced nicotinamide adenine; PCMB, *p*-chloromercuribenzoate.

TABLE 1

Hepatic Microsomal Electron Transport Components and Fatty Acid Hydroxylation in the Harvest Mouse, Micromys minutus

Characteristic	n = 6[a]
Body Weight (g)	8.9 ± 1.6[b]
Liver Weight (g)	0.31 ± 0.07[b]
Microsomal protein (mg/g liver)	9.1 ± 1.3
Cytochrome P-450 (nmol/mg microsomal protein)	1.22 ± 0.11
Cytochrome b_5 (nmol/mg microsomal protein)	0.61 ± 0.08
NADPH-cytochrome *c* reduction (μmol/mg microsomal protein/min)	0.38 ± 0.03
Decanoate hydroxylation	
nmol/mg microsomal protein/min	10.92 ± 0.56
nmol/nmol P-450/min	9.41 ± 0.48

[a]Since the weight of each liver of the harvest mouse is only 0.3 g, the amount of microsomal protein from each liver is not enough for detemination of hepatic microsomal transport components and assay for fatty acid hydroxylation. Twenty-four livers were thereby divided into six pools (each pool: four livers), and six microsomal preparations (n = 6) were obtained.

[b]n = 24 in this table.

TABLE 2

Substrate Specificity of Fatty Acid Hydroxylation by Harvest Mouse Liver Microsomes

Chain length	Hydroxylation activity (nmol/mg microsomal protein/min)	Distribution of hydroxy isomers (%)		ω / ω -1-hydroxylation
		ω	ω-1	
8	1.06(10)[a]	35	65	0.54
10	10.92(100)	76	24	3.17
12	7.80(71)	48	52	0.92
13	5.46(50)	81	19	4.26
14	9.14(84)	58	42	1.38
16	3.65(33)	59	41	1.44
18	0.83(8)	59	41	1.44
18:1	3.75(34)	100	0	—

[a]Values in parentheses are percentages of the specific activity of decanoate hydroxylation.

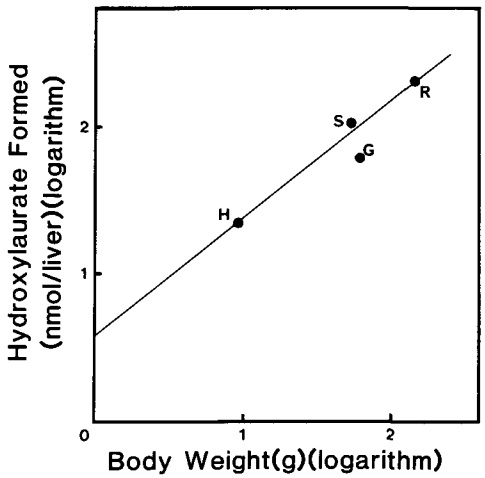

FIG. 1. Relation between body weight (X) of laboratory animals (harvest mouse (H), musk shrew(S), Mongolian gerbil (G) and rat(R)) and total laurate hydroxylation activity (Y) in their liver. The slope of regression line for body weight of the animals on the total laurate hydroxylation activity in the liver was 0.787 (the correlation coefficient r = 0.971). Details are given in the text.

the hydroxylation activity *vs* chain length of fatty acids is biphasic at decanoate and myristate. Although the $\omega/(\omega$-1)-hydroxylation ratio was constant (1.38-1.44) for the saturated fatty acids ranging in chain length from 14 to 18 carbons, the ratio of the fatty acids from 8 to 13 carbon atoms in length varied in many ways. Since the hydroxylation activity of medium-chain fatty acids is high in musk shrew and gerbil liver microsomes (7,8), we examined the relationship between total hydroxylation activity of decanoate and laurate in the liver of the harvest mouse, the musk shrew, the Mongolian gerbil and the rat, and their body weight. A highly significant correlation (r=0.971) was observed between total hydroxylation activity of laurate in the livers of these laboratory animals and their body weight (Fig. 1). The equation relating the body weight (X) to total laurate hydroxylation activity (Y) was as follows: log Y = 0.589 + 0.787log X. However, no correlation was observed for total decanoate hydroxylation activity in the liver (data not shown).

Properties of the microsomal decanoate hydroxylation. Using decanoate as a substrate, various properties of decanoate hydroxylase of harvest mouse liver microsomes were investigated. The variation of hydroxylation activity with pH was determined (Fig. 2), and pH optimum was observed at pH 7.5 for total hydroxylation activity. However, the ω-hydroxylation increased with increasing pH while the (ω-1)-hydroxylation decreased with increasing pH. The $\omega/(\omega$-1)-hydroxylation ratio increased with increasing pH.

The requirements of reduced pyridine nucleotides for decanoate hydroxylation are shown in Table 3. NADPH was the favored electron donor. NADH had little effect on decanoate hydroxylation. Although NADH was added together with NADPH, a synergistic effect of NADH was not observed, but some additive effect of NADH was

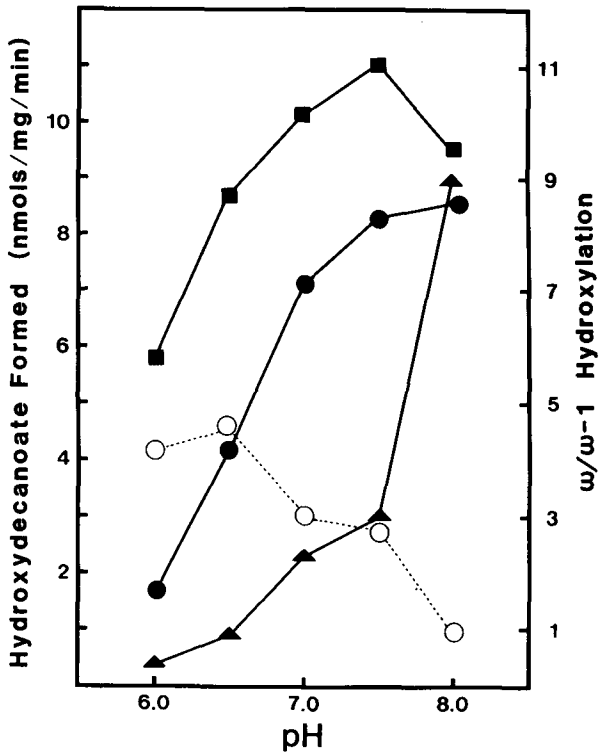

FIG. 2. Effect of pH on decanoate hydroxylation by harvest mouse liver microsomes. The amount of microsomal protein was 109 μ g. ●, ω-hydroxylation; ○, (ω-1)-hydroxylation; ■, total hydroxylation (the sum of ω- and (ω-1)-hydroxylation); ▲, ω/ω-1-hydroxylation ratio.

observed. Molecular oxygen was also required for decanoate hydroxylation. Table 4 shows the effect of inhibitors on decanoate hydroxylation activity. PCMB and carbon monoxide were potent inhibitors of ω- and (ω-1)-hydroxylation of decanoate. Menadione inhibited both the ω- and (ω-1)-hydroxylation to the same extent, but SKF 525-A, metyrapone, and NaN_3 inhibited only the (ω-1)-hydroxylation and showed no effect on ω-hydroxylation. KCN showed a somewhat stimulatory effect on the ω-hydroxylation but an inhibitory effect on the (ω-1)-hydroxylation.

TABLE 3

Effect of Reduced Pyridine Nucleotides on Decanoate Hydroxylation by Harvast Mouse Liver Microsomes

Reduced pyridine nucleotide	Relative hydroxylation activity	Distribution of hydroxy isomers (%)	
		ω	ω-1
NADH 500 μ M	7	—	—
NADPH 50 μ M	45	77	23
100 μ M	72	75	25
500 μ M	100[a]	82	18
NADH (500 μ M)			
+ NADPH 50 μ M	57	87	13
+ NADPH 100 μ M	97	85	15
+ NADPH 500 μ M	113	87	13
None	2	—	—
NADPH (500 μ M), anaerobic (helium)	5	—	—

[a]Actual hydroxylation activity:10.56 nmol/mg microsomal protein/min.

DISCUSSION

It was demonstrated in this study that the fatty acid hydroxylase in harvest mouse liver microsome, like the hydroxylase in the liver microsomes of other species, catalyzed the hydroxylation of fatty acids at the ω- and (ω-1)-positions. It was also demonstrated that cytochrome P-450 is involved in the fatty acid hydroxylase system of harvest mouse liver microsomes, because carbon monoxide, metyrapone and SKF 525-A were potent inhibitors of decanoate hydroxylation, and molecular oxygen and NADPH were required for the hydroxylation. It was found in this study that several inhibitors dramatically changed the ω/(ω-1)-hydroxylation ratio. The ratio was also changed with increasing pH. Thus, these findings support the hypothesis that different cytochrome P-450 species are involved in the hepatic microsomal hydroxylation of fatty acids at ω- and (ω-1)-positions in the harvest mouse. In order to prove the involvement of different cytochrome

TABLE 4

Effect of Inhibitors on Decanoate Hydroxylation by Harvest Mouse Liver Microsomes

Inhibitor	Decanoate hydroxylation activity (nmol/mg microsomal protein/min)			ω /ω-1 hydroxylation
	ω	ω - 1	ω & ω - 1	
None (control)	8.69(100)[a]	3.22(100)	11.91(100)	2.70
PCMB[b] (0.1 mM)	1.65(19)	0.49(15)	2.14(18)	3.37
CO-O_2 (1:1, v/v)	1.64(19)	0.27(8)	1.91(16)	6.07
CO-O_2 (9:1, v/v)	0.21(2)	0.15(5)	0.36(3)	1.40
Menadione(0.01 mM)	5.49(63)	2.13(66)	7.64(64)	2.58
KCN (2 mM)	10.73(123)	1.06(33)	11.79(99)	10.12
SKF 525-A (1 mM)	8.44(97)	2.52(78)	10.96(92)	3.35
Metyrapone (0.1 mM)	8.80(101)	1.80(56)	10.60(89)	4.89
NaN_a (1 mM)	8.42(97)	0.63(20)	9.05(76)	13.37

[a]Values in parentheses are percentages of the specific activity to control.

[b]*p*-Chloromercuribenzoate.

TABLE 5

Hydroxylation Activity of Fatty Acids by Various Microsomes

Enzyme source	Cytochrome P-450 (nmol/mg microsomal protein)	Hydroxylation activity (nmol/nmol cytochrome P-450/min)			Reference
		C_{10}	C_{12}	C_{16}	
Harvest Mouse					
Liver Microsomes (MS)	1.22 ± 0.11	9.41(100)	6.44(100)	3.07(100)	—
Shrew liver MS	0.79 ± 0.14	8.01(85)	8.92(139)	2.09(68)	(7)
Gerbil liver MS	1.60 ± 0.16	1.62(17)	4.03(63)	1.89(62)	(8)
Rat(Wistar) liver MS	0.87 ~ 1.22	3.45(37)	2.80(43)	1.06(35)	(7)
Rabbit intestine MS	0.40	0.83(9)	1.83(28)	0.78(25)	(19)

P-450 species in harvest mouse liver microsomes, solubilization and purification of cytochrome P-450 species should be done in the future.

Although the fatty acid hydroxylase in harvest mouse liver microsomes is a typical cytochrome P-450-dependent monooxygenase, several characteristics of data in this study should be noted. First, the hepatic microsomal protein (9.1 ± 1.3 mg/g liver) of the harvest mouse is much higher than that in the musk shrew (2.7 ± 0.6 mg/g liver) (7) and the Mongolian gerbil (2.4 ± 0.5 mg/g liver) (8). The high value for microsomal protein of the harvest mouse may be an advantage of the experimental animal model. Secondly, although the cytochrome P-450 content in various microsomes of laboratory animals showed different values (Table 5) (7,8,19), the hydroxylase activity (based on cytochrome P-450) of decanoate and palmitate was much higher in liver microsomes of the harvest mouse than in liver or intestine microsomes of other species. It may be postulated that the cytochrome P-450 species catalyzing ω- and (ω-1)-hydroxylation of fatty acids are present in harvest mouse liver microsomes in higher concentrations.

Finally, it is noteworthy that the hydroxylation activity curve *vs* chain length was biphasic at decanoate and myristate in harvest mouse liver microsomes. Although from inhibitor and cofactor studies it was demonstrated that ω- and (ω-1)-hydroxylation of fatty acids were catalyzed by different cytochrome P-450 species in liver microsomes of the harvest mouse, the fact mentioned above demonstrates also the involvement of at least two cytochrome P-450 species specific for fatty acid hydroxylation.

Since some constants (0.743 for basal metabolic rate; 0.73 for heat production; 0.73 for O_2 flow, etc.) for the hepatic and physiological allometric equations (20,21) are similar to our data (0.787) in the equation relating body weight of laboratory animals to total laurate hydroxylation activity in liver, the similarity of the constants suggests a close correlation between the rate of laurate hydroxylation in liver and the energy metabolism in the possible laboratory species. The physiological significance of ω- and (ω-1)-oxidation of fatty acids under normal conditions is still unknown. Neither starved nor diabetic animals were used for our previous and present studies. Therefore, our present results could shed more light on the physiological significance of ω- and (ω-1)-oxidation of fatty acids in laboratory animals under normal conditions.

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Isolation of a Guanine-Malondialdehyde Adduct from Rat and Human Urine

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A 1:1 adduct of guanine with malondialdehyde (MDA) was isolated from rat and human urine. This compound was shown to be identical to a synthetic adduct prepared according to the procedure of Seto *et al.* (*Bull. Chem. Soc. Jpn. 58,* 3431–3435, 1985). The UV, NMR and other characteristics of the compound were consistent with the tricyclic pyrimidinopurine structure proposed by these investigators. Its endogenous origin is indicated by its presence in the urine of rats fed an MDA-free diet, and by the observation that its excretion increased following iron or carbon tetrachloride administration. It may serve as a marker for nucleic acid modification caused by lipid peroxidation *in vivo*.
***Lipids 25,* 82–85 (1990).**

In previous experiments in this series, chromatographic analysis of rat and human urine has revealed the presence of several acid hydrolyzable MDA derivatives in addition to small amounts of the free compound (Fig. 1). Two major metabolites were identified as *N*-ε-(2-propenal)lysine (1) and its *N*-α-acetylated derivative (2) (Fig. 1, Compounds 1 and 7), and two minor metabolites as MDA adducts with serine (3) and ethanolamine (4) (Fig. 1, Compounds 2 and 6). The presence of these compounds in urine reflects reactions of MDA with the amino groups of proteins and phospholipid bases.

The reactivity of MDA with amino groups suggested that one or more of three unidentified minor MDA derivatives in urine (Fi.g 1, Compounds 3–5) might be an addition product with the nucleic acid base adenine, guanine or cytosine. MDA adducts with these bases were prepared and their chromatographic behavior was compared with that of the unidentified MDA derivatives isolated from urine. This paper describes the identification of one of these metabolites as an MDA adduct with guanine (Fig. 1, Compound 3).

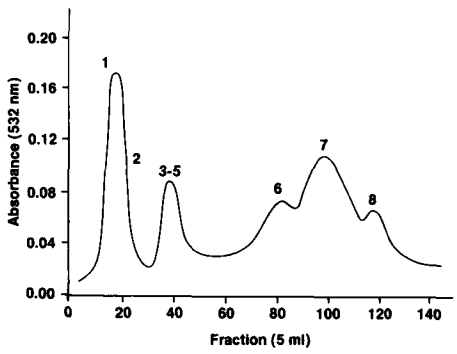

FIG. 1. Elution profile of MDA derivatives obtained by anion exchange chromatography of 50 ml of human urine as previously described (2). 1 = *N*-e-(2-propenal)lysine; 2 = *N*-(2-propenal)ethanolamine; 6 = *N*-(2-propenal)serine; 7 = *N*-α-acetyl-*N*-ε-(2-propenal)lysine; 8 = free MDA; 3 = guanine adduct; 4 and 5 are unidentified. MDA was determined spectrophotometrically as the TBA derivative (12) in every fifth fraction after hydrolysis at pH 3.0 for 30 min at 95°C.

EXPERIMENTAL

Synthesis of a guanine-MDA adduct. The reaction of guanine and its nucleosides with MDA has been reported to yield a number of different products. Moschel and Leonard (5) found that guanosine reacted with various substituted MDA compounds to form tricyclic guanosine derivatives. Reiss *et al.* (6) reported that MDA reacted with adenine and guanine to yield compounds with the fluorescence properties of aminoiminopropenes. Lee *et al.* (7) prepared a similar fluorescent MDA adduct with guanosine. Basu *et al.* (8) obtained a 2:1 adduct of MDA with guanine, and reported the following order of MDA reactivity with DNA bases: deoxyguanosine > deoxyadenosine ≫ deoxycytidine > thymidine. Nair and coworkers (9) prepared 3:1 adducts of MDA with adenine and cytosine nucleosides and a 1:1 adduct with adenosine. Seto *et al.* (10) reported that adenine and cytosine failed to react with MDA, but obtained tricyclic reaction products with guanine and guanosine.

A modification of the procedure of Seto *et al.* (11) was adopted for the synthesis of a guanine-MDA adduct. MDA was generated by stirring a solution of 25 mmol of tetramethoxypropane in 1 M HCl at 40°C for 40 min. Guanine (5 mmol in 25 ml of 1 M HCl) was added, and the solution was titrated to pH 4.0 ± 0.2 (final volume 62 ml). After stirring for 1 hr at 40°C, 200 ml of acetonitrile was added and the mixture was kept at 0°C for 16 hr. The supernatate was decanted and the solid material collected by centrifugation at 2000 × *g* for 5 min. The precipitate was washed with cold, absolute ethanol until the washings were no longer yellow, then extracted three times with 20 ml of water at 65°C. The combined extracts were centrifuged, and the supernate was passed through a Millex®-GS 0.22 μm filter unit.

The filtrate was chromatographed on an anion exchange column as described elsewhere (2), and MDA in the eluates was determined spectrophotometrically as the TBA derivative (12) after heating at 95°C for 30 min in the presence of TBA. The MDA profile indicated the presence of two products (Fig. 2). Fractions 30–50, corresponding to those containing three unidentified MDA compounds in urine (Fig. 1) were concentrated *in vacuo* at 37°C and subjected to size exclusion chromatography on Sephadex G-10 (2). Those fractions testing positive for MDA (180–220 ml) were pooled and taken to dryness at 37°C. The reaction products were separated from residual NaCl by extracting three times with 20 ml of anhydrous methanol.

The combined extracts were evaporated to dryness, and the residue in 2 ml of 50% methanol was applied to a 1.2 × 10 cm column of AG® 50W-X8 cation exchange

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Abbreviations: MDA, malondialdehyde; TBA, thiobarbituric acid; HPLC, high performance liquid chromatography; R_T, retention time

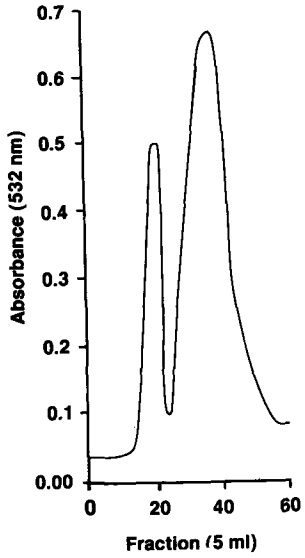

FIG. 2. Elution profile of MDA derivatives obtained by anion exchange chromatography of the reaction products of guanine with MDA.

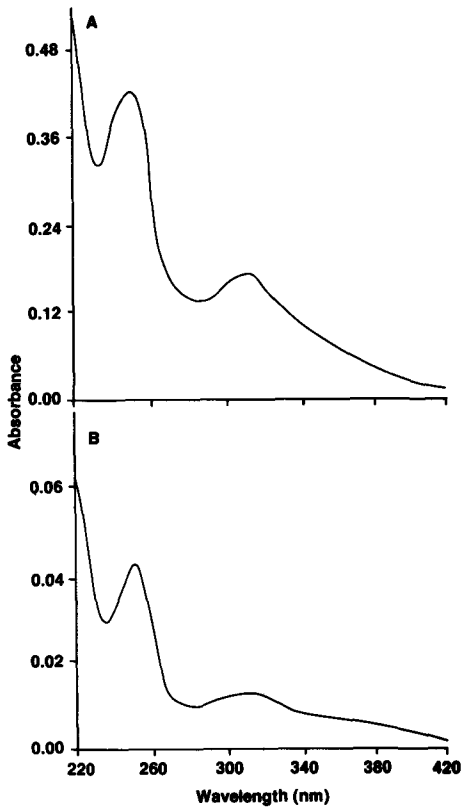

FIG. 3. UV absorbance spectra in water of the synthetic guanine-MDA adduct (A) and the adduct isolated from 1 liter of rat urine (B).

resin (200–400 mesh) in the sodium form (Bio-Rad Laboratories, Richmond, CA). The column was developed with 50 ml of water followed by 60 ml of a saturated aqueous solution of KCl. The last 50 ml, which contained a compound that yielded MDA on acid hydrolysis, was evaporated to dryness at 37°C, and the residue was extracted three times with 20 ml of anhydrous methanol. After removing the solvent at 37°C, the residue was dissolved in water and subjected to final purification by reverse-phase HPLC using a Waters μBondapak C_{18} analytical column developed with water at 1.2 ml/min.

Isolation of a guanine-MDA adduct from urine. Anion exchange chromatography of 50 ml aliquots of rat urine was carried out as described elsewhere (2). Fractions 30–50 (Fig. 1) were pooled, reduced in volume and subjected to size exclusion chromatography (2). A peak in the elution profile for MDA analogous to that for the synthetic guanine-MDA adduct was collected and the residue was purified by reverse-phase HPLC (2) using water as the mobile phase. The flow rate was increased from 1.2 ml to 2.2 ml/min at 48 min and a 50 to 60 min fraction was collected. The fraction was reduced in volume to 200 μl and subjected to final purification on a reverse-phase HPLC column (2) developed with 18% methanol at a flow rate of 1 ml/min. A peak for UV absorbance was collected in the 5.5 to 6.5 min fractions.

Effect of iron administration. Four adult rats, weighing about 500 g, were fed an MDA-free diet containing hydrogenated coconut oil as the fat source (13) for 2 days, then were injected intraperitoneally with 9 mg Fe as iron nitrilotriacetate per kg body weight. This treatment has been shown to produce a marked increase in total MDA excretion (13). Urine collected during the following 48 hr was subjected to the isolation procedure described for the guanine-MDA adduct and the relative amounts of the adduct excreted by Fe-treated animals, and controls were assessed from the elution profiles obtained by G-10 column chromatography.

RESULTS

The synthetic adduct yielded single coinciding peaks for UV absorbance at 254 nm and TBA reactivity at R_T 50–60 min when chromatographed on the reverse-phase μBondapak C_{18} column using water and at R_T 6.0 min using 18% methanol. Its UV absorption spectrum (Fig. 3A) was similar to that of the guanine-MDA adduct reported by Seto *et al.* (11). In view of the multiplicity of products obtained by reacting MDA with guanine reported by other investigators, and the fact that two products yielding MDA on acid hydrolysis were obtained in the present study, the synthetic adduct was subjected to further characterization. The UV spectrum (Fig. 3A) lacks the peak at 280 nm characteristic of enaminals formed by reactions of MDA with free amino groups (14). The coupling constants in ^1H NMR (Fig. 4) also are inconsistent with an enaminal structure. After heating the adduct in boiling water at pH 3.0 for 30 min in the presence of TBA, the hydrolystate was analyzed for MDA as the TBA-MDA complex using an HPLC procedure (13) and for guanine using reverse-phase HPLC. The observed molar ratio of MDA to guanine was 1.00:0.91. These findings support the pyrimidopurinone structure of a guanine MDA adduct assigned by Seto *et al.* (11) (Fig. 5).

On reverse-phase chromatography, the urinary compound co-eluted with the synthetic adduct using either water or 18% methanol as solvent (Fig. 6). Hydrolysis of the isolate at 97°C in 1 ml of HCl, pH 3.0 ± 0.2, for 30 min yielded MDA and guanine in a molar ratio of 1.00:0.92. The isolate from 1 liter of rat urine exhibited

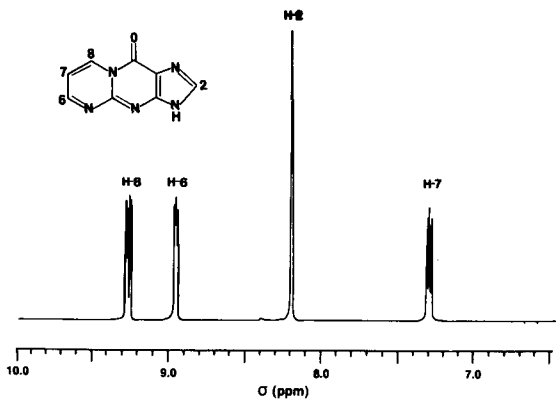

FIG. 4. Nuclear magnetic resonance spectrum (400 MHz) of the synthetic guanine-MDA adduct. ^1H NMR (D_2O) δ (ppm) 7.3 (1H, *dd*, $J_{6,7}$ = 4.1 Hz and $J_{7,8}$ = 7.2 Hz, C-7(H)), 8.2 (1H, *s*, C-2(H)), 8.96 (1H, *dd*, $J_{6,8}$ = 2.2 and $J_{6,7}$ = 4.1 Hz, C-6(H)), and 9.28 (1H, *dd*, $J_{6,8}$ = 2.2 Hz and $J_{7,8}$ = 7.1 Hz, C-8(H)). A Bruker WH-400 instrument was used.

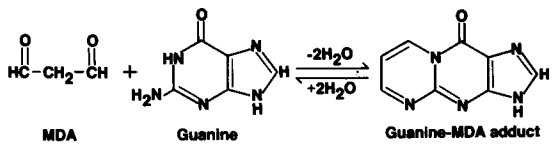

FIG. 5. Structure of the guanine-MDA adduct proposed by Seto *et al.* (11) and confirmed in this study.

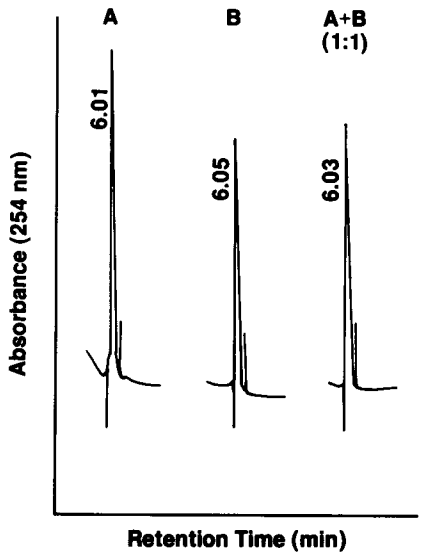

FIG. 6. Retention times obtained by HPLC of the synthetic guanine-MDA adduct (A), the urinary isolate (B) and an equal volume of a 1:1 mixture. Peak amounts were 131, 107 and 114, respectively. Reverse-phase C_{18} μBondapak column, mobile phase 18% methanol, flow rate 1 ml/min, UV detection at 254 nm.

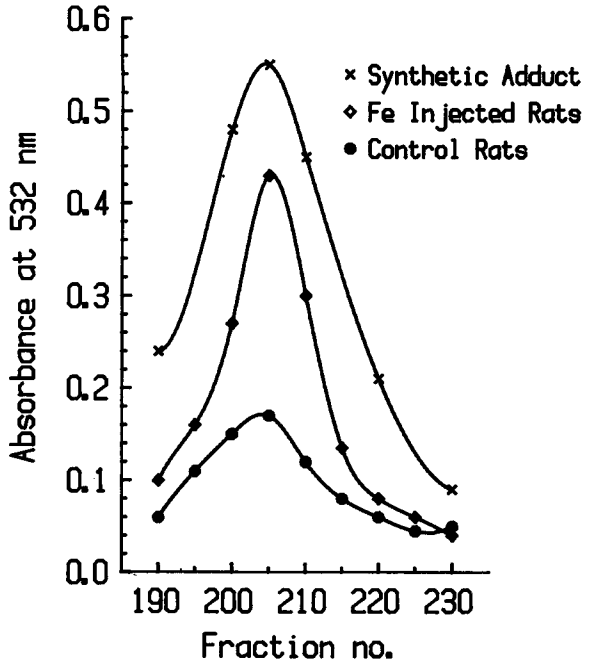

FIG. 7. Elution profiles obtained by Sephadex G-10 chromatography (2) of concentrates of the guanine-MDA adduct in composite 48-hr urine samples from 5 rats injected with iron nitrilotriacetate (diamond) and 5 uninjected controls (closed circle) expressed in terms of TBA-MDA equivalents. The elution profile for the synthetic guanine-MDA adduct is also shown (X).

a UV spectrum corresponding to that of the synthetic adduct (Fig. 3B).

The Sephadex G-10 elution profiles for the Fe-injected animals; the controls and the synthetic adduct are shown in Fig. 7. The guanine-MDA fractions from the Fe-injected animals contained about 4 times as much MDA as the control fractions. An analytical procedure recently developed for the quantitative determination of the guanine-MDA adduct (unpublished) was applied to rat urine. Excretion of the adduct by 500-g rats fed an MDA-free diet was increased 3-fold after intraperitoneal administration of 9 mg Fe/kg as Fe nitrilotriacetate, and 2.5-fold after stomach intubation with 1 ml CCl_4/kg.

DISCUSSION

An endogenous origin of the guanine-MDA adduct is indicated by its presence in the urine of rats during the last 24 hr of a 48 hr fast, during consumption of an MDA-free diet, and by increases in its excretion following administration Fe or CCl_4, which are well known catalysts of lipid peroxidation *in vivo*. Analysis of the *in vitro* digestion products of several foods has revealed that MDA is present mainly as the lysine adduct *N*-ε-(2-propenal)lysine (15), and has failed to detect an MDA derivative with the chromatographic properties of the guanine adduct.

MDA has been reported to react with DNA to form interstrand and DNA-protein crosslinks (16) and to bind to the guanine and cytosine bases of DNA in rat skin fibroblasts grown in culture (17). It is unlikely that the adduct in urine is the product of a reaction with free

guanine, as guanine is relatively insoluble at physiological pH and occurs at very low concentrations as the result of rapid metabolism by guanine phosphoribosyl transferase. Urinalysis has failed to reveal any adducts of MDA with free amino compounds. The guanine adduct is unlikely to arise from MDA generated in the cyclooxygenase reaction, as endogenous MDA excretion in the rat is reduced only about 20% by salicylate inhibition of this enzyme (13).

The presence of an MDA derivative of guanine in urine constitutes strong evidence of nucleic acid modification caused by lipid peroxidation *in vivo* and is consistent with the mutagenicity of MDA in bacterial and mammalian cells (18). Guanine is released from nucleic acids mainly in the form of nucleosides and partially as the free base. The presence in urine of an MDA adduct with d-guanosine, a possible marker for lipid peroxidative damage to DNA specifically, has been investigated without success (unpublished data). However, Cundy *et al.* (19) have reported the presence in urine of 8-hydroxydeoxyguanosine. Kasai *et al.* (20) identified 8-hydroxyguanine in DNA exposed to agents that produce oxygen radicals. The oxidizing species appears to be the hydroxyl radical (21), which is also strongly implicated in the initiation of lipid peroxidation. Determination of the 8-hydroxy and MDA derivatives of guanine in urine thus may be a means of assessing damage to nucleic acids caused directly and indirectly by radicals of molecular oxygen.

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Biochemical and Functional Abnormalities in Hypercholesterolemic Rabbit Platelets

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This study was designed to elucidate changes in rabbit platelet lipids induced by a cholesterol rich diet and to explore the possible correlation of these lipid changes with platelet abnormalities. Pronounced biochemical alterations were observed when serum cholesterol levels of 700–1000 mg% were reached. Hypercholesterolemic (HC) platelets contained 37% more neutral lipids and 16% less phospholipids than the controls. Lysolecithin, cholesterol esters and phosphatidylinositol (PI) levels were increased in HC platelets, and the levels of phosphatidylcholine (PC) were decreased. The cholesterol/phospholipid molar ratio of lipidemic platelets increased from 0.55 ± 0.011 to 0.89 ± 0.016 ($P < 0.01$) in eight weeks. HC platelets had 90% more arachidonic acid (AA) in the PI than normal platelets. No significant changes in AA of PC were observed.

Platelet function was monitored by the uptake and release of [^{14}C]serotonin in platelet rich plasma (PRP), using varying concentrations of collagen as an aggregating agent. The uptake of [^{14}C]serotonin in HC and normal platelets ranged from 78–94%. The percent of [^{14}C]serotonin released from normal and HC platelets was proportional to the concentration of collagen. However, lipidemic platelets were hyperreactive to low concentrations of collagen. Incorporation of 50 μM acetylsalicylic acid into the aggregating medium suppressed the release of [^{14}C]serotonin in normal PRP by more than 90%, but had only a partial effect on lipidemic PRP.
***Lipids 25,* 86–92 (1990).**

Despite considerable epidemiological evidence that cholesterol affects the development of atherosclerosis, mechanisms involved in this relationship remain elusive. Accumulation of cholesterol and fat in tissues could result in altered levels of lipid and protein in cells which may play a significant role in atherogenesis. It has been demonstrated that cholesterol enhances aggregation of platelets (1). Accumulation of cholesterol in platelets *(in vitro)* was the main cause of increased platelet reactivity to aggregating agents and of increased serotonin release (2). Changes in the other platelet lipids, specifically phospholipids (PL), have been suggested to also influence platelet aggregation (2,3).

The purpose of this study was to evaluate the interrelationships among platelet cholesterol, PL metabolism, and function of platelets during diet induced hypercholesterolemia (HC) in rabbits.

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Abbreviations: AA, arachidonic acid; ASA, acetylsalicylic acid; BHT, butylated hydroxytoluene; CE, cholesterol esters; EDTA, disodium ethlenediaminetetraacetic acid; FA, fatty acid(s); FC, free cholesterol; FC/PL, free cholesterol/phospholipid molar ratio; HC, hypercholesterolemia(s); LL, lysolecithin; NL, neutral lipid(s); PC, phosphatidylcholine; PI, phosphatidylinositol; PL, phospholipid(s); PO, peanut oil; PPP, platelet poor plasma; PRP, platelet rich plasma; TLC, thin-layer chromatography; U.I., unsaturation index.

METHODS

Animals and diet. New Zealand white rabbits (R&R Rabbitry, Stanwood, WA) weighing 2–4 kg were used. One experimental group was given a diet supplemented with 4% peanut oil (PO) (4) (Planters, East Hanover, NJ) and 1% cholesterol (Dyets, Bethlehem, PA). The diet of a second set of animals was supplemented with 2% PO and 0.5% cholesterol. A third group of control rabbits was given a diet supplemented with 4% PO only. The corresponding control animals were fed normal Purina Laboratory Chow (Ralston Purina Co., Inc., St. Louis, MO). The diets were fed for a period of 8–12 weeks. Blood samples were drawn every two weeks from animals on the higher cholesterol diet and every four weeks from animals on the lower cholesterol diet. Blood was collected from an ear artery with a 21 gauge Butterfly® infusion set (Abbott Hospitals, Inc., North Chicago, IL). Samples were processed immediately.

Platelet lipids. Ten to 14 ml of blood were collected into heparin (5 units/ml, final concentration) from control and experimental groups. Samples were centrifuged at 22°C for 20 min at 100 × *g*. The resultant platelet rich plasma (PRP) was transferred into 12 × 75 mm Falcon tubes (Falcon, Oxnard, CA), and platelets were counted by phase microscopy. The platelets were harvested from PRP by centrifugation at 2500 × *g* for 30 min at room temperature. The platelets were washed three times with buffered saline containing 0.1% glucose, pH 7.4, and were frozen at −20°C under N_2 until further analysis. Platelet poor plasma (PPP) was kept frozen (−20°C) if not used immediately.

Lipids were extracted from washed platelets by the methods of Folch-Pi *et al.* (5) and Dalal *et al.* (6). Purified lipid extract containing butylated hydroxytoluene (BHT, 50 mg/liter) to prevent autooxidation of lipids was evaporated to dryness under nitrogen until constant weight was achieved. Total lipids were fractionated by one dimensional TLC using $CHCl_3/CH_3OH/13.5N$ NH_4OH/CH_3COOH (70:40:5:1, by volume) as solvent system (containing BHT). Precoated silica-gel H plates of 0.25 mm were used. The fractionated PL and neutral lipids (NL) were visualized in an iodine chamber and quantitated according to the modified techniques of Hill *et al.* (7) and Parker and Peterson (8). Lipid phosphorus was determined by Bartlett's technique (9). The cholesterol (free and esterified) was estimated by a modified method of Pearson *et al.* (10). Total protein was estimated by the method of Lowry *et al.* (11).

The percentage distribution of the various chain length fatty acids (FA) was determined for the purified phosphatidylinositol (PI) and phosphatidylcholine (PC) of platelets. Acid catalyzed transmethylation was used to form the methyl esters, which were then quantitated by TLC using a modified solvent system of hexane/diethyl ether/acetic acid (80:18:2, by volume) (12). Appropriate lipid and FA standards (Sigma Diagnostic, St. Louis, MO)

were used as reference samples. Unsaturation index (U.I.) was calculated by summing the pcercentage of individual unsaturated FA (18:1, 18:2 and 20:4) times the number of double bonds (1, 2 and 4, respectively).

Measurement of [^{14}C]serotonin uptake and release. Nine volumes of blood were collected into a plastic tube containing one volume of 0.11 M sodium citrate; PRP was prepared by centrifuging at 100 × *g* for 20 min at room temperature. PRPs (obtained from 3-4 control and 3-4 diet animals) were separately pooled. Platelet concentration was determined with phase microscopy. The platelet count of both control and diet PRP was adjusted with PPP to achieve a final concentration of 280,000 ± 7000 per microliter.

[^{14}C]Serotonin (Amersham/Searle Corp., Arlington Heights, IL) 57.4 mCi/mmol was dissolved in 0.01N hydrochloric acid (final concentration 50 μCi/5 ml HCl) and kept at −20°C. Disodium ethylenediaminetetraacetic acid (EDTA) (Merck Sharp & Dohme, West Point, PA) was diluted to 134 mmol/l with 66% aqueous ethanol. Triton-X100 (Packard Instrument Co., Downers Grove, IL) was diluted to 20% volume in millipore filtered deionized water. Lyophilized collagen (calf skin) (Sigma Diagnostic) reconstituted with 1 ml of deionized water was used as an aggregating agent, at concentrations indicated in Results. Fourteen ml of adjusted PRP was incubated with [^{14}C]serotonin (10–15 μl/ml PRP) at room temperature for 1 hr. The incubated mixture was then divided into six aliquots, each containing 2 ml labelled PRP. EDTA (0.2 ml) and 0.1 ml Triton-X100 were added to tube one, which was then kept in an ice bath. Collagen, 0.1 ml, containing 200 μg, 40 μg, 20 μg or 10 μg, respectively, was added to each of four tubes, and the mixtures were stirred mechanically for 10 min at 37°C. The reaction was stopped by adding 0.2 ml of EDTA and transferring to an ice bath. A blank of PPP was prepared by centriguging the labelled PRP (tube six); 0.1 ml of saline and 0.2 ml EDTA were then added to the PPP. All tubes except number one were then centrifuged at 2500 × *g* for 15 min at 4°C. Then 0.2 ml from tube one and 0.2 ml of supernatant from each tube (2–6) were spread on to glass fiber filter paper and counted (Beckman Model LS9000), using 15 ml of Distol (Amersham/Searle Corp.) scintillation fluid to determine the radioactivity of the resultant materials. The serotonin taken up by the platelets was determined by subtracting the amount remaining in the centrifuged PPP from the total amount added.

The aforementioned method is a modified procedure of the techniques reported elsewhere (13,14). It was adopted to avoid trying to measure platelet aggregation in the nearly opaque plasma of HC rabbits. Collagen was chosen as the agonist, because it gave an acceptable dose response curve.

Statistical analysis. The results were expressed as mean ±SEM and were compared using Student's *t*-test for unpaired samples.

RESULTS

Effects of 4% peanut oil on platelet lipids. Serum cholesterol and free cholesterol/phospholipid molar ratio (FC/PL) of platelets of both control and PO fed animals are shown in Table 1. The addition of 4% PO did not change the lipid composition of platelets. Serum cholesterol was slightly higher than normal after two and eight weeks of PO feeding. Platelet function, monitored by the percent uptake and release of [^{14}C]serotonin using varying concentrations of collagen, showed little variation between the control and PO fed rabbits. The uptake of [^{14}C]serotonin was 86% in control and 78% in PO platelets. The shapes of the dose response curves were comparable. The percentages of [^{14}C]serotonin released from PO fed platelets were 3–8% higher than the corresponding control values with all doses of collagen.

Effects of higher cholesterol diet. Serum cholesterol concentrations are shown in Figure 1. They fluctuated very little in the control rabbits. Average serum cholesterol levels increased rapidly in rabbits fed the higher cholesterol (1%) diet. The mean platelet lipid levels of control animals were comparable to those reported by others

TABLE 1

Platelet Lipids in Rabbits Fed 4% Peanut Oil or Control Diet

Week	Number of animals	Free cholesterol ($\mu g/10^9$ platelets)	Phospholipids ($\mu g/10^9$ platelets)	FC/PL[a]	Serum cholesterol (mg%)
		4% peanut oil			
0	8	112 ± 10.3[b]	400 ± 7.8	0.56	85.0 ± 10.9
2	4	100 ± 10.2	425 ± 9.8	0.47	113.8 ± 7.5[c]
4	4	102 ± 10.2	390 ± 6.4	0.52	103.2 ± 4.7
6	4	94 ± 14.1	366 ± 10.5	0.51	100.8 ± 10.4
8	4	109 ± 4.6	410 ± 4.0	0.53	105.0 ± 3.5[c]
		Control			
2	4	100 ± 6.7	375 ± 3.5	0.53	94.2 ± 5.0
4	4	92 ± 8.2	390 ± 4.9	0.47	91.0 ± 7.0
6	4	106 ± 4.5	410 ± 3.0	0.51	95.0 ± 13.0
8	4	97 ± 7.7	385 ± 8.0	0.51	93.5 ± 4.0

[a] FC/PL = free cholesterol/phospholipid molar ratio.

[b] Each value is the average ±SEM.

[c] Significantly different from time zero value $p \leq 0.05$.

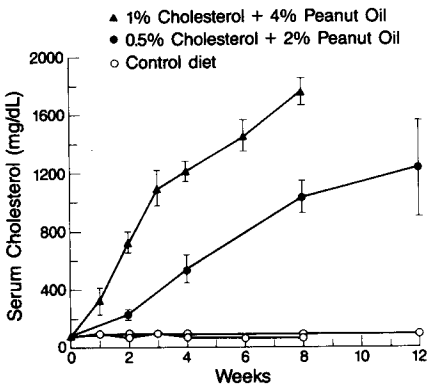

FIG. 1. Serum cholesterol (mean ±SEM) in rabbits fed a diet with 0.5% (n = 4) or 1% (n = 20) cholesterol supplemented with 2% or 4% peanut oil, respectively, and controls (n = 4 − 11).

(Table 2) (15). After eight weeks of diet, HC platelets (Table 2) contained 37% more NL than their normal counterparts, due to an increase in free and esterified cholesterol (FC and CE). This, together with a reduction in PL, resulted in the FC/PL of HC platelets increasing within two weeks from control levels of 0.55 ± 0.011 to 0.64 ± 0.021 and continuing to increase progressively until reaching 0.89 ± 0.016 ($P < 0.01$) at 8-9 weeks. CE levels sequentially increased, showing a five-fold elevation at the end of the 8-9 week period. The levels of triglycerides showed small fluctuations from the normal control values. Individual PL of HC platelets changed markedly compared to the control platelets. HC platelets showed increases in PI (45 ± 1.5 vs 25 ± 1.6 $\mu g/10^9$ plts.), lysolecithin (LL) (16 ± 3.5 vs 8 ± 1.6 $\mu g/10^9$ plts.), and unidentified lipids (43 ± 2.5 vs 20 ± 5.0 $\mu g/10^9$ plts.) and a decrease in PC (88 ± 12 vs 132 ± 20 $\mu g/10^9$ plts.)

TABLE 2

Platelet Lipid Composition of Rabbits Fed 1% Cholesterol Plus 4% Peanut Oil or Control Diet

Week	Number of animals	Free cholesterol ($\mu g/10^9$ platelets)	Phospholipids ($\mu g/10^9$ platelets)	FC/PL[a]	Cholesterol esters ($\mu g/10^9$ platelets)	Triglycerides ($\mu g/10^9$ platelets)
			Diet rabbits			
0	31	106 ± 2.1[b]	384 ± 5.7	0.55 ± 0.011	4.5 ± 2.5	13.0 ± 1.5
2	20	105 ± 2.4	327 ± 4.1[c]	0.64 ± 0.021[c]	11.5 ± 2.0[d]	16.0 ± 1.7
4	20	112 ± 5.7	315 ± 4.8[c]	0.71 ± 0.018[c]	12.8 ± 3.0[d]	17.0 ± 1.7
6	20	130 ± 2.6[c]	322 ± 4.0[c]	0.81 ± 0.034[c]	19.0 ± 2.3[d]	15.0 ± 3.0
8-9	20	145 ± 2.3[d]	324 ± 4.6[d]	0.89 ± 0.016[d]	23.2 ± 2.0[d]	17.0 ± 2.6
			Control rabbits			
2	11	102 ± 3.5	373 ± 7.0	0.55 ± 0.015	6.0 ± 3.0	14.0 ± 1.3
4	11	99 ± 3.0	370 ± 5.5	0.53 ± 0.010	5.0 ± 2.0	10.0 ± 0.8
6	11	110 ± 2.8	415 ± 3.5	0.53 ± 0.016	8.0 ± 4.5	14.0 ± 2.0
8-9	11	105 ± 2.7	410 ± 5.0	0.51 ± 0.016	10.0 ± 5.0	15.0 ± 1.3

[a]FC/PL = free cholesterol/phospholipid molar ratio.

[b]Each value is the average ±SEM.

[c]Significantly different from time zero value $p \leq 0.05$.

[d]Significantly different from time zero value $p \leq 0.01$.

TABLE 3

Percent Fatty Acid Distribution of Platelet Phosphatidylcholine and Phosphatidylinositol in Control and HC Platelets

	Phosphatidylcholine		Phosphatidylinositol	
	Control	1% C[a] + 4% PO	Control	1% C + 4% PO
Week	0	8-9	0	8-9
Number of animals	31[b]	20	31	20
Fatty acids				
16:0	26.9 ± 0.9[c]	26.0 ± 0.8	7.5 ± 0.2	8.5 ± 1.7
18:0	18.6 ± 1.0	20.9 ± 1.4	52.4 ± 1.2	38.0 ± 1.2[d]
18:1	14.5 ± 0.8	16.2 ± 1.6	5.0 ± 0.2	3.0 ± 2.1
18:2	33.9 ± 2.9	29.0 ± 2.5	2.1 ± 0.24	5.2 ± 0.41[d]
20:4	4.6 ± 0.4	6.0 ± 0.8	23.5 ± 0.36	45.5 ± 0.56[d]
U.I.[e]	101	98	103	195

[a]C = cholesterol.

[b]Five to eight determinations: platelets pooled from 3-4 rabbits for each determination.

[c]Each value is the average of ±SEM.

[d]Significantly different from time zero value $P \leq 0.001$.

[e]Unsaturation Index; calculated by sum of percentage of individual unsaturated fatty acid times number of the corresponding double bonds (15). (18:1, 18:2 and 20:4) (1, 2 and 4).

after 8-9 weeks of the diet. The FA compositions of platelet PI and PC are shown in Table 3. There was a sequential accumulation of arachidonic acid (AA) (20:4) in the PI of HC platelets which reached a maximum (45.5 ± 0.56 vs 23.5 ± 0.36) (wt%) at the end of 8-9 weeks. The higher cholesterol diet also induced a reduction in the levels of 18:0 and an elevation of 18:2 in the PI. The increases in 18:2 and 20:4 caused a pronounced elevation in the U.I. of the PI molecule. FA levels of PC in HC platelets showed no variation from their corresponding control values. The U.I., which measures degree of unsaturation, remained unaltered. The protein content (not shown) of HC platelets did not differ throughout the duration of diet from their respective control values.

Platelet function was monitored at two-week intervals. The uptake of [^{14}C]serotonin in HC and normal platelets ranged from 80-92%. The shapes of the dose response curves in HC platelets were similar to those produced by control platelets (Fig. 2). The percent of [^{14}C]serotonin released from control and HC platelets was proportional to the concentration of collagen. However, HC platelets consistently released more of the absorbed [^{14}C]serotonin in response to the two lowest doses of collagen than did control platelets.

Effects of the lower cholesterol diet. With this diet the average serum cholesterol levels increased more slowly than those observed with the higher cholesterol diet (Fig. 1). The changes in the levels of various PL, FC/PL and CE of lower cholesterol diet platelets were similar to those seen in rabbits on the higher cholesterol diet (Table 4). The changes became pronounced when the serum cholesterol reached a level of 1030.0 ± 106.4 mg/dl (eight weeks). At this time the effects of cholesterol on platelet lipid content were similar to those observed during the first four weeks of the higher cholesterol diet (when the serum cholesterol was 1209.9 ± 71.6 mg/dl). Increases in platelet PI and AA were moderate (20% and 15%, respectively) at four weeks and reached maximums (96% and 93%, respectively) when the diet had been continued for eight weeks. Changes in the levels of FA and in the PC and PI and their U.I. followed the same pattern as seen with the higher cholesterol diet (Table 5).

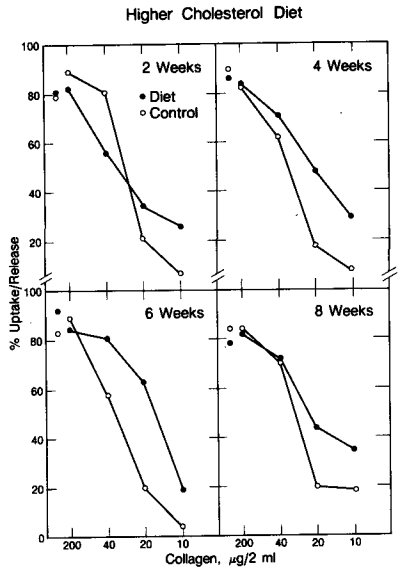

FIG. 2. Effects of various concentrations of collagen on the uptake and release of radioactivity from ^{14}C-labelled PRP from diet (1% cholesterol + 4% peanut oil) and control rabbit platelets (unconnected points represent % uptake). Each point is the result obtained with pooled PRP from 3-4 rabbits.

The percentage uptake of [^{14}C]serotonin for control and HC platelets was 78-94% (Fig. 3). The dose/release response curves were similar at four weeks, but thereafter HC platelets showed greater differences from controls with the two lowest doses of collagen than were found on the higher cholesterol diet. Incorporation of 110 μM acetylsalicylic acid (ASA) into the aggregating medium at 12 weeks suppressed the release of [^{14}C)serotonin from both control and diet PRP by more than 90%. However, when only 50 μM ASA was incorporated into the aggregating medium at 15 weeks, the release of [^{14}C]-serotonin was partially affected in the hypercholesterolemic PRP, but nearly completely suppressed in control platelets.

TABLE 4

Platelet Lipid Composition of Rabbits Fed 0.5% Cholesterol Plus 2% Peanut Oil or Control Diet

Week	Number of animals	Free cholesterol ($\mu g/10^9$ platelets)	Phospholipids ($\mu g/10^9$ platelets)	FC/PL[a]	Cholesterol esters ($\mu g/10^9$ platelets)	Triglycerides ($\mu g/10^9$ platelets)
			Diet rabbits			
4	4[b]	80	300	0.53	6.0	14
8	4	102	315	0.65	12.0	20
12	4	114	320	0.71	11.0	16
15	4	126	340	0.74	16.0	17
			Control rabbits			
4	4	75	310	0.48	<4.0	12
8	4	88	345	0.51	<2.0	18
12	4	95	366	0.52	<3.0	13
15	4	108	379	0.57	7.0	14

[a]Free cholesterol/phospholipid molar ratio.

[b]One determination of pooled platelets.

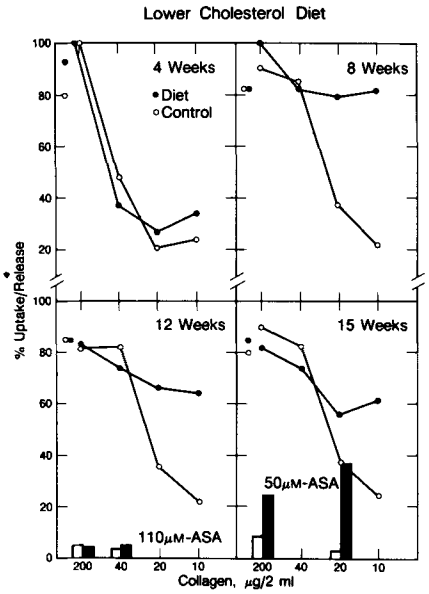

FIG. 3. Effects of various concentrations of collagen on the uptake and release of radioactivity from ^{14}C-labelled PRP from diet (0.5% cholesterol + 2% peanut oil) and control rabbit platelets (unconnected points represent % uptake). The bars indicate release of ^{14}C from the same platelets in the presence of the indicated amount of ASA; open bars represent control platelets, the solid bars HC platelets. Each point is the result obtained with pooled PRP from four rabbits.

Correlations among changes in lipids, morphology, count (16) and function in HC rabbit platelets are shown in Table 6. Platelet microcytosis occurred early and coincided with low platelet PL and high platelet CE with the 1% cholesterol diet, but neither lipid abnormality was striking when microcytosis developed after four weeks of the 0.5% cholesterol diet. Platelet aggregation became abnormal when, in addition to the above abnormalities, the FC/PL became elevated on the two diets (at four and eight weeks, respectively). However, there was no obvious difference between the effects of the two diets to explain the greater hyperreactivity to low doses of collagen that was present in platelets from rabbits fed the 0.5% cholesterol diet. Thrombocytosis occurred as a delayed phenomenon in rabbits fed the 1% cholesterol diet, but platelet counts remained normal on the 0.5% cholesterol diet despite the presence of similar biochemical abnormalities, albeit of less severity.

DISCUSSION

The present studies show that increases in the cholesterol content of platelets have effects on platelet PL metabolism and platelet function. LL and PI were high not only as a percent of total lipid phosphorus but also in absolute amounts. The FC/PL of HC platelets increased progressively throughout the course of the diets. This was due to an accumulation of FC and a decline in total PL.

The mechanisms by which dietary lipids affect platelet behavior have been associated with the changes in the FA distribution of PL (17). Our data showed increased levels of platelet PI and its AA. These changes may have been important factors in determining platelet hypersensitivity to collagen, because more substrate PI would be available for the action of phospholipase (17), and for each molecule of PI hydrolyzed, more molecules of AA would be generated. There is substantial evidence (18) that phospholipase induced hydrolysis of PI is stimulated in hypersensitive platelets, suggesting that degradation of PI and production of AA could play a significant role in the membrane modification, aggregation and release reactions. The FA composition of HC platelet PC remained unaltered, giving further credence to the concept that PI of platelet PL is one of the major sources of AA.

The enhanced release of platelet-bound [^{14}C]serotonin by low concentrations of collagen we observed in HC platelets could have been due to an accelerated generation of free AA and its various oxygenated derivatives, such as prostaglandins G_2 and H_2 and thromboxane A_2.

TABLE 5

Percent Fatty Acid Distribution of Platelet Phosphatidylcholine and Phosphatidylinositol in Control and HC Platelets

	Phosphatidylcholine		Phosphatidylinositol	
	Control	0.5% C[a] + 2% PO	Control	0.5% C + 2% PO
Week	8	8	8	8
Number of animals	4[b]	4	4	4
Fatty acids				
16:0	27.0	29.5	13.0	6.5
18:0	19.0	20.0	60.0	42.0
18:1	15.0	13.5	6.0	8.0
18:2	32.0	28.0	4.0	8.0
20:4	6.0	7.0	17.0	33.0
U.I.[c]	104	97.5	82	156

[a]C = cholesterol.

[b]One determination of pooled plataelets.

[c]Unsaturation Index: calculated by sum of percentage of individual unsaturated fatty acid times number of the corresponding double bonds (18:1, 18:2 and 20:4) (1, 2 and 4) (15).

TABLE 6

Comparison of Numerical, Morphological, Functional, and Biochemical Abnormalities in Rabbits Fed Diets of 1% Cholesterol plus 4% Peanut Oil or 0.5% Cholesterol (C) plus 2% Peanut Oil (PO)

Week	Platelet count[a]	Platelet volume[a]	[^{14}C]Serotonin release	Free cholesterol	Total phospholipid	Cholesterol esters	FC/PL[b]
			1% C + 4% PO				
2	—[c]	↓[d]	?[e]	—	↓	↑[f]	↑
4	—	↓	↑	—	↓	↑	↑
6	↑	↓	↑	↑	↓	↑	↑
8	↑	↓	↑	↑	↓	↑	↑
			0.5% C + 2% PO				
4	—	↓	—	?	?	?	—
8	—	↓	↑	↑	↓	↑	↑
12	—	↓	↑	↑	↓	↑	↑

[a] Ref. 16.

[b] FC/PL = free cholesterol/phospholipid molar ratio.

[c] — Indicates no change from control value.

[d] ↓ Represents decrease from control value.

[e] ? Indicates questionable difference from control value.

[f] ↑ Represents increase from control value.

These products play a significant role in hemostasis and thrombosis by affecting platelet function (19). Likewise, high levels of AA in aortic phospholipids, specifically PI, have been suggested to be responsible for an elevated production of prostacyclin (15). The failure of aspirin to completely inhibit serotonin release from HC platelets may have been due to formation of proaggregatory PGE_2 and an endoperoxide-type product of arachidonate, PGG_2. These products, in combination or individually, are known to potentiate irreversible aggregation and release reaction induced by collagen (20).

The increased level of unidentified lipids at the origin of the TLC plate could have been by-product(s) of the degradation of PI (e.g., phosphatidic acid or hydroperoxidized olefinic lipids) (21). However, no specific attempt was made to identify these lipids.

There was a sequential appearance of platelet microcytosis and hypersensitivity to collagen in HC animals which correlated with sequential changes in CE, PL, and the FC/PL. Since cholesterol and PL are mainly membrane components, diminution in size and hyperreactivity, as well as short survival of platelets *in vivo* (16) may be related to a structural defect in the platelet membrane. It has been reported that platelets from HC animals may contain less (22) or more (23) than the normal amount of protein, findings which imply that platelet volume changes are mediated by modifications in the fragmentation of megakaryocyte cytoplasm into platelets. In the present study, the amount of protein per platelet was normal, suggesting that, under these experimental conditions, small size of the cells may have been determined partly by the physical constraint of platelet volume by a rigid membrane. Thrombocytosis occurred only as a late manifestation of the most severe HC, indicating that it may have been mediated by effects of severe HC on other tissues, such as liver, spleen, or bone marrow, or by the more severe platelet biochemical abnormalities.

The present study demonstrates that atherogenic diets have profound and complex effects on platelet cholesterol, phospholipids, and fatty acids. These are associated with abnormalities of platelet function, size, number and survival, but cause and effect relationships have not been established. Exact mechanisms responsible for these disturbances in platelet molecular and structural integrity and the possibility that they play a role in the pathophysiology of atherosclerosis remain to be tested.

ACKNOWLEDGMENT

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Uptake and Metabolism of Dolichol and Cholesterol in Perfused Rat Liver

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The uptake of dolichol and cholesterol by perfused rat liver was studied. When these radioactive lipids were incorporated into egg phosphatidylcholine liposomes, both dolichol and cholesterol appeared initially in the supernatant and in the microsomal fraction and, later on, in the mitochondrial-lysosomal fraction. The lipids taken up were esterified to some extent, but no phosphorylation of dolichol occurred. Incorporation of dolichol and cholesterol into lipoproteins increased the efficiency of uptake, which was receptor-mediated in this case. Accumulation of these lipids occurred in lysosomes followed by a transport to the endoplasmic reticulum (ER). Both labeled dolichol and cholesterol appeared in the bile. In the case of dolichol, the majority of this radioactivity was not associated with the original substance itself, and probably represented lipid-soluble catabolites. In the case of cholesterol, most of the radioactivity was associated with bile acids. It appears that, in contrast to the receptor-mediated uptake of lipoproteins from the perfusate, the uptake of liposomal lipids involves a different mechanism. After association with the plasma membrane, the lipids enter into the cytoplasm and are transported to the ER and later to the lysosomes.
***Lipids 25,* 93–99 (1990).**

Dolichol, like cholesterol, is synthesized primarily in the endoplasmic reticulum, but to some extent the biosynthesis of these two lipids also takes place in the peroxisomes (1,2). Dolichol synthesis in the endoplasmic reticulum (ER) and its metabolic regulation are necessary for maintaining an appropriate cellular supply of this lipid, which is present in all membranes. Dolichyl-P is also synthesized *de novo* in the ER and, furthermore, a CTP-kinase present in the ER is capable of dolichol phosphorylation, at lease under *in vitro* conditions (3,4).

The metabolic turnover of dolichol has been studied to only a limited extent. The half-life of this lipid is about 100 hr in microsomes and about 120 hr in lysosomes, i.e., similar to the half-lifes of cholesterol and phospholipids at these locations (5). Dolichol accumulates in most tissues during aging (6,7), but various investigations with different tissues have not succeeded in identifying catabolic enzymes or breakdown products of dolichol (8,9).

The presence of dolichol in the blood makes it probable that this lipid is actively transferred into the cells of the liver, where it mixes with the endogenous pool of dolichol. In the present investigation the uptake and distribution of dolichol in the liver during perfusion have been examined and compared with that of cholesterol. This system also proved to be useful for obtaining information about the transport and metabolism of this lipid in hepatocytes.

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MATERIAL AND METHODS

Chemicals. Dolichol-19 and dolichol-23 were isolated from bovine pituitary gland (10), and dolichol-19 was labeled with sodium [^{3}H]borohydride (2.1 TBq/mmol, Amersham) according to Keenan and Kruczek (11). The labeled dolichol was purified chromatographically. Before each experiment the [^{3}H]dolichol-19 was repurified by preparative thin-layer chromotography (TLC). Dolichol-23 was phosphorylated according the Danilov and Chojnacki (12). [1a,2a(n)-^{3}H]Cholesterol (1.6 TBq/mmol) was purchased from Amersham, cholesterol was obtained from Sigma Chemical Co. (St. Louis, MO) and ergosterol from Steraloids Inc. (Wilton, NH).

Preparation of liposomes. Seven and a half mg egg phosphatidylcholine (Lipid Products, South Nutfield, England) in chloroform/methanol (CM), 2:1, was mixed with 2.2 MBq [^{3}H]dolichol-19 (0.37 TBq/mmol) or 1.5 MBq [^{3}H]cholesterol (0.37 TBq/mmol). The solvent was evaporated under nitrogen and then *in vacuo.* Two ml 50 mM phosphate buffer, pH 7.4, containing 0.9% NaCl was added to the lipid residue, and the suspension was subjected to pulsed sonication in an ice-water bath under nitrogen for 25 min. Power output was about 150 W. After sonication, the slightly opalescent suspension was centrifuged at 100,000 *g* for 30 min and the middle part, 1.5 ml, was used in the experiments. An electron microscopic investigation of this fraction using negative staining showed only unilamellar vesicles. The liposomes were stored in an ice-water bath for a maximum of 30 min before use.

Preparation of lipoproteins enriched in [^{3}H]dolichol-19 or [^{3}H]cholesterol. 1.1 MBq [^{3}H]Dolichol-19 (0.37 TBq/mmol) or 0.7 MBq [^{3}H]cholesterol (0.37 TBq/mmol) in CM 2:1 were evaporated to dryness under nitrogen in Teflon tubes. Five ml rat serum was subsequently added to the tubes and incubated for 10 hr at 12°C with continuous magnetic stirring. The total lipoprotein fraction was then prepared by adjusting the serum to a density of 1.30 g/ml by addition of solid KBr (13). Ethylenediaminetetraacetic acid (EDTA) was added to give a final concentration of 0.01% (w/v). Centrifuge tubes containing 15 ml of this solution were overlayered with 24 ml 0.9% NaCl adjusted to a density of 1.25 g/ml with KBr. Centrifugation was then performed in a vertical rotor (VTi 50, Beckman) at 200,000 *g* for 3 hrs at 12°C. The top 4 ml (lipoproteins) were removed. In the case of dolichol, 15–20% of the added lipid was incorporated into the lipoproteins and, in the case of cholesterol, 20–25% was recovered in lipoprotein-bound form after this procedure. Dialysis of the lipoproteins was performed overnight, and the lipoproteins were then stored at 12°C for a maximum of 3 hr before use.

Liver perfusion. The perfusion apparatus employed was described earlier (13). The gas introduced into the

arterial pool was humidified at 37°C and contained 95% O_2 and 5% CO_2. The pH was continuously monitored and maintained at 7.4 by addition of 150 mM $NaHCO_3$. The flow rate through the liver was maintained at around 15 ml/min.

Preparation of livers for perfusion. Male albino rats (Sprague-Dawley) weighing 180–220 *g* were used. The surgical preparation of the rat liver for isolated organ perfusion was essentially the same as described earlier (13). The first 50 ml of the perfusate was discarded. After an equilibration period of 15 min, liposomes or lipoproteins enriched with [^{3}H]dolichol-19 or [^{3}H]cholesterol were added to the arterial pool and the perfusate volume was adjusted to 60 ml. After an additional 15, 150 or 180 min, the liver was disconnected from the perfusion system and washed via the vena porta cannula with 100 ml 0.9% NaCl. The liver was then cut into small pieces, and these were washed 7–10 times in 0.25 M sucrose. The last wash did not contain any radioactivity. The pieces of liver were then homogenized in 0.25 M sucrose.

Liver fractionation. The liver homogentate was centrifuged at 350 g for 10 min. The resulting supernatant was centrifuged at 2,800 *g* for 20 min. The resulting pellet was washed three times and then suspended in 0.25 M sucrose. In a number of experiments, this mitochondrial-lysosomal fraction was further subfractionated on a metrizamide gradient (14) in order to isolate lysosomes. The exogenous lipids were found to be almost exclusively associated with the lysosomes, in agreement with previous investigations (15). For this reason, and because of inadequate recoveries, lysosomes were not separated in routine experiments.

The postmitochondrial supernatant was centrifuged at 10,000 *g* for 20 min. The microsomes were prepared from the resulting supernatant by centrifugation at 105,000 *g* for 60 min, and the microsomal pellet was suspended in 0.25 M sucrose. "Supernatant 1" represented the top portion of the post-microsomal supernatant (12% of the total supernatant volume). "Supernatant 2" was obtained by centrifuging the rest of this supernatant at 105,000 *g* for 180 min.

Lipid extraction. Dolichyl phosphate was isolated as described earlier using 1 nmol dolichyl-P-23 as internal standard (16), with alkaline hydrolysis prior to lipid extraction. When dolichol and dolichyl esters were to be extracted, 1–3 nmol dolichol-23 and dolichyl-23-palmitate were added as internal standards. Recoveries were between 75–85%. Chloroform and methanol were added to obtain a chloroform/methanol/water (CMW) ratio of 1:1:0.3, and lipids were subsequently extracted at 37°C for 1 hr under nitrogen with magnetic stirring. The extracts were adjusted to a final CMW ratio of 3:2:1 and the tubes were centrifuged. The lower phases were evaporated under nitrogen and dissolved in 4 ml chloroform. These extracts were placed onto silica gel (230–400 mesh) columns (0.8 × 2.5 cm), the columns were eluted with 7.5 ml chloroform and, after evaporation of the solvent, the lipids eluted were dissolved in 200 µl hexane for HPLC. When cholesterol and cholesteryl esters were to be extracted, 100–200 µg ergosterol were added as internal standard (17). These lipids were extracted as above and, after evaporation of the solvent, dissolved in 200 µl chloroform/methanol (2:1) and analyzed by HPLC.

Cholesterol was separated from bile acids after alkaline hydrolysis in 10 ml methanol/water/60% KOH (1:1:0.5) for two hr at 98°C. Cholesterol was extracted with 5 ml hexane, a procedure which was repeated six times. The remaining suspension was then acidified with 5 M HCl and bile acids were subsequently extracted with diethyl ether. This extract was found to contain no cholesterol upon analysis by HPLC. HPLC analyses of the lipids were performed as described previously (13,16,18).

RESULTS

Lipid uptake. The two major possibilities for distributing exogenous lipids in the circulation for uptake studies are to employ liposomes or lipoproteins enriched with a specific lipid. Dolichol incorporated into phosphatidylcholine liposomes previously was found to be effectively taken up by isolated hepatocytes, and in

TABLE 1

Uptake of Lipids During Perfusion[a]

	Perfusion time			
	15 min		150 min	
Addition	dpm/g liver (× 10^{-3})	% of total	dpm/g liver (× 10^{-3})	% of total
Liposomal				
Dolichol	265 ± 31	2.8	572 ± 55	6.3
Cholesterol	125 ± 13	2.8	351 ± 37	7.8
Lipoprotein-bound				
Dolichol	113 ± 10	12.8	279 ± 25	29.1
Cholesterol	108 ± 9	24.1	236 ± 25	52.8

[a][^{3}H]Dolichol-19 or [^{3}H]cholesterol were present during the preparation of liposomes, which were subsequently added to the perfusion medium. The preparation of lipoproteins containing radioactive dolichol or cholesterol is described in the Materials and Methods section. The values given represent the total radioactivity in the homogenate as percentage of the total perfusate radioactivity. The values are the means ± SD of six experiments.

this manner the intracellular membranes could be enriched in polyisoprenols (19). When livers were perfused with liposomes containing [^{3}H]dolichol-19 or [^{3}H]cholesterol, both these lipids were taken up into the liver after only 15 min of perfusion and, by continuing the perfusion for as long as 150 min, the total uptake amounted to 6–8% of the total radioactivity added to the perfusate (Table 1). As expected, the total lipoprotein fraction isolated from rat blood and enriched in radioactive dolichol or cholesterol was more effectively taken up than were liposomes (20). After 150 min perfusion, 30% and 52%, respectively, of these lipids were found in the liver in this case.

Uptake and distribution of dolichol. After uptake into the liver, liposomal dolichol exhibited a characteristic distribution pattern (Table 2). During the first 15 min it was recovered in higher concentrations in microsomes than in the mitochondrial-lysosomal fraction and was also present at a high concentration in the supernatant fraction. After perfusion for 150 min, the main increase, in comparison with a 15-min perfusion, was observed in the mitochondrial-lysosomal fraction (six-fold), and at this time radioactivity was also easily measurable in the lipid extract from the bile. About 10% of the exogenous dolichol was esterified with a fatty acid and these esters were initially found in both isolated organelles and the supernatant fractions. Dolichol esters were highly enriched in the mitochondrial fraction after perfusion for 150 min.

The intracellular distribution of dolichol was different when the liver was perfused with lipoproteins (Table 3). After perfusion for only 15-min, the lipid labeling was

TABLE 2

Intracellular Distribution and Esterification of Liposomal Dolichol[a]

	Perfusion time			
	15 min		150 min	
	Dolichol	Dolichyl-FA	Dolichol	Dolichyl-FA
Preparation	dpm/mg protein			
Homogenate	1,280 ± 135	142 ± 15	2,925 ± 310	221 ± 20
Microsomes	1,610 ± 150	148 ± 13	2,545 ± 220	170 ± 15
Mitochondria	1,020 ± 95	172 ± 19	6,740 ± 710	1,280 ± 130
Supernatant 1	870 ± 93	316 ± 34	1,235 ± 98	48 ± 5
Supernatant 2	592 ± 51	161 ± 18	1,060 ± 102	54 ± 6
Bile			722 ± 82[b]	ND[c]

[a]Egg lecithin liposomes containing [^{3}H]dolichol-19 were added to the perfusion medium and the livers were homogenized either 15 or 150 min later. Supernatant 1 represents the top layer of supernatant obtained after preparation of microsomes. The values are the means ± SD of six experiments.

[b]dpm/ml.

[c]Not detectable.

TABLE 3

Intracellular Distribution and Esterification of Dolichol Added to the Perfusate Medium in Lipoprotein-Bound Form[a]

	Perfusion time			
	15 min		150 min	
	Dolichol	Dolichyl-FA	Dolichol	Dolichyl-FA
Preparation	dpm/mg protein			
Homogenate	570 ± 48	60.1 ± 7.2	1,280 ± 110	240.0 ± 28.1
Microsomes	424 ± 45	24.3 ± 2.9	1,811 ± 205	203.8 ± 19.6
Mitochondria	642 ± 59	105.0 ± 9.1	7,280 ± 690	1,210.0 ± 114.7
Supernatant 1	154 ± 13	27.8 ± 3.2	223 ± 25	37.2 ± 4.1
Supernatant 2	170 ± 15	19.4 ± 2.4	195 ± 22	31.9 ± 3.3
Bile			150 ± 14[b]	ND[c]

[a]Lipoprotein-containing [^{3}H]dolichol-19 was prepared as described in the Materials and Methods section. The values are the means ± SD of seven experiments.

[b]dpm/ml.

[c]Not detectable.

highest in the mitochondrial-lysosomal fraction and after perfusion for 150 min, a more than ten-fold increase in labeling appeared in the mitochondria, in parallel with a four-fold elevation in the microsomes. Characteristically, the level of label in the supernatant was low. As seen with exogenous liposomal dolichol, dolichol added in lipoprotein-bound form was also esterified with a fatty acid, but to a lower extent, and these esters were present at relatively high concentrations in the mitochondrial-lysosomal fraction. Radioactivity was also recovered in the bile.

Uptake and distribution of cholesterol. After perfusion for 15 min with labeled cholesterol incorporated in liposomes, radioactivity was recovered mainly in the microsomal fraction (Table 4). In the following two hours cholesterol labeling increased both in the microsomes and in the supernatant fraction, but the largest elevation, about eight-fold, was associated with the mitochondrial lysosomal fraction. The exogenous cholesterol was esterified in the liver, but to a lower extent (2–3%) than dolichol. Considerable labeling of bile cholesterol could also be observed after perfusion for 150 min.

The distribution of labeled cholesterol in the liver was different when perfusion was performed with lipoproteins (Table 5). After a short perfusion, extensive labeling was found in the mitochondrial-lysosomal fraction and, in addition, the microsomes and the level of [^{3}H]cholesterol in both these fractions increased considerably during the subsequent perfusion. Furthermore, exogenous cholesterol was also transported to the bile for secretion. As was the case with liposomal cholesterol, this lipid taken up into the liver in lipoprotein-bound form

TABLE 4

Intracellular Distribution and Esterification of Liposomal Cholesterol[a]

	Perfusion time			
	15 min		150 min	
Preparation	Cholesterol	Cholesteryl-FA	Cholesterol	Cholesteryl-FA
	dpm/mg protein			
Homogenate	682 ± 72	15.3 ± 1.3	1,910 ± 210	42.1 ± 4.4
Microsomes	1,530 ± 165	22.9 ± 2.8	3,630 ± 415	56.4 ± 6.1
Mitochondria	185 ± 21	12.6 ± 1.4	1,510 ± 165	33.8 ± 3.9
Supernatant 1	225 ± 24	11.3 ± 1.2	480 ± 52	12.1 ± 1.4
Supernatant 2	210 ± 19	34.3 ± 3.6	550 ± 61	33.5 ± 3.2
Bile			460 ± 52[b]	ND[c]

[a]Liposomes containing [^{3}H]cholesterol were added to the perfusion medium. The values are the means ± SD of five experiments.

[b]dpm/ml.

[c]Not detectable.

TABLE 5

Intracellular Distribution and Esterification of Cholesterol Added to the Perfusion Medium in Lipoprotein Bound Form[a]

	Perfusion time			
	15 min		150 min	
Preparation	Cholesterol	Cholesteryl-FA	Cholesterol	Cholesteryl-FA
	dpm/mg protein			
Homogenate	580 ± 62	22.1 ± 2.1	1,250 ± 180	64.3 ± 7.1
Microsomes	675 ± 72	32.7 ± 2.9	1,813 ± 195	32.1 ± 3.4
Mitochondria	710 ± 77	31.5 ± 3.3	1,440 ± 152	38.3 ± 4.1
Supernatant 1	289 ± 32	3.1 ± 0.4	252 ± 27	2.9 ± 0.3
Supernatant 2	205 ± 19	32.7 ± 3.1	138 ± 15	11.4 ± 1.2
Bile			305 ± 29[b]	ND[c]

[a]Lipoproteins containing [^{3}H]cholesterol were added to the perfusate either 15 min or 150 min before fractionation. The values are the means of ± SD of eight experiments.

[b]dpm/ml.

[c]Not detectable.

was esterified to a small extent, without any clear differences in the intracellular distribution of esterified cholesterol.

Dolichol phosphorylation. In attempt to detect dolichol phosphorylation, the livers were perfused with liposomal [^{3}H]dolichol-19 which lead to an accumulation of dolichol in microsomes, where CTP-kinase is localized (Table 6). After such perfusion for 150 min, only 2–3% of the labeled dolichol was phosphorylated and, consequently, this pathway does not appear to contribute to any major extent to the maintenance of dolichyl-P levels.

Esterified lipids in the perfusate. Under *in vivo* conditions, a considerable portion of blood lipids are esterified with a fatty acid before being secreted from the liver. The majority of dolichol in blood is present in esterified form (21) and esterification is known to occur in the ER-Golgi system (18). However, one cannot exclude the possibility that esterification may occur in the blood by some as yet uncharacterized mechanism. In order to study esterification processes, the livers were perfused with liposomal [^{3}H]dolichol-19 or [^{3}H]cholesterol and the appearance of labeled esterified lipids in the perfusate was monitored (Fig. 1). During the three-hr period studied, a continuously increasing concentration of [^{3}H]dolichyl ester could be observed in the perfusate, but no esterified cholesterol was found.

Excretion to the bile. Since both dolichol and cholesterol are secreted into the bile, we compared the rates of the appearance of total radioactivity (Fig. 2). When liposomal dolichol was added to the perfusate, the total radioactivity in the bile appeared at a high rate during the first hour, followed by a gradual decrease. On the other hand, when liposomal cholesterol was added, the highest rate of secretion was attained only after 90 min of perfusion and the rate remained at this level during the next 90 min.

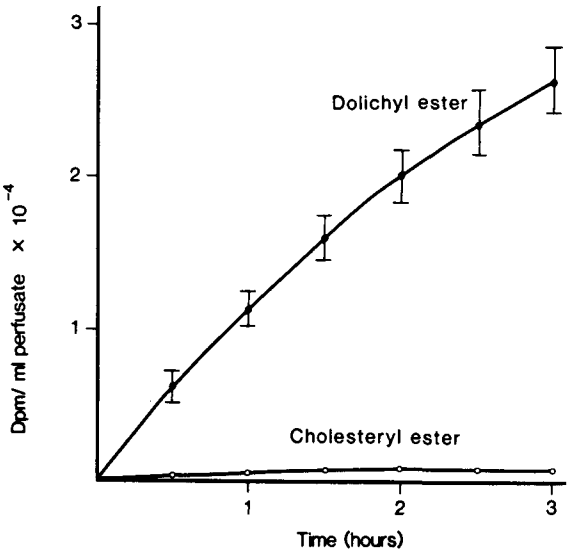

FIG. 1. Determination of newly synthesized dolichyl-FA and cholesteryl-FA in the perfusate. Liposomal [^{3}H]dolichol-19 or [^{3}H]-cholesterol were added to the perfusate, and at the various time-points aliquots were taken for analysis of dolichyl and cholesteryl esters. The values represent the means ± SD of five experiments.

TABLE 6

Esterification and Phosphorylation of Liposomal [^{3}H]Dolichol in Perfused Rat Liver[a]

Compound	Amount μg/g liver	Radioactivity dpm/μg polyprenol
Dolichol	34.5 ± 5.1	15,360 ± 1,720
Dolichyl ester	10.5 ± 1.5	3,860 ± 410
Dolichyl phosphate	15.2 ± 1.2	540 ± 62

[a]Liposomal [^{3}H]dolichol-19 was added to the perfusate. The labeling and levels of dolichol, dolichyl ester and dolichyl phosphate were determined after 150 min of perfusion. The values are the means ± SD of five experiments.

The content of the bile was analyzed in order to identify the nature of the substances excreted (Table 7). After perfusion for 180 min with liposomal [^{3}H]dolichol-19, only 4% of the radioactivity recovered in bile was associated with the intact lipid, i.e., 96% of the labeling was not associated with dolichol. In the corresponding case of liposomal [^{3}H]cholesterol, only 3–4% of the radioactivity was present in the form of cholesterol and the rest was associated with bile acids.

The lipid extract of the bile collected during perfusion of the liver with [^{3}H]dolichol-19 was chromatographed on a silica gel column and about 50% of the radioactivity could be eluted with chloroform. Most of the remaining radioactivity could be eluted subsequently with methanol, and was thus not related to dolichol. This radioactivity was not associated with dolichyl-P, in agreement with previous findings that bile does not contain this derivative (13), and it was not further characterized. The metabolites eluted with chloroform were analyzed further by reversed phase HPLC (Fig. 3). The majority of the labeled lipid compounds eluted during the initial three min, long before dolichols. These compounds are probably shorter breakdown products of the polyisoprenols.

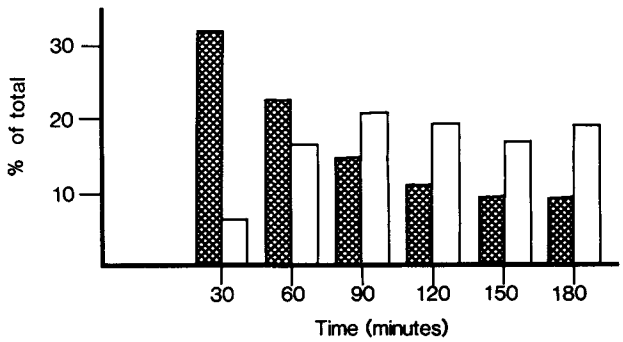

FIG. 2. Appearance of radioactivity in the bile after administration of [^{3}H]dolichol-19 or [^{3}H]cholesterol containing liposomes in the perfusate. Aliquots of bile were taken every 30 min. The individual values for radioactivity are given as percentage of the total radioactivity excreted in the bile during 180-min perfusion. The patched bars illustrate the values after administration of liposomal dolichol and the open bars represent the values after cholesterol administration.

TABLE 7

Metabolites of Dolichol and Cholesterol Excreted Into the Bile[a]

	Dolichol	Dolichol metabolites	Cholesterol	Bile acid
Liposomal lipid present in the perfusate		% of total		
Dolichol	4.1 (0.15 ± 0.02)[b]	95.9		
Cholesterol			3.4 (5.2 ± 0.6)[b]	96.6

[a]The livers were perfused with [^{2}H]dolichol-19 or [^{3}H]cholesterol containing liposomes, and the radioactivity in the bile after 150 min was identified. Dolichol metabolites were separated from dolichol and bile acids were determined as described in the Materials and Methods section. The values are the means of ± SD six experiments.

[b]μg/g liver/150 min.

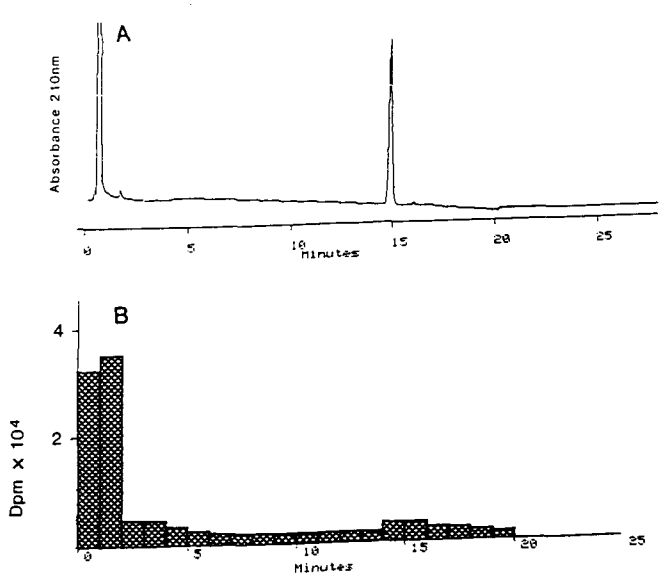

FIG. 3. Isolation of bile lipids by HPLC after perfusion of the liver with liposomes containing [^{3}H]dolichol-19. (A) Dolichol-19 standard. (B) Distribution of radioactivity in the extract of bile lipids after perfusion.

DISCUSSION

The perfused liver is a most useful system for studying lipid uptake and metabolism. One can supply lipids in different forms in order to investigate the effectiveness of the uptake. The mechanism of the entrance into the liver cell, the intracellular distribution as a function of time and excretion back into the circulation and into the bile can all be investigated.

Calculations of dolichol turnover indicate that a breakdown mechanism such as those present for other lipids in the liver exists, but to date no experimental data on this degradation is available. Like other tissues, the liver synthesizes dolichol and, to a limited extent, this lipid is excreted into both the blood and the bile. Dolichol in the blood is associated with lipoproteins and, consequently, may enter into liver by a mechanism similar to that by which cholesterol is taken up. Dolichol injected into the blood is taken up by the liver and remains in the lysosomes for a period of six days (22). On the other hand, isolated hepatocytes take up liposomes by a mechanism which does not appear to involve receptors (19).

Autoradiographic studies performed as previously described demonstrated that the components of liposomes are distributed evenly in the hepatocytes and not restricted to reticuloendothelial cells (22). Dolichol of high specific radioactivity was found in the supernatant and also in the microsomes. Lysosomal accumulation of this lipid occurred only at later times, after perfusion for 150 min. As was the case with the free alcohol, the esterified form, which is produced in the ER, accumulated to a great extent in the lysosomes at later times. In comparison, the uptake of dolichol bound to lipoproteins instead of liposomes clearly involves a mechanism which is quite different. In this case entrance of the lipid probably proceeded via the mechanism established for low density lipoproteins (LDL). It was previously demonstrated that upon mixing with serum, dolichol is incorporated into LDL (8). Even initially, the level of labeled dolichol was higher in lysosomes than in microsomes and low in the supernatant.

Cholesterol uptake shows behavior similar to that seen with dolichol, which indicates that the type of lipid carrier used for administration is more important for uptake than is lipid structure. In spite of minor differences, such as low concentrations in the supernatant and limited esterification, liposomal cholesterol initially also accumulated preferentially in the microsomes; whereas cholesterol in lipoprotein-bound form was taken up via the receptor mechanism and was associated with the lysosomes.

The large number of investigations concerning the mechanism for LDL uptake have clarified the pathway leading to the formation of lysosomes containing lipoproteins (23). After intralysosomal hydrolysis, components such as dolichol and cholesterol enter the cytoplasm and are, at least to some extent, transported to the endoplasmic reticulum for esterification. The experiments performed in this study, together with previous data, demonstrate that the uptake of liposomes does not involve lipoprotein receptors and is not related primarily

to lysosomes. Probably, fusion of liposomes with plasma membranes occurs. We have no experimental evidence for such phenomenon, but since liposomal lipids appear in the cytoplasm in non-membrane bound form (24), an endocytic mechanism can not be involved. These lipids may be bound to carrier proteins, such as those known to exist for phospholipids (25), or be present in micelles in association with phospholipids. The cytoplasmic lipid appears to move to the ER, followed by transport to other intracellular locations.

Since lipids taken up in lipoprotein-bound form are enclosed in the lumen of the endosomes, their intracellular movement is associated with that of the lysosomes. It remains, however, to be determined what is the targeting signal for movement of lipids taken up from liposomes. It has been proposed previously that the fatty acid moiety of esterified dolichol is required for the transport of the newly synthesized lipid from the ER to the lysosomes (18), where a specific esterase renders dolichol available for further transport and/or metabolism (26). The accumulation of dolichyl esters in the lysosomes is in agreement with this suggestion, since esterification is exclusively a microsomal process.

About 25% of rat liver dolichol is phosphorylated and participates in glycoprotein synthesis (27). Dolichyl-P is normally synthesized *de novo* as a product of the mevalonate pathway (28), but under certain conditions, such as in human hepatocellular carcinoma, the CTP-kinase phosphorylates dolichol to an appreciable extent (29). The absence of phosphorylation during the liposomal uptake of dolichol during perfusion demonstrates, with previous experiments (30), that the kinase does not contribute to any large extent to the maintenance of the dolichyl-P pool under normal conditions.

Dolichol produced in the liver is discharged into the circulation, where it is found in lipoprotein-bound form (13). We do not know whether the lipid taken up into hepatocytes in perfused liver reenters the circulation, since the exogenous lipid is radioactively labeled. However, 5% of the dolichol in the perfusate was found to be esterified, which might reflect hepatic esterification.

Dolichol, but not its esterified or phosphorylated form, is discharged into the bile. This pathway is considered to be of great importance for the turnover of this lipid (9). Interestingly, a large portion of the lipid-soluble, biliary radioactivity recovered after administration of [^{3}H]dolichol-19 was not associated with dolichol itself, but eluted earlier than dolichol on HPLC. This may reflect the metabolic breakdown searched for, but not previously found. It will be an important task in the future to identify the compounds in this fraction in order to establish that catabolism of dolichol does occur in the liver cell.

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Synergistic Activation of CTP:Phosphocholine Cytidylyltransferase by Phosphatidylethanolamine and Oleic Acid

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CTP:phosphocholine cytidylyltransferase present in rat liver cytosol was activated almost 30-fold when assayed in the presence of liposomes containing 60 mole % dioleoyl phosphatidylethanolamine (DOPE). During the assay, some of the DOPE was degraded to lysoPE and oleic acid. Whereas cytidylyltransferase activity was not affected when assayed in the presence of liposomes containing lysoPE, liposomes containing oleic acid activated the enzyme. Activation by oleic acid could be eliminated by the addition of fatty acid-free bovine serum albumin (BSA) to the assay. When cytidylyltransferase activity was measured in the presence of both BSA and liposomes containing DOPE, enzyme activity was increased almost 20-fold, as compared with assays performed in the absence of added lipid. The 1.5-fold difference in cytidylyltransferase activity observed when cytosol was assayed with DOPE containing liposomes in the absence or presence of BSA (30-fold stimulation vs 20-fold stimulation) cannot be explained by the loss of activation attributable to oleic acid alone. Activation of the enzyme in the presence of liposomes containing DOPE and oleic acid is several-fold greater than the sum of the activations caused by the individual compounds. These data suggest that PE and oleic acid act synergistically in activating the cytidylyltranferase.

Lipids 25, 100–107 (1990).

In most tissues, regulation of phosphatidylcholine (PC) biosynthesis occurs via modulation of the activity of CTP:phosphocholine cytidylyltransferase (EC 2.7.7.15) (1–4). This enzyme catalyzes the reversible formation of CDPcholine and pyrophosphate from CTP and phosphocholine. The enzyme is ambiquitous in that it resides in both cytosolic and membranous portions of cells (5,6). While membrane-associated cytidylyltransferase is very active, the cytosolic form of the enzyme is relatively inactive (7). The activity of the cytosolic cytidylyltransferase can be greatly enhanced *in vitro* in the presence of certain lipid species (8–10).

We have proposed a regulatory mechanism for the control of PC biosynthesis based on the ability of the cytidylyltransferase to "sense" the lipid composition of membranes (7). Our hypothesis predicts that when the cytidylyltransferase is in the presence of membranes deficient in PC, a redistribution/activation of the cytosolic enzyme occurs (7). This activation would enhance the rate of PC production, resulting in an increase in the PC content of membranes. Our hypothesis further suggests that after the PE deficient membranes are repaired with respect to PC, the membrane bound enzyme dissociates, and returns to the cytosol where it exists in an inactive form (7,11). Support for this hypothesis comes from studies demonstrating that as the rate of PC biosynthesis changes in cells, a redistribution of the cytidylyltransferase between cytosolic and membrane-bound forms occurs (12–15). Additional support has been obtained by examining the distribution and activity of the enzyme in cells having an altered lipid composition (16).

Recently we began a series of experiments aimed at further testing our hypothesis. While performing these experiments we observed that rat liver cytidylyltransferase was activated by liposomes containing dioleoyl phosphatidylethanolamine (DOPE). Although this is consistent with some previous reports (7,12,17,18), several investigators have reported little or no activation of the enzyme by phosphatidylethanolamine (PE) (8,19,20).

In the present paper, we report on the ability of PE molecules to activate the cytidylyltransferase both in crude cytosolic and highly purified preparations. Our findings indicate that whereas highly purified cytidylyltransferase is only slightly activated by PE, the activity of the enzyme in cytosol is greatly enhanced by some PE species. The activation of the enzyme in cytosol appears to be the result of direct activation by PE, and by fatty acids released from the phospholipid. In addition, we have observed that PE and fatty acid molecules act synergistically in activating the enzyme.

EXPERIMENTAL

Radioactive reagents. CDP-[methyl-^{14}C]choline and phospho[methyl-^{14}C]choline were purchased from New England Nuclear (Boston, MA). Di[1-^{14}C]oleoyl phosphatidylcholine was obtained from Amersham. Di[1-^{14}C]oleoyl phosphatidylethanolamine was prepared by the transphosphatidylation of di[1-^{14}C]oleoyl phosphatidylcholine as described previously (21).

Lipids. Phospholipids were purchased from Avanti (Birmingham, AL) and their purity verified by thin-layer chromatography. Thin-layer chromatography was performed using silica gel 60 plates (Merck and Co., Rahway, NJ) and either $CHCl_3$/MeOH/NH_4OH (65:35:5, by vol) or $CHCl_3$/MeOH/H_2O/acetone/acetic acid (50:10:5:20:10, by vol) as developing solvents. Liposomes were prepared by the method of Papahadjopoulos *et al.* (22). Suspensions of phosphatidylethanolamine (PE) were prepared using the same method. It should be noted that liposomes can be prepared from PE/PC mixtures until the PE content reaches approximately 70 mole %, at which time hexagonal phase arrays are formed (23,24).

Preparation and storage of rat liver cytosol. Freshly

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Abbreviations: BSA, bovine serum albumin; DOPC, dioleoyl phosphatidylcholine; DOPE, dioleoyl phosphatidylethanolamine; DPPE, dipalmitoyl phosphatidylethanolamine; EDTA, ethylenediaminetetraacetic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

obtained female rat liver was placed in 150 mM NaCl, 50 mM Tris-HCl, 1 mM EDTA, 2 mM dithiothreitol and 0.25% sodium azide buffer (pH 7.4), cut into small pieces, and then homogenized with a Brinkman Polytron (Brinkman Instruments, Ltd., Toronto, Canada). The cells were then disrupted with 20 strokes in a Dounce homogenizer (type B pestle). This homogenate was subjected to centrifugation at 10,000 × *g* for 10 min at 2°C. The 10,000 × *g* supernatant was then subjected to centrifugation at 37,100 rpm in a Beckman 50.2 Ti rotor (Beckman, Fullerton, CA) for 60 min at 2°C. The resulting supernatant (cytosol preparation) was divided into small aliquots and stored at −20°C before use. We have observed that storage of the enzyme preparation for greater than two months resulted in a gradual decline in activity when assayed in the presence of fatty acid free-bovine serum albumin (BSA).

Cytidylyltransferase assay. CTP:phosphocholine cytidylyltransferase was assayed as described previously (7). For some experiments, up to 5 mg/ml of fatty acid-free BSA (Sigma Chemical Co., St. Louis, MO) was preincubated with the assay mixture containing all components except phosphocholine for 10 min at room temperature.

A purified preparation of CTP:phosphocholine cytidylyltransferase was kindly provided by Professor Claudia Kent, Purdue University, West Lafayette, IN. In this paper we distinguish between the "cytosolic enzyme" and the "purified enzyme". The term cytosolic enzyme is used to refer to activity observed in isolated cytosol, while purified enzyme refers to the activity of the highly purified preparation.

Measurement of DOPE/DOPC degradation. To determine the amount of lipid degradation that occurred during assays of cytidylyltransferase activity, assays were performed in the presence of liposomes containing trace amounts of radiolabeled lipid. After stopping the reactions, lipids were extracted by the procedure of Bligh and Dyer (25), using 0.9% NaCl and 10 mM HCl in the aqueous phase. Lipid extracts were then applied to silica gel 60 thin-layer plates and the plates developed using $CHCl_3$/MeOH/H_2O/acetone/acetic acid (50:10:5:20:10, by vol). Radiolabeled lipid species were detected by autoradiography and quantitated by liquid scintillation counting (26).

Miscellaneous determinations. Protein concentrations were determined by the procedure of Lowry *et al.* (27) with BSA as the standard. Phospholipid concentrations were determined by the procedure of Rouser *et al.* (28).

RESULTS

The specific activity of cytosolic CTP:phosphocholine cytidylyltransferase was extremely low when assayed in the absence of exogenous lipid or in the presence of DOPC liposomes (Fig. 1). However, when assayed in the presence of liposomes containing DOPE, the activity was greatly enhanced (Fig. 1). This activation was found to be dependent on both the DOPE content of the liposomes (Fig. 1A) and the amount of exogenous lipid present (Fig. 1B). Maximal activation was found using liposomes composed of 60 mole % DOPE and 40 mole % DOPC. Using small unilamellar vesicles of this composition, half maximal activation of the cytosolic cytidylyltransferase occurred at a concentration of approximately 0.2 mM with respect to lipid phosphate. Maximal activation of the enzyme was routinely achieved using 1 mM liposomes; therefore, this concentration of lipid was used throughout the remainder of these studies.

A time course for the production of CDPcholine by cytosolic cytidylyltransferase assayed in the presence of DOPE/DOPC (60:40, mole %) is presented in Figure 2. Although the time course appeared to be fairly linear at short times, a clear increase in the rate of CDPcholine production occurred at later times, suggesting a progressive activation of the enzyme. When fatty acid-free BSA was added to the assay the time course became linear, suggesting that BSA either inhibited the production of an activator or removed an

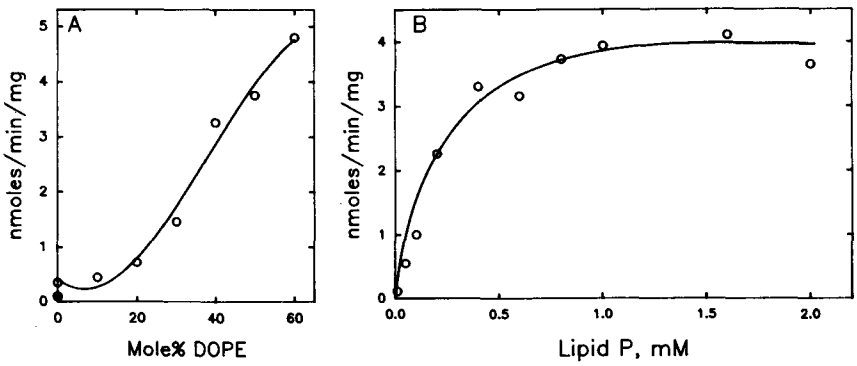

FIG. 1. Activation of cytosolic CTP:phosphocholine cytidylyltransferase by dioleoyl phosphatidylethanolamine. In A, cytosolic cytidylyltransferase activity was measured either in the presence of 1 mM liposomes containing a mixture of DOPC and DOPE (open circles), or in the absence of exogenous lipid (closed circle). In B, cytosolic cytidylyltransferase activity was measured in the presence of liposomes containing 60 mole % DOPE and 40 mole % DOPC. Data points are the average of triplicate determinations. The standard error in the assay points is smaller than the symbol size.

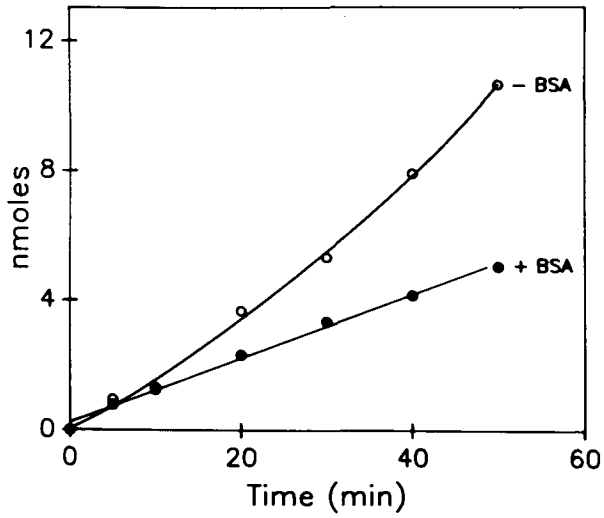

FIG. 2. Time course of cytidylyltransferase assay. The amount of CDPcholine produced during cytosolic cytidylyltransferase assays performed in the presence of 1 mM liposomes containing 60 mole % DOPE and 40 mole % DOPC was measured in the absence (open circles) or presence (filled circles) of 5 mg/ml fatty acid-free BSA. Data points are the average of triplicate determinations. The standard error in the assay points is smaller than the symbol size.

TABLE 1

Degradation of Radiolabeled Phospholipids by Rat Liver Cytosol[a]

Percent of total counts			
Starting with di[1-^{14}C]oleoyl PC			
DOPC	LysoPC	Fatty acid	Other[b]
99.46 ± 0.08	0	0.53 ± 0.09	0.02 ± 0.02
Starting with di[1-^{14}C]oleoyl PE			
DOPE	LysoPE	Fatty Acid	Other[b]
93.41 ± 0.05	2.45 ± 0.07	3.95 ± 0.05	0.11 ± 0.03

[a]Cytidylyltransferase assays were performed in the presence of liposomes containing trace amounts of either di[1-^{14}C]oleoyl phosphatidylcholine or di[1-^{14}C]oleoyl phosphatidylethanolamine, as described in the Experimental Section. After stopping the reactions, lipids were extracted and separated by thin-layer chromatography. The radiolabeled lipids were then extracted from the silica gel and the amount of radioactivity quantitated by liquid scintillation counting. The results are presented as the fraction of counts found in each lipid as compared to the starting material. Approximately 60,000 counts per assay were recovered. Data points are the average of triplicate determinations ± one standard deviation.

[b]Other refers to the fraction of counts that were not located with the identified lipids.

course became linear, suggesting that BSA either inhibited the production of an activator or removed an activator from the enzyme.

Since both oleic acid and lysoPE have been reported to be activators of the cytosolic cytidylyltransferase (8,29), we examined the ability of rat liver cytosol to degrade DOPC and DOPE. Liposomes composed of 60 mole % DOPE and 40 mole % DOPC were prepared containing either di[1-^{14}C]oleoyl PC or di[1-^{14}C]oleoyl PE. The liposomes were then incubated under cytidylyltransferase assay conditions except that unlabeled phosphocholine was used. After incubation for 30 min at 37°C, lipids in the samples were extracted, separated by thin-layer chromatography, and the amount of radioactivity present in the various species measured. As shown in Table 1, DOPE was degraded much more rapidly than DOPC. From these data we calculated that starting with liposomes composed of 60 mole % DOPE and 40 mole % DOPC, the liposome composition after incubation with cytosol was 52.74 mole % DOPE, 39.58 mole %, DOPC, 4.90 mole % oleic acid and 2.77 mole % lysoPE. This calculation assumes that all the lipid remained associated with the liposomes.

We found that liposomes containing 1–10 mole % lysoPE do not activate the enzyme (data not shown). Since the maximal lysoPE content of the DOPE/DOPC liposomes after the cytidylyltransferase assay was less than 3 mole %, it seems unlikely that the production of lysoPE during the assay resulted in activation of the enzyme. Although low levels of lysoPE present in liposomes did not activate the enzyme, in agreement with the work of Choy and Vance (8), we have observed activation of the cytidylyltransferase by high concentrations (mM) of lysoPE when the lipid was presented in solution, and no liposomes were present (data not shown). The significance of cytidylyltransferase activation by high levels of lysoPE is unknown.

It has been reported that free oleic acid activates cytosolic cytidylyltransferase (13,17,30,31). We observed that oleic acid can also activate the cytosolic cytidylyltransferase when presented in liposomes (Fig. 3). As mentioned above, during assays in the presence of DOPE/DOPC liposomes (60:40), the free oleic acid content of the liposomes increases to 4.9 mole %. Since liposomes containing more than twice that amount of oleic acid did not activate the cytosolic cytidylyltransferase to the same extent as the DOPE/DOPC vesicles (compare Figs. 1 and 3), it seemed unlikely that activation by oleic acid alone could completely account for the observed activation by DOPE/DOPC vesicles (Fig. 1). To demonstrate this more convincingly, we sought a method of specifically inhibiting the activation of the cytidylyltransferase by oleic acid. As displayed in Figure 3, the addition of fatty acid-free BSA to an assay mixture containing oleic acid/DOPC vesicles almost completely reversed the activation of the enzyme by oleic acid. Under our assay conditions, BSA at a final concentration greater than 1 mg/ml abolished most fatty acid dependent activation. When BSA was included in assays with liposomes containing other phospholipids known to activate the cytidylyltransferase (e.g., phosphatidylglycerol, phosphatidylinositol and phosphatidic acid), no inhibition of enzyme activity was observed (data not shown). This suggests that the ability of fatty acid-free BSA to inhibit cytidylyltransferase activity was due to its binding to fatty acids, and not to a nonspecific interaction with liposomes.

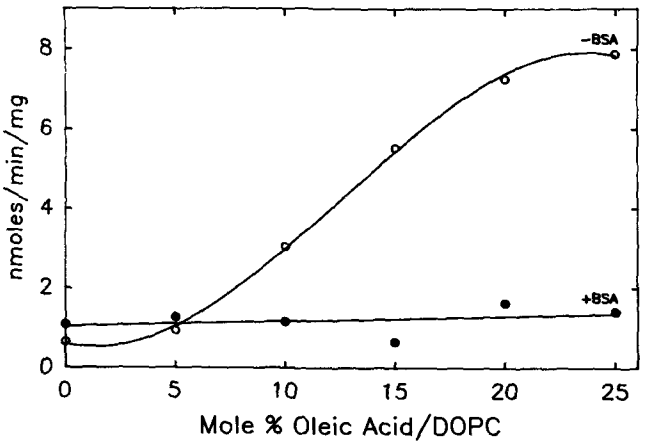

FIG. 3. Effect of fatty acid-free BSA on the oleic acid activation of cytosolic cytidylyltransferase. Cytosolic cytidylyltransferase activity was measured in the presence of 1 mM liposomes containing a mixture of DOPC and oleic acid in the absence (open circles) or presence of 5 mg/ml fatty acid-free BSA (closed circles). Data points are the average of duplicate determinations.

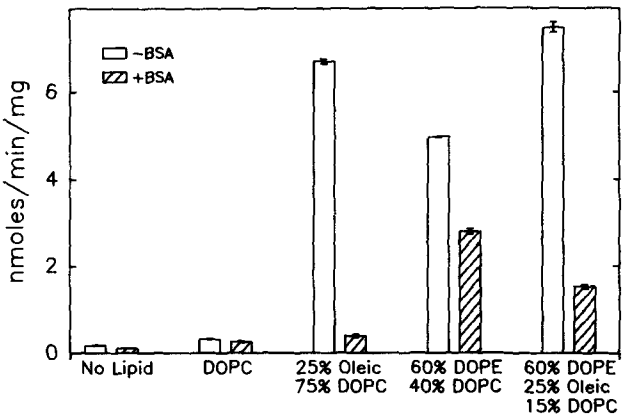

FIG. 4. Effect of fatty acid-free BSA on the activation of cytosolic CTP:phosphocholine cytidylyltransferase by DOPE, DOPC and oleic acid. Cytosolic cytidylyltransferase activity was measured in the presence of 1 mM liposomes. In some cases 5 mg/ml fatty acid-free BSA was preincubated with the assay mixture as described in the Experimental section (hatched bars). The lipid composition of the different liposome preparations used is listed as mole % of total lipid below the Figure. In one instance pure DOPC liposomes were utilized. Data points are the average of triplicate determinations ± one standard deviation.

We observed little affect on the activity of cytosolic cytidylyltransferase when assays were performed in the absence of added lipid or in the presence of pure DOPC liposomes, regardless of the presence of fatty acid-free BSA (Fig. 4). Small decreases in the activity of the cytosolic cytidylyltransferase when assayed in the presence of BSA or DOPC vesicles and BSA (Fig. 4) may result from abstraction of small amounts of fatty acids from the enzyme. In samples incubated with liposomes containing 25 mole % oleic acid, 94% of the activity was abolished by the addition of BSA. When BSA was included in assays performed in the presence of DOPE/DOPC (60:40) liposomes, approximately 50% of the lipid dependent activity was lost (Fig. 4). This suggested that the enhanced activity observed in the presence of liposomes containing DOPE/DOPC (60:40) was only partially due to production of oleic acid.

To determine more specifically the affect of lysoPE and oleic acid production on cytosolic cytidylyltransferase activity in the presence of DOPE containing liposomes, liposomes of various compositions were used (Table 2). The concentration of the individual lipid species was adjusted to mimic that present after incubation of DOPE/DOPC (60:40) vesicles with the cytidylyltransferase assay mixture. The presence of lysoPE in the various liposome populations did not significantly affect enzyme activity compared with controls having no lysoPE (compare -BSA samples A and G, B and E, C and F, D and H). Using liposomes composed of DOPC/oleic acid (95.1:4.9, mole %) in the absence of BSA, cytidylyltransferase activity was only slightly enhanced compared to assays performed using pure DOPC vesicles (compare -BSA samples A and H). This small increase in activity cannot account for the stimulation of cytidylyltransferase activity observed in the presence of DOPE (compare -BSA samples A and B). Adding oleic acid to liposomes containing DOPE increased the activity of the enzyme more than when the oleic acid is presented in the absence of DOPE (compare -BSA samples A and H with B and C).

As shown in Table 2, activation by oleic acid was inhibited in the presence of fatty acid-free BSA. All assays performed in the presence of DOPE containing liposomes had approximately the same activity when fatty acid-free BSA was included in the mixture (compare + BSA samples B, C, E and F), suggesting that this level of activity is due exclusively to an interaction between DOPE and the cytosolic cytidylyltransferase. Because these assays were performed using cytosol as a source of the enzyme, the possibility that other factors present in the cytosol also played a role in activation cannot be ruled out.

We believe that these data can best be explained by the synergistic activation of cytosolic cytidylyltransferase by DOPE and oleic acid. The effect of oleic acid alone on the activity is shown by sample H when assayed in the absence of BSA. In this sample the activity was 0.521 nmol/min/mg, as compared to the DOPC control, which had an activity of 0.269 nmol/min/mg. The effect of DOPE alone on activity is shown by sample B when assayed in the presence of BSA (to eliminate the effect of oleic acid). This sample had an activity of 1.20 nmol/min/mg, as compared to the DOPC control, which had an activity of 0.074 nmol/min/mg. If the effects of DOPE and oleic acid were additive, we would expect that assays in the presence of DOPE/DOPC liposomes and absence of BSA would have an activity of approximately 1.8 nmol/min/mg. However, the observed activity (sample B) is 3.90 nmol/min/mg. Therefore, the two compounds work synergistically to activate the cytosolic cytidlylytransferase.

TABLE 2

Effect of Various Lipid Mixtures on Cytosolic Cytidylyltransferase Activity[a]

Sample	Liposome composition (mole %)				Specific activity (nmole/min/mg)	
	DOPC	DOPE	LysoPE	Oleic Acid	−BSA	+BSA
A	100	—	—	—	0.269 ± 0.004	0.074 ± 0.023
B	40	60	—	—	3.90 ± 0.06	1.20 ± 0.04
C	39.59	52.74	2.77	4.90	4.79 ± 0.03	1.21 ± 0.12
D	92.33	—	2.77	4.90	0.632 ± 0.066	0.012 ± 0.008
E	44.49	52.74	2.77	—	3.98 ± 0.111	1.55 ± 0.05
F	42.36	52.74	—	4.90	4.51 ± 0.05	1.34 ± 0.02
G	97.23	—	2.77	—	0.363 ± 0.047	0.00 ± 0.008
H	95.10	—	—	4.90	0.521 ± 0.025	0.060 ± 0.048

[a]Cytosolic cytidylyltransferase activity was measured in the presence of 1 mM liposomes. In some cases 5 mg/ml fatty acid-free BSA was preincubated with the assay mixture (+BSA), as described in the Experimental Section. Data points are the average of triplicate determinations ± one standard deviation.

Activation of cytidylyltransferase by hexagonal phase PE. Because simple lipid mixtures containing greater that 60 mole % PE tend to form hexagonal phase arrays rather than lamellar structures (23,24), the ability of hexagonal phase DOPE suspensions to activate the cytosolic cytidylyltransferase was determined. When the enzyme was assayed in the presence of a 1 mM suspension of pure DOPE, its specific activity was 2.52 nmole/min/mg, as compared with 4.77 nmol/min/mg when assayed with liposomes containing DOPE/DOPC (60:40). Thus, the hexagonal phase DOPE activated the enzyme approximately half as well as liposomes containing 60 mole % DOPE and 40 mole % DOPC.

When cytosolic cytidylyltransferase was assayed in the presence of liposomes composed of oleic acid/DOPE/DOPC (25:60:15, mole %), its activity was enhanced as compared with assays using DOPC liposomes (Fig. 4). However, the level of activation was not equal to the sum of assays performed with oleic acid/DOPC and DOPE/DOPC containing liposomes independently. When oleic acid/DOPE/DOPC liposomes were incubated with cytosol and BSA, the activity of the cytidylyltransferase was increased approximately six-fold over the DOPC control, but was lower than that observed with DOPE/DOPC vesicles in the presence of BSA (Fig. 4). These observations are consistent with the idea that in the presence of BSA, oleic acid was removed from the liposomes, causing an increase in the mole % DOPE until a breakdown of liposome structure occurred, and phase arrays formed.

Synergistic activation of cytosolic cytidylyltransferase by oleic acid and dipalmitoyl phosphatidylethanolamine (DPPE). Recently we have found that some species of PE are unable to activate cytosolic cytidylyltransferase independently (Sleight and Dao, unpublished data). To determine if one of these lipids, DPPE, can act together with oleic acid to stimulate the enzyme, the experiment presented in Figure 5 was performed. As shown in Figure 5, when the cytosolic cytidylyltransferase was assayed in the presence of DPPE and increasing amounts of oleic acid, its activity was stimulated above that observed when oleic acid/DOPC vesicles were used. As expected, this stimulation was completely inhibited when the assay was performed in the presence of fatty acid-free BSA. Activation of the cytidylyltransferase by a mixture of DPPE and oleic acid is greater than the sum of the activations by the individual lipids (Fig. 5, dotted line). Although best fit, second order curves are presented in Figure 5 to compare activations by the various lipid species, it appears that activation in the presence of DPPE and increasing amounts of oleic acid (assayed in the absence of BSA) occurs sigmoidally. When a similar experiment was performed using palmitic acid in place of oleic acid, the addition of palmitic acid/DPPE vesicles had no affect on cytidylyltransferase activity (data not shown). These data are consistent with a synergistic activation of the enzyme by DPPE and unsaturated fatty acid; however, the degree of this synergistic activation is much less than that observed using DOPE. When the enzyme was assayed in the presence of DPPE and equal amounts of palmitic and oleic acid, it was activated as if only half as much oleic acid were used alone (data not shown). A possible explanation for this finding is that both the activating unsaturated fatty acid and nonactivating saturated fatty acid compete for a fatty acid binding site located on the enzyme.

Activation of purified cytidylyltransferase by lipids. To further demonstrate the ability of liposomes containing DOPE to activate the cytidylyltransferase, and to rule out the possibility that degradation of PE was a prerequisite for activation, we examined the affects of lipids on a highly purified preparation of the enzyme. When the purified enzyme was incubated in the presence of liposomes containing DOPE/DOPC (60:40) and trace amounts of di[1-^{14}C]oleoyl PE and dioleoyl phosphatidyl[^{3}H-methyl]choline, no degradation of radiolabeled lipids was observed. Therefore, the purified enzyme contained no phospholipase activity. In agreement with previous studies (19,32), the purified enzyme had significant activity in the absence of lipid (data not shown). It has been suggested that the relatively high activity observed in purified enzyme preparations in the absence of added lipid may be due to a lipid or lipid(s) that copurify with the enzyme (19,32); however, there is no experimental evidence to support this. Our current studies indicate that addition of BSA to the purified enzyme significantly de-

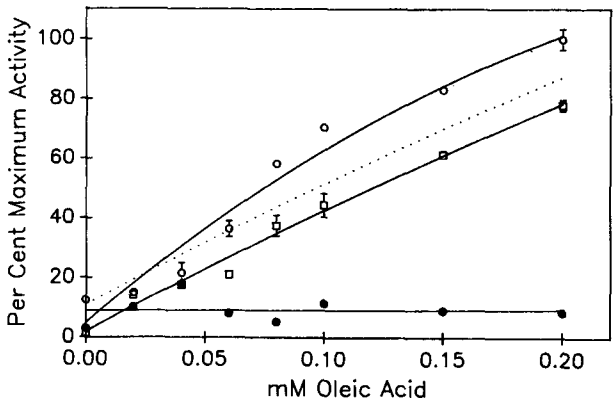

FIG. 5. Synergistic activation of cytosolic cytidylyltransferase activity by DPPE and oleic acid. Cytosolic cytidylyltransferase activity was measured in the presence of increasing concentrations of oleic acid. The circles indicate assays performed in the presence of 1 mM DPPE and increasing amounts of oleic acid. Squares indicate assays performed in the presence of oleic acid/DOPC liposomes (1 mM final lipid concentration). Filled symbols indicate assays performed in the presence of 5 mg/ml fatty acid-free BSA. Data points are the average of duplicate determinations. When the range of the data points exceeded the size of the symbols, error bars are used to indicate the range. The dotted line represents the theoretical activity of the cytidylyltransferase if activation by DPPE and oleic acid was simply additive.

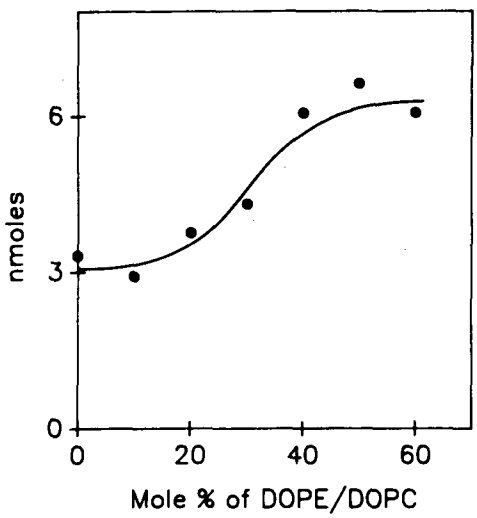

FIG. 6. Activation of a purified cytidylyltransferase by DOPE. Cytidylyltransferase activity was measured in a highly purified preparation of the enzyme isolated from rat liver cytosol. The assay was performed in the presence of 5 mg/ml fatty acid-free BSA and 1 mM liposomes containing DOPE and DOPC. Data points are the average of duplicate determinations.

creases its activity when assayed in the absence of added lipid (data not shown). This suggests that fatty acids are associated with the purified enzyme (see Discussion). Because of this, we have chosen to perform all of our cytidylyltransferase assays using purified enzyme in the presence of 5 mg/ml BSA.

When the purified enzyme was incubated in the presence of DOPE/DOPC (60:40) vesicles, a one-fold increase in activity was observed (Fig. 6). This activation was dependent on the amount of DOPE present in the liposomes (Fig. 6). In Figure 7, a comparison of the activation of the cytosolic cytidylyltransferase with that of the purified enzyme, and with a mixture of cytosol and purified enzyme, is presented. One feature that distinguishes the two preparations is the ability of DOPE/DOPC liposomes to activate the cytosolic enzyme to a much greater extent than the purified enzyme. To determine if a factor that potentiates the activation of the cytosolic cytidylyltransferase was present in the cytosol and absent in the purified enzyme, a mixture of the two preparations was made and assayed. The activity of the mixed preparation was identical to the sum of the activities when each of the preparations were assayed separately (Fig. 7). This observation indicates that the purified enzyme was not acted upon by some unknown component of the cytosol.

DISCUSSION

Three mechanisms for the regulation of cytidylyltransferase activity have been postulated: (i) sensitivity to lipid environment (7,8,12,13,16,19,20,29,33,34); (ii) phosphorylation/dephosphorylation (35); and (iii) alteration of substrate pool sizes (35–37). Of these mechanisms, sensitivity to lipid environment has been studied in the most detail. In this paper, we describe the ability of both suspensions of pure PE and liposomes containing PE to activate CTP:phosphocholine cytidylyltransferse. Our findings indicate that DOPE molecules can activate both the purified enzyme and the enzyme as it exists in the cytosol. We have demonstrated that activation of cytosolic cytidylyltransferase can be explained, in part, by the breakdown of PE molecules and subsequent activation of the enzyme by unsaturated fatty acids. Lastly, we have provided evidence suggesting that PE and oleic acid act synergistically in the activation of the cytosolic cytidylyltransferase.

Conflicting reports concerning the ability of PE molecules to activate cytidylyltransferase have appeared (7,8,12,17–20). Using several different preparations of rat liver cytosol, we have consistently found that both liposomes and lipid suspensions containing DOPE are able to activate the enzyme. We suggest that the inability of some laboratories to demonstrate activation by PE may be due to (i) use of liposomes having extremely low PE/PC ratios; (ii) use of hexagonal PE suspensions at low concentrations (8,19,20); (iii) use of a protocol for isolation or storage of cytosol that inactivates phospholipases; or (iv) use of PE species containing only saturated fatty acids.

Degradation of phospholipids by the cytosol. We have demonstrated that phospholipase activity in

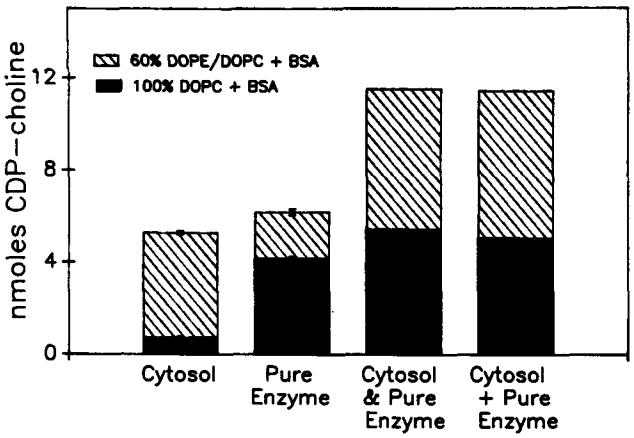

FIG. 7. Comparison of cytosolic cytidylyltransferase activity and a highly purified preparation of the enzyme. Activity was assayed in the presence of 1 mM liposomes composed of either DOPC (solid bar) or DOPE/DOPC (60:40, mole %) (hatched bar). All incubations were performed in the presence of 5 mg/ml fatty acid-free BSA. The amount of enzyme used was chosen so that it produced approximately 5 nmoles of CDPcholine in the presence of the DOPE/DOPC containing liposomes. The notation "cytosol & pure" enzyme indicates an assay performed in which the activity of a mixture of the cytosol and pure enzyme was measured. The notation "cytosol & pure" enzyme indicates the level of activation as an arithmetic sum of the individual cytosol and pure enzyme activities. Data points are the average of at least triplicate determinations ± one standard deviation.

rat liver cytosol selectively degraded DOPE compared with DOPC (Table 1). Because degradation of the di[1-^{14}C]oleoyl phosphatidylethanolamine resulted in the release of 1.6-fold more oleic acid than lysoPE, we suspect that either a phospholipase B or a combination of phospholipases A_1 and A_2 are responsible for the hydrolysis. The specificity of the phospholipase activity for DOPE as compared with DOPC is consistent with the known substrate specificity of several well studied enzymes (38).

Most studies concerning the lipid sensitivity of cytidylyltransferase activity have been performed using crude cytosol preparations as a source of the enzyme. Because the production of small amounts of fatty acids during cytidylyltransferase assays is likely to significantly alter the activation state of the enzyme, we suggest that many previous studies may require reinterpretation.

Differences between the activity of the cytosolic and purified preparations of cytidylyltransferase. At levels of cytosolic and purified enzyme which produce approximately identical amounts of CDPcholine in the presence of DOPE/DOPC (60:40) liposomes, different amounts of CDPcholine are produced when assays are performed in the presence of pure, nonactivating DOPC liposomes (Fig. 7). These data suggest that there is a fundamental difference in the state of the enzyme in the two preparations. The most likely differences in the enzyme preparations are either in their phosphorylation state and/or their association with lipid. To date, no information exists comparing the phosphorylation state or the lipid association of the purified enzyme with the enzyme in isolated cytosol. The purification of cytidylyltransferase requires the addition of large amounts of oleic acid (19). Our findings that (i) the enzyme is very active in the absence of exogenously supplied lipids, and that (ii) this activity can be greatly reduced by the addition of fatty acid-free BSA, suggests that oleic acid remains associated with the enzyme.

Possible role of PE and fatty acids in activating the cytidylyltransferase in vitro *and* in vivo. Weinhold and co-workers have demonstrated that free fatty acids promote the translocation of cytidylyltransferase from the cytosol to microsomes (30). In addition, when the free fatty acid content of microsomes is increased in the absence of cytosol, there is a concomitant increase in microsomal cytidylyltransferase activity (30). Therefore, fatty acids appear to increase both the affinity of the soluble form of the enzyme for membranes, and the activity of the membrane bound form of the enzyme. Several studies have been performed to determine the relationship between cytidylyltransferase binding and membrane lipid composition (1-4,38,39). To date, no clear relationship has been established, and several conflicting pieces of data have been published.

Our current working hypothesis is that the cytidylyltransferase has distinct binding sites for phospholipids and fatty acids. These sites may have overlapping specificity. For example, the fatty acid site may bind weakly to phospholipids or neutral lipids, particularly if the lipids contain unsaturated fatty acids. The binding of lipids in the cytosol may cause an interconversion between the L and H forms of the enzyme. In addition, changes in membrane lipid composition may signal the enzyme to reversibly bind to specific membranes. Finally, certain changes in membrane lipid composition may allow binding of specific phospholipids and fatty acids to the enzyme's lipid binding sites, resulting in activation.

Why would such a regulatory mechanism benefit cells, and how might PE play a role? We have previously proposed that regulation of PC biosynthesis occurs via a mechanism in which inactive cytidylyltransferase recognizes and binds to membranes deficient in PC (7). Since PC deficient membranes must, by definition, be rich in other phospholipid species, we proposed that soluble cytidylyltransferase recognizes PC deficient membranes by associating with activating species of lipid which appear at an increased concentration on the cytosolic leaflet of the membrane (7). The majority of PE molecules are most likely located on the cytosolic face of membranes (40). Thus, it seems reasonable that changes in the mole % PE present in membranes may play an important role in determining the rate of cellular PC biosynthesis. The intracellular abundance of fatty acids may play a central role in determining the rate at which various phospholipids are produced, allowing cells to make the most essential lipid repairs first. Alternatively, when fatty acid levels are low, the cells may reduce phospholipid biosynthesis and use the fatty acids for fuel.

ACKNOWLEDGMENTS

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METHODS

Separation of the Enantiomers of 1-Alkyl-2-Acyl-*rac*-Glycerol and of 1-Alkyl-3-Acyl-*rac*-Glycerol by High Performance Liquid Chromatography on a Chiral Column[1]

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High performance liquid chromatographic separations of two enantiomeric pairs of 1-alkyl-2-acyl-*rac*-glycerol (1-alkyl-2-acyl- and 3-alkyl-2-acyl-*sn*-glycerols) and 1-alkyl-3-acyl-*rac*-glycerol (1-alkyl-3-acyl- and 3-alkyl-1-acyl-*sn*-glycerols) as 3,5-dinitrophenylurethanes (3,5-DNPUs) were carried out on a chiral stationary phase, N-(*R*)-1-(α-naphthyl)ethylaminocarbonyl-(*S*)-valine chemically bonded to γ-aminopropyl silanized silica (Sumipax OA-4100). Good separation of the enantiomers of 1-hexadecyl-2-hexadecanoyl-*rac*-glycerol was easily achieved within 10 min using hexane/ethylene dichloride/ethanol (80:20:1, v/v/v) as a mobile phase. Separation of the enantiomers of 1-hexadecyl-3-hexadecanoyl-*rac*-glycerol was more difficult and required about 80 min to achieve satisfactory peak resolution (0.8) using hexane/ethylene dichloride/ethanol (250:20:1, v/v/v) as a mobile phase.
***Lipids 25,* 108–110 (1990).**

Enantiomer separations of acylglycerols (1), alkylglycerols (2), diacylglycerols (3,4) and dialkylglycerols (4) by high performance liquid chromatography (HPLC) of their 3,5-dinitrophenylurethanes (3,5-DNPUs) on chiral stationary phases have recently been reported by us. In alkyl and acylglycerols having one or two long alkyl chains, monoalkylmonoacylglycerol enantiomers remained to be resolved by the HPLC method. This paper describes a direct and accurate method for the analysis of 1-alkyl-acylglycerol enantiomers as their 3,5-DNPU derivatives using HPLC on a chiral stationary phase.

MATERIALS AND METHODS

Alkylacylglycerols were synthesized in our laboratory by acylation of monoalkylglycerols with acyl chlorides (5). Hexadecanoyl chloride (Kanto Chemicals, Tokyo), 3-hexadecyl-*sn*-glycerol (Bachem AG, Bubendorf, Switzerland; purity indicated as higher than 99%) and 1-hexadecyl-*rac*-glycerol (Nakarai Chemicals, Kyoto; purity indicated as higher than 97%) were used for the synthesis. The purity of the hexadecanoyl chloride was about 98% as judged by gas chromatographic analysis of the methyl esters obtained by reaction of methanol with acid chloride. 1-Hexadecyl-hexadecanoylglycerols were prepared by the reaction of the hexadecylglycerol, 80 mg, in dry carbon tetrachloride (0.5 ml) with hexadecanoyl chloride (35 mg) in dry carbon tetrachloride (1.5 ml) in the presence of dry pyridine (100 μl) at ambient temperature overnight. The products were extracted with diethyl ether and fractionated by thin-layer chromatography (TLC) on Silica Gel 60G plates impregnated with boric acid (6) using hexane/diethyl ether (6:4, v/v) for developing. The bands were detected under ultraviolet (UV) light after spraying with Rhodamine 6G-ethanol solution. The 1-hexadecyl-2-hexadecanoylglycerol and 1-hexadecyl-3-hexadecanoylglycerol fractions were isolated by extraction with diethyl ether.

HPLC analysis of the alkylacylglycerols was carried out as 3,5-DNPUs with a Shimadzu LC-6A instrument (Shimadzu, Kyoto, Japan) equipped with a chiral column (25 cm × 4 mm i.d.) packed with 5 μ particles of N-(*R*)-1-(α-naphtyl)ethylaminocarbonyl-(*S*)-valine chemically bonded to γ-aminopropyl silanized silica, Sumipax OA-4100 (Sumitomo Chemical Co., Osaka, Japan). The 3,5-DNPUs were prepared from 1 mg of alkylacylglycerol and about 2 mg of 3,5-dinitrophenyl isocyanate without heating, as previously described (1,3). HPLC analysis was done isocratically using a mixture of the HPLC grade solvents hexane/ethylene dichloride/ethanol as a mobile phase at a constant flow rate at ambient temperature. Peaks were monitored at 0.02 AUFS with a Shimadzu SPD-6A variable wavelength UV detector. Peak areas and retention times were obtained with a Shimadzu integrator C-R2AX.

RESULTS AND DISCUSSION

*Separation of 1-alkyl-2-acyl-*rac*-glycerol.* Figure 1 shows typical chromatograms of 1-hexadecyl-2-hexadecanoyl-*rac*-glycerol and 3-hexadecyl-2-hexadecanoyl-*sn*-glycerol as 3,5-DNPU derivatives on the chiral column OA-4100 using solvent A, hexane/ethylene dichloride/ethanol (80:20:1, v/v/v) for elution. The chromatogram of the racemate gave two clearly separate peaks with a 1:1 peak area ratio (Fig. 1A). The retention time of the second peak agreed with that of 3-hexadecyl-2-hexadecanoyl-*sn*-glycerol (Fig. 1B). The identification of the latter peak was also verified by its overlap upon co-injection of the racemate with 3-hexadecyl-2-hexadecanoyl-*sn*-glycerol prepared from 3-hexadecyl-*sn*-glycerol. Consequently, the peaks in Figure 1A were assigned to 1-hexadecyl-2-hexadecanoyl-*sn*-glycerol (I) and 3-hexadecyl-2-hexadecanoyl-*sn*-glycerol (II), respectively. The characteristic chromatographic data are listed in Table 1.

*Separation of 1-alkyl-3-acyl-*rac*-glycerol.* Figure 2 shows the typical chromatograms of 1-hexadecyl-3-hexadecanoyl-*rac*-glycerol and 3-hexadecyl-1-hexadecanoyl-*sn*-glycerol as their 3,5-DNPU derivatives on the chiral column OA-4100. The chromatogram of the racemate shows two separated peaks with approximately equal peak areas (Fig. 2A). Good enantiomer separation was achieved using solvent B, hexane/ethylene chloride/ethanol (250:20:1, v/v/v) for elution. The peak resolution was about 0.8, as shown in both Figure 2 and Table 1. Identification of 1-hexadecyl-3-hexadecanoyl-*sn*-glycerol (III) and 3-hexadecyl-1-hexadecanoyl-*sn*-glycerol (IV) was confirmed by

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Abbreviations: HPLC, high performance liquid chromatography; TLC, thin-layer chromatography; Vr, retention volume; VrA, Vr when eluted with the solvent A; UV, ultraviolet; 3,5-DNPU, 3,5-dinitrophenylurethane.

TABLE 1

Chromatographic Separation of 1-Hexadecyl-Hexadecanoylglycerols on a Chiral Column, OA-4100

Peak no.	*sn*-Position		Ratios of solvent for developing						
			A 80:20:1			B 250:20:1			
	Alkyl	Acyl	Vr[a]	α[b]	Rs[c]	Vr	α	Rs	VrB/VrA[d]
I	1	2	6.85			40.26			5.88
				1.16	1.84		1.14	2.50	
II	3	2	7.94			45.89			5.78
III	1	3	6.87			37.42			5.45
				1.03	0.42		1.05	0.79	
IV	3	1	7.11			39.35			5.53

[a]Vr, retention volume (ml) corrected by subtracting the column void volume (2.68 ml).
[b]α, Separation factor (the ratio of the capacity ratios).
[c]Rs, peak resolution.
[d]VrA denotes Vr when solvent A was used, VrB when solvent B used for elution.

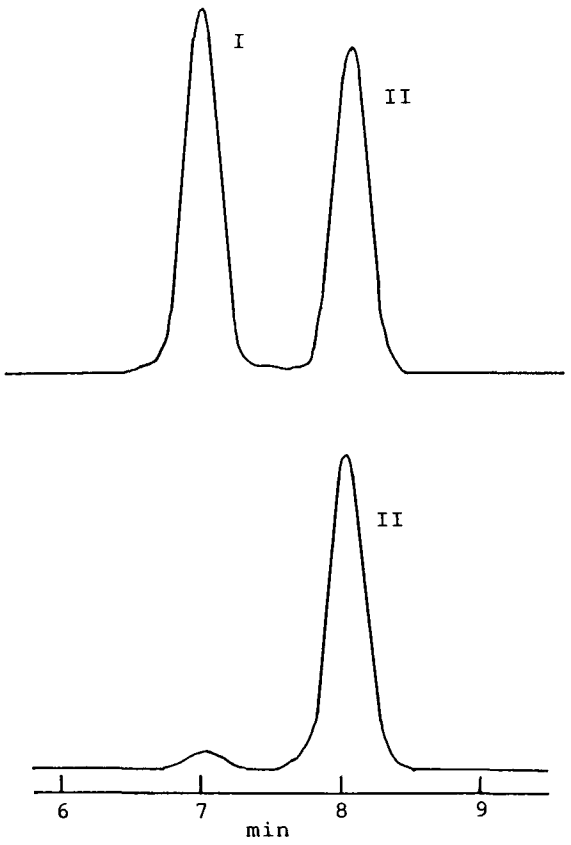

FIG. 1. Enantiomer separation of 1-hexadecyl-2-hexadecanoyl-*sn*-glycerol (I) and 3-hexadecyl-2-hexadecanoyl-*sn*-glycerol (II) as 3,5-DNPUs on a chiral column, OA-4100. Upper, racemate; lower, 3-hexadecyl-2-hexadecanoyl-*sn*-glycerol prepared from 3-hexadecyl-*sn*-glycerol. Hexane/ethylene dichloride/ethanol, 80:20:1, v/v/v. Flow rate, 1 ml/min. Detection 254 nm.

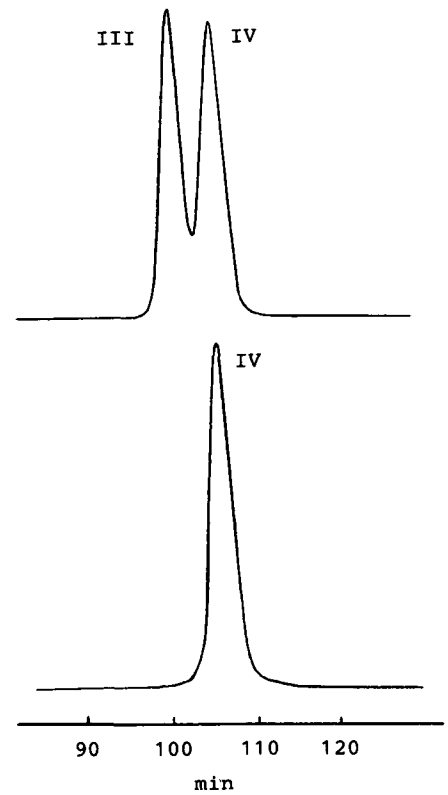

FIG. 2. Enantiomer separation of 1-hexadecyl-3-hexadecanoyl-*sn*-glycerol (III) and 3-hexadecyl-1-hexadecanoyl-*sn*-glycerol (IV) as 3,5-DNPUs on a chiral column, OA-4100. Upper, racemate; lower, 3-hexadecyl-1-hexadecanoyl-*sn*-glycerol prepared from 3-hexadecyl-*sn*-glycerol. Hexane/ethylene dichloride/ethanol, 250:20:1, v/v/v. Flow rate, 0.5 ml/min. Detection 254 nm.

co-injection of the racemate with IV prepared from 3-hexadecyl-*sn*-glycerol. The HPLC separation was more difficult as the degree of asymmetry at the C-2 carbon of glycerol of III and IV is much lower than for I and II, and the other enantiomers. Obviously, the 1,3-disubstituted glycerols, such as III and IV, are more symmetric in structure than the 1,2-disubstituted glycerols I and II.

Chromatographic data. Table 1 shows that the retention times obtained with solvent B are much longer than those obtained with solvent A. This can be expected

because elution time generally increases with decreasing polarity of the mobile phase in normal phase HPLC. Table 1 shows that solvent A eluted 1-hexadecyl-hexadecanoylglycerols in the order I plus III, < IV < II, with partial overlap occurring between I plus III and IV. Solvent B eluted in the order III < IV < I < II, with partial overlap occurring between IV and I. In this case, Vr_{II}/Vr_{I} and Vr_{IV}/Vr_{III} (Vr = volume retention) are about 1.15 and 1.04, respectively, in both A and B. The ratios VrB/VrA (Vr eluted with solvent B or A, respectively) are 5.8 for I and II and 5.5 for III and IV (Table I). Poor separation was attained for a mixture of I, II, III and IV under conditions of isocratic elution (Table 1). Gradient elution with a change from solvent A to B may therefore improve the separation. Also, preliminary separation of the 1,3- and 1,2(2,3)-isomers by TLC on silicic acid or HPLC on achiral columns (7) before HPLC on the chiral column may be useful to obtain better separation of the mixture.

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COMMUNICATIONS

Lability of Red Blood Cell Membranes to Lipid Peroxidation: Application to Humans Fed Polyunsaturated Lipids

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Red blood cell membranes (RBCM) were used to estimate human red blood cell lability to lipid peroxidation *in vitro*. RBCM were prepared from blood collected from humans fed diets with either 3 or 15% polyunsaturated fatty acids for 80 days. RBCM were isolated by centrifugation, and oxidative stress was induced by *in vitro* incubation with 0.1 or 0.5 mM tert-butyl hydroperoxide (t-BOOH) in the presence of 0.5 mg added hemoglobin. Lipid Peroxidation was evaluated by measurement of thiobarbituric acid-reactive substances (TBARS). Lipid peroxidation correlated with the protein content of RBCM in both noninduced and t-BOOH-induced lipid peroxidation systems. TBARS production was dependent on the amount of t-BOOH added to the RBCM. The production of TBARS by RBCM incubated with 0.5 mM t-BOOH was correlated with the arachidonic acid content in the red blood cells (RBC) from which RBCM were prepared. The methodology developed was useful for comparative estimations of the lability of RBCM to lipid peroxidation.
***Lipids 25,* 111–114 (1990).**

Over the past few years, a growing number of physiopathological states have been associated with the production of oxidative species (1,2). The relevance of oxyl radical-mediated reactions to human health has not been fully determined, and this area requires further investigation. Lipid peroxidation, a major and common occurrence under oxidative conditions, can be assessed either as a cause for, or a consequence of, many pathological states. Blood can reflect the lability of the whole animal to oxidative conditions, and it is also a major target of oxyl radical attack. Polyunsaturated fatty acids (PUFA) and antioxidants are the main constituents that determine red blood cell membrane (RCBM) lability to oxidative reactions. The lability of RBCM to lipid peroxidation *in vitro* may reflect the lability of other cell membranes to oxidative damage *in vivo*. RBC and RBCM have been used extensively in mechanistic studies of oxidative damage (3–6). Koster and Slee (7) studied lipid peroxidation as measured by thiobarbituric acid-reactive substances (TBARS) of RBCM induced by test-butylhydroperoxide (t-BOOH), and cumene hydroperoxide. The evaluation of RBCM lability to oxidative damage under various dietary conditions has not received much attention. Stocks *et al.* (8) have broadly surveyed the lipid peroxidation susceptibility of red blood cells (RBC) from healthy and diseased persons.

This paper describes a simple method to determine comparative lability to oxidative damage to human RBCM by evaluation of their lability to *in vitro* lipid peroxidation. The methodology was applied to RBCM prepared from blood collected from subjects provided diets with known levels of PUFA and vitamin E. This investigation was part of a project to study the effects of dietary PUFA on some biochemical parameters in humans.

MATERIALS AND METHODS

Blood samples. Blood samples were obtained from eight 29- to 45-year-old males selected after examination and the determination that they were healthy. The subjects were maintained at the Human Nutrition Suite, Western Human Nutrition Research Center, ARS, USDA, Presidio of San Francisco, CA. Diets were formulated to provide 15% of the calories from protein and 55% of the calories from carbohydrates. For the first 15 days, all subjects consumed a stabilization diet that contained 6% of the calories as PUFA, with 30% of the total calories as fat, and 3 mg of vitamin E per day. On day 16, the subjects were divided into two groups. One group was provided a high level (12.9 calorie %) of PUFA in the diet and the other group was provided a low level (3.5 calorie %) of PUFA. The PUFA in the diets was varied by adjusting the levels of two types of vitamin E-stripped safflower oil (Eastman Kodak Co., Kingsport, TN); one type contained 74.5% oleic acid, and the other 75.3% linoleic acid. The percentage of calories as fat remained at 30%, and the vitamin E level of the diets remained at 3 mg per day. The subjects were provided these diets for the subsequent 80 days. Overnight fasting blood samples were collected by venipuncture on days 1, 18, 29, 58 and 80. After each draw of blood, the RBC were packed by centrifugation and immediately frozen until use.

Isolation of RBC membranes. Two-milliliter aliquots of the thawed RBC were lysed with four volumes of water and centrifuged for 30 min at 22,000 × *g*. The pelleted RBCM were resuspended with 10 ml 4 mM phosphate buffer, pH 7.4, using a Potter-Elvejhem homogenizer, and centrifuged at 22,000 × *g* for 30 min. The wash procedure was repeated, and the pellet was resuspended in 2.5 ml 40 mM phosphate buffer, pH 7.4. This preparation was used as the RBCM.

Lipid peroxidation assay. Lipid peroxidation assays were carried out in 12-ml glass serum bottles. A 1-ml aliquot of RBCM suspension was added to 0.9 ml 40 mM phosphate buffer, pH 7.4, and 0.1 ml 5 mg hemoglobin/ml buffer. Lipid peroxidation was induced by addition of either 0.1 or 0.5 mM t-BOOH. Immediately after t-BOOH addition, the bottles were sealed and incubated at 37°C with continuous shaking at 120 cycles/min. After 30 min,

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Abbreviations: GC, gas chromatography; PUFA, polyunsaturated fatty acids; RBC, red blood cells; RBCM, red blood cell membranes; TBARS, thiobarbituric acid-reactive membranes; t-BOOH, tert-butyl hydroperoxide.

0.5-ml aliquots were added to 0.1 ml 4% (w/v) butylated hydroxytoluene in ethanol in order to minimize degradation of unoxidized lipids (5). Products of lipid peroxidation were assayed by measurement of TBARS.

TBARS analysis. TBARS were analyzed as previously reported (9), with modifications for application to RBCM. Each 0.5-ml RBCM aliquot was added to 0.5 ml 3% sodium dodecyl sulfate. After mixing, 2 ml 0.1 N HCl, 0.3 ml 10% phosphotungstic acid, and 1.0 ml 0.7% 2-thiobarbituric acid were added. The mixture was heated for 20 min in boiling water, and then the TBARS were extracted into 5 ml *n*-butanol. After a brief centrifugation, the fluorescence of the butanol layer was measured at 515 nm excitation and 555 nm emission. Malonaldehyde standards were prepared from 1,1,3,3-tetramethoxypropane.

Protein determination. Protein content of the RBCM was evaluated by the method of Bradford (10) using bovine serum albumin as a standard.

Fatty acyl group profiling. Lipid from RBC was extracted into isopropanol and chloroform according to the method of Rose and Oklander (11). The fatty acyl group composition of RBC lipids was measured by gas liquid chromatography after transesterification with a methanol/hydrochloric acid mixture in the presence of butylated hydroxytoluene and margaric acid as an internal standard. The fatty acid methyl ester composition was determined on a Shimadzu 95A gas chromatograph equipped with a 30 meter × 0.25 mm fused silica SP 2330 column (Supelco, Bellfonte, PA) and a flame ionization detector.

Statistical analysis. Statistical analysis was done using routines available in Minitab (Pennsylvania State University, 1982) and in Statview 512+ (Brainpower, Inc., Calabazas, CA).

RESULTS

RBC peroxidation methodology. To develop the method for measurement of RBCM lability to lipid peroxidation, RBCM were isolated from a single blood sample. The production of TBARS increased linearly with the amount of protein when the RBCM were incubated either without an inducer or with t-BOOH (Fig. 1). Production of TBARS was also dependent upon the amount of t-BOOH added to the incubation mixtures (Fig. 1).

Hemoglobin can catalyze hydroperoxide decomposition, thus accelerating lipid peroxidation and the production of TBARS (12,13). Although the procedure used to prepare the membranes decreased the hemoglobin content, differing amounts of hemoglobin remained in the various preparations. The amount of hemolglobin added to the RBCM increased TBARS production in RBCM incubated either without or with added t-BOOH. In RBCM incubated without t-BOOH, the addition of 0.1–0.5 mg of hemoglobin increased TBARS production from 3% to 120%. In RBCM incubated with 0.5 mM t-BOOH, addition of hemoglobin from 0.1–5 mg increased TBARS production from 2% to 500%. Hemoglobin at 0.5 mg per sample of RBCM was chosen to normalize its content in the RBCM preparations. The effect of this added hemoglobin on TBARS production was never less than 5%. With added hemoglobin, the reproducibility of the lipid peroxidation assay was good, with a variation of less than 10% for individual samples of RBCM.

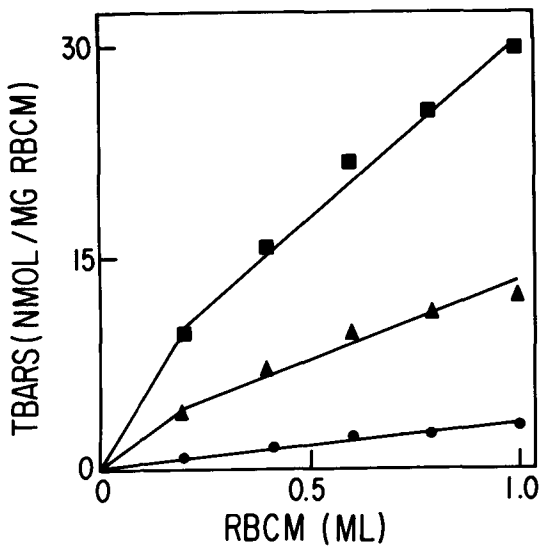

FIG. 1. Effect of the amount of RBCM on TBARS formation. RBCM suspensions (2.1 mg protein/ml) were incubated for 30 min at 37°C with either no addition (●), 0.1 mM t-BOOH (▲), or 0.5 mM t-BOOH (■). Values are means of 3 aliquots of a single incubated sample, and the S.E.M. were less than 5%.

Lability of RBCM from humans provided diets with two levels of PUFA. TBARS production by RBCM was not significantly changed during the experimental dietary period. TBARS production by RBCM over the course of the study was linearly related to RBCM protein content (Fig. 2). Considering that additional hemoglobin was added to the membranes during the 30-min incubation, the dependence on protein content was probably associated with the total amount of peroxidizable components in the RBCM. The linear relationship to protein concentration was observed both in RBCM incubated without an inducer (Fig. 2A) and in RBCM incubated with 0.1 mM t-BOOH (Fig. 2B) or 0.5 mM t-BOOH (Fig. 2C). A concentration of 0.5 mM t-BOOH increased the mean TBARS per milligram of RBCM protein 2.1-fold over TBARS produced by 0.1 mM t-BOOH incubated with the RBCM. The levels of α-tocopherol (0.65 ± 0.11 mg/dl) and γ-tocopherol (0.11 ± 0.05 mg/dl) were not modified by the diets.

The content of palmitic, stearic, oleic, linoleic, and arachidonic acid in the RBC were not significantly modified by the diets. Considering these five major fatty acids as totaling 100%, the amounts were approximately 25, 22, 18, 13 and 22% for 16:0, 18:0, 18:1, 18:2 and 20:4, respectively. Based on our experiences in modeling lipid peroxidation processes (14), we tested many possible correlations of the data. Models of the form TBARS vs (PUFA × PEROXIDIZABILITY)/TOCOPHEROLS gave no statistically significant correlations. For models of the form TBARS vs PUFA × PEROXIDIZABILITY, only one model involving arachidonic acid produced a significant correlation (Fig. 3). When 0.5 mM t-BOOH was added to RBCM, TBARS production was linearly related ($r = 0.36$, $p < 0.03$) to the amount of arachidonic acid in the RBC from which the RBCM were prepared (Fig. 3). TBARS from RBCM incubated without t-BOOH or with 0.1 mM t-BOOH was not related to the RBC arachidonic acid content.

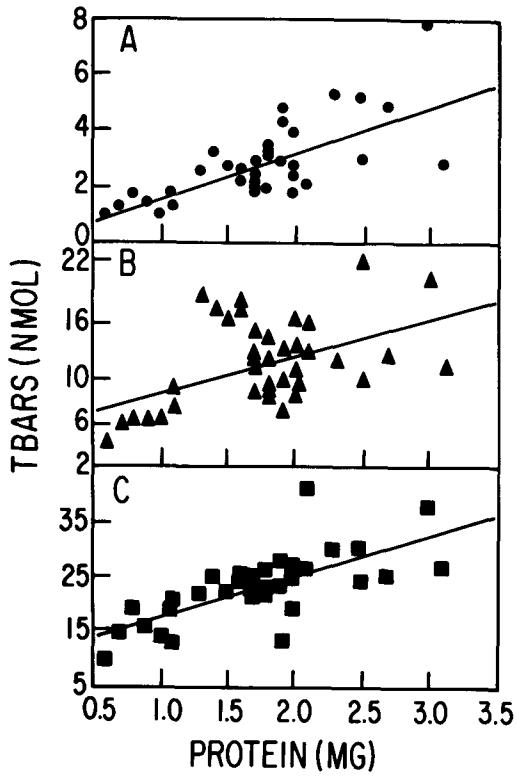

FIG. 2. TBARS production by RBCM as a function of protein content. RBCM were incubated (A) without an inducer, (B) with 0.1 mM t-BOOH, or (C) with 0.5 mM t-BOOH. The points are values obtained for each blood sample drawn from each subject over the course of the study. Regression analysis gives (A) $r = 0.69$, $p > 0.001$, (B) $r = 0.50$, $p > 0.01$, and (C) $r = 0.70$ $p > 0.001$.

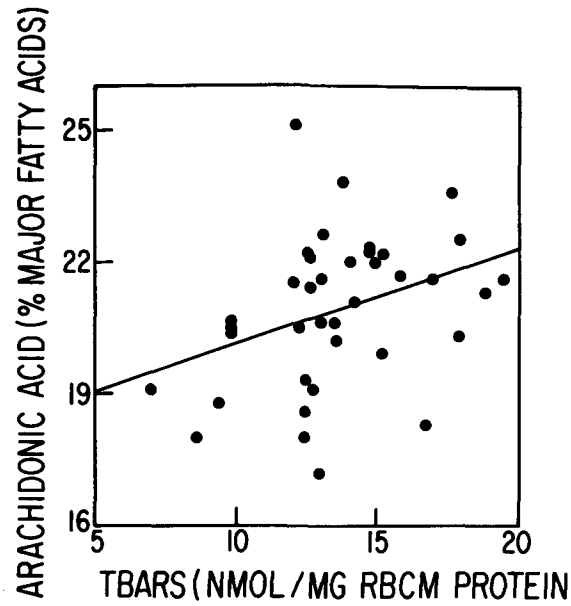

FIG. 3. Relationship between TBARS produced by RBCM incubated for 30 min at 37°C in the presence of 0.5 mM t-BOOH and the percentage of arachidonic acid in the 16:0, 18:0, 18:1, 18:2 and 20:4 fatty acids in the RBC from which RBCM were isolated. Points are values obtained for each blood sample drawn from each subject.

DISCUSSION

There is a growing need for experimental systems to evaluate oxidative lability of PUFA in humans. Lability of RBCM to lipid peroxidation can be influenced by a large number of biochemical components. RBC are highly sensitive to oxidative damage (3). They are rich in highly unsaturated lipids and are continuously exposed to a high oxygen concentration. Hemoglobin and iron in RBC are powerful catalysts of oxidative reactions. Consequently, the appropriate substrates and catalysts in RBC ensure that a significant rate of oxyl radical production could occur *in vivo*. RBCM may reflect *in vivo* oxidative stress, including decreased levels of antioxidants such as vitamin E. When insufficient dietary antioxidants are consumed, oxidative damage to PUFA will occur. By testing the oxidative lability of RBCM to peroxidation *in vitro*, it may be possible to visualize some of the effects of prooxidant conditions in humans. Even though arachidonic acid was not a dietary variable in this study, the correlation of RBCM lability with the arachidonic acid content in the RBC stresses the importance of dietary PUFA. Increasing the relative amounts of PUFA in the diet reportedly decreases the incidence of atherosclerosis and coronary diseases (15), while high fat diets are associated with increased mortality and cancer (16). In antioxidant-deficient experimental animals, there is evidence that a high level of PUFA increases lipid peroxidation rats (17,18).

In this study, variations in PUFA content in the human diet did not accelerate the rate of oxidative reactions in RBCM. This result was shown by the lack of effect on *in vitro* TBARS production by RBCM incubated either without inducer or with 0.1 mM t-BOOH. Under the stronger oxidative conditions provided by incubation with 0.5 mM t-BOOH, TBARS production by RBCM correlated with the amount of arachidonic acid in the RBC. Under conditions of low *in vitro* oxidant challenge, the physiological antioxidant defenses were able to overcome the oxidative stress, but when exposed to greater oxidative stress, the antioxidant defenses were overwhelmed, and lipid peroxidation became dependent on the PUFA substrate. The method used in this study for application to blood samples from human subjects provided different diets may be applicable to other studies of *in vivo* oxidative stress in humans.

ACKNOWLEDGMENTS

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Molecular Species Composition of Phosphatidylcholine from *Crypthecodinium cohnii* in Relation to Growth Temperature

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The molecular species composition was determined for phosphatidylcholine (PC) isolated from the marine dinoflagellate *Crypthecodinium cohnii* grown at three different temperatures. At all three temperatures the didocosahexaenoyl species comprised about 25% of the PC with 14:0/22:6 and 16:0/22:6 also being of major importance; these three species comprised 75–82% of the total. Another 20 species were identified, including several short chain disaturated species. Only small differences in the composition of PC were found in response to growth at 16, 23 and 27°C. On dropping the growth temperature from 27°C to 16°C the largest changes were a decrease of 8.9% in saturated/saturated species and an increase of 5.3% in saturated/PUFA species; the 22:6/22:6 content only increased slightly (by 1.9% to 25.4%). This unusual molecular species composition is discussed.
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Crypthecodinium cohnii is a marine dinoflagellate in which ca. 30% of the constituent fatty acids are docosahexaenoic acid, 22:6(n-3), and other polyunsaturated fatty acids (PUFA) are usually present at <1% of the total (1,2). Since this alga is non-photosynthetic, the glycolipids characteristic of photosynthetic membranes are absent and phospholipids are the predominant polar lipids. Most of the 22:6(n-3) in *C. cohnii* is present in the phospholipids, particularly in phosphatidylcholine (PC), the major phospholipid class (64%), in which it comprises 50–60% by weight of the total fatty acids (3). The other major fatty acids present in PC are 14:0 and 16:0, with small amounts of 12:0, 18:0 and 18:1(n-9), and several minor components present at <0.5% (3).

The present study had two aims. First, to determine the molecular species present in PC derived from *C. cohnii,* since the unique fatty acid composition suggested the presence of unusual species such as 22:6/22:6. Second, to determine both whether and how the molecular species composition of PC altered in relation to the homeoviscous adaptation of membranes, which is characteristic of poikilotherms grown at different temperatures.

MATERIALS AND METHODS

Materials. Phospholipase C from *Bacillus cereus* (Sigma type XIII), butylated hydroxytoluene (BHT) and 3,5-dinitrobenzoylchloride were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). Didocosahexaenoin was obtained from BAST of Copenhagen, Copenhagen V, Denmark.

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Abbreviations: BHT, butylated hydroxytoluene; HPLC, high performance liquid chromatography; HPTLC, high performance thin-layer chromatography; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PUFA, polyunsaturated fatty acid; TLC, thin-layer chromatography; molecular species, e.g., 14:0/22:6, 1-myristoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine.

Merck thin-layer chromatography (TLC) and high performance thin-layer chromatography (HPTLC) plates coated with silica gel 60, Analar grade glacial acetic acid, methyl acetate, propan-1-ol, propan-2-ol and pyridine were purchased from BDH Ltd. (Poole, Dorset, U.K.). All other solvents of high performance liquid chromatography (HPLC) grade were from Rathburn Chemicals (Walkerburn, Peeblesshire, Scotland).

Ultrasphere ODS and Ultrasphere C8 HPLC columns (25 × 0.46 cm, 5 micron particle size) were obtained from Altex/Beckman (Beckman Instruments U.K. Ltd., High Wycombe, Bucks, U.K.).

Growth of organism. Stock cultures of *Crypthecodinium cohnii* (Seligo) Javornicky (4), strain WH-d, were maintained axenically at 27°C in the dark on MLH medium (4) solidified with 1.5% (w/v) agar. Cells for lipid analysis were cultivated in 5 × 200 ml of MLH medium (in the dark) with gentle aeration at 16 ± 1°C, 23 ± 1°C and 27 ± 1°C. Cell density was measured using a haemocytometer, and cells were harvested at densities of 4.6×10^5/ml (13 days, 16°C), 8.2×10^5/ml (5 days, 23°C) and 6.8×10^5/ml (3 days, 27°C). Cells were collected by centrifugation at 7500 g for 15 min, and the resulting pellets freeze-dried.

Extraction and purification of lipids. Total lipid was extracted from the freeze-dried pellets essentially by the method of Folch *et al.* (5). Solvents routinely contained 0.01% (w/v) BHT, and between preparative procedures samples were stored at −20°C under N_2. Neutral lipids were separated from polar lipid by TLC using hexane/diethyl ether/acetic acid (70:30:1, v/v/v). The polar lipid remaining on the origin was eluted from the absorbent with chloroform/methanol/water (5:5:1, v/v/v), dried by rotary evaporation under vacuum at 30°C, and finally under N_2. PC was purified by TLC using methyl acetate/propan-2-ol/chloroform/methanol/0.25% (w/v) KCl (25:25:25:10:9, v/v/v/v/v/) (6) alongside an authentic PC standard. Lipids were visualized under UV light after spraying the chromatogram with 0.1% (w/v) 2,7-dichlorofluorescein in methanol containing 0.01% (w/v) BHT. PC was eluted from the silica gel as before. The purity of PC was checked by two-dimensional HPTLC in chloroform /methanol/water/0.88 ammonia (130:70:8:0.5, v/v/v/v), then in chloroform/acetone/methanol/acetic acid/water (10:4:2:2:1, v/v/v/v/v) (7).

Preparation of 3,5-dinitrobenzoyl derivatives. PC was hydrolyzed with phospholipase C (from *Bacillus cereus*) and the resultant 1,2-diacylglycerols reacted with 3,5-dinitrobenzoylchloride to give the 3,5-dinitrobenzoyl derivatives (8).

Separation of molecular species. Molecular species were separated by HPLC on Beckman ODS and C8 columns (25 × 0.46 cm, 5 μ particle size) using three isocratic solvent systems: methanol/propan-2-ol 95:5, v/v; acetonitrile/propan-2-ol 80:20, v/v; methanol/water/acetonitrile 93:5:2, v/v/v (9). Peaks were detected at 254 nm, and peak areas were measured using a Shimadzu CR 3A (Shimadzu Corp., Kyoto, Japan) recording integrator. Retention

TABLE 1

Molecular Species Composition of Phosphatidylcholine from *C. cohnii* Grown at Different Temperatures

	Mole %			Significance[a]
	16°C	23°C	27°C	16°C vs 27°C
12:0/12:0	0.5 ± 0.1	1.1 ± 0.1	0.6 ± 0.2	ns
12:0/14:0	0.9 ± 0.1	1.7 ± 0.4	2.4 ± 0.2	$p < 0.005$
14:0/12:0	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.2	ns
14:0/14:0 } 12:0/16:0 }	0.8 ± 0.1	2.4 ± 0.3	7.2 ± 0.2	$p < 0.0001$
14:0/16:0	0.3 ± 0.1	0.9 ± 0.1	1.0 ± 0.3	$P < 0.05$
16:0/16:0	0.2 ± 0.1	0.7 ± 0.2	0.4 ± 0.1	ns
Sat/sat	3.1	7.2	12.0	
12:0/16:1	0.4 ± 0.1	—	0.7 ± 0.2	ns
14:0/16:1	0.3 ± 0.1	—	0.7 ± 0.2	ns
14:0/18:1	4.9 ± 0.6	3.9 ± 0.5	2.8 ± 0.2	$p < 0.005$
16:0/18:1	1.7 ± 0.1	2.3 ± 0.5	2.8 ± 0.4	$p < 0.01$
Sat/monoene	7.2	6.2	7.0	
14:0/20:5	tr	—	—	
14:0/22.6	35.7 ± 1.0	29.9 ± 0.7	28.7 ± 0.6	$p < 0.001$
16:0/20:5	0.7 ± 0.1	0.9 ± 0.1	2.5 ± 0.5	$p < 0.005$
16:0/22:6	22.3 ± 1.1	24.3 ± 0.4	23.4 ± 1.2	ns
16:0/22.5	tr	0.3 ± 0.1	—	ns
18:0/22.6	0.3 ± 0.1	0.4 ± 0.1	0.8 ± 0.1	$p < 0.005$
18:0/22.5	1.5 ± 0.2	0.7 ± 0.1	—	$p < 0.01$
Sat/PUFA	60.5	56.5	55.2	
18:1/18:1	0.2 ± 0.1	0.7 ± 0.1	1.3 ± 0.3	$p < 0.005$
16:1/22:6	tr	0.2 ± 0.1	—	ns
18:1/20:5	—	—	0.4 ± 0.1	ns
18:1/22:6	3.5 ± 0.8	4.4 ± 0.4	0.8 ± 0.3	$p < 0.005$
18:1/22:5	tr	—	—	ns
Monoene/PUFA	3.5	4.6	1.2	
22:6/22:6	25.4 ± 0.2	24.3 ± 0.8	23.5 ± 1.0	$p < 0.05$

[a]The significance of the changes in composition between 16 and 27°C were calculated using an ANOVA t-test program. All values are the mean of three determinations ± 1 S.D. All compositions are corrected to 100%, tr < 0.1%. Molecular species are abbreviated as follows: e.g., 14:0/22:6 is 1-myristoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine. The most saturated fatty acid is assumed to be on position 1 of the glyceride.

times relative to 16:0/22:6 were calculated and peaks were assigned from plots of Log_{10} (relative retention time × 10) vs the effective carbon number on the C-1 position of the glyceride constructed from known standards (9).

RESULTS AND DISCUSSION

Three molecular species, 22:6/22:6, 14:0/22:6 and 16:0/22:6, made up 75–82% of the PC in *C. cohnii* grown at 16, 23 and 27°C (Table 1). Another 20 species were detected, of which only 14:0/12:0 + 12:0/14:0, 14:0/14:0 + 12:0/16:0, 14:0/18:1, 16:0/20:5 and 18:1/22:6 were present at levels of greater than 2.0% at any one of the temperatures. Typical HPLC separations are shown in Figure 1.

The large amount of the highly unsaturated 22:6/22:6 species (23.4–25.0%) is of particular interest and is unexpected, since an earlier study found that docosahexaenoic acid comprised only ca. 55% of the total fatty acids present in PC from *C. cohnii* grown at 27°C (3), and this was also the case under the growth conditions used here (Table 2). The large amounts of didocosahexaenoate PC were balanced by the presence of disaturated species, and at 27°C these comprised 12.0% of the total the most important of which were 14:0/14:0 + 12:0/16:0 (unfortunately, none of the three solvent systems resolved these two species). Thus, in this particular case the fatty acid composition does not give a true indication of the range of molecular species present.

Dipolyunsaturated molecular species of phosphoglycerides are increasingly being found in a variety of tissues, including PC from fish muscle (10), PC and phosphatidylethanolamine (PE) from cod roe (9), PE and phosphatidylserine (PS) from rat erythrocyte (11), PC and

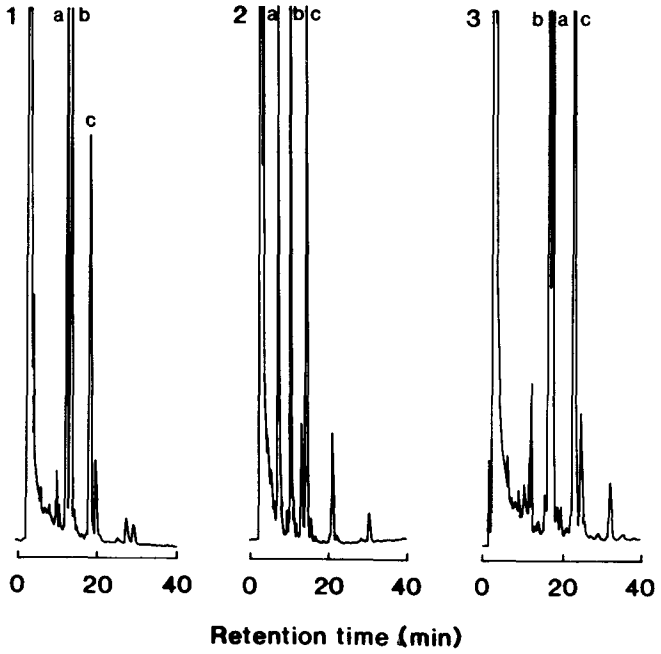

FIG. 1. Molecular species separations of the 3,5-dinitrobenzoyl derivatives of PC from *C. cohnii* grown at 16°C. The solvent systems are: (1) methanol/propan-2-ol, 95:5 (v/v), 1.0 ml/min; (2) acetonitrile/propan-2-ol, 80:20 (v/v), 1.0 ml/min; and (3) methanol/water/acetonitrile, 93:5:2 (v/v/v), 1.2 ml/min. The absorbance range at 254 nm was 0.04 units with ca. 20 μg of material injected. The three main molecular species are indicated: (a) 22:6-22:6, (b) 14:0-22:6, and (c) 16:0-22:6.

TABLE 2

The Fatty Acid Composition of Phosphatidylcholine from *C. cohnii* Grown at Different Temperatures Calculated from the Molecular Species Composition

	Mole %		
	16°C	23°C	27°C
12:0	2.1	2.7	3.8
14:0	21.3	18.8	21.4
16:0	12.8	15.2	17.0
16:1	0.4	0.1	0.3
18:0	0.9	1.2	0.4
18:1	5.2	5.8	4.7
20:5(n-3)	0.5	1.5	1.5
22:5(n-3)	0.9	0.5	—[a]
22:6(n-3)	55.9	53.1	50.0
Total saturates	37.1	37.9	42.6
Total monoenes	5.6	5.9	5.0
Total PUFA	57.3	55.1	51.5

[a]Not detected.

PE from rat testes (12) and especially in PC, PE and PS from mammalian retina (13) and fish brain and retina (14). The presence of such species is contrary to the earlier view of phosphoglyceride structure, which was that a saturated, or sometimes a monounsaturated, fatty acid is strongly preferred on the *sn*-1 position of the glyceride, with an unsaturated fatty acid strongly preferred on the *sn*-2 position. The present data establish that this is not always the case, even when the overall fatty acid composition of the phosphoglyceride indicates no great excess of PUFA. The molecular species of PC from *C. cohnii* are particularly unusual in that large amounts of diPUFA, and disaturated species are present rather than the expected pairings of saturated with polyunsaturated fatty acids. The function of such diPUFA species in the biomembrane is presently unclear. However, it may be relevant that most galactolipid species in the chloroplast membranes of plants are also diPUFA (15).

The fatty acid and molecular species compositions of PC from *C. cohnii* are also unusual with respect to the length profile of the fatty acids. In mammals, C16 and C18 saturated and monounsaturated fatty acids, together with C20 and C22 PUFA, are the major constituents of phosphoglycerides. Shorter chain saturates like 12:0 and 14:0 are rarely found in phosphoglycerides, though some fish lipids contain a few percent of 14:0 (16). Computer modeling studies have shown that 22:6(n-3) has two energetically favored conformations, an "angle-iron" form and a helical form (17), both of which effectively shorten the fatty acid to approximately the same length as an extended 14:0 or 16:0 chain. Thus, it can be argued that only 4.1% of the fatty acids in PC from *C. cohnii* are effectively longer than 16:0. The phospholipid bilayer from the biomembrane of *C. cohnii* may therefore be narrower than "typical" mammalian cell membranes, and could be an interesting model system for studies on membrane function, e.g., it may have a higher curvature.

There was an increase of 5.5% in saturated fatty acids and a decrease of 5.8% in PUFA in PC between 16 and 27°C (Table 2). *C. cohnii* grows optimally at 27°C, yet even at this quite high temperature almost a quarter of the PC is the highly unsaturated di22:6 species. On decreasing the temperature to 16°C, the molecular species composition of PC, the major membrane phospholipid in this organism, is significantly, but not markedly, altered. Over the 11°C temperature decrease there is an increase of 1.9% in 22:6/22:6 content, a decrease of 8.9% in disaturated species and a decrease of 1.1% in 18:1/18:1. There is no change in the proportion of saturated/monounsaturated species. Saturated/PUFA species (mainly 14:0/22:6 and 18:0/22:5) increase by 5.3%, through 16:0/20:5 and 18:0/22:6 decrease by 1.8%, respectively (Table 1). Whether these changes alone are sufficient to bring about the alterations in membrane fluidity required to maintain membrane function at the lower temperature is not known, although the changes noted here are of a similar magnitude to those found in the membranes of trout on thermal acclimation from 20°C to 5°C (18). It is nonetheless surprising that the content of the major unsaturated species in the membrane, 22:6/22:6, is virtually unaltered by growth at the different temperatures. *C. cohnii* also contains an unusual sterol, dinosterol, which was found to be less soluble in model membranes than cholesterol (19), and this may be related to the high content of 22:6-rich phosphoglyceride species in *C. cohnii* membranes. Modulation of the amounts of sterols could also have a role in temperature adaptation.

In conclusion, PC from *C. cohnii* shows several interesting features. The molecular species composition is unusual, with didocosahexaenoate comprising a quarter of the total, while small amounts of disaturated species were also present, particularly at 27°C. The response to

changes in growth temperature was also unusual. Thermal acclimation was largely achieved by altering the proportion of disaturated and saturated/PUFA species, leaving the large amounts of 22:6/22:6 virtually unaltered. The role of this highly unsaturated species in biomembrane function remains to be clarified.

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Synergism Between Penicillin G and the Antimicrobial Ether Lipid, *rac*-1-Dodecylglycerol, Acting Below Its Critical Micelle Concentration

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***rac*-1-Dodecylglycerol (DDG) and penicillin G (Pen G) act synergistically to dramatically lower the minimum inhibitory concentration (MIC) of each other in four Gram-positive bacteria studied. At one-half its MIC, DDG ether lowered the MIC of Pen G 10- to 80-fold. Under the same conditions, Pen G lowered the MIC of DDG 4- to 7.5-fold.**

The critical micelle concentration of DDG was determined to be 7.93 mg/ml (0.0305 mM), which is approximately two-fold greater than the minimum inhibitory concentration of DDG determined in the presence of a protein-free chemically defined medium. This finding suggests that DDG is not killing bacteria through its detergent action. Pen G also did not alter the critical micelle concentration of DDG, which indicates that the synergism between these two agents is not related to micelle formation.
Lipids 25, 119–121 (1990).

The alkyl ether lipid, *rac*-1-dodecylglycerol (DDG) is a relatively potent antibacterial agent (1-3) that hinders microbial viability at least in part through a metabolic mode of action. Thus far, DDG has been shown to promote peptidoglycan hydrolysis by stimulating an autolytic enzyme (1,2), to inhibit peptidoglycan synthesis (3), and to inhibit lipid and lipoteichoic acid synthesis (4). Pen G and other β-lactams act in similar metabolic areas (5-8) except that Pen G stimulates rather than inhibits lipid synthesis and excretion (9,10). The tolerance of certain organisms, such as *Streptococcus mutans* BHT, to Pen G may be related to the organism's ability to produce and excrete copious amounts of lipid and lipoteichoic acid when exposed to Pen G, thus shielding the cell from further action by Pen G. Precedence for this idea has already been established in certain Gram-positive bacteria [*Staphylococcus aureus, Streptococcus faecalis (Enterococcus hirae)* and *Bacillus subtilus*] where the minimum inhibitory concentrations of several β-lactams were observed to increase in direct proportion to the lipid content of these organisms (11). As an extension of these observations, it was reasoned that DDG, being a hydrophilic lipid and an inhibitor of normal lipid synthesis, might decrease the normal hydrophobic environment of the membrane sufficiently to change the bacteria's response to Pen G. The results show that DDG and Pen G together do indeed produce a significant synergistic decrease in the minimum inhibitory concentration, and that this synergism is not related to any physical phenomenon associated with micelle formation.

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MATERIALS AND METHODS

Culture conditions. Cultures of specimens investigated were kindly supplied by the Department of Microbiology and Immunology, Temple University School of Medicine. Bacterial growth was monitored by determining the optical density of the cultures with the use of a Spectronic-21 (Baush and Lomb Inc.) set at 675 nm. The optical density was corrected for Beers Law (12) and is expressed as the adjusted optical density (AOD).

For comparative studies with gram positive bacteria, a complex organic medium was prepared by dissolving 30 g of Todd-Hewitt powder, 18 g of dextrose and 5 g of yeast extract in deionized distilled water to a final volume of 1 liter. It was then autoclaved at 121°C for 15 min.

The effect of DDG, synthesized by the method of Baumann and Mangold (13), and Pen G on bacterial growth was studied using the "Checkerboard" technique (14). Growth medium (5 ml), containing graded combinations of DDG and Pen G, was inoculated with 50 μl of a log phase culture of 0.5 AOD. The growth was assessed turbidimetrically after an overnight incubation at 37°C. The minimum inhibitory concentration (MIC) is defined as the lowest concentration of compound(s) which results in no evidence of growth. The results are expressed in the form of isobolograms. Synergy is defined as no growth of bacteria in the presence of two antibiotics, each of which is at a concentration less than 1/2 of its MIC (15). The degree of synergism is also indicated by the hypobolic shape of a graph (14,16) derived from a plot of the MIC produced by different combinations of concentrations of the two inhibitors.

Determination of the critical micelle concentration (CMC). The CMC of DDG was determined by measuring the shift of fluorescence spectrum of the dye N-phenyl-1-naphthylamine in the presence and absence of varying concentrations of DDG as described by Kovatchev *et al.* (17). The emission properties of the dye in the chemically defined medium in the presence and absence of 0.1M DDG are shown in Figure 1. In the presence of DDG micelles, the fluorescence emission has a maximum at 418 nm upon excitation at 350 nm. In the presence of DDG below its critical micelle concentration, the emission maximum is at 440 nm. The plots of N-phenyl-1-naphthylamine fluorescence vs DDG concentrations do not change below the CMC. The validity of the method was verified by using detergents with known CMC's (18).

RESULTS

Effect of DDG and Pen G on the minimum inhibitory concentration. Determination of the minimum inhibitory concentration (MIC) of Pen G and DDG alone and in various combinations using four Gram-positive test bacteria produced data which, when plotted, yielded hypobolic

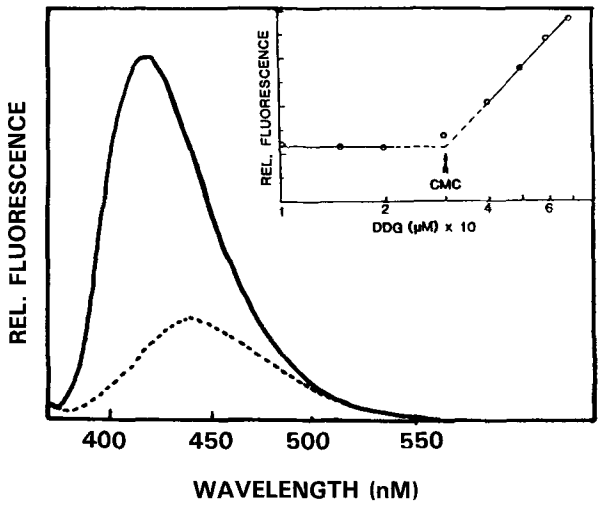

FIG. 1. Determination of the critical micelle concentration (CMC) of DDG. The figure shows the relative (uncorrected) fluorescence emission spectrum of N-phenyl-1-naphthylamine in presence (—) and in absence (----) of 100 mM DDG in a chemically defined medium. The inset shows a typical determination of CMC of DDG where the concentrations of the compounds are plotted on logarithmic scale.

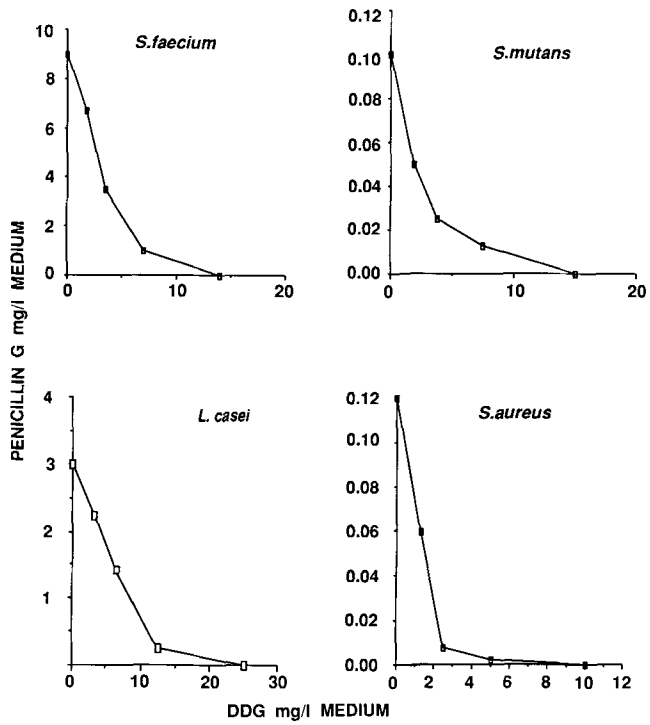

FIG. 2. Synergism between Pen G and DDG demonstrated using the Gram-positive organisms *Lactobacillus casei, Staphylococcus aureus, Streptococcus mutans BHT,* and *Streptococcus faecium* ATCC 9790. The minimum inhibitory concentrations were determined for Pen G and DDG alone and together. Synergy is defined as no growth of bacteria in the presence of two antibiotics, each of which is at a concentration less than one-half of its MIC (15). Each of the graphs is representative of experiments repeated 4 times.

curves (Fig. 2). The hypobolic shape of the curves is characteristic of a relatively strong synergism between two antibacterial agents for each organism (14-16). For example, the MIC for Pen G is 9 mg/l and for DDG it is 14 mg/l with *S. faecium* as the test organism. The MIC for DDG of 14 mg/l determined in these studies in the presence of a complex medium is higher than the previously published value of 4 mg/l (1), which was determined in the presence of a protein-free chemically defined medium. The DDG probably adsorbs to the proteins in the complex medium. A complex medium was used in these studies since the chemically defined medium was designed to meet the growth requirements of *S. faecium* and would not necessarily be suitable for the other bacteria of this study. With DDG at one-half its MIC (7 mg/l), the MIC for Pen G is lowered to 1 mg/l. At one-half the MIC of Pen G, the MIC for DDG is lowered to 3 mg/l. This mutual lowering of the MIC is clearly more than a simple additive effect and is a good example of antimicrobial synergism. Three other test organisms gave similar results (Fig. 2). At one-half the MIC of DDG, the MIC of Pen G is decreased 10-fold in *S. mutans*, 10-fold in *L. casei*, and 80-fold in *S. aureus*. At one-half the MIC of Pen G, the MIC of DDG is decreased 7.5-fold in *S. mutans*, 4-fold in *L. casei*, and 7-fold in *S. aureus*. Gram-negative bacteria were not tested because neither of these antimicrobial agents is highly active against most Gram-negative microorganisms (1).

Critical Micelle Concentration (CMC) of DDG. The CMC of DDG was found to be 0.0305 mM, i.e. 7.93 mg DDG/l (Fig. 1). This concentration is approximately two-fold higher than the MIC of DDG for *S. faecium* 9790 ATCC using a chemically defined protein-free growth medium (1). The CMC of DDG was not altered by varying concentrations of Pen G. The CMC of DDG in the presence of Pen G at 0, 2, 4, and 8 mg/l was 0.0305 mM, 0.0300 mM, 0.0305 mM, and 0.0310 mM respectively.

DISCUSSION

The ability of DDG at very low concentrations to lower the MIC of Pen G by as much as 80-fold is a striking and potentially useful property. It may be especially useful against bacteria, such as the cariogenic *S. mutans*, which are tolerant to Pen G. Furthermore, feeding studies with mice appear to show no detrimental effects of DDG on animal tissues (19).

The mechanism by which Pen G and DDG achieve their synergy could be multifocal since both compounds adversely interact with bacteria at a number of sites. However, it is clear from the present study that DDG is not acting through a detergent-like action, which requires micelle formation. DDG, is active well below its critical micelle concentration. Moreover, the critical micelle concentration is not altered by Pen G in concentrations up to 8 mg/l. Therefore, the synergism between DDG and Pen G on the growth of *S. faecium* is apparently not due to a change in a physical property of DDG. These data support our previously proposed contention that DDG is not destroying bacterial cells through a purely physical phenomenon associated with micelle formation.

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Solubilization and Partial Purification of Cholinephosphotransferase in Hamster Tissues

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CDPcholine:1,2-diacylglycerol cholinephosphotransferase (EC 2.7.8.2) is located on the cytoplasmic side of the endoplasmic reticulum, and catalyzes the final step in the synthesis of phosphatidylcholine via the CDPcholine pathway. The enzyme was solubilized from hamster liver microsomes by 3% Triton QS-15, and partially purified by DEAE-Sepharose chromatography and Sepharose 6B chromatography. The microsomal and partially purified enzymes displayed similar pH profile, and both showed absolute requirement for Mg^{++} or other divalent cations. The Km values of CDPcholine were similar between microsomal and partially purified enzyme, whereas the Km value for diacylglycerol was substantially lowered when the enzyme was partially purified. Hamster heart cholinephosphotransferase was not solubilized by Triton QS-15.
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Phosphatidylcholine is the major membrane phospholipid in mammalian tissues (1). Beyond its structural role in the membrane morphology, phosphatidylcholine may be involved in the modulation of membrane-bound enzymes (2,3). Recently, the participation of phosphatidylcholine in signal transduction has been implicated (4). Three pathways have been identified for the biosynthesis of phosphatidylcholine in mammalian tissues (5). In the heart and liver, the CDPcholine pathway is the major pathway for the *de novo* syntheis of phosphatidylcholine (6,7). The final step of this pathway is the conversion of CDPcholine and diacylglycerol to phosphatidylcholine and CMP, catalyzed by CDPcholine: 1,2-diacylglycerol cholinephosphotransferase (EC 2.7.8.2) (7).

The utilization of diacylglycerol by cholinephosphotransferase was found to be influenced by the nature of saturated and unsaturated acyl groups at the C-1 and C-2 positions of the glycerol moiety (8,9). The specificity of choline phosphotransferase towards the selection of diacylglcerol groups may contribute to the characteristic composition of fatty acyl chains in membrane phosphatidylcholine (8,9). The enzyme is located on the cytoplasmic side of the endoplasmic reticulum (10). Attempts to solubilize this enzyme in order to study its regulation have been met with limited success (11,12).

In this present study, we report the solubilization and partial purification of cholinephosphotransferase from hamster liver microsomes. The enzyme in the hamster heart was also characterized and compared with the liver enzyme.

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MATERIALS AND METHODS

Materials. CDP-[*methyl*-^{14}C] choline was obtained from NEN division, Dupont Co. (Dorval, Que.). CDPcholine and 1,2-diacylglycerol (pig liver) were purchased from Serdary Research Laboratories (London, Ont.). Octyl glucoside, taurocholic acid, Triton QS-15, Triton X-100, Tween 20, phosphatidylcholine, phosphatidylethanolamine and lysophosphatidylcholine were obtained from Sigma Chemical Co. (St. Louis, MO). CHAPS and CHAPSO were purchased from Pierce Chemical Co. (Rockford, IL). DEAE-Sepharose (fast flow) and Sepharose 6B were the products of Pharmacia Fine Chemicals (Dorval, Que.). All other chemicals were reagent grade and were obtained through the Canlab Division of Travenol Canada Inc. (Winnipeg, Man.).

Syrian golden hamsters (110 ± 10 g) were maintained on Purina hamster chow and tap water *ad libitum*, in a light- and temperature-controlled room.

Preparation of subcellular fractions. Hamster livers and hearts were homogenized in 0.25 M sucrose - 10 mM Tris-HCl (pH 7.4) in the presence of 5 mM EDTA - 2 mM 2-mercaptoethanol. The homogenate was centrifuged at 10,000 *g* for 10 min and the resulting supernatant was recentrifuged at 100,000 *g* for 60 min. The microsomal pellet was washed once and suspended in 10 mM Tris-HCl (pH 7.4) - 2 mM 2-mercaptoethanol as previously described (13). Protein concentration was determined by the method of Lowry *et al.* (14).

Determination of cholinephosphotransferase activity. Cholinephosphotransferase activity was assayed as described previously (15). Briefly, the reaction mixture contained 10 mM Tris-HCl (pH 8.5), 10 mM magnesium chloride, 1 mM EDTA, 0.4 mM CDP-[^{14}C]choline (1.0 μCi/μmol), 1.0 mM diacylglycerol (prepared in 0.015% Tween 20 by sonication), and 0.2 - 1.0 mg protein to a final volume of 1.0 ml. The reaction was initiated by the addition of labelled CDP-choline, and the mixture was incubated at 37°C for 15 min. The reaction was terminated by the addition of 3 ml of chloroform/methanol (2:1, v/v) to the mixture. Water (0.5 ml) was added to the mixture to cause phase separation. The lower (organic) phase was washed twice with 2 ml of 40% methanol, and the radioactivity in the lower phase after the wash was determined by liquid scintillation counting using channels ratio calibration method. Analysis of the lower phase by thin-layer chromatography revealed that over 95% of the radioactivity was associated with the phosphatidylcholine fraction.

Solubilization of cholinephosphotransferase. The washed microsomes were resuspended in 10 mM Tris-HCl (pH 7.4) - 2 mM 2-mercaptoethanol with 3% Triton QS-15. The mixture was homogenized with a dounce homogenizer at 4°C and then centrifuged at 100,000 *g* for 60 min. The resulting clear supernatant was employed as a source for further purification.

Partial purification of cholinephosphotransferase. The solubilized enzyme preparation was applied to a DEAE-Sepharose (fast flow) column (1.5 × 15 cm) equil-

ibrated with 25 mM Tris-succinate (pH 6.0) - 2 mM 2-mercaptoethanol. Subsequent to the application of the sample (containing 200 - 250 mg protein), the column was washed with 100 ml of 25 mM Tris-HCl (pH 6.0) - 2 mM 2-mercaptoethanol followed by 0.5 M KCl in the same buffer. Fractions (5 ml) were collected after sample application, and were assayed for cholinephosphotransferase activity.

The most active fractions (fractions 29 and 30) from the DEAE-Sepharose column were pooled (25.20 mg protein) and applied to a Sepharose 6B column (2.5 × 40 cm) which was equilibrated with 25 mM Tris-succinate (pH 6.0)-0.5 M KCl-5% glycerol. The column was washed with the same buffer, and fractions (3.5 ml) were collected. The fractions were assayed for enzyme activity.

RESULTS AND DISCUSSION

Since cholinephosphotransferase is tightly bound to the microsomal membrane, attempts were made to solubilize the enzyme in both the liver and heart microsomes with detergents. A number of neutral and ionic detergents were employed for this study, including octyl glucoside, CHAPS, CHAPSO, taurocholate, Triton X-100 and Tween 20. These detergents were found to be ineffective for the solubilization of the enzyme from both sources.

Alternatively, cholinephosphotransferase from hamster liver microsomal fraction was solubilized by Triton QS-15. In this study, the liver microsomes were suspended in 3% Triton QS-15 (with a detergent-to-protein ratio of 1:1.5), and the mixture was centrifuged at 100,000 *g* for 60 min. A substantial amount (20%) of the original activity in the microsomal fraction was found in the supernatant after centrifugation. The enzyme activity in the supernatant was greatly enhanced (up to 3-fold) by dialysis in 10 mM Tris-HCl, pH 7.4. Further studies revealed that Triton QS-15 (0.5-3.0%) inhibited cholinephosphotransferase activity in a dose-dependent manner. However, Triton QS-15 was not effective in the solubilization of the enzyme from hamster heart microsomes. Only 1-4% of the original activity in the heart microsomal fraction was found in the supernatant after Triton QS-15 treatment.

Comparison of the liver and heart enzymes from the microsomal fractions revealed that they had similar pH profiles and absolute requirements for Mg^{++} or other divalent cations. However, the stability of the liver enzyme was quite different from the heart enzyme. Upon storage at 4°C for 24 h, the liver enzyme was slightly activated, whereas a 26% loss of activity was observed in the heart enzyme (Fig. 1). Kinetic studies revealed that the Km values of CDPcholine for both enzymes were similar, but the Km values for diacylglycerol were different between the liver and heart enzymes (Table 1). Incidentally, the hamster liver cholinephosphotransferase displayed a much higher Km for diacylglycerol than that observed in the rat liver enzyme (11, 16). Although such discrepancy might have arisen from the different detergent used for the emulsion of diacylglcerol or the type of diacylglycerol employed for the assay (11,16), it is also possible that the affinity of the enzyme for diacylglycerol is species specific.

The solubilized enzyme preparation from the hamster liver was subjected to further purification. An aliquot of the enzyme preparation, containing 200-250 mg protein, was applied to a DEAE-Sepharose column (1.5 × 15 cm) equilibrated with 25 mM Tris succinate- 2 mM 2-mercaptoethanol, pH 6.0. The column was washed with 100 ml of the same buffer, and cholinephosphotransferase activity was subsequently eluted from the column with 0.5 M KCl in 25 mM Tris-succinate (pH 6.0) - 2 mM 2-mercaptoethanol (Fig. 2). The fractions containing highest cholinephosphotransferase activities (fractions 29 and 30) were pooled and applied to a Sepharose 6B column (2.5 × 40 cm) equilibrated with 25 mM Tris-succinate (pH 6.0)-0.5 M KCl-5% glycerol. The enzyme activity was eluted from the column near the void volume. Further purification of cholinephosphotransferase by other chromatographic techniques was not successful. A summary of the purification is depicted in Table 2.

The characteristics of the partially purified hamster liver cholinephosphotransferase were studied and compared with those of the microsomal enzyme. The microsomal and partially purified enzymes displayed similar pH profile, and both showed absolute requirements for Mg^{++} or Mn^{++}. Maximum enzyme activity was obtained in the presence of 10 mM Mg^{++}. When the partially purified enzyme was stored at 4°C for 24 h, 85-90% of the enzyme activity was recovered. Kinetic studies revealed that the Km value of CDP-choline was similar to that of the microsomal enzyme (Table 1), but the Km value for diacylgly-

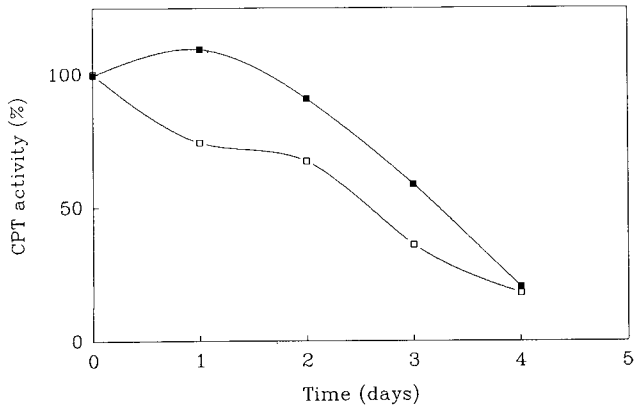

FIG. 1. Stability of cholinephosphotransferase in hamster liver and heart microsomes. Hamster liver (■) and heart (□) microsomes (3 mg/ml protein) were stored at 4 °C for various time periods. Enzyme activities were determined, and were expressed as percentage of activity obtained from fresh microsomes. Each point is the mean of two separate experiments, each determined in duplicate.

TABLE 1

Km Values of CDPcholine and Diacylglycerol for Cholinephosphotransferase of Hamster Liver and Heart

	Km	
	CDP-Choline	Diacylglycerol
	μM	μM
Liver microsomes	94	460
Heart microsomes	70	280
Partially purified enzyme from liver	85	160

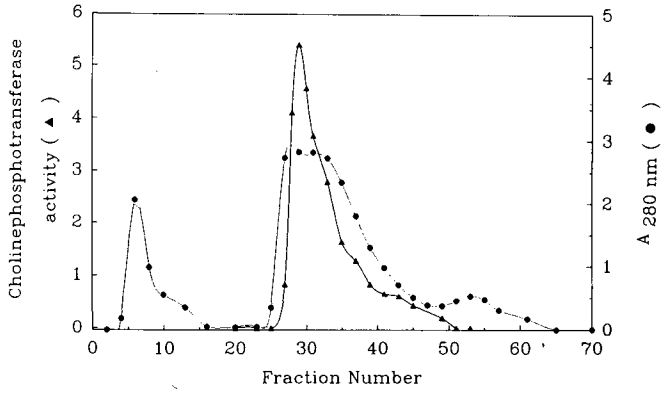

FIG. 2. Elution profile of cholinephosphotransferase from hamster liver microsomes by DEAE-Sepharose chromatography. Solubilized hamster liver microsomes were applied to a DEAE-Sepharose column (1.5 × 15 cm) equilibrated with 25 mM Tris-succinate (pH 6.0) -2 mM 2-mercaptoethanol. The column was washed with 100 ml of the same buffer and subsequently washed with 0.5 M KCl in the same buffer. Fractions (5 ml) were collected and the enzyme activity was expressed as nmol of product formed/min.

TABLE 2

Purification of Cholinephosphotransferase from Hamster Liver

	Total activity nmol/min	Protein mg	Specific activity nmol/min/mg	fold
Microsomes	299.00	598.00	0.50	—
Solubilized microsomes	59.50	238.00	0.25	0.50
DEAE-Sepharose[a] chromatography	47.88	25.20	1.90	3.80
Sepharose 6B[b] chromatography	16..78	4.56	3.68	7.36

[a]After DEAE-Sepharose chromatography, only fractions 29 and 30 (containing 25.20 mg protein) were pooled and the total enzyme activity was calculated from this pooled sample.

[b]After Sepharose 6B chromatography, the total enzyme activity was calculated from fractions 21 and 22 (containing 4.56 mg protein).

cerol was substantially lowered when the enzyme was partially purified. The partially purified cholinephosphotransferase did not display any absolute requirement for neutral lipids or phospholipids (15). However, enzyme activity was activated (25%) by 0.5 mM phosphatidylcholine or phosphatidylethanolamine but was severely inhibited (>90%) by 0.5 mM lysophosphatidylcholine. The inhibition by lysophosphatidylcholine has been suggested as an important mechanism for the regulation of cholinephosphotransferase activity (17). The ability to solubilize and partially purify cholinephosphotransferase in hamster liver will enable us and other investigators to closely examine the control mechanism of this enzyme and its role in the regulation of phosphatidylcholine biosynthesis.

ACKNOWLEDGMENT

This study was supported by the Medical Research Council of Canada. P.C.C. is a Medical Research Council Scientist. K.O. was a recipient of a Studentship from the Manitoba Health Research Council.

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ERRATUM

"Altered Arachidonic Acid Content in Polymorphonuclear and Mononuclear Cells from Patients with Allergic Rhinitis and/or Asthma" by Ross E. Rocklin, Lori Thistle, Leo Gallant, M. S. Manku and David Horrobin, *Lipids 21*, 17-20, 1986. In Table 2, the difference in linoleic acid levels between control lymphocytes and those of atopic patients was significant at $p < 0.05$, and not at $p < 0.01$. The difference in arachidonic acid levels between the same two groups was not significant as opposed to being significant at $p < 0.05$ as stated in the table.

Lipids

Notices
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February 1990

Fourteenth Annual Meeting of The American Society of Preventive Oncology (ASPO), Hyatt Regency-Bethesda, Bethesda, MD, March 19–21, 1990. The meeting is intended for professionals in clinical, educational and research disciplines which contribute to comprehensive approaches to cancer prevention. Topics: Hormones and Cancer, Squamous Cell Cancers: Similarities and Contrasts of Etiology and Prevention. For further information contact: Richard R. Love, M.D., American Society of Preventive Oncology, 1300 University Avenue - 7C, Madison, WI 53706. Telephone: (608) 263-6919.

81st Annual Meeting of the American Oil Chemists' Society, Baltimore, MD, April 22–25, 1990. The technical program will include over 400 papers and posters. A tentative list of speakers and titles is printed in the January 1990 issue of the new AOCS journal *INFORM.* Major symposia will discuss the biochemistry of phospholipids, the relationship of dietary proteins and health, milk lipids in infant nutrition, dietary aspects of blood cholesterol and of cancer, plant and fungal sterols, anaytical methods, and related topics. For more information, call Jean Bremer at AOCS. Phone: (217) 359-2344.

AOCS Mission Statement

To be a forum for the exchange of ideas, information and experience among those with a professional interest in the science and technology of fats, oils and related substances in ways that promote personal excellence and provide for a high standard of quality.

Declaration by the Governing Board

The American Oil Chemists' Society is organized for charitable, educational and scientific purposes. It does not have as its purpose the promotion of any product, manufacturers, laboratory or business. Members of the Society, and employees of the Society, do not and may not speak for or on behalf of the Society without the expressed permission of the Governing Board. This prohibition includes the use of the American Oil Chemists' Society logo or letterhead when making a statement of a technical or economic nature. Members of the Society, or employees of the Society, speak only for themselves when giving opinion or making statements concerning technical matters and economic matters.

Lipid Peroxidation in Rat Tissue Slices: Effect of Dietary Vitamin E, Corn Oil-Lard and Menhaden Oil

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Rats were fed for 5 weeks either 10% (w/w) menhaden oil (MO) or a 10% corn oil-lard (COL) mixture (1:1) in diets with ≤5 IU or ≤2 IU/kg vitamin E, respectively, or the same diets supplemented with d-α-tocopheryl succinate to a total of 35 and 180 IU vitamin E/kg, respectively. Slices of liver and heart from these rats were used to study lipid peroxidation *in vitro*. Thiobarbituric acid-reactive substances (TBARS) were measured in the medium after incubation of the slices at 37°C for 1 hr in the absence (uninduced) and presence of 0.5 mM tert-butyl hydroperoxide (induced). The release of TBARS from slices of heart and liver from rats fed either lipid decreased with increasing levels of dietary vitamin E. At the same level of dietary vitamin E, TBARS release was greater for slices of liver and heart from the MO-fed rats than from the COL-fed rats. Application of the TBARS data to a model simulating the experimental conditions showed a good correlation ($r = 0.95$, $p < 0.001$) between experimental and simulated values. Of the 16:0-22:6 fatty acids measured in liver from MO-fed rats, 15.4% was n-6 fatty acids and 29.9% was n-3 fatty acids; in liver from COL-fed rats, the respective values were 37.4% and 3.7%. Liver and kidney vitamin E levels were unaffected by the dietary lipid. This study demonstrated that a dietary fish oil increased the susceptibility of rat liver and heart to *in vitro* lipid peroxidation, and that vitamin E decreased TBARS in tissues from rats fed COL to lower levels than for tissues from rats fed MO. The results suggest that there might also be an increased requirement for dietary antioxidants by humans using fish oil supplements.
***Lipids 25,* 125-129 (1990).**

Consumption of marine oils has been linked to a low incidence of coronary heart disease (1,2). Recent studies have documented a number of health benefits from fish oil supplements, including decreased platelet aggregation and production of thromboxane (3) and lowered blood pressure (3,4). Consequently, fish oils and concentrates of n-3 fatty acids from fish oils have been widely promoted for the prevention of heart disease. However, fish oils contain highly unsaturated n-3 fatty acids that are very susceptible to lipid peroxidation. A high dietary intake of n-3 fatty acids might overwhelm the normal antioxidant defenses, thus increasing the need for dietary antioxidants. Commercially available n-3 fatty acids are mostly fish oils sold in 1-g gelatin capsules, and vitamin E is usually added at 1 IU/g as a stabilizer (5).

Although several studies of lipid peroxidation in tissues from animals fed fish oil have been published (6-8), comparable levels of polyunsaturated fatty acids (PUFA) or dietary vitamin E were not used for the various diets within a single study. In the study reported here, rats were fed three levels of vitamin E in diets that contained either 10% menhaden oil (MO) or 10% corn oil-lard (1:1) (COL). Susceptibility to lipid peroxidation of liver and heart slices incubated with and without tert-butyl hydroperoxide (t-BHP) was studied.

METHODS

Diets and animals. To a basal fat-free, vitamin E- and selenium-deficient diet (Teklad Test Diets, Madison, WI) was added 0.2 ppm selenium as sodium selenite. To this diet was added either 10% menhaden oil (MO) (Zapata Haynie, Inc., Reedville, VA) containing 50 IU α-tocopherol/kg oil or 10% tocopherol-stripped corn oil and tocopherol-stripped lard (1:1, COL) containing ≤20 IU α-tocopherol/kg oil. The low-vitamin E diets (estimated as approximately 5 IU/kg of the MO diet and ≤2 IU/kg of the COL diet) were supplemented with d-α-tocopheryl succinate (Henkel Corporation, Chicago, IL) to either 35 or 180 IU/kg. The MO and COL diets contained 3.3% and 3.7% PUFA, respectively. Diets were stored in small aliquots at −20°C and were provided daily to avoid autoxidation.

Male Sprague-Dawley rats (Simonsen Laboratories, Inc., Gilroy, CA) 21 days of age were divided into six groups of six animals each. After the various diets were fed to the respective groups for five weeks, the rats were killed by decapitation.

Peroxidation in slices. Liver and heart were excised, rinsed in Krebs-Ringer-phosphate buffer, and slices were prepared as previously described (8). Slices weighing approximately 100 mg were placed into serum vials with 5 ml Krebs-Ringer-phosphate buffer, pH 7.4. The slices were incubated at 37°C for 1 hr in a shaker-water bath with or without the inclusion in the vials of 0.5 mM t-BHP for induced and uninduced lipid peroxidation measurement, respectively. Thiobarbituric acid-reactive substances (TBARS) were assayed in the medium as previously described (9). Studies were limited to liver and heart because of the time required to carry out these assays.

Other assays. Vitamin E in liver and kidney was determined by the method of Taylor *et al.* (10). Tissues were homogenized in 5 volumes of isotonic KCl, saponified and extracted with hexane. Vitamin E in the hexane extract was determined fluorometrically at 290 nm excitation and 330 nm emission. For analysis of fatty acids, liver lipids were extracted using the methodology of Burton *et al.* (11). Total lipids extracted were directly transesterified using acetylchloride in methanol/benzene as detailed by Lepage and Roy (12). The methyl esters were purified over ODS reverse and solid-phase extraction columns (Analytichem International, Harbor City, CA) and eluted with 5% diethyl ether in hexane. The fatty acids were analyzed by gas chromatography (Hewlett Packard model 5890) with a DB-225 30 m × 0.24 mm capillary column (J & W Scientific, Folsom, CA) and identified by known

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Abbreivations: COL, corn oil-lard; MO, menhaden oil, t-BHP, tert-butyl hydroperoxide; TBARS, thiobarbituric acid-reactive substances.

standards. Analyses of tissues were limited because of the limited amounts of tissues available.

Simulation modeling of lipid peroxidation. A model for simulating the experimental variables (13) was modified as: TBARS = (LPC × I × FO)/(BA + log E). "LPC" is a composite of factors involved in lipid peroxidation that were not otherwise expressed. "I" is the inducer (t-BHP) factor calculated from the average ratio of induced/uninduced TBARS; I was equal to 5. "FO," the fish oil factor, was calculated from the ratio of TBARS in tissues from MO-fed rats/TBARS in tissues from COL-fed rats. The FO factor thus obtained was 2 for liver and 1.5 for heart. The "log E" term denotes the logarithmic value of dietary vitamin E. "BA" represents basal tissue antioxidants excluding vitamin E. BA was assigned a value of 1 (equivalent to log 10 mg d-α-tocopheryl succinate or 12 IU vitamin E/kg diet).

Statistical analysis. Data were analyzed by the Student's t-test and ANOVA using routines available in MINITAB (14). Tukey's test (15) was used to separate means when F ratios were significant. Linear regression analysis was employed to assess relationships between TBARS and vitamin E.

RESULTS

Peroxidation in tissue slices. During uninduced lipid peroxidation, TBARS in the incubation medium of either liver or heart slices from both COL- and MO-fed rats decreased significantly with increasing levels of dietary vitamin E (Table 1). The t-BHP-induced release of TBARS from heart slices decreased significantly with increasing dietary vitamin E, whereas the release of TBARS from liver slices was decreased significantly only by the highest level of dietary vitamin E in both COL- and MO-fed rats. At any single level of dietary vitamin E, TBARS release was significantly higher by slices of liver or heart from MO-fed rats than by those from COL-fed rats.

Fatty acid composition of liver. For the liver fatty acid compositions shown in Table 2, data from all dietary vitamin E groups of rats fed the same lipid were combined, since the level of dietary vitamin E did not affect the fatty acid composition. The fatty acid composition of liver reflected the dietary lipid composition. Livers from MO-fed rats contained significantly more 20:5n-3, 22:5n-3, and 22:6n-3 fatty acids but significantly less 18:2n-6 and 20:4n-6 fatty acids than did livers from COL-fed rats. The ratio of n-3/n-6 fatty acids was higher in liver from MO-fed rats (1.95) than from COL-fed rats (0.1).

Tissue vitamin E. The content of liver and kidney vitamin E increased with increasing levels of dietary vitamin E (Table 3). Increasing dietary vitamin E approximately 30-fold (to 180 IU/kg) increased liver vitamin E 3.5- and 2.7-fold in the COL- and MO-fed rats, respectively; in kidney, the respective increases were 6.5- and 11-fold. With two exceptions, the source of lipid was without effect on tissue vitamin E. At the lowest level of dietary vitamin E, there was 33% more ($p < 0.01$) liver vitamin E in the MO-fed rats than in the COL-fed rats. At the highest level of vitamin E, kidney vitamin E in MO-fed rats was 34% higher ($p < 0.01$) than in COL-fed rats.

Correlations of measurements. TBARS released from liver slices during both uninduced and induced lipid peroxidation were inversely related to the log of dietary vitamin

TABLE 1

Lipid Peroxidation in Slices of Liver and Heart from Rats Fed Diets Containing COL or MO and Three Levels of Vitamin E[a]

Measurement[b] tissue	Dietary lipid	Dietary vitamin E (IU/kg) ⩽5	35	180
Uninduced TBARS				
Liver	COL	37 ± 3.7[b,a]	12 ± 1.5[b]	6 ± 0.8[c]
	MO	58 ± 7.1[a]	33 ± 3.9[b]	18 ± 2.0[c]
	p[c]	<0.05	<0.001	<0.001
Heart	COL	14 ± 0.7[a]	5.5 ± 0.4[b]	3.3 ± 0.3[c]
	MO	17 ± 1.1[a]	9.5 ± 0.8[b]	4.9 ± 0.3[c]
	p	<0.05	<0.005	<0.01
Induced TBARS				
Liver	COL	89 ± 6.3[a]	74 ± 6.2[a]	45 ± 2.7[b]
	MO	175 ± 11[a]	157 ± 8.7[a]	130 ± 4.5[b]
	p	<0.001	<0.001	<0.001
Heart	COL	51 ± 1.3[a]	31 ± 1.4[b]	19 ± 0.9[c]
	MO	63 ± 3.0[a]	43 ± 1.0[b]	31 ± 1.9[c]
	p	<0.01	<0.001	<0.001

[a] Weanling rats were fed diets containing 10% corn oil-lard (COL) or 10% menhaden oil (MO) with ⩽5, 35 or 180 IU vitamin E/kg for 5 wk. Tissue slices were incubated at 37°C for 2 hr in the absence (uninduced) or presence (induced) of 0.5 mM t-BHP.

[b] Units are nmol malonaldehyde equivalents/g tissue for TBARS released into the medium. Values are means ± SEM for 6 rats. Data in the same row not sharing a common superscript letter are significantly different ($p < 0.05$) by ANOVA and Tukey's test.

[c] Significance of difference for data in the same column.

TABLE 2

Fatty Acid Composition (%) of Liver from Rats Fed COL- or MO-Containing Diets[a]

Fatty acid	COL	MO	p[b]
16:0	20.1 ± 0.7[c]	23.2 ± 0.7	<0.01
16:1n-7	1.8 ± 0.2	3.3 ± 0.4	<0.01
18:0	17.2 ± 0.2	16.2 ± 0.6	NS
18:1n-9	15.4 ± 0.7	10.9 ± 0.5	<0.001
18:2n-6	14.9 ± 1.0	5.9 ± 0.5	<0.001
18:3n-6	0	0	
20:4n-6	22.5 ± 0.4	9.4 ± 0.8	<0.001
20:5n-3	0.4 ± 0.2	8.0 ± 0.6	<0.001
22:5n-3	0.2 ± 0.1	4.7 ± 0.1	<0.001
22:6n-3	3.1 ± 0.2	17.2 ± 1.0	<0.001
UI[d]	159	230	

[a]Weanling rats fed 10% corn oil-lard (COL) or 10% menhaden oil (MO) diets containing ≤5, 35 or 180 IU vitamin E/kg for 5 wk.

[b]Significance of difference by Student's t-test between data for COL- and MO-fed rats.

[c]Data were combined from each of the three dietary vitamin E groups for liver from rats fed either COL or MO. Values are means ± SEM for 6 rats.

[d]Unsaturation index (UI) = sum of percentages of individual fatty acids × number of double bonds.

TABLE 3

Vitamin E in Liver and Kidney from Rats Fed COL- or MO-Containing Diets[a]

Tissue	Dietary lipid	Dietary vitamin E (IU/kg) ≤5	35	180
Liver	COL	4.7 ± 0.3[b,a]	8.2 ± 0.4[b]	16.8 ± 1.1[c]
	MO	6.3 ± 0.3[a]	8.9 ± 0.3[b]	16.9 ± 0.6[c]
	p[c]	<0.01	NS	NS
Kidney	COL	2.1 ± 0.6[a]	6.0 ± 0.8[b]	13.3 ± 0.6[c]
	MO	1.6 ± 0.1[a]	5.7 ± 0.8[b]	17.8 ± 0.6[c]
	p	NS	NS	<0.01

[a]Weanling rats fed 10% corn oil-lard (COL) or 10% menhaden oil (MO) diets with ≤5, 35 or 180 IU vitamin E/ig for 5 wk.

[b]Values are means ± SEM for 4 rats from each dietary group. Units are μg vitamin E/g tissue.

[c]Significance of the difference by ANOVA and Tukey's test for data in the same row. NS, not significant.

TABLE 4

Linear Regression Analyses of TBARS and Vitamin E

Measurements regressed	t-BHP addition	Dietary lipid	Linear regression[a] N	r	p
Liver TBARS vs liver vitamin E	—	COL	12	−0.71	<0.01
	—	MO	12	−0.80	<0.01
	+	COL	12	−0.74	<0.01
	+	MO	12	−0.74	<0.01
Liver TBARS vs log dietary vitamin E	—	COL	18	−0.89	<0.001
	—	MO	18	−0.84	<0.001
	+	COL	18	−0.81	<0.001
	+	MO	18	−0.70	<0.01

[a]r, Linear correlation coefficient; N, number of pairs of observation.

E and to the liver vitamin E content, regardless of the type of dietary lipid used (Table 4). The inverse correlation tended to be stronger when logarithmic values of dietary vitamin E rather than liver vitamin E levels were regressed against liver TBARS.

Simulation modeling of lipid peroxidation. Simulation modeling of TBARS release by liver slices was performed using an I value of 5, an FO value of 2, a BA value of 1, an LPC value of 30 and the log of dietary vitamin E. As illustrated in Figure 1, the experimental and simulated release of TBARS from liver slices was highly correlated ($r = 0.95$, $p < 0.001$).

For simulation of cardiac lipid peroxidation, an FO factor of 1.5 and an LPC of 15 were used, and BA and I remained at 1 and 5, respectively. The log E value was derived from dietary vitamin E concentrations. There was a significant correlation ($r = 0.99$, $p < 0.001$) between experimental and simulated TBARS release from heart slices.

Simulation also was performed using tissue concentrations (μg vitamin E/g tissue) rather than logarithmic values of dietary vitamin E. Consequently, instead of using a logarithmic value, BA was restored to 12 IU vitamin E/kg. In this simulation of peroxidation in liver slices, the value of LPC was increased to 300 and all other factors remained the same; a significant correlation was obtained ($r = 0.95$, $p < 0.001$).

DISCUSSION

The present study was undertaken because of a growing interest in and increased human consumption of highly unsaturated n-3 PUFA in marine oils. This study compared the susceptibility of slices of liver and heart from rats fed lipids with different PUFA compositions. Tissue slices have long been employed in biochemical studies such as neurochemical and protein synthesis research (16). This laboratory has shown that the tissue slice system is useful for studies of xenobiotic-induced lipid peroxidation (9,17-19). The present study used TBARS as the index of lipid peroxidation since good correlations have been reported between TBARS and release of total ethane

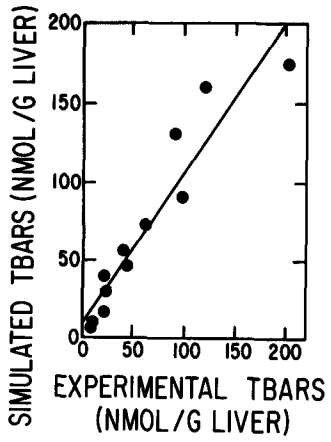

FIG. 1. Linear regression of simulated values with experimental values of TBARS for liver slices from rats fed COL and rats fed MO; $r = 0.95$, $p < 0.001$ ($N = 12$).

and pentane from liver and kidney slices incubated with bromotrichloromethane (17) and between TBARS and total aldehydes measured as cyclohexanedione-reactive substances in liver and testes slices (18).

A unique feature of the present design was that while the compositions of dietary fatty acids were different, the levels of lipids and total PUFA were comparable for the two diets. Moreover, the effect of MO and COL on lipid peroxidation in tissue slices were compared at three dietary vitamin E levels that ranged from low (≤5 IU/kg) to high (180 IU/kg). The results show that slices of liver and heart from MO-fed rats released significantly more TBARS into the medium in both uninduced and induced lipid peroxidation than did those from COL-fed rats, regardless of dietary vitamin E levels. The results were in accord with those (13) obtained during uninduced and iron-induced lipid peroxidation in homogenates of liver and kidney from rats fed COL and MO. Thus, dietary MO at a moderate level (10%, w/w) unequivocally enhanced the susceptibility of tissues to lipid peroxidation *in vitro*. Other investigators have reported similar susceptibilities. Mounie *et al.* (7) observed 1.9-fold higher uninduced and 3-fold higher NADPH-induced lipid peroxidation in microsomes prepared from fish oil-fed rats than in microsomes from corn oil-fed rats. However, the two diets used (7) varied greatly in both the total PUFA and vitamin E contents. Lamers *et al.* (6) reported an increase of approximately 10-fold in cardiac lipid peroxidation after coronary artery occlusion over that measured before occlusion. In that study, the effects on peroxidation of supplements of fish oil were compared to those of lard supplements; lard has a much lower unsaturation index than does corn oil. Recently, Nalbone *et al.* (20) reported a drastically higher accumulation of lipofuscin-like or ceroid material in hearts from rats fed primarily salmon oil than in hearts from rats fed primarily corn oil or lard. In this study, the dietary lipid content was kept constant, whereas PUFA and vitamin E content were not.

Increasing dietary vitamin E from the lowest level to 180 IU/kg decreased uninduced lipid peroxidation in livers from the COL- and MO-fed rats by 85% and 69%, respectively. The t-BHP-induced release of TBARS from liver was decreased 49% and 26% for the COL group and MO group, respectively. Vitamin E supplementation had a similar but smaller effect in heart than in liver. Thus, vitamin E was less effective in decreasing TBARS in tissues from MO-fed than from COL-fed rats. The results were in agreement with those (13) based on measurements of TBARS, conjugated dienes, headspace hexanal and total volatiles during iron-induced lipid peroxidation in homogenates of liver and kidney from rats fed COL or MO.

This study also demonstrated that MO-containing diets significantly increased the liver content of 20:5n-3 (eicosapentaenoic acid) and 22:6n-3 (docosahexaenoic acid) and significantly decreased 18:2n-6 (linoleic acid) and 20:4n-6 (arachidonic acid). As fatty acid composition of rat tissues closely follows that of the diet (21,22), these changes are a reflection of the composition of the dietary lipid. The unsaturation index is indicative of the relative rate of peroxidation since it is calculated from the reactivity of each fatty acid of the tissue (23). Thus, the enhanced susceptibility to lipid peroxidation induced by t-BHP of tissue from rats fed fish oil compared to rats fed COL may be related to the higher unsaturation index of the former than the latter.

The source of lipid did not significantly affect the vitamin E content of liver and kidney except for the liver from rats fed the MO or COL diets with ≤5 IU vitamin E/kg and for the kidney from rats fed the MO or COL diets with 180 IU vitamin E/kg. In both exceptions, the higher vitamin E content was in tissues from rats fed MO. The vitamin E content of the basal diets was not equal (<5 IU/kg for the MO diet and ≤2 IU/kg for the COL diet), and this small difference may explain the lower vitamin E content in livers from COL-fed rats than in livers from MO-fed rats. However, such a small difference is unlikely to account for the significantly higher vitamin E content of kidneys from MO-fed rats than of kidneys from rats fed the COL diet with 180 IU vitamin E/kg. Meydani *et al.* (24) reported that livers from mice fed fish oil had less vitamin E than those of corn oil-fed mice. This effect was observed only in liver of young mice; kidney and lung tocopherol content did not differ between treatment groups over the range of 30–100 ppm dietary vitamin E. The reason for the discrepancy in findings between the present study and that of Meydani *et al.* (24) is unknown. The question whether supplementary fish oils can decrease tissue vitamin E remains to be resolved.

Simulation modeling has recently been employed in nutritional sciences (25,26). A modeling equation was developed previously (13) to simulate the experimental conditions for lipid peroxidation studies in tissue homogenates. The model was sufficiently flexible to allow data fitting for various experimental conditions. The simulation also served to rationalize the interpretation of the present results and to demonstrate that various factors involved in the lipid peroxidation process can be included to give a broad perspective of oxidant and antioxidant interactions.

In conclusion, with an improved experimental design as stated above, this study clearly demonstrated that liver and heart from rats fed MO-containing diets were more susceptible to lipid peroxidation *in vitro* than were those from COL-fed rats. Vitamin E decreased the susceptibility to lipid peroxidation in livers and hearts from rats fed either dietary lipid. Vitamin E more effectively decreased TBARS in tissues from rats fed diets high in n-6 PUFA than in tissues from rats fed diets high in n-3 PUFA. This might suggest an increased dietary requirement for vitamin E by people who consume fish oil supplements.

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Preparation of Deuterated Methyl 6,9,12-Octadecatrienoates and Methyl 6,9,12,15-Octadecatetraenoates

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Methyl 6,9,12-octadecatrienoate-15,15,16,16-d_4 was obtained by Wittig coupling between 6,6,7,7-tetradeutero-3-nonenyltriphenylphosphonium iodide, 8, and the aldehyde ester, methyl 9-oxo-6-nonenoate. Methyl 6-oxohexanoate, obtained by ozonolysis of cyclohexene, was coupled in a Wittig reaction with [2-(1,3-dioxan-2-yl)ethyl]triphenylphosphonium bromide to give methyl 8-dioxanyl-6-octenoate. This compound was transacetalized to methyl 9,9-dimethoxy-6-nonenoate, which was then hydrolyzed to the aldehyde ester. For the preparation of compound 8, the tetrahydropyranyl ether of 2-pentynol was deuterated with deuterium gas and tris-(triphenylphosphine)chlororhodium. The tetradeuterated tetrahydropyranyl ether was converted to the bromide with triphenylphosphine dibromide, and the bromide was coupled with 3-butynol by means of lithium amide in liquid ammonia to give 3-nonynol-6,6,7,7-d_4. Hydrogenation over Lindlar's catalyst converted the deuterated alkynol to 3-nonenol-6,6,7,7-d_4. This deuterated alkenol was converted to the bromide with triphenylphosphine dibromide, then to the iodide with sodium iodide in acetone, and finally to 8 with triphenylphosphine in acetonitrile. Methyl 6,9,12,15-octadecatetraenoate-12,13,15,16-d_4 was obtained by Wittig coupling between methyl 9-oxo-6-nonenoate and 3,4,6,7-tetradeutero-3,6-nonadienyltriphenylphosphonium iodide, 15. For the preparation of compound 15, the bromide obtained from the reaction of 2-pentynol with triphenylphosphine dibromide was coupled with 3-butynol with lithium amide in liquid ammonia. The resulting 3,6-nonadiynol was deuterated with deuterium gas in the presence of P-2 nickel, and the resultant deuterated nonadienol was converted to 15 through the bromide and iodide. The final products were separated from isomers formed during the synthetic sequences by silver resin chromatography.
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For our study of the metabolism of fatty acids in humans (1,2), we have synthesized various monoenoic (3), dienoic (4), and trienoic (5,6) acids labeled with various numbers of deuterium atoms. Some of these have been omega-3 and others omega-6 fatty acids. In this paper, we describe the synthesis of the omega-6 fatty acid, methyl 6,9,12-octadecatrienoate-15,15,16,16-d_4; (6,9,12-18:3-d_4); and the omega-3 fatty acid, methyl 6,9,12,15-octadecatetraenoate-12,13,15,16-d_4 (6,9,12,15-18:4-d_4). These acids may be metabolic intermediates in the conversion of linoleate (9,12-18:2) and linolenate (9,12,15-18:3) to arachidonate (5,8,11,14-20:4) and eicosapentaenoate (5,8,11,14,17-20:5), respectively. The deuterated compounds were prepared to compare the relative conversion rates of these compounds. The deuterium atoms were placed in those positions from which it is unlikely that they would be lost during these conversions.

Abbreviations: DHP, dihydropyran; EE, diethyl ether; GLC, gas liquid chromatography; NMR, nuclear magnetic resonance; PE, petroleum ether (35–60°C); *p*-TSA, *p*-toluenesulfonic acid; THF, tetrahydrofuran; THP, tetrahydropyranyl.

EXPERIMENTAL

Reagents. [2-(1,3-Dioxan-2-yl)ethyl]triphenylphosphonium bromide, butyl lithium, triphenylphosphine, dihydropyran, sodium borohydride, ethyl magnesium bromide, 3-butynol, Lindlar's catalyst (5% Pd on calcium carbonate poisoned with lead acetate), and ethylenediamine were obtained from Aldrich Chemical Company (Milwaukee, WI). 2-Pentynol was purchased from Farchan Laboratories (Gainseville, FL) and tris-(triphenylphosphine)chlororhodium was obtained from Strem Chemicals (Newburyport, MA).

Procedures. A 50 m × 0.25 mm CPS-2 capillary column (Quadrex Corp., New Haven, CT) was used for analyzing binary mixtures of geometric isomers. For other analyses, a 6 ft × 4 mm column packed with 3% EGSS-X on 100/120 GasChrom Q or a 6 ft × 4 mm column packed with 3% OV101 on 80/100 Supelcoport was employed (Supelco, Bellefonte, PA). The fractionating column used is a concentric tube unit, Ace 9331 (Ace Glass Inc., Vineland, NJ), with approximately 40 theoretical plates.

^{13}C NMR. ^{13}C nuclear magnetic resonance (NMR) spectra were recorded with a Bruker WM 300 WB pulsed Fourier transform spectrometer (Brucker Instruments, Inc., Karlsruhe, West Germany) operating at 75.5 MHz and ambient temperature. Typically, 2500 transients were collected from solutions in $CDCl_3$, which served as both the internal lock and secondary reference, using 5 mm tubes. Sweep widths of 200 ppm and 8 K real data points limited acquisition time to 0.54 seconds and were used to obtain chemical shift values within ± 1.85 Hz, i.e., ±0.05 ppm. A pulse width of 3 μs (40°) was employed with no delay between pulses. Decoupling power was held to ca. 1 W to provide adequate broadband decoupling power while minimizing sample heating.

The signal from carbons bearing two deuterium atoms or one deuterium atom and a double bond is diminished to such an extent that it is usually not detected.

Mass spectroscopy. Mass spectra were determined on a Finnigan 4500 mass spectrometer (Finnigan Corp., Sunnyvale, CA) using isobutane chemical ionization with data processing of the isotope distribution against standards (7).

Silver resin chromatography. The macroreticular resin used for the separations was Rohm and Haas XN1010 sulfonic acid resin ground to the mesh sizes indicated. For the Ag/H columns, the indicated percentage of hydrogen ions was displaced by Ag ions. For the Ag/Na columns, the hydrogen ions were displaced by sodium ions and then the indicated percentage of sodium ions was displaced by Ag ions (8,9).

SYNTHETIC PROCEDURES

*Methyl 9-oxo-*cis*-6-nonenoate.* Methyl 9,9-dimethoxy-*cis*-6-nonenoate (5.14 g, 22.3 mmol) was dissolved in a

mixture of water (20 ml) and acetonitrile (50 ml). The apparatus was flushed with N_2 and concentrated HCl (10 drops) was added. Progress of the reaction was monitored by gas liquid chromatography (GLC) on EGSS-X. After about 1.5 hr, solid Na_2CO_3 was added, the liquid was decanted and concentrated on the rotary evaporator. The organic layer was extraacted into diethyl ether (EE) (20 ml), which was washed with H_2O (10 ml) and dried (Na_2SO_4). Removal of the drying agent and solvent gave a product mixture (3.78 g, 92% yield) which contained about 5% conjugated aldester and 8% acetal ester. Analysis by infrared did not show any conjugation, i.e., absorbance at 1690 cm^{-1}.

2-(2'-Pentynyloxy)tetrahydropyran, **(1)**. 2-Pentynol (50.24 g, 590 mmol) and *p*-toluenesulfonic acid (*p*-TSA) (1.9 g, 10 mmol) were mixed in a 250-ml, three-necked flask equipped with a mechanical stirrer, a thermometer, and a dropping funnel. While a stream of N_2 was passed through the apparatus, dihydropyran (60 g, 710 mmol) was added dropwise over 30 min with the temperature maintained at 20–30°C by intermittent use of an ice bath. The amber liquid was placed on a column (3 × 50 cm) containing silica gel (120 g) in petroleum ether, 35–60°C (PE), and eluted with 600 ml of PE. Yield was 98%. ^{13}C NMR (ppm): C-1, 54.5; C-2, 87.8; C-3, 75.0; C-4, 12.4; C-5, 13.6. Tetrahydropyranyl (THP) group: C-2, 96.5; C-3, 30.2; C-4, 19.0; C-5, 25.3; C-6, 61.8.

2-(2',2',3',3'-Tetradeuteropentanyloxy)tetrahydropyran, **(2)**. Compound **1** (48.5 g, 289 mmol) in benzene (1000 ml) was treated with deuterium gas at atmospheric pressure in the presence of tris-(triphenylphosphine) chlororhodium (5 g) in the manner described previously (3). Benzene (ca. 850 ml) was removed on the rotary evaporator, PE (300 ml) was added and the mixture was filtered through a pad of Celite to remove the catalyst. The flask and precipitate were washed with PE, which was combined with the filtrate. Removal of the solvent on the rotary evaporator yielded a mixture (49.36 g) which was distilled to give the product (39.12 g, 77% yield), b.p. 49–77°C at 0.5–1.3 torr. Mass spectral analysis: 0.1% d_1, 5.7% d_3, 90.6% d_4, 2.6% d_5, 0.95% d_6. ^{13}C NMR (ppm): C-1, 67.3; C-4, 22.1; C-5, 13.8. THP group: C-2, 98.6; C-3, 30.6; C-4, 19.5; C-5, 25.4; C-6, 62.0.

1-Bromopentane-2,2,3,3-d_4, **(3)**. Triphenylphosphine (66.7 g, 255 mmol) was dissolved in methylene chloride (200 ml) in a 500-ml, three-necked flask equipped with a mechanical stirrer, a burette or dropping funnel, a low temperature thermometer, and a calcium chloride drying tube. A stream of N_2 was passed through the apparatus, immersed in an ice bath, as bromine (12.7 ml, 39.7 g, 248 mmol) was added at a temperature of 2–5°C. Compound **2** (39 g, 221 mmol), dissolved in methylene chloride (20 ml), was added over 15 min at a temperature of −2°C. The cooling bath was removed, and one hour later the clear amber liquid was washed with water (2 × 100 ml) and dried (Na_2SO_4 and Drierite). Solvent (125 ml) was removed by distillation and the remaining material was diluted with PE (300 ml). The filtrate from filtration of this mixture was passed through a column (3 × 50 cm) packed with silica gel (110 g) in PE. A total of 850 ml of eluate was collected. Solvent was removed on a fractionating column and the residue was distilled to give product **3**, (29.72 g, 86.5% yield), b.p. 110–125°C. ^{13}C NMR (ppm): C-1, 33.6; C-4, 21.6; C-5, 13.8.

3-Nonynol-6,6,7,7-d_4, **(4)**. Liquid ammonia (ca. 800 ml) was charged into a 2-l, three-necked flask equipped with a mechanical stirrer, a dry ice cooled condenser, and immersed in a box of Vermiculite insulating material. As catalyst for the formation of lithium amide, $Fe(NO_3)_3.9H_2O$ (0.5 g) was added. Lithium metal (3.5 g, 500 mmol) was added in small pieces over 20 min. After the grey slurry appeared, 3-butynol (17.6 g, 250 mmol) in tetrahydrofuran (THF) (50 ml) was added over 25 min. Then, compound **3** (26 g, 167 mmol) in THF (50 ml) was added dropwise over 30 min. Four hours later the stirrer was stopped, the Vermiculite was removed, and ammonia was allowed to evaporate overnight. Ammonia remaining in the reaction mixture was removed by a stream of inert gas while the reaction vessel was in a 35°C bath. The flask was cooled in an ice bath and water (250 ml) was added dropwise and the temperature rose to 22°C. The reaction mixture was transferred to a separatory funnel with EE (100 ml). The aqueous layer was separated, acidified with 5N H_2SO_4 (120 ml) and extracted with EE (2 × 100 ml). The combined ether extracts were washed with saturated NH_4Cl (100 ml), 5% H_2SO_4 (60 ml) and water (2 × 75 ml) and dried (Na_2SO_4). After removal of the drying agent and EE, the residue was distilled under reduced pressure to give unreacted 3-butynol (2.12 g), b.p. 33–62°C at ca. 75 torr, and product **4** (16.64 g, 69% yield) b.p. 105–120°C at ca. 75 torr. ^{13}C NMR (ppm): C-1, 61.3; C-2, 23.1; C-3, 76.2; C-4, 82.6; C-5, 18.4; C-8, 21.9; C-9, 13.9.

3-Nonenol-6,6,7,7-d_4, **(5)**. Compound **4** (16.5 g, 115 mmol) and ethyl acetate (100 ml) were placed in a 250-ml, round bottom flask equipped with a magnetic stirring bar, and connected to an apparatus for maintaining one atmosphere of pressure. The apparatus was evacuated and flushed with H_2 (three times), then Lindlar's catalyst (1 g) and quinoline (0.4 ml) were added. The flask was flushed with H_2 and the stirrer was started. After absorption of hydrogen had ceased, the reaction mixture was filtered through a pad of Celite. Ethyl acetate was removed by distillation at atmospheric pressure and then compound **5** was obtained (16.07 g, 97% yield), b.p. 112–118°C at ca. 75 torr. ^{13}C NMR (ppm): C-1, 62.2; C-2, 30.7; C-3, 124.9; C-4, 133.2; C-5, 27.0; C-8, 22.2; C-9, 13.9.

1-Bromo-3-nonene-6,6,7,7-d_4, **(6)**. In a manner similar to the conversion of **2** to **3**, compound **5** was converted to compound **6** in 75% yield, b.p. 110–120°C at 55 torr. ^{13}C NMR (ppm): C-1, 32.5; C-2, 30.8; C-3, 125.7; C-4, 133.1; C-5, 27.1; C-8, 22.3; C-9, 14.0.

1-Iodo-3-nonene-6,6,7,7-d_4, **(7)**. A solution of **6** (17 g, 81 mmol) in acetone (50 ml) was added with stirring to a solution of NaI (15.25 g, 101 mmol) in acetone (100 ml). The mixture was heated at the reflux temperature for 2 hr; the white solid was removed by filtration; and the filtrate was concentrated on the rotary evaporator. The mixture was diluted with H_2O (50 ml) and extracted with EE (2 × 50 ml). The ether layer was washed with H_2O (10 ml) and dried (Na_2SO_4). After removal of the drying agent and solvent, the residue was distilled to give compound **7** (17.07 g, 82% yield), b.p. 75–80°C at 0.7 torr. ^{13}C NMR (ppm): C-1, 5.4; C-2, 31.5; C-3, 127.6; C-4, 132.6; C-5, 27.1; C-8, 22.2; C-9, 14.0.

*6,6,7,7-Tetradeutero-*cis*-3-nonenyltriphenylphosphonium iodide,* **(8)**. Compound **7** (17 g, 66 mmol), triphenylphosphine (20 g, 76 mmol) and methyl myristate (0.8 ml) (internal standard) were mixed in acetonitrile (150 ml)

in a 250-ml, round bottom flask equipped with a magnetic stirring bar. The mixture was heated with stirring at the reflux temperature and the change in the ratio of 7 to methyl myristate was determined by periodic sampling of the reaction mixture, followed by GLC analysis on the EGSS-X column. When most of 7 had reacted, acetonitrile was removed on the rotary evaporator and the residue was triturated several times with fresh portions of EE to give compound 8 (32.06 g, 93% yield). ^{13}C NMR (ppm): C-1, 23.1; C-2, 19.6; C-3, 125.0; C-4, 128.0; C-5, 26.3; C-8, 22.4; C-9, 13.3; aromatic ring; C-1, 117.1; C-2, 132.9; C-3, 130.0; C-4, 134.6.

Methyl 6,9,12-octadecatrienoate-15,15,16,16-d_4, **(9)**. Compound 8 (2.46 g, 4.7 mmol) was dissolved in THF (10 ml) in a 50-ml, three-necked flask equipped with a mechanical stirrer, a low temperature thermometer, and a $CaCl_2$ drying tube. While N_2 was passed through the apparatus, it was cooled in an ice bath and butyl lithium (2.5M, 2 ml, 5 mmol) was added. Temperature rose from 6°C to 22°C and a deep orange color developed. Methyl 9-oxo-*cis*-6-nonenoate (0.86 g, 4.7 mmol) in THF (5 ml) was added at 5–15°C. The ice bath was removed and a pale orange color developed as a precipitate formed. The sample was washed with saturated NaCl solution (25 ml) and dried (Na_2SO_4). Removal of the solvent and drying agent gave a thick orange liquid which was passed through a column (1.3 × 20 cm) containing silica gel (ca. 5 g) with hexane. Evaporation of the solvent gave the product mixture (0.6 g, 43% yield, ca. 80% triene by gas chromatography. Mass spectral analysis: 0.6% d_2, 4.2% d_3, 92.1% d_4, 3.0% d_5, 0.1% d_6. ^1H NMR ($CDCl_3$): δ 0.88 (*t*, 3H, $C\underline{H}_3$), δ 1.2–1.4 (*m*, 4H, $CH_3C\underline{H}_2$, $-C\underline{H}_2-$), δ 1.6 (*m*, 2H, $C\underline{H}_2CH_2COO-$), δ 2.05 (*m*, 4H, $C\underline{H}_2-C=$), δ 2.31 (*t*, 2H, $C\underline{H}_2COO-$), δ 2.80 (*m*, 4H, $=C-C\underline{H}_2C=$), δ 3.66 (*s*, 3H, $OC\underline{H}_3$), δ 5.35 (*m*, 6H, $\underline{H}C=C\underline{H}$). ^{13}C NMR (ppm): C-1, 174.0; C-2, 34.1; C-3, 24.7; C-4, 29.2; C-5, 27.0; C-6, 129.7; C-7, 128.4; C-8, 25.8; C-9, 128.2; C-10, 128.5; C-11, 25.8; C-12, 127.7; C-13, 130.5; C-14, 27.1; C-17, 22.4; C-18, 14.1; OCH_3, 51.5.

1-Bromo-2-pentyne, **(10)**. Triphenylphosphine (137 g, 523 mmol) was dissolved in methylene chloride (300 ml) in a one-liter three-necked flask equipped with a mechanical stirrer, a low temperature thermometer, a burette or dropping funnel, and a $CaCl_2$ drying tube. While a slow stream of N_2 was passed through the apparatus immersed in an ice bath, bromine (26.5 ml, 82.3 g, 514 mmol) was added dropwise over one hour while the temperature varied between 0 and 15°C. 2-Pentynol (39.86 g, 475 mmol) dissolved in CH_2Cl_2 (50 ml) was added to the slurry over 30 min while the temperature was maintained below 8°C. The ice bath was removed, and two hours later the reaction mixture was diluted with PE (400 ml). The precipitate that formed was stirred two times with fresh portions of PE (200 ml). Solvent was removed through a concentric tube fractionating column at atmospheric pressure to leave a solid and liquid. The solid was removed by filtration and was washed with PE (50 ml). The PE wash was combined with the filtrate, and solvent was again removed through the fractionating column. The liquid residue was then fractionated under reduced pressure to yield 1-bromo-2-pentyne (33.34 g), b.p. 36–53°C at 20–30 torr and 1,3-dibromo-2-pentene (17.52 g) b.p. 66–68°C at 22–29 torr. An additional 15 g of 1-bromo-2-pentyne was recovered from the dry ice cooled traps. Total yield of 1-bromo-2-pentyne was 48 g (68% yield), while the dibromide was formed in 16% yield. When the reaction was allowed to proceed for 21 hr, the yield of 1-bromo-2-pentyne was 23%, while the yield of the dibromide was 46%. Hence, it is important that the reaction be worked up promptly.

The chemical shifts reported previously (6) for 1-bromo-2-pentyne are in error. Heteronuclear carbon-proton correlations have confirmed the following assignments: ^{13}C NMR ($CDCl_3$, ppm): C-1, 15.6; C-2, 74.6; C-3, 89.4; C-4, 12.6; C-5, 13.5.

For the dibromide, mass spectroscopy gave a formula $C_5H_8Br_2$. Proton NMR: δ 1.11 [*t*, $C\underline{H}_3CH_2-$]; δ 2.51 [*q*, $CH_3C\underline{H}_2-$]; δ 3.28 [*t*, $-CBr=C\underline{H}$]; δ 4.04 [*d*, $-C\underline{H}_2Br$]. ^{13}C NMR (ppm): C-1, 35.1; C-2, 123.3; C-3, 135.9; C-4, 30.2; C-5, 13.1.

3,6-Nonadiynol, **(11)**. This compound was prepared in the same manner as previously described (6). ^1H NMR ($CDCl_3$) δ 1.12 (*t*, $C\underline{H}_3-$); δ 2.17 (*m*, $CH_3C\underline{H}_2-$) δ 2.44 (*m*, $-C\underline{H}_2CH_2OH$); δ 2.59 (*bs*, $-O\underline{H}$); δ 3.14 (*m*, $C\equiv C-C\underline{H}_2C\equiv C$); δ 3.69 (*t*, $C\underline{H}_2OH$). ^{13}C NMR ($CDCl_3$, ppm): C-1, 61.0; C-2, 23.0; C-5, 9.6; C-7, 81.95; C-8, 12.2; C-9, 13.7. Unassigned: 76.8, 76.6, 73.4.

3,6-Nonadienol-3,4,6,7-d_4, **(12)**. This compound was prepared in the same manner as the undeuterated compound as previously described (6). When 100 ml of ethanol was used as solvent, the deuterium distribution was 1.2% d_0, 2.2% d_1, 8.9% d_2, 24.4% d_3, 62.1% d_4, 1% d_5. When only 30 ml of ethanol was used as the solvent, the scatter was less: 0.6% d_0, 0.9% d_1, 3.3% d_2, 19.6% d_3, 72.1% d_4, 3.4% d_5. ^{13}C NMR (ppm): C-1, 62.0; C-2, 30.6; C-5, 25.3; C-8, 20.3; C-9, 14.1. Proton NMR showed about 0.3 CH= per molecule.

1-Bromo-3,6-nonadiene-3,4,6,7-d_4, **(13)**. This compound was prepared in 77% yield in the manner previously described for the undeuterated compound (6). ^{13}C NMR (ppm): C-1, 32.3; C-2, 30.7; C-5, 25.4; C-8, 20.4; C-9, 14.2. Both proton and ^{13}C NMR show some CH= groups.

1-Iodo-3,6-nonadiene-3,4,6,7-d_4, **(14)**. Compound 14 was prepared in 90% crude yield in the manner previously described for the undeuterated compound (6).

3,4,6,7-Tetradeutero-3,6-nonadienyltriphenylphosphonium iodide, **(15)**. This compound was prepared in 85% yield in the manner previously described for the undeuterated compound (6).

Methyl cis-*6*,cis-*9*,cis-*12*,cis-*15-octadecatetraenoate-12,13,15,16-*d_4, **(16)**. A slurry of compound 15 (8.66 g, 16.8 mmol) in THF (100 ml) was prepared in a 250-ml, three-necked flask equipped with a mechanical stirrer, a low temperature thermometer, a dropping funnel, and a $CaCl_2$ tube. A stream of N_2 was passed through the apparatus and it was cooled in an ice-salt bath to 3°C. Butyl lithium in hexane (2.5 M, 10 ml, 25 mmol) was added and the slurry turned to a very dark red solution. Methyl 9-oxo-*cis*-6-nonenoate (3.78 g, 20.5 mmol) in THF (10 ml) was added over 2 min at a temperature of 3–11°C. The color became much lighter. The ice bath was removed and two hours later the reaction mixture was transferred to a separatory funnel and washed with saturated NaCl solution (100 ml) and dried (Na_2SO_4). From this point the sample was handled under a N_2 atmosphere and TBHQ was added as an antioxidant whenever a sample was stored. Removal of the drying agent and solvent yielded a dark red liquid (14.06 g), which was extracted with

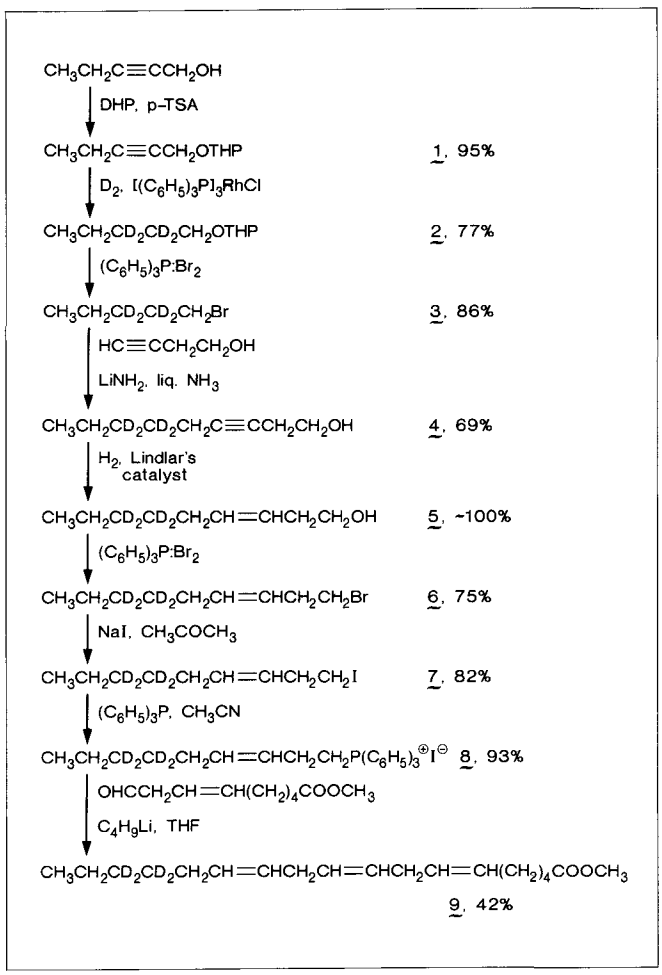
hexane and the hexane extract was passed through a column (1.5 × 20 cm) packed with silica gel (15 g) in hexane (degassed with He). Removal of the solvent gave a mixture of products (1.5 g, 31% yield, ca. 60% tetraene by gas chromatography). The all-*cis* isomer (300 mg) was isolated from a series of 200 microliter samples eluted through a column (0.8 × 70 cm) of Amberlyst XN1010 resin, 80/120 mesh, 70% Ag/Na, using 5% CH_3CN in methanol as eluant. The product was better than 98% pure as measured on the CPS-2 capillary column. Mass spectral analysis: 1.39% d_1, 6.46% d_2, 24.55% d_3, 67.03% d_4, 0.52% d_5. ^1H NMR ($CDCl_3$): δ 0.96 (*t*, 3H, $C\underline{H}_3$), δ 1.39 (*m*, 2H, $C\underline{H}_2CH_2CH_2COO-)_1$ δ 1.63 (*m*, 2H, $C\underline{H}_2CH_2COO-$), δ 2.07 (*m*, 4H, $C\underline{H}_2C=C$), δ 2.31 (*t*, 2H, $C\underline{H}_2COO-$), δ 2.81 (*m*, 6H, $C=C-C\underline{H}_2-C=C$), δ 3.65 (*s*, 3H, $OC\underline{H}_3$), δ 5.35 (*m*, 4H, $-C\underline{H}=C\underline{H}-$). ^{13}C NMR (ppm): C-1, 174.0; C-2, 34.0; C-3, 24.6; C-4, 29.1; C-5, 26.9; C-6, 129.6; C-7, 9, 10, 128.1-128.2; C-8, 11, 14, 25.3-25.6; C-17, 20.4; C-18, 14.2; OCH_3, 51.4.

RESULTS AND DISCUSSION

For the synthesis of these compounds, we used acetylenic coupling in liquid ammonia or Grignard coupling of alkynes, and, for the last coupling, the Wittig reaction. Using these reactions and separating geometric isomers as formed during the reaction sequences, isomers other than the all *cis* isomer could be prepared.

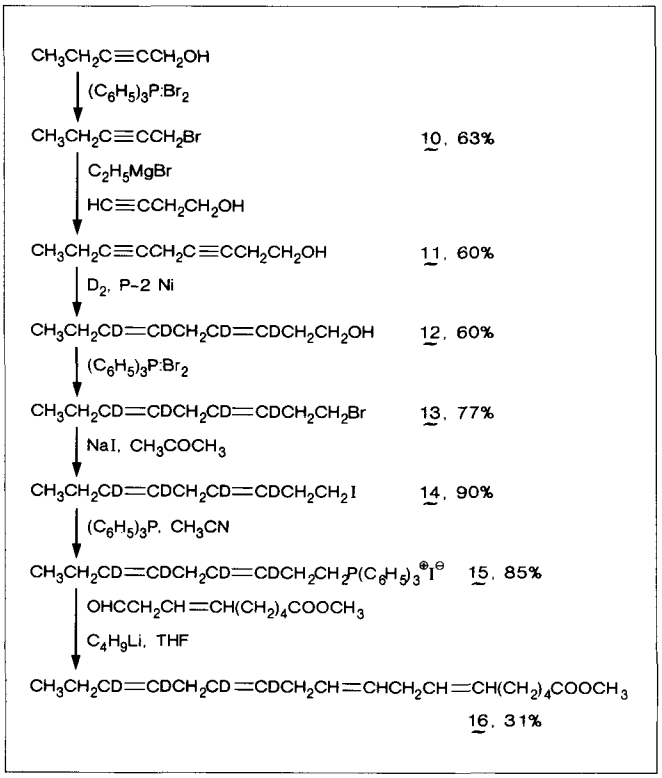
The Wittig reaction involves the reaction of an aldehyde with a phosphonium salt to generate a double bond between the two units. For the synthesis of both the triene and the tetraene, the same aldehyde ester, methyl 9-oxo-6-nonenoate, was used. To prepare this aldehyde ester, cyclohexene was ozonized in glacial acetic acid and the ozonide was reduced with sodium acetate to give 6-oxohexanoic acid in 62% yield (10). This product was converted to the acetal ester, methyl 6,6-dimethoxyhexanoate, in 74% yield with trimethyl orthoformate, methanol and HCl (10). Hydrolysis of this acetal ester with HCl in aqueous acetonitrile gave methyl 6-oxohexanoate in 80% yield (5). To elongate the chain, [2-(1,3-dioxan-2-yl)ethyl]-triphenylphosphonium bromide was condensed with methyl 6-oxohexanoate in a Wittig reaction with butyl lithium to give methyl 8-dioxanyl-6-octenoate in 71% yield (5). This was transacetalized with methanol and *p*-toluenesulfonic acid to methyl 9,9-dimethoxy-6-nonenoate in 93% yield (5). Analysis by GLC indicated 70% *cis* and 30% *trans* isomers. These geometric isomers were separated by silver resin chromatography (8,9) on a 100% Ag/H column. While there are many methods described in the literature (11-13) for hydrolyzing a β, γ unsaturated acetal to the aldehyde without conjugating the double bond with the carbonyl group, no method was found that works as well as it is described. Best results were obtained with a rapid hydrolysis with concentrated HCl in aqueous acetonitrile. Thus, we were able to form methyl 9-oxo-6-nonenoate in 92% yield with only 5-10% conjugation.

The phosphonium salt required for the preparation of the trienoic ester, 6,6,7,7-tetradeutero-3-nonenyltriphenylphosphonium iodide, 8, was prepared by a sequence of reactions as presented in Scheme 1. 2-Pentynol was converted to the tetrahydropyranyl ether (THP), 1, with dihydropyran and *p*-toluenesulfonic acid so that there



SCHEME 1. Synthesis of methyl 6,9,12-octadecatrienoate-15,15,16,16-d_4.

would be no replaceable hydrogens available during the deuteration reaction. Deuteration with deuterium gas in the presence of Wilkinson's catalyst, tris-(triphenylphosphine)chlororhodium, gave the tetradeuterated THP ether, 2, in 77% yield and containing 90% d_4. This ether was converted with triphenylphosphine dibromide to 1-bromopentane-2,2,3,3-d_4, 3, in 86% yield. Low molecular weight alkyl bromides tend to codistil with most solvents, so it is necessary to use a fractionating column for the separation of the solvent and product. This bromide, 3, was coupled with 3-butynol with lithium amide in liquid ammonia to give 3-nonynol-6,6,7,7-d_4, 4, in 69% yield. Hydrogenation of 4 with hydrogen in the presence of Lindlar's catalyst gave 3-nonenol-6,6,7,7-d_4, 5, in essentially 100% yield. The hydroxy group was then replaced by bromine to give 1-bromo-3-nonene-6,6,7,7-d_4, 6, in 75% yield. Reaction of 6 with sodium iodide in acetone gave the corresponding iodo compound, 7, in 82% yield, and further reaction with triphenylphosphine in acetonitrile gave the phosphonium salt, 8, in 93% yield. Reaction of this phosphonium iodide, 8, with methyl 9-oxo-*cis*-6-nonenoate gave a mixture of isomers (*cis/trans* = 80/20) of methyl 6,9,12-octadecatrienoate-15,15,16,16-d_4, 9, in about 42% yield. Isomers were separated on a 62% Ag/Na column (8,9).



SCHEME 2. Synthesis of methyl 6,9,12,15-octadecatetraenoate-12,13,15,16-d_4.

A similar sequence of reactions for the preparation of the tetraenoic ester, **16**, was unsuccessful for several reasons. First, reaction of the THP ether of 2-pentenol-2,3-d_2 with triphenylphosphine dibromide yields a mixture of 1-bromo-2-pentene-d_2 and 3-bromo-1-pentene-d_2; second, difficulty in hydrolyzing to and isolating 2-pentenol-d_2; third, difficulty in isolating 1-bromo-2-pentene-d_2 from the reaction of the alcohol with triphenylphosphine dibromide; and fourth, additional isomers formed in later reactions. The synthetic scheme that was carried out successfully is presented in Scheme 2.

2-Pentynol was converted to 1-bromo-2-pentyne, **10**, in 63% conversion by reaction with triphenylphosphine dibromide in methylene chloride. If the reaction time is prolonged, 1,3-dibromo-2-pentene is formed at the expense of 1-bromo-2-pentyne. Compound **10**, which is a *powerful lachrymator*, codistils with the solvents present, so an efficient fractionating column must be used to remove solvents. The bromide, **10**, was condensed with 3-butynol in a Grignard reaction to give **11**, 3,6-nonadiynol, in 49% yield. By-products formed during the synthetic sequence deactivate the Lindlar catalyst. Usually repeated additions of fresh catalyst are required, resulting in the use of relatively large amounts of catalyst. We chose to use P-2 Nickel, which is generated by the reaction of sodium borohydride with nickel acetate in ethanol containing ethylenediamine (14). In spite of all the replaceable hydrogens present, we were able to prepare the tetradeuterated compound **12** containing 60–70% d_4 in 62% yield. We were able to convert **12** to the bromide **13** in 77% yield by reaction with triphenylphosphine dibromide in CH_2Cl_2, to the iodide, **14**, in 90% yield by reaction with NaI in acetone and to the phosphonium iodide, **15**, in 85% yield by reaction with triphenylphosphine in acetonitrile. Reaction of **15** with methyl 9-oxo-*cis*-6-nonenoate and butyl lithium in THF gave a crude product mixture of **16** in 30% yield. The all *cis* isomer was isolated by silver resin chromatography on a 70% Ag/Na XN1010 column (8,9) with 5% CH_3CN in methanol as eluant. ^{13}NMR chemical shifts are consistent with both the structure and configuration assigned.

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Phosphatidylcholine Synthesis in the Rat: The Substrate for Methylation and Regulation by Choline

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Two lines of evidence led us to reexamine the possibility that methylation of phosphoethanolamine and its partially methylated derivatives, in addition to methylation of the corresponding phosphatidyl derivatives, plays a role in mammalian phosphatidylcholine biosynthesis: (a) Results obtained by Salerno and Beeler with rat [Salerno, D. M. and Beeler, D. A. (1973) *Biochim. Biophys. Acta 326,* 325–338] appear to strongly support such a role for methylation of phosphobases; (b) Such reactions have recently been shown to play major roles in phosphatidylcholine synthesis by higher plants [see Datko, A. H. and Mudd, S. H. (1988) *Plant Physiol. 88,* 854–861 and references therein]. We found that, following continuous labeling of rat liver with L-[*methyl*-^{3}H]methionine for 10.4 min (intraperitoneal administration) or for 0.75 min (intraportal administration), virtually no ^{3}H was detected in methylated derivatives of phosphoethanolamine, but readily detectable amounts of ^{3}H were present in the base moiety of each methylated derivative of phosphatidylethanolamine. Thus, there was no indication that phospho-base methylation makes a significant contribution. Studies of cultured rat hepatoma cells showed definitively for the first time in a mammalian system that choline deprivation up-regulates the rate of flow of methyl groups originating in methionine into phosphatidylethanolamine and derivatives. Even under these conditions, methylation of phosphoethanolamine bases appeared to play a negligible role.
***Lipids 25,* 135–142 (1990).**

Early studies of the pathways for synthesis of phosphatidylcholine (PtdCho) indicated that in the rat the required methyl groups may be introduced by successive methylations of phosphatidylethanolamine (PtdEA), phosphatidylmonomethylethanolamine (PtdMEA) and phosphatidyldimethylethanolamine (PtdDMEA). Thus, 15 to 20 min after intraperitoneal administration of [*methyl*-^{14}C]-methionine, substantial amounts of label were found in the base moieties of each of these compounds in liver (1,2); and liver microsomes incubated with *S*-adenosyl-[*methyl*-^{14}C]methionine ([*Me*-^{14}C]AdoMet) also incorporated label into the base moieties of each (3,4). During such experiments, no indication was obtained of the functioning of a hypothetical alternative pathway for methylations utilizing as substrates the phosphobases, phosphoethanolamine (P-EA), phosphomonomethylethanolamine (P-MEA), and phosphodimethylethanolamine (P-DMEA). At short times after intraperitoneal injection of [*Me*-^{14}C]-methionine the specific activity of P-Cho remained very low relative to that of PtdCho (4,5). With microsomal systems, addition of P-EA had no effect on the accumulation of methyl groups originating in AdoMet in phospholipids (4,6).

Later investigations confirmed the presence in mammalian tissues of the methylation pathway utilizing the phosphatidyl-bases [see reviews by Ansell and Spanner (7), Pelech and Vance (8), and Crews (9)]. However, two subsequent findings suggest that the conclusion—this is the sole pathway—needs to be reevaluated: (a) Following intraportal administration of a brief pulse of [*Me*-^{3}H]-methionine to *in situ* rat livers, Salerno and Beeler (10) found that the specific radioactivity of P-Cho rose rapidly to a maximum of 399 dpm/nmol at 30 sec, then declined within 5 min to low equilibrium values (10–14 dpm/nmol). In contrast, the specific activity of PtdCho rose slowly, reaching 18 dpm/nmol at 5 min and a maximum of 36 dpm/nmol at 60 min. These results were "interpreted to indicate the direct methylation of P-EA to P-Cho." The authors suggested "the general belief that the only substrate for methylation is the complete PtdEA molecule . . . must be reexamined and perhaps revised" (10). To the best of our knowledge, these results have not been reinvestigated. (b) Investigations of PtdCho biosynthesis in higher plants have revealed that in all species studied the initial methyl group may be introduced by the methylation of P-EA (11–14). Subsequent methyls are introduced solely at the phospho-base level [duckweed (13) and sugarbeet (12)], solely at the phosphatidyl-base level [soybean (14)], or at both levels [carrot (14) and barley (11)]. The required AdoMet-dependent methyltransferase activities have been demonstrated (15). We have now applied the techniques which permitted elucidation of the role of phospho-base methylation in higher plants to a reinvestigation of *in situ* rat liver. Further, we studied cultured rat hepatoma cells, a system in which one could clarify and take advantage of the possible regulatory role of Cho.

MATERIALS AND METHODS

Radiolabeled chemicals. [^{14}C]EA was obtained commercially from ICN; [*Me*-^{14}C]P-Cho and [*Me*-^{14}C]methionine, from NEN; [*Me*-^{14}C]Cho, [^{14}C]CDP-Cho, [^{3}H]Cho, [^{3}H]EA, [EA-^{14}C]PtdEA and L-[*Me*-^{3}H]methionine, from Amersham. Before use in labeling experiments, the L-[*Me*-^{3}H]methionine was purified by passage through a column of Dowex 50-NH_4^+ (14). [^{14}C]Betaine [also obtained originally from Amersham (16)] was a gift from J. D. Finkelstein. [^{14}C]Sphingomyelin was a gift from A. Gal and R. Brady. [^{3}H]MEA, [^{3}H]DMEA, [^{14}C]MEA and [^{14}C]DMEA were synthesized by CH_3I treatment of either [^{3}H]EA or [^{14}C]EA (13). [^{14}C]GP-EA was prepared by mild alkaline deacylation of [^{14}C]PtdEA (13). [^{3}H]P-Cho was isolated from *Lemna* following long-term labeling with [^{3}H]Cho (17). [^{3}H]P-MEA, [^{3}H]P-DMEA,

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Abbreviations: EA, ethanolamine; MEA, *N*-methylethanolamine; DMEA, *N,N*-dimethylethanolamine; Cho, choline. Phosphate esters are designated by the prefix, P- (e.g. P-EA or P-Cho); the corresponding phosphatidyl derivatives, by the prefix, Ptd (e.g. PtdEA or PtdCho); and the corresponding glycerylphospho-base derivatives, by the prefix, GP- (e.g. GP-EA). CDP-Cho, cytidine diphosphocholine; AdoMet, *S*-adenosylmethionine.

[^{14}C]P-MEA and [^{14}C]P-DMEA were isolated from *Lemna* following short-term labeling with either [^{3}H]EA or [^{14}C]EA, essentially as described (13). Aliquots of chloroform/methanol soluble fractions were used as sources of combinations of internal radiolabeled markers as follows: *Lemna* labeled long-term with L-[*Me*-^{3}H]-methionine for combined [^{3}H]PtdCho and [^{3}H]neutral lipid (17); soybean cells labeled short-term with L-[*Me*-^{3}H]methionine for combined [^{3}H]PtdMEA, [^{3}H]-PtdDMEA, [^{3}H]PtdCho and [^{3}H]neutral lipid (14); hepatoma cells labeled with [*Me*-^{14}C]Cho for combined [^{14}C]PtdCho and [^{14}C]sphingomyelin (see below).

Experiments with intact rats. Male Sprague-Dawley rats, maintained on a normal laboratory diet, and weighing 95–150 g were used for all experiments with intact animals. Injected L-[*Me*-^{3}H]methionine had a specific radioactivity of 184 × 10^{6} dpm/nmol.

Time course of accumulation of hepatic radioactivity after intraperitoneal L-[methyl-^{3}*H*]*methionine injection.* L-[Me-^{3}H]Methionine (approximately 7.3 × 10^{6} dpm in 0.5 ml sterile 0.15 M NaCl) was injected intraperitoneally into each of several rats. At the desired time the animal was stunned, and the liver was quickly excised, rinsed, blotted, minced and homogenized in cold 5% trichloroacetic acid. After centrifugation, radioactivity in the soluble fraction was determined directly. Radioactivity in the insoluble fraction was determined after solubilization by repeated extraction with 70% formic acid.

Intraperitoneal labeling with L-[Methyl-^{3}*H*]*methionine.* A 140 g rat was injected intraperitoneally with L-[*Me*-^{3}H]]methionine (142 × 10^{6} dpm dissolved in 0.5 ml sterile 0.15 M NaCl). The liver was excised 10.4 min later, quickly blotted, and homogenized in a chilled stainless-steel Waring blender in methanol/chloroform/2 M formic acid (12/5/3, v/v/v) initially chilled almost to dry-ice temperature (18). After centrifugation, the insoluble residue was rinsed by resuspension twice in methanol/chloroform (2/1, v/v), once in methanol, and twice in 10% trichloroacetic acid. After removal of trichloroacetic acid by extraction with ether, these washes were combined with the initial supernatant solution, and the whole was taken essentially to dryness. The resulting syrupy residue was dissolved in methanol/chloroform (2/1, v/v) and separated into methanol/water soluble and chloroform/methanol soluble fractions (19). The washed insoluble residue was dissolved in 70% formic acid for determination of radioactivity.

Intraportal labeling with L-[methyl-^{3}*H*]*methionine.* A 100 g rat was anesthetized with halothane and the portal vein exposed. L-[*Me*-^{3}H]Methionine (170 × 10^{6} dpm in 0.2 ml sterile 0.15 M NaCl) was infused into the portal vein at a constant rate over 25 sec. At 30 sec the liver was excised and within seconds frozen by compression to a thin wafer between aluminum blocks cooled in liquid nitrogen (20). The frozen tissue was crumbled into a cold stainless-steel Waring blender vessel, extracted, and separated into fractions as described above.

Cell culture. H-4 rat hepatoma cells (H-4-II-E rat hepatoma from the American Type Culture Collection, CRL 1548) were continuously grown in Eagle's modified minimum essential medium in 75 cm^2 culture flasks (Costar), with 25 ml of medium added to each flask. Added methionine was decreased from 100 μM to 25 μM (see Results). The medium was supplemented with fetal calf serum which had been heat-inactivated at 56°C for 30 min. To limit the amount of Cho added with the serum, the latter was passed through a column of Dowex 50-NH_4^+ (100–200 mesh, 40 ml resin volume for each 115 ml serum). A preliminary experiment with [*Me*-^{14}C]-Cho demonstrated that this procedure removed at least 99.7% of the Cho from the serum. The final concentration of treated serum in the growth medium was 5%. For maintenance, cells were subcultured twice a week at a split ratio of 1:2 or 1:3 (see Results). Cells used in the individual experiments had been pregrown for at least two weeks in Dowex 50-NH_4^+-treated serum with the specified amount of exogenous Cho. To initiate these experiments, nearly confluent monolayer cell cultures were generally split 1:10.

Labeling and harvesting of cells. To label cells, the medium in which they were growing was removed by aspiration and replaced by a chemically similar medium (i.e. one in which the constituents were present at the same original concentrations as had been present in the growth medium) but with the specified radiochemical added. The cells were maintained in this medium under growth conditions for periods specified in the individual experiments. At harvest, medium was removed by aspiration and the cells were washed twice with Dulbecco's phosphate-buffered saline without Ca^{2+} or Mg^{2+}. The cell layer was flooded with 3 ml ice-cold methanol/2 M formic acid (12/3, v/v), then loosened by scraping. The entire suspension was transferred to a centrifuge tube by use of two additional 1 ml washes of methanol/2 M formic acid. After centrifugation, the supernatant fluid was taken to dryness. A single aliquot of 4.8 ml methanol/chloroform (2/1, v/v) was used to extract, first, the resulting residue, then second (sequentially) the initial residue resulting from the prior centrifugation (each for 30 min). The residues were then combined and extracted with methanol, then trichloroacetic acid to yield a washed insoluble fraction. These washes were combined with the methanol/chloroform extract as described above and separated into methanol/water soluble and chloroform/methanol soluble fractions (19).

Cellular turnover of choline. Hepatoma cells were pregrown in media supplemented with 7 or 21 μM Cho. At "time 0" these cells were subcultured in duplicate into media supplemented with 0 or 21 μM Cho. At 120 hr, the media were changed to chemically similar media with [^{3}H]thymidine added at 200 × 10^{3} dpm/ml, and [*Me*-^{14}C]Cho added at 20 × 10^{3} dpm/ml. At 144 hr, these media were removed and the cells were washed with nonradioactive media. One of each duplicate set of cells was harvested; the other was incubated in its nonradioactive medium to 168 hr, then harvested.

Chromatography and electrophoresis. Except as otherwise noted, paper chromatography was carried out on Whatman No. 1 paper using the descending method. Solvents were: solvent A, phenol, 125 g:1-butanol, 125 ml/88% HCOOH, 6.8 ml/H_2O, 25.7 ml (the entire solution saturated with KCl and the chromatography paper predipped in 1 M KCl and dried); solvent B, 2-propanol/88% HCOOH/H_2O (7:1:2, v/v/v); solvent C, 2-propanol/29% NH_4OH/H_2O (7:1:2, v/v/v); solvent D, methanol/88% HCOOH/H_2O (80:14.8:5.2, v/v/v) (ascending development for approximately 6 hr); solvent E, chloroform/methanol/acetic acid/H_2O (65:35:8:4, by vol)

(ascending development, overnight, on Whatman SG 81 silica gel chromatography paper). Electrophoresis at pH 7.0, 3.7 or 1.9 was performed as described (17).

Other. Mild alkaline deacylation of phosphatidyl compounds was carried out with 0.2 M methanolic NaOH (21) to form the corresponding glycerylphospho-bases. Free bases were formed by acid hydrolysis of phosphatidyl compounds or of glycerylphospho-bases (17), or by phosphatase treatment of phospho-bases (13). Methods for protein determination, utilization of authentic internal radioactive standards as markers, location of radioactive compounds on chromatograms and electrophoretograms, elution of such materials, and calculation of the amounts of radioactivity in particular compounds which had been subjected to several sequential purification steps have been described (13-15,17).

Analyses. Compounds were purified by serial chromatographic procedures, usually using a radiolabeled authentic internal marker (^{3}H-labeled for ^{14}C-containing unknowns, and vice versa) to follow the behavior of the material under investigation.

Chloroform/methanol fraction. Chromatography of aliquots of this fraction with solvent E separated peaks of sphingomyelin, PtdCho, and combined PtdEA, PtdMEA and PtdDMEA (14). For samples resulting from labeling with L-[*Me*-^{3}H]methionine or $^{32}P_i$ the peaks of phosphatidyl compounds were eluted separately, and each was subjected to mild alkaline deacylation. Materials labeled from L-[*Me*-^{3}H]methionine were then acid hydrolyzed. The derived free bases were chromatographed in solvent B, then separated by chromatography with solvent A (13,14). [^{32}P]GP-Cho resulting from the mild alkaline deacylation was chromatographed with solvent D. [^{32}P]GP-EA, [^{32}P]GP-MEA and [^{32}P]GP-DMEA were separated by chromatography with solvent C (14).

Methanol/water soluble fraction—(a) Materials labeled from [Me-^{14}*C*]*Cho.* [^{14}C]P-Cho, [^{14}C]Cho and [^{14}C]betaine were separated by electrophoresis at pH 7.0. [^{14}C]P-Cho was then chromatographed with solvent C; [^{14}C]Cho, solvent A (13). [^{14}C]Betaine remained near the origin during pH 7.0 electrophoresis. It was further purified by chromatography with solvent B, then electrophoresis at pH 1.9 (movement toward negative pole). [^{14}C]CDP-Cho was separated by chromatography with solvent B (13).

(b) Materials labeled from L-[Me-^{3}*H*]*methionine.* [^{3}H]P-MEA, [^{3}H]P-DMEA and [^{3}H]P-Cho were initially purified by loading an aliquot of a methanol/water soluble fraction onto Dowex-50-H$^+$. For samples originating as liver extracts, an amount equivalent to 20% of one liver (diluted to 100 ml) was loaded upon a 15.7 cm^3 column. The column was washed with water, 50 ml, then eluted with 0.1 M HCl, 100 ml. For samples originating as cell extracts, approximately 95% of the methanol/water soluble fraction from a single flask of cells was diluted and loaded onto a column containing 1.5 cm^3 of resin. The column was washed with water to a total volume of 15 ml flow-through plus wash, then eluted with 10 ml 0.1 M HCl. The phospho-bases were recovered together in the combined flow-through, water wash and 0.1 M HCl eluates (15). They were then electrophoresed at pH 7.0 and chromatographed with solvent B (traveling together during these procedures). Material eluted from the chromatogram with solvent B was treated with phosphatase to form the corresponding free bases. The latter were electrophoresed at pH 7.0. The ^{3}H content of combined P-MEA, P-DMEA and P-Cho was determined during the last step. To determine the P-bases individually, the phosphatase treatment and the final pH 7.0 electrophoresis were omitted and the methylated phospho-bases were separated by chromatography with solvent C (13). [^{3}H]MEA, [^{3}H]DMEA and [^{3}H]Cho were initially separated as a group from combined [^{3}H]-labeled methionine (and/or methionine sulfoxide) by electrophoresis at pH 7.0. The combined free bases were further purified by chromatography with solvent B. Methionine (which remained near the origin of the electrophoretogram) was eluted and converted to its sulfoxide by treatment with dimethylsulfoxide. Dimethylsulfoxide was removed by Dowex 50-H$^+$ chromatography and methionine sulfoxide recovered (22), chromatographed with solvent B, and reduced with mercaptoethanol to methionine (22). The latter was chromatographed with solvent B.

RESULTS

Intraperitoneal administration of L-[methyl-^{3}*H*]*methionine.* To determine a suitable time period at which to search in liver for ^{3}H-labeled methylated derivatives of P-EA following intraperitoneal injection of L-[*Me*-^{3}H]-methionine, the time course of accumulation of hepatic radioactivity was investigated. After 1 min, 1.3% of the total administered intraperitoneal dose of radioactivity was found in liver; after 4.5 min, 4.8%; after 9 min, 7.0%; and after 20 min, 10.7%. Based on these results, a 10-min labeling period was selected (see Discussion).

The distribution of ^{3}H in selected compounds in liver 10.4 min after intraperitoneal administration of L-[^{3}H]methionine is shown in Table 1, left column. An ample amount of [^{3}H]methionine (not distinguished by the

TABLE 1

Distribution of ^{3}H in Livers After Intraperitoneal or Intraportal Injection of L-[*Methyl*-^{3}H]Methionine[a]

Compound	Injection (time of labeling, min)	
	Intraperitoneal (10.4)	Intraportal (0.75)
	Radioactivity, % of total in liver	
Met/Met sulfoxide[b]	24.2	58.0
PtdMEA	0.7	0.42
PtdDMEA	3.0	1.46
PtdCho	31.1	1.92
P-MEA	0.0	n.d.[c]
P-DMEA	<0.02	n.d.[c]
P-Cho	0.05	n.d.[c]
MEA + DMEA + Cho	<0.02	<0.02

[a] Rats were injected either intraperitoneally or intraportally with L-[*Me*-^{3}H]methionine. Labeled compounds listed in this and other tables were analyzed (sometimes with minor variations) as detailed in Methods. These methods did not distinguish methionine from methionine sulfoxide. Total liver ^{3}H contents were 11.0 and 22.2 × 10^6 dpm after the intraperitoneal and intraportal injections, respectively.

[b] Met = methionine.

[c] Not detected. Total ^{3}H in the three methylated phospho-bases (analyzed as a group) was <0.004% the liver total.

method used from [^{3}H]methionine sulfoxide) remained. Among the products, some 31% of the total hepatic radioactivity was found in [^{3}H]PtdCho, with lesser, but readily detectable amounts of [^{3}H]PtdMEA and [^{3}H]PtdDMEA. The sensitivity for detection of the latter compounds is illustrated by the fact that after acid hydrolysis of the [^{3}H]PtdMEA the discrete peak of [^{3}H]MEA into which it had been converted was prominent and easily quantitated after chromatography with solvent A, the final purification step employed (data not presented). The only ^{3}H-containing methylated derivative of P-EA detected was [^{3}H]P-Cho, the ^{3}H content of which was only 0.14% that of the combined ^{3}H-containing methylated phosphatidylethanolamine derivatives [i.e. 0.05 × 100%/(31.1 + 3.0 + 0.7) = 0.14%]. ^{3}H-Containing methylated derivatives of free EA were not detected.

Brief intraportal administration of L-[methyl-^{3}*H*]*methionine.* The results of this experiment are reported in Table 1, right column. Compatible with the shorter time of labeling, more of the total tissue ^{3}H was in the form of [^{3}H]methionine, less in metabolites. [^{3}H]PtdCho was again quantitatively the most important measured product, but both [^{3}H]PtdDMEA and [^{3}H]PtdMEA were labeled more strongly in proportion to [^{3}H]PtdCho than had been the case 10.4 min after intraperitoneal injection. No ^{3}H was detected in methylated derivatives of P-EA, with an upper limit of 0.11% that of the combined phosphatidyl derivatives. Again, no ^{3}H was detected in free base derivatives of EA.

Experiments with cultured rat hepatoma cells; establishment of a standard methionine concentration. A preliminary experiment was carried out to help establish a standard amount of methionine to add to the growth medium, and the required frequency of medium changes. It was desired to keep the concentration of methionine high enough, and the medium changes frequent enough, to avoid depletion of this amino acid or marked changes in its concentration with time after subculture. On the other hand, it was desired to lower the methionine concentration as much as possible so that the specific radioactivity of this compound could be kept high during labeling experiments. The H-4 rat hepatoma cells were pregrown for several weeks in medium with Dowex 50-NH_4^+-treated serum supplemented with 7 μM Cho (see below). At "time 0" such cells were split 1:10 into flasks in the same medium, but in which L-[*Me*-^{14}C]-methionine was added at either 100 μM (the standard concentration in Eagle's modified minimum essential medium), 25 μM, or 10 μM. No differences in growth rates were noted. After 65 hr, the concentrations of ^{14}C in the media were, respectively, 93, 81 and 65% of the initial concentrations. It was concluded that addition of 25 μM methionine, provided the growth medium was changed at least once in three days, met the above considerations concerning the concentration of this amino acid. In subsequent experiments methionine was added at 25 μM.

Depletion and repletion with Cho. When hepatoma cells were subcultured into media with serum freed of Cho by treatment with Dowex 50-NH_4^+, during the initial passage the cells grew more slowly than normal, and more floating cells were observed than usual, regardless of whether Cho had been added back to the medium. One week after a 1:10 split, such cells had usually attained confluence. During a second passage, cells growing without added Cho, or at 2.1 μM Cho, progressively slowed their growth and generally failed to attain confluence. Cells growing with Cho added back at 7 μM (the usual concentration of Cho in Eagle's modified minimum essential medium), 21 μM, or 2100 μM continued to grow at the initial rate, and could be passed indefinitely.

To gain some insight into the effects upon Cho metabolism of growth over this range of supplemental Cho, cells were grown to isotopic equilibria in media to which [*Me*-^{14}C]Cho was added at initial concentrations of either 2.1, 21, or 2100 μM at the time of subculture and at each refeeding. Analyses of the [*Me*-^{14}C]Cho contents of the media (see footnote to Table 2) showed that under these circumstances, even with refeeding at intervals no longer than 3 days, with the lowest Cho supplement the concentration of this compound in the medium fell markedly with time due to cellular uptake. With 21 μM Cho, this fluctuation was less severe, and at 2100 μM Cho an essentially constant medium concentration was maintained. Analyses of Cho-containing compounds in the cells (Table 2) revealed that with the lowest supplement of Cho virtually all the cellular Cho moieties were in the form of either PtdCho or sphingomyelin. As Cho was raised to 21 μM, P-Cho increased in its contribution to total cellular Cho. At 2100 μM Cho, P-Cho increased further,

TABLE 2

Distribution of ^{14}C in Hepatoma Cells Labeled to Isotopic Equilibrium with Various Concentrations of [*Methyl*-^{14}C]Choline[a]

Compound	[*Me*-^{14}C]Cho added to medium, μM		
	2.1	21	2100
	Radioactivity, % of total in cells		
PtdCho	65.7	44.6	27.2
P-Cho	0.18	28.5	42.7
Cho	<0.09	0.38	15.9
CDP-Cho	<0.11	n.a.[b]	<0.14
Betaine	<0.1	0.0	5.5
Sphingomyelin	22.6	13.6	4.5
Total Cho moieties	88.5	87.1	95.8

[a]The H-4 rat hepatoma cells were pregrown in media containing Dowex 50-NH_4^+-treated serum supplemented with either 21 or 2100 μM Cho. At "time 0" the former cells were used to inoculate flasks supplemented with either 2.1 or 21 μM [*Me*-^{14}C]Cho; the latter cells, a flask with 2100 μM [*Me*-^{14}C]Cho. The initial concentrations of ^{14}C in the media of these three cultures were approximately 9.3, 10 and 430 × 10^3 dpm/ml, respectively. The media were removed and replaced with fresh media at the following times in hours after inoculation (concentration of [*Me*-^{14}C]Cho in the medium at the time of each change given in parentheses as a percent of the original concentration of [*Me*-^{14}C]Cho): (a) 2.1 μM [*Me*-^{14}C]Cho: 71 (15%); 119 (12%); harvest at 143 (8%); (b) 21 μM [*Me*-^{14}C]Cho: 94 (69%); 142 (42%); harvest at 166 (53%); (c) 2100 μM [*Me*-^{14}C]Cho: 70 (100%); 118 (100%); harvest at 142 (100%). At harvest, total cellular ^{14}C was 335, 244 and 232 × 10^3 dpm in the three samples. The ^{14}C contents of the specified compounds were determined as described in Methods for samples resulting from labeling with [*Me*-^{14}C]Cho. During analysis of [^{14}C]P-Cho in the cells grown at 21 μM [*Me*-^{14}C]Cho, chromatography with solvent C was omitted and replaced by chromatography with solvent B, treatment with phosphatase, and repeat chromatography with solvent B.

[b]Not analyzed.

and free Cho became prominent. Since the Cho-containing compounds analyzed accounted for 87-96% of total cellular radioactivity, under no condition was there indication of extensive degradation of Cho moieties to non-Cho-containing compounds.

To determine the effects of similar circumstances upon the absolute concentrations of some of the compounds of interest, cells were grown to isotopic equilibria in $^{32}P_i$ (Table 3). As supplemental Cho increased, the concentration of PtdCho increased from 45 nmol/mg protein to 127, then remained constant. PtdEA concentration decreased from 55 to 32, then remained constant. PtdMEA and PtdDMEA were always relatively low. Likewise, the concentration of sphingomyelin remained essentially unchanged.

Rates of accumulation in total EA derivatives of methyl groups originating in methionine. Hepatoma cells growing at a range of concentrations of Cho were exposed to L-[*Me*-^{3}H]methionine over 24 hr periods (from either day 4 to day 5 or day 6 to day 7 after subculture) (Table 4). In all samples, the great majority of total cellular radioactivity was found in the washed insoluble pellet (range, 95.4 to 97.0%). Among the EA derivatives, the most prominent was [^{3}H]PtdCho, with, in each case, readily detectable amounts of ^{3}H-labeled PtdMEA, PtdDMEA and sphingomyelin. Relatively small amounts of ^{3}H-labeled phospho-bases were sometimes detected, and ^{3}H was not found in methylated derivatives of free EA. An estimate of the proportion of methyl groups originating in methionine which accumulate in total EA derivatives

TABLE 3

Concentrations of Phosphatidylethanolamine Derivatives and Sphingomyelin in Hepatoma Cells Grown in Various Concentrations of Choline[a]

Compound	Cho added to medium, μM		
	0	21	2100
	Cellular content, nmol/mg protein		
PtdEA	55	32	30
PtdMEA	8[b]	6[b]	6[b]
PtdDMEA	<2[c]	<2[c]	<2[c]
PtdCho	45	127	126
Sphingomyelin	15[d]	15	18[d]

[a]The H-4 rat hepatoma cells were pregrown as described in the footnote to Table 2. At "time 0" cells pregrown, respectively, in 1.7, 21 or 2100 μM Cho were used to inoculate, in triplicate, flasks supplemented with either 0, 21 or 2100 μM Cho. In all media, the inorganic phosphate was labeled with ^{32}P of measured specific radioactivity. In each flask the spent medium was replaced with fresh medium at approximately 92, 165, and 191 hr after inoculation. At 213 hr the triplicate sets of cells were harvested and combined. The ^{32}P contents of the specified compounds were determined as described in Methods for samples resulting from labeling with $^{32}P_i$.

[b]These values are approximations due to the low amount of ^{32}P migrating with the peak of [^{3}H]GP-MEA, and imperfect resolution of this peak from the larger peak of [^{14}C,^{32}P]GP-EA.

[c]No definite peak of ^{32}P accompanied the peak of [^{3}H]GP-DMEA.

[d]These preparations were carried through chromatography with solvent E, only.

TABLE 4

Distribution of ^{3}H in Hepatoma Cells Grown in Various Concentrations of Choline and Labeled for 24 Hours with L-[*Methyl*-^{3}H]Methionine[a]

Cho added to medium, μM	0	0	7	7	21	21	2100
Time labeling started, days	4	6	4	6	4	6	6
Total cellular ^{3}H, dpm × 10^{-3}	1143-1236	1556-1590	1132	1074	1218	1912	2242-2228
Percent of cellular ^{3}H in:							
Insoluble pellet	95.6-95.9	96.0-96.2	96.4	95.4	96.5	97.0	96.0-96.4
PtdMEA	.037-.040[b]	.027	.037[b]	.041[b]	.031[b]	.012	.016
PtdDMEA	.085-.093[b]	.109	.059[b]	.119[b]	.055[b]	.060	.044
PtdCho	.555-.580	.641-.682	.377	.575	.218	.241	.197-.230
Sphingomyelin	.079-.084	.100-.108	.022	.050	.025	.018	.014-.015
P-MEA + P-DMEA + P-Cho	n.a.[c]	.003	n.a.	n.a.	n.a.	.066	.028
MEA + DMEA + Cho	n.a.	n.a.	n.d.[d]	n.a.	n.d.	n.a.	n.d.
Total in EA derivatives	.764[e]	.905	.460[e]	.739[e]	.311[e]	.397	.302
Adjusted relative rate[f]	.798	.942	.477	.775	.322	.409	.314

[a]The H-4 rat hepatoma cells were pregrown in media supplemented with either 7, 21 or 2100 μM Cho. At "time 0" these cells were subcultured into flasks supplemented with the same concentration of Cho as that in which they had been pregrown, except that the cells pregrown in 7 μM Cho were used to inoculate the flask with 0 μM Cho supplement. Replicate flasks were prepared as appropriate. On day 3 the spent medium in each flask was replaced with fresh medium. Flasks specified were labeled from day 4 to 5 by a 24 hr incubation in fresh media containing L-[*Me*-^{3}H]methionine. Other flasks were refed on day 5, and labeled by exposure to L-[*Me*-^{3}H]methionine for 24 hr from day 6 to 7. L-[*Me*-^{3}H]Methionine was present at 25 μM in each flask, and contained from 235 to 270 × 10^3 dpm/ml in the various flasks. In those instances in which samples from replicate flasks were carried through the entire analytical procedures, the values for each replicate are presented. In other instances, replicates were combined during the analytical procedures.

[b]These compounds were not carried through the entire purification procedures. The values listed may be overestimated by 10-30%.

[c]Not analyzed.

[d]Not detected.

[e]Calculated taking into account the probable overestimates of the values for PtdMEA and PtdDMEA.

[f]Percent in "total in EA derivatives" ÷ percent in "insoluble pellet."

is provided on the next-to-the-bottom line: "total in EA derivatives." These values are the sums of the ^3H contents of all compounds listed (except, of course, the "insoluble pellet"). To adjust these values for possible differences in the rates of growth of the cells, each was divided by the percent of ^3H in the insoluble pellet for that sample. (The latter should reflect chiefly the rate of increase in protein methionine, with lesser contributions from increases in methylated protein amino acids and in the methylated nucleotides of RNA and DNA.) These "adjusted relative rates" are displayed on the bottom line. For cells labeled at comparable times after splitting, as Cho supplementation was increased, these values decreased. For example, for cells labeled from day 6 to day 7, the "adjusted relative rate" values were 0.942, 0.775, 0.409 and 0.314 as Cho increased from 0 to 2100 μM.

Lack of metabolic degradation of Cho moieties. The decrease in the "adjusted relative rate" values as Cho supplementation increased (Table 4) could be due either to a decreased rate of utilization of methyls originating in methionine for methylation of EA derivatives (considered as a group), or to an increased rate of metabolic degradation of the base moieties of these EA derivatives. To distinguish these possibilities, cells growing with supplements of either 0 or 21 μM Cho were labeled for 24 hr with [*Me*-^{14}C]Cho, then chased by transfer to nonradioactive medium and growth for an additional 24 hr. Degradation of Cho moieties would result in a loss of the ^{14}C content of total Cho moieties during this chase. To permit adjustment of the ^{14}C content of each sample for differences in the amounts of cells present during the labeling period, and correction for cell loss during the chase, during the initial labeling with [*Me*-^{14}C]Cho each batch of cells was labeled also with [^{3}H]thymidine. The latter was expected to accumulate in cellular DNA in proportion to the number of cells present. Indeed, in all samples more than 99.2% of the total cellular ^3H was found in the washed methanol/chloroform insoluble pellet. During the chase there was a greater loss of cellular ^3H in the culture growing without supplemental Cho (to 66.3% of the initial value) than in that growing in 21 μM Cho (to 84.3%). This is consistent with the expected enhanced loss of cells in the absence of Cho. For each culture there was a decrease of ^{14}C in the combined Cho moieties (i.e. those of Cho, P-Cho, PtdCho and sphingomyelin, together) which exactly paralleled the decrease of ^3H, indicating there was little or no metabolic degradation of Cho and that loss of ^{14}C in these moieties was that expected from loss of cells only.

Labeling of Cho-deprived cells for 10 min with L-[methyl-^{3}*H*]*methionine.* Hepatoma cells were subcultured into media supplemented with either 0 or 21 μM Cho and, during day 6, were labeled for 10 min with L-[*Me*-^{3}H]methionine of high specific radioactivity (Table 5). As expected, compared with the results of 24 hr labeling of similar cells (Table 4), more of the ^3H was found in the methanol/water soluble fractions (presumed methionine) and less in the insoluble pellets and in the phosphatidyl compounds. Nevertheless, each of the latter was present in readily detectable amounts. Relative to PtdCho, both PtdMEA and PtdDMEA were more highly labeled than had been the case in the 24 hr experiments. Again, no significant amount of ^3H was found in any methylated derivative of P-EA. Upper limits for the ^3H contents of the combined methylated phospho-bases as a percent of the ^3H contents of the combined methylated phosphatidyl-bases were 1.1% and 0.9% in the two cell samples.

TABLE 5

Distribution of ^3H in Hepatoma Cells After a 10 Min Labeling with L-[*Methyl*-^{3}H]Methionine[a]

Compound or fraction	Choline added to medium, μM	
	0	21
	Radioactivity, % of total in cells	
Methanol/water soluble	78.6	68.4
Insoluble pellet	20.2	30.4
PtdMEA	0.0080[b]	0.0058[b]
PtdDMEA	0.0239[b]	0.0216[b]
PtdCho	0.0555[b]	0.0291[b]
P-MEA + P-DMEA + P-Cho	<0.00095[c]	<0.00053[c]

[a]The H-4 rat hepatoma cells were pregrown and subcultured in triplicate for labeling as described in the footnote to Table 4 for cells with 0 and 21 μM Cho. Labeling was by a 10 min exposure to L-[*Me*-^{3}H]methionine (approximately 13.5 × 10^6 dpm/ml medium) during day 6. After harvest, the extracts from the triplicate sets of cells were combined and analyzed. Total cellular ^3H was 5.64 and 8.23 × 10^6 dpm for cells grown at 0 and 21 μM Cho, respectively.

[b]These compounds were represented by well-defined peaks of ^3H accompanying the authentic ^{14}C internal markers during the final purification step (chromatography with solvent A).

[c]No ^3H was detected accompanying the authentic ^{14}C-labeled internal markers during the final purification step (pH 7.0 electrophoresis of derived free methylated bases).

DISCUSSION

Lack of a role for phospho-base methylation in in situ *liver.* Rats were injected with L-[*Me*-^{3}H]methionine of high specific radioactivity, and the presence of ^3H-labeled methylated derivatives of P-EA was sought in the liver. Following intraperitoneal administration, a labeling period of some 10 min was used because preliminary experiments had demonstrated that accumulation of radio-label in the liver was virtually linear for this long. Thus, ample L-[*Me*-^{3}H]methionine should be present continuously, and any early intermediate would be expected to be labeled relatively strongly. The intraportal injection closely mimicked the experimental approach of Salerno and Beeler (10) and utilized a time which, according to the results of these authors, should be optimal for detection of ^3H in P-Cho. The continuous mode of labeling had the further advantage—if methylation of both phospho- and phospatidyl-bases had been found to contribute to PtdCho synthesis, the relative amounts of radioactivity in the phospho-, as compared to the phosphatidyl-, ethanolamine derivatives at short labeling time would have provided an estimate of the relative contributions of these two pathways. In these experiments no significant amount of ^3H was found in either P-MEA or P-DMEA. A very small amount of [^{3}H]P-Cho was detected after the intraperitoneal injection, but not after the intraportal injection. In both experiments, not only was [^{3}H]PtdCho a major product, but [^{3}H]PtdMEA and

[^{3}H]PtdDMEA were also demonstrated, with relatively greater amounts of label in the latter partially methylated compounds at the shorter period. Together, these findings are entirely consistent with the conclusion that the methylations involved in PtdCho synthesis occur at the level of phosphatidyl-bases (4,5), and offer no support for a significant role in rat liver of a putative pathway involving methylation of phospho-bases. The reason(s) for this striking difference between the results of Salerno and Beeler (10) and our present results are uncertain. The methods employed during the present investigation were chosen because of their demonstrated capacity to purify each EA derivative and to accurately quantitate its content of radioactivity (13-15,17). With respect to the crucial methylated phospho-bases, during the present experiments a number of radioactive impurities accompanied these compounds during purification up to the phosphatase treatment. The latter step, which removed the base moieties from compounds negatively charged at neutral pH and converted them to compounds positively charged, with subsequent reelectrophoresis, provided a very powerful tool to remove such radiolabeled impurities. This sequence was not included among the purification steps used by Salerno and Beeler (10). It seems at least possible that the samples of P-Cho studied by these authors were contaminated by ^{3}H-containing impurities.

Regulation by Cho. Administration of exogenous Cho markedly down-regulates the rate of methylation of P-EA in plants (23,24). However, the role of Cho in regulating the methylations involved in PtdCho synthesis in mammalian systems has remained controversial (8,25): with intact rats, several investigators observed that Cho deficiency was accompanied by an increase in the rate of incorporation of radiolabel originating in the methyl group of methionine into PtdCho (26-30), whereas other investigators observed a decrease in this rate (31-33). Not only have the results of such experiments differed, but in general it has not been possible to allow for possible variations in pool sizes, specific radioactivities of precursor intermediates, and so forth, so that such measurements could be interpreted in terms of synthetic rates (8). During the present experiments with hepatoma cells, Cho deprivation was achieved by growth in a medium without added Cho and with serum freed of Cho by treatment with Dowex 50-NH_4^+. During growth in such a medium, the cells were shown to become depleted of Cho. The cellular concentration of PtdCho decreased and that of PtdEA increased (Table 3). Labeling with L-[*Me*-^{3}H]methionine was performed under conditions which permitted unambiguous interpretation of the results in terms of the true relative rates of methylation: (i) The cells were maintained in a relatively constant concentration of external L-methionine, thus avoiding overall methyl depletion. The latter condition in whole rats led to alterations in AdoMet and S-adenosylhomocysteine with accompanying perturbations in the rate of PtdCho biosynthesis (25). (ii) The labeling period was long enough so that cellular pools of soluble methionine and other metabolic intermediates should have come to isotopic equilibrium with the external methionine. Thus, even after 10 min labeling, some 20 to 30% of the ^{3}H in the methionine which had been taken up had already entered the insoluble pellet (Table 5), indicating a turnover time for cellular soluble methionine that is short relative to 24 hr. This conclusion is confirmed by the observation that after 24 hr labeling at least 95% of the ^{3}H taken up had entered the insoluble pellet (Table 4). (iii) It was possible to adjust for differences in the number of cells used in different experiments, and for differences in their growth rates by the use of "adjusted relative rates." Fortunately, these adjustments were very small (Table 4). (iv) It was demonstrated experimentally that during either Cho depletion or moderate repletion, no significant metabolic degradation of Cho moieties took place in the hepatoma cells. Thus differences in accumulation of ^{3}H in Cho moieties were due to differences in synthetic rates, rather than in rates of degradation.

The results of these experiments provide the first definitive demonstration in a mammalian system that Cho-deprivation up-regulates the relative rate of utilization of methyl groups originating in methionine for PtdCho biosynthesis (Table 4). An increase of approximately three-fold was observed over cells which were completely Cho-repleted. The observed increment in the concentration of PtdEA (Table 3) may contribute to, possibly even wholly account for, this increase. Under certain conditions a rise in the concentration of the substrate, PtdEA, leads to a disproportionally great increase in the rate of its methylation by PtdEA *N*-methyltransferase, the rate which is limiting in the overall pathway to PtdCho (34). An additional possibility is that there is derepression of PtdEA *N*-methyltransferase. The major portion of the increase in rate of PtdCho synthesis occurred as medium Cho rose from 0 to 21 μM. Over this range, cellular P-Cho increased strikingly, but free Cho increased only slightly (Table 2). P-Cho thus becomes a plausible candidate for the effector of any regulatory event(s) which occur. Again in this system, even during Cho deprivation, synthesis of PtdCho appeared to take place solely by methylation of phosphatidyl-bases; no evidence was found to indicate any role for methylation of phospho-bases (Table 5).

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The Effect of Alfalfa-Corn Diets on Cholesterol Metabolism and Gallstones in Prairie Dogs

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Cholesterol gallstones were present in prairie dogs fed alfalfa plus corn with and without exogenous cholesterol (0.4%). The diets fed to the animals for eight weeks contained alfalfa plus corn in fixed proportions of 50:50, 85:15 and 15:85 (w/w). At sacrifice, all animals were healthy but had not gained weight; no deaths occurred during the experiment. Cholesterol gallstones were present in all groups. In the absence of exogenous cholesterol, the highest stone incidence was found in the animals which received the lowest fiber (highest corn) diets (alfalfa plus corn, 50:50, 67%; alfalfa plus corn, 15:85, 83%). Cholesterol gallstone incidence was 100% when exogenous cholesterol was added to the alfalfa plus corn diets (50:50 and 15:85). No pigment gallstones were detected in any animal. Liver and plasma cholesterol concentrations were highest in the animals receiving alfalfa plus corn (15:85) plus 0.4% cholesterol (4.29 mg/g, and 356 mg/dl, respectively). These values were lowest in animals receiving 85% alfalfa plus 15% corn without cholesterol (2.19 mg/g and 88 mg/dl, respectively). Lithogenic indices were below 1.00 in all groups. Biliary bile acids were mainly amidates of cholic acid and chenodeoxycholic acid, with the former predominating. Thus, gallstones can be formed in prairie dogs in the absence of exogenous cholesterol; gallstone incidence is reduced by dietary fiber.
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Animal models have been employed to study the etiology and course of various diseases of man. Many models of cholesterol gallstone disease exist, for example, the hamster (1–4), prairie dog (5–12), mouse (13,14), tupaia (15,16) and squirrel monkey (17). Initially, these models were used in attempts to gain an understanding of the mechanisms involved in the formation of lithogenic bile, crystal aggregation, nucleation/antinucleation and stone dissolution. More recently, much attention has focused on the prevention of gallstone recurrence (18,19). This is due to the fact that new treatment modalities, such as contact dissolution with methyl *tert*-butyl ether (20) and extracorporeal shockwave lithotripsy (21), leave the patients with intact, functioning gallbladders. Under these conditions, gallstone recurrence rates have been estimated to amount to 10% per year, similar to those found after medical therapy (19). Consequently, it may be assumed that animal models will remain important for studies of gallstone prevention as well as recurrence.

Our laboratory has worked extensively with the prairie dog model of cholesterol cholelithiasis. We have shown that hydrophobic and hydrophilic bile acids, both natural and synthetic, prevent gallstones when administered in a lithogenic diet (8,9). The model was reasonably reproducible as soon as it was understood that the previous dietary intake of the animals affected the incidence of gallstones (5). [We have further found that dietary and seasonal variations may affect stone incidence, not only in the prairie dog, but also in the hamster (unpublished).] We examined the effect of alfalfa-corn diets on cholesterol gallstone formation with the expectation of developing a new prairie dog model of cholelithiasis requiring no dietary cholesterol. We also thought it important to determine whether the lithogenic stimulus was due to the alfalfa (mainly fiber), corn (whole grain) or a combination of the two.

This manuscript describes the effects of altering the proportions of alfalfa plus corn (with and without dietary cholesterol) on cholesterol metabolism and gallstones in prairie dogs. The data suggest that increasing the dietary fiber (alfalfa) reduces the incidence of gallstones in this model.

MATERIALS AND METHODS

Animals and diets. Thirty-six male prairie dogs (Cynomys ludovicianus) were obtained from R-Zoo, Neshboro, WI. The animals were received when their average weight was between 800 and 900 g. They were quarantined for two weeks, during which time they were fed a cholesterol-free, pelleted alfalfa plus corn diet (50:50 by wt). The animals were divided at random into six experimental groups (six animals/group) as follows: 1) alfalfa (A) + corn (C) (50:50); 2) A + C (50:50) + 0.4% cholesterol (CH); 3) A + C (85:15); 4) A + C (85:15) + 0.4% CH; 5) A + C (15:85); and 6) A + C (15:85) + 0.4% CH. All animals were fed the pelleted diets *ad libitum* for eight weeks and kept on an alternating 12-hr light/dark cycle. The animals were fasted for 24 hr prior to sacrifice to assure adequate quantities of bile in the gallbladder for determination of biliary lipids. The prairie dogs were anesthetized with 100–120 mg of ketamine hydrochloride (Bristol Labs, Syracuse, NY) and 20 mg of xylazine (Haver Lockhart, Shawnee, KA), and killed by exsanguination. Blood was obtained for serum cholesterol determination. The gallbladder was examined visually to determine the presence or absence of cholesterol gallstones (usually 1–2 mm). The gallbladder was removed and fresh bile aspirated with a Hamilton syringe. Gallbladder bile was removed and immediately examined by polarized light microscopy (Olympus MCHAP microscope, Olympus Corp., Lake Success, NY) to determine the presence of cholesterol crystals. The contents of the gallbladder (gallstones) were crushed and examined by polarized light microscopy to verify the presence of cholesterol. The liver was excised, weighed, and aliquots were taken for determination of cholesterol concentration.

Gallstone analysis by Fourier Transform Infrared spectrometry. The gallstones were rinsed with saline, followed

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Abbreviations: FTIR, Fourier Transform Infrared; GLC, gas liquid chromatography; GLC-MS, gas liquid chromatography-mass spectrometry; PEDR, Perkin-Elmer diffuse reflectance.

by distilled water. They were dried in a vacuum oven desiccator at 80°C for 3-4 days. Most of the stones looked white, but a few had a slightly yellowish appearance. The dried stones were ground thoroughly with KBr to a fine powder. A Perkin-Elmer diffuse reflectance (PEDR) accessory (Perkin-Elmer, Norwalk, CT) attached to a Perkin-Elmer 1710 Fourier Transform Infrared (FTIR) spectrometer was used to analyze the sample by infrared spectrometry. For diffuse reflectance, samples wre diluted to 5% or less in KBr; particle size was 10-20 microns to minimize peak distortions.

Biliary lipids. Gallbladder bile was immediately centrifuged at 2000 × *g* for 10 min. Aliquots (50 μl) were obtained for determination of cholesterol, phospholipids and bile acids (6). Cholesterol saturation of bile (lithogenic index) was determined using the methods of Carey (22) and Kuroki *et al.* (23).

Gas liquid chromatography (GLC) and gas liquid chromatography-mass spectrometry (GLC-MS). Quantitative analysis of cholesterol in liver, plasma and bile was carried out by GLC (Hewlett-Packard model 5830A) as described earlier (6). Biliary bile acids in an aliquot of fresh bile were analyzed as the methyl ester acetates using a SPB-5 capillary column (25 m, Supelco Inc., Bellefonte, PA). The conditions for this analysis were as follows: injector temp, 270°C; detector, 270°C; auxiliary, 270°C; temp 1, 200°C for 2 min; and temp 2, 270°C for 25 min (rate, 10°/min). The identity of bile acid derivatives was confirmed by GLC-MS on a Hewlett-Packard 5992B mass spectrometer using conditions described earlier (6).

Reference compounds. 5α-Cholestane (Alltech Inc., Deerfield, IL) was used as an internal standard for quantitation of cholesterol. 3α,7α-Dihydroxy-12-keto-5β-cholanoic acid (Steraloids, Wilton, NH) was the internal standard for bile acid analysis.

Statistical calculations. All data are reported as the mean ± SEM. Analysis of variance was used to obtain the F statistic. Students' unpaired *t*-test was used for values where the F statistic was significant (24). Fisher's exact test was used to determine the significance of gallstone incidence between groups.

RESULTS

All of the prairie dogs used in this experiment were bred in captivity. They were fed alfalfa plus corn *ad libitum* from weaning until arrival at our animal facility where they were kept during a two-week quarantine period. The composition of the various diets is shown in Table 1. The average initial weight for the animals was similar (Table 2). Food intake was similar for all groups and remained fairly constant throughout the experiment. The animals appeared healthy, yet animals in all groups lost weight, with the largest losses being in groups 3-6.

The animals were fasted for 24 hr prior to sacrifice, at which time the gallbladder mucosa and gallbladder bile were examined for the presence of cholesterol gallstones and cholesterol crystals. Previously reported data for animals fed Purina chow is presented for comparison (10). Incidence of gallstones and crystals is summarized in Table 3. Cholesterol gallstones were present in all experimental groups, i.e., in diets with and without exogenous cholesterol. In animals given no dietary cholesterol (groups 1, 3 and 5), gallstone incidence increased with increasing amounts of corn in the diet. The highest gallstone incidence (83%) was obtained for group 5 (alfalfa

TABLE 1

Approximate Composition of Experimental Diets[a]

Diet	Component (%)						
	Carbohydrate	Protein	Fat	Fiber	Vitamin	Mineral	Other
Alfalfa + corn (50:50)	55	14	2	15	1	3	10
Alfalfa + corn (85:15)	45	16	2	24	1	2	10
Alfalfa + corn (15:85)	66	11	3	6	1	4	9
Purina chow	45	23	5	5			22

[a]Analyses of the ingredients of the Alfalfa/corn diets supplied by Dr. R. Rose, Teklad, Madison, WI.

TABLE 2

Data on Prairie Dogs[a]

Group number	Number of animals	Diet	Average initial weight (g)	Average final weight (wt. loss) (g)	Food intake (g/day)
1	6	Alfalfa + corn (50:50)	875 ± 47	846 ± 22 (−29)	35 ± 1
2	6	Alfalfa + corn (50:50) + 0.4% cholesterol	878 ± 51	858 ± 37 (−20)	39 ± 1
3	6	Alfalfa + corn (85:15)	886 ± 29	806 ± 21 (−80)	39 ± 1
4	6	Alfalfa + corn (85:15) + 0.4% cholesterol	857 ± 38	745 ± 38 (−112)	37 ± 1
5	6	Alfalfa + corn (15:85)	971 ± 39	909 ± 66 (−62)	38 ± 2
6	6	Alfalfa + corn (15:85) + 0.4% cholesterol	848 ± 37	748 ± 43 (−100)	38 ± 1

[a]Animals were divided into six experimental groups and fed the experimental diets for eight weeks. See Experimental section for details. Numbers are average ± SEM.

plus corn, 15:85, w/w), while the lowest incidence (33%) was obtained for animals in group 3 (alfalfa plus corn, 85:15, w/w). In groups fed exogenous cholesterol, gallstone incidence was 100% when the amount of alfalfa was 15% or 50%. However, gallstone incidence was reduced to only 33% when alfalfa comprised 85% of the diet (group 4). The incidence of cholesterol crystals was similar to that of the gallstones. Animals fed chow had no stones or crystals. No pigment gallstones were detected in any experimental animal.

The infrared spectra of the gallstones showed that they contained 95% or more of pure cholesterol. There was small absorbance in the 1700–1500 cm^{-1} region, which was ascribed to bilirubinate and protein.

Table 4 summarizes the cholesterol concentrations in liver, plasma and bile. Liver and plasma cholesterol levels were highest in the group that received alfalfa plus corn (15:85) plus 0.4% cholesterol (group 6; 4.29 mg/g and 356 mg/dl, respectively). The lowest values were present in the animals receiving the highest amount of alfalfa with and without 0.4% cholesterol (1.97 mg/g and 88 mg/dl in group 4, and 2.19 mg/g and 83 mg/dl in group 3, respectively). The highest biliary cholesterol level was present in animals receiving 85% alfalfa plus 15% corn plus 0.4% cholesterol (group 4, 3.40 mg/ml). Animals fed chow had the lowest values for biliary cholesterol.

The results summarized in Table 5 show the effect of the different alfalfa plus corn diets, with or without cholesterol, on the biliary lipids and cholesterol saturation of bile (lithogenic indices). Although a majority of animals had cholesterol gallstones, none of the groups had lithogenic indices above unity. The mole % cholesterol ranged from 2.45 (group 1) to 3.35 (group 3). The mole % phospholipid averaged 2–3 times that of cholesterol. Total lipid (g/dl) levels were similar in all alfalfa-corn groups (range 10.29–14.82), but were lower on chow (6.6 g/dl).

The biliary bile acid composition was analyzed at sacrifice (Table 6). In contrast to previous studies with high cholesterol diets (5), exogenous cholesterol did not alter the chenodeoxycholic acid/cholic acid ratio in either group 2 or group 4. In group 6, the ratio was significantly higher than in groups 1–4. The amounts of deoxycholic acid and lithocholic acid remained low, ranging from 1.3–3.4% and 0.2–1.4%, respectively.

DISCUSSION

It was recently suggested that (in the absence of excess dietary cholesterol) suppression of cholesterol synthesis could lead to gallstone dissolution (25). We thought it of interest to develop a new animal model of cholesterol cholelithiasis which did not require dietary cholesterol. One such model has been known since 1951, namely, the hamster model of Dam. Cholesterol gallstones in these hamsters were induced using fat-free diets containing

TABLE 3

Incidence of Gallstones and Cholesterol Crystals in Prairie Dogs at Sacrifice[a]

Group number	Number of animals	Diet	Cholesterol gallstones		Cholesterol crystals	
			Animals	%	Animals	%
1	6	Alfalfa + corn (50:50)	4/6	67	5/6	83
2	6	Alfalfa + corn (50:50) + 0.4% cholesterol	6/6[b]	100	6/6	100
3	6	Alfalfa + corn (85:15)	2/6	33	3/6	50
4	6	Alfalfa + corn (85:15) + 0.4% cholesterol	2/6	33	3/6	50
5	6	Alfalfa + corn (15:85)	5/6	83	5/6	83
6	6	Alfalfa + corn (15:85) + 0.4% cholesterol	6/6[b]	100	6/6	100
7[c]	8	Purina chow	0/8[d]	0	0/8[e]	0

[a] See Table 2 for experimental details.

[b] Differs from group 4, $p < 0.03$ by chi square test.

[c] Previously reported (10).

[d,e] Differs from groups 1, 2, 5, 6, $p < 0.01$ by chi square test.

TABLE 4

Cholesterol Concentration in Prairie Dogs[a]

Group number	Number of animals	Diet	Liver (mg/g)	Plasma (mg/dl)	Bile (mg/ml)
1	6	Alfalfa + corn (50:50)	2.30 ± 0.12	102 ± 8	2.00 ± 0.16
2	6	Alfalfa + corn (50:50) + 0.4% cholesterol	2.51 ± 0.24	151 ± 38	2.49 ± 0.32
3	6	Alfalfa + corn (85:15)	2.19 ± 0.11	83 ± 4	2.88 ± 0.65
4	6	Alfalfa + corn (85:15) + 0.4% cholesterol	1.97 ± 0.07	88 ± 4	3.40 ± 1.41
5	6	Alfalfa + corn (15:85)	2.83 ± 0.15	134 ± 21	2.52 ± 0.56
6	6	Alfalfa + corn (15:85) + 0.4% cholesterol	4.29 ± 0.17	356 ± 122[b]	2.96 ± 0.30
7[c]	8	Purina chow	2.90 ± 0.20	180 ± 50	0.90 ± 0.20[d]

[a] See Table 2 for experimental details; numbers are average ± SEM.

[b] Differs from group 4, $p < 0.01$.

[c] Previously reported (10).

[d] Differs from groups 1–6, $p < 0.01$.

glucose (3,4). The absence of essential fatty acids greatly increased hepatic cholesterol secretion, and this was associated with a corresponding increase in hepatic cholesterol synthesis. The Dam hamster model has decreased in popularity because the essential fatty acid deficiency resulted in cirrhotic liver damage and, in many cases, death from diarrhea induced by intestinal infections. In addition, the dihydroxy bile acids, CDCA and UDCA, which are employed in gallstone dissolution therapy in humans, are completely ineffective in this model (3,4).

The possibility of producing gallstones in prairie dogs fed a cholesterol-free diet was based upon a previous study dealing with dietary conditioning and gallstone induction (5). It was found that in a group of four prairie dogs, maintained with only alfalfa and corn *ad libitum*, one animal had cholesterol gallstones and three had biliary cholesterol crystals at the end of an eight-month feeding period. Hepatic microsomal HMG-CoA reductase was greatly elevated in this group of animals (in comparison with chow controls, which do not develop gallstones spotaneously), suggesting increased cholesterol synthesis/secretion (5). In the eight-month study mentioned above, a loose mixture of alfalfa and corn was fed, although the contribution of each component to the total food intake was unknown. In the present experiment with various proportions of alfalfa and corn, the dietary components were ground and pelleted, so that the contribution of each component to the diet could be predetermined. We fed the three experimental diets (in which the proportion of corn to alfalfa was 15, 50 or 85%) without cholesterol in order to develop the cholesterol-free lithogenic diet. We also added cholesterol (0.4%) in order to determine whether corn or alfalfa provided the more effective lithogenic stimulus in the presence of dietary cholesterol. The results obtained indicate that the diets with the greatest proportion of alfalfa (fiber) (85%) were the least lithogenic, both with and without exogenous cholesterol.

All groups of animals lost weight during the eight-week feeding period. The weight loss could not be correlated with changes in food intake (which remained quite constant throughout the experiment); moreover, all of the animals appeared healthy and there was no diarrhea. It had previously been reported that rats maintained with alfalfa diets gained less weight than animals receiving cellulose (26,27). Although the weight loss might be ascribable to an unidentified dietary insufficiency, we think it more likely that we are dealing with a seasonal variation in food intake. We recently found that prairie dogs, from the same supplier and maintained with a 50% corn plus 50% alfalfa diet plus cholesterol for two months had a similar food intake, but gained about 200–300 g per animal (5). We have further found that dietary and

TABLE 5

Effect of Diet on Lipid Composition in Prairie Dogs[a]

Group number	Number of animals	Diet	Mole % biliary lipid			Total lipid (g/dl)	Lithogenic index
			Cholesterol	Phospholipids	Bile acids		
1	6	Alfalfa + corn (50:50)	2.45 ± 0.30	8.25 ± 1.32	89.30 ± 1.46	11.18 ± 1.07	0.66 ± 0.09
2	6	Alfalfa + corn (50:50) + 0.4% cholesterol	3.17 ± 0.25	8.45 ± 0.65	88.40 ± 0.78	10.29 ± 0.79	0.83 ± 0.06
3	6	Alfalfa + corn (85:15)	3.35 ± 0.42	11.92 ± 1.55	84.72 ± 1.91	11.13 ± 2.26	0.71 ± 0.06
4	6	Alfalfa + corn (85:15) + 0.4% cholesterol	2.63 ± 0.41	7.41 ± 0.68	89.96 ± 1.07	14.82 ± 4.42	0.68 ± 0.06
5	6	Alfalfa + corn (15:85)	2.93 ± 0.37	10.41 ± 1.13	86.66 ± 1.45	11.17 ± 1.42	0.66 ± 0.05
6	6	Alfalfa + corn (15:85) + 0.4% cholesterol	2.94 ± 0.43	10.14 ± 1.52	86.91 ± 1.94	13.53 ± 0.58	0.65 ± 0.05
7[b]	8	Purina chow	1.70 ± 0.40[c,d]	3.50 ± 0.50[e]	94.80 ± 0.80[e]	6.60 ± 0.90[e]	0.67 ± 0.06

[a]See Table 2 for experimental details; numbers are average ± SEM.

[b]Previously reported (10).

[c]Differs from groups 5 and 6, $p < 0.03$.

[d]Differs from groups 2 and 6, $p < 0.01$.

[e]Differs from groups 1–6, $p < 0.01$.

TABLE 6

Bile Acid Composition in Prairie Dogs at Sacrifice (%)[a]

Group number	Number of animals	Diet	Cholic acid	Chenodeoxycholic acid	Deoxycholic acid	Others
1	6	Alfalfa + corn (50:50)	87.2 ± 3.4	7.0 ± 1.9	3.4 ± 1.9	2.4 ± 2.2
2	6	Alfalfa + corn (50:50) + 0.4% cholesterol	88.3 ± 1.4	9.0 ± 1.7	2.5 ± 0.1	0.2 ± 0.0
3	6	Alfalfa + corn (85:15)	91.7 ± 1.1	5.6 ± 0.5	2.2 ± 0.7	0.5 ± 0.2
4	6	Alfalfa + corn (85:15) + 0.4% cholesterol	93.1 ± 1.5	3.8 ± 1.0	2.3 ± 0.3	0.9 ± 0.8
5	6	Alfalfa + corn (15:85)	84.1 ± 2.2	13.3 ± 2.1	2.4 ± 0.6	2.3 ± 0.1
6	6	Alfalfa + corn (15:85) + 0.4% cholesterol	66.5 ± 13.5	31.7 ± 14.4	1.3 ± 0.8	0.5 ± 0.4
7[b]	8	Purina chow	89.0 ± 2.0	7.9 ± 1.0	3.0 ± 0.7	0.1 ± 0.1

[a]See Table 2 for experimental details; numbers are average ± SEM.

[b]Previously reported (10).

seasonal variations interact to affect stone incidence, not only in the prairie dog but also in the hamster (unpublished observation).

Cholesterol gallstones and biliary cholesterol crystals were found in all groups of animals after eight weeks, regardless of whether cholesterol was present in the diet or not. The lowest incidence of cholesterol gallstones was obtained with the diet of the higher fiber content (85% alfalfa). Presumably, the fiber interferes differentially with the absorption of bile acids and cholesterol from the intestine (28-31). Several parameters of cholesterol metabolism were measured at sacrifice. The cholesterol saturation of all biles was below 100%, on the average; these values do not enable us to arrive at a definite conclusion as to why the diet highest in fiber produced the lowest incidence of gallstones. It should be mentioned that certain epidemiological studies suggested that dietary fiber might be useful for gallstone prevention in certain rural populations (30). Gallstones in the presence of lithogenic indices below 1.0 have been reported in other species (32,33). In order for gallstones and crystals to form, the bile may have been supersaturated with cholesterol at some time(s) during the feeding period. Alternatively, cholesterol may nucleate from bile via a vesicular mechanism (34). An experiment in which the bile is sampled periodically is needed to clarify this point. Similarly, it would be of great interest to find out whether the composition or concentration of nucleating and antinucleating factors are altered by the alfalfa/corn diet (as opposed to chow or semipurified diets). We were unable to demonstrate the formation of pigment stones (35) or biliary sludge in the gallbladders of prairie dogs maintained on alfalfa plus corn diets or any of the other diets studied in our laboratory (36). This may be due to differences in age and/or genetic variability of the animals.

The tissue cholesterol concentrations summarized in Table 4 are similar to the values found in prairie dogs fed rodent chow. There were several exceptions—group 6 [alfalfa plus corn (15:85) plus 0.4% cholesterol], with elevated levels of liver and plasma cholesterol; and groups 3 and 4, with reduced levels of plasma cholesterol. The biliary cholesterol and total biliary lipids (Table 5) were increased with alfalfa plus corn diets, compared to chow-fed controls studied earlier (10). Thus, the alfalfa plus corn diets may interfere with cholesterol absorption which would be in accord with the elevated cholesterol synthesis with this type of diet, observed previously (5).

In the prairie dog, as well as in the hamster, the biliary bile acid composition (particularly the cholic acid/chenodeoxycholic acid ratio) usually changes with increasing cholesterol content of the diet (1,5). Increasing the dietary cholesterol apparently inhibits the 7α-hydroxy-4-cholesten-3-one 12α-hydroxylase so that the CA/CDCA ratio decreases (37). In the present study, this effect of exogenous cholesterol was much less apparent. In all groups fed alfalfa and corn with or without cholesterol (except group 6), the bile contained mainly taurocholate; taurochenodeoxycholate ranged from 5.6 to 13.3%. (In previous experiments with the semipurified lithogenic diet, the percentage of CDCA was as high as 45%). In group 6, which had the lowest concentration of dietary fiber, CDCA was 31.5% and CA 66.5%. In all groups, the proportions of secondary bile acids were low, presumably because the 7α-hydroxylase remained active (38).

In summary, we report a new regimen to form gallstones in prairie dogs without exogenous cholesterol. Variations in amounts of dietary fiber appear to reduce gallstone incidence in an almost linear fashion. Cholesterol levels in tissues also are reduced when increased amounts of fiber are added to the diet. Further studies to elucidate the mechanism of action of these dietary manipulations are under way.

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Glyceroamidothiophosphates of Cholecalciferol (Vitamin D_3)

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The hexamethyltriamide of phosphorous acid activated by the addition of iodine at the optimum molar ratio 1.05:0.05 was used as a phosphorylating reagent to synthesize cholecalciferyl-3-*O*-(*N,N*-dimethylamido)thiophosphate derivatives of 1,3-benzylidene-*rac*-glycerol, 1,2-isopropylidene-*rac*-glycerol, 1,3-dioleoyl-*rac*-glycerol, and 1,2-dioleoyl-*rac*-glycerol in a one-pot procedure in high overall yields (81–86%). The compounds represent new model types of phospholipid structures which, in addition to glycerol and a steroid fragment, contain a biologically important linkage other than an oxygen-phosphorus bond.
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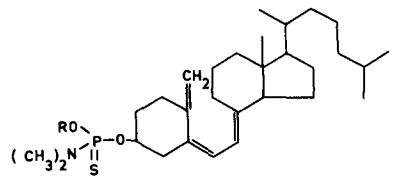

FIG. 1. R = 1,3-benzylidene-*rac*-glyceryl; 1,2-isopropylidene-*rac*-glyceryl; 1,3-dioleoyl-*rac*-glyceryl; 1,2-dioleoyl-*rac*-glyceryl.

As constituents of plasma membranes, some lipids (steroids, tocopherols, etc.) have been shown to perform regulatory functions as essential as those of phospholipids (1–4). The occurrence of phosphatidylsteroids (steroid and diglyceride joined through a phosphate bridge) in biological systems has been suggested (5).

Because present purification techniques still do not permit complete resolution of molecular spieces of natural mixtures of compounds, phospholipids of defined structures can often only be obtained by synthetic routes. Recent advances in the chemistry of naturally occurring and model phospholipids have been stimulated by their need in biochemical and membrane research, in the preparation of new drugs, and by their usefulness as effective carriers of antibiotics, antigens, enzymes and hormones (6–8). The synthesis of phospholipids which contain another biologically active lipid (steroid, tocopherol, etc.) as a second structural component (instead of an amino alcohol, carbohydrate, etc.) seems to be a reasonable goal both from a physicochemical and pharmacological point of view (5,9–11). Glyceroamidothiophosphates containing a steroid moiety have so far not been described in literature.

This paper deals with the synthesis of cholecalciferyl-3-*O*-(*N,N*-dimethylamido)thiophosphate derivatives with the general formula shown in Figure 1. These compounds represent a new phospholipid model structure in which a biologically important element other than oxygen, is bonded to phosphorus, in addition to the phosphagen fragment.

EXPERIMENTAL

The *tris*-(*N,N*-dimethyl)amide of phosphorous acid 1 was prepared and freshly distilled as described (12). Cholecalciferol **a** and 1,2-isopropylidene-*rac*-glycerol **c** had a purity of over 98% (Fluka). 1,3-Benzylidene-*rac*-glycerol **b** was prepared according to established procedures (13). 1,3-Dioleoyl-*rac*-glycerol *d* and 1,2-dioleoyl-*rac*-glycerol *e* were prepared as described (14). All other reagents were of a purity better than 98% (Janssen). Benzene (Merck) was dried over sodium and freshly distilled. Reaction conditions were kept strictly anhydrous.

Analytical thin-layer chromatography (TLC) on precoated aluminum sheets of silica gel $60F_{254}$ (Merck) with chloroform as mobile phase was routinely used for monitoring all processes. High performance liquid chromatography (HPLC) was done (LDC Consta Metric III system, equipped with LKB 2142 Refractive Index Detector) using a NUCLEOSIL silica gel column (500 × 10 mm) and n-heptane/ethyl acetate 8:2 (v/v) as mobile phase.

^{13}C NMR spectra were recorded on a Varian XL-300 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); a positive sign is downfield from the standard. IR spectra were recorded on a Perkin-Elmer 298 spectrometer. Peak positions are reported in cm^{-1}. For 1aS, 1abS,1acS and 1adS, satisfactory microanalyses were obtained: C, ± 0.23; H, ± 0.11; N, ± 0.18; P, ± 0.12.

Cholecalciferyl-3-O-bis *(N,N-dimethylamido)thiophosphate, 1aS.* 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 75°C in a stream of argon for about 15 min until the precipitate dissolved. The solution was cooled to room temperature (20–25°C), cholecalciferol (*a*; 0.769 g, 2.0 mmol) was added, and the reaction mixture was kept under these conditions for 3 hr to give *1a*. Then sulfur (0.067 g, 2.1 mmol) was added, and the mixture was stirred for 30 min. The solvent was removed under vacuum and the thiophosphate *1aS* (see Fig. 2) was isolated by HPLC in analytically pure form. Yield of *1aS*: 1.00 g (93%); n^{40} = 1.5194; Rf ($CHCl_3$ = 0.64; $C_{31}H_{55}N_2OPS$ (534.9). ^{13}C NMR-{^1H} ($CDCl_3$) δ 11.8, 11.9 ppm (each *s*, C-18), 22.4 (*s*, C-26), 22.7 (*s*, C-27), 72.5, 73.6 (each *d*, C-3, *J* = 4 Hz), 108.0, 112.4 (each *s*, C-19), 115.8, 117.4 (each *s*, C-7), 120.8, 122.4 (each *s*, C-6), 134.2, 134.3 (each *s*, C-8), 142.0, 144.3 (each *s*,

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Abbreviations: HPLC, high performance liquid chromatography; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography; TMS, tetramethylsilane.

C-5), 144.6, 148.9 (each *s*, C-10), cholecalciferyl-3-O-fragment; 36.8 (*t*, CH_3N, J = 4 Hz). ^{31}P NMR- {^1H} ($CDCl_3$ δ 81.6, 81.8 ppm (each *s*, 1:1). IR (KBr, film) ν 3030 (CH=), 1630 (C=C), 1025, 815 (PO-C, P-OC), 750 (P-N), 700 cm^{-1} (P=S).

1, 3-Benzylidene- rac *-glycero-2-O-(cholecalciferyl-3-O)-(N,N-dimethylamido)thiophosphate, 1abS, representative procedure).* Using cholecalciferol (**a**; 0.769 g, 2.0 mmol), the intermediate *1a* was prepared as described for *1aS*. Then 1,3-benzylidene-*rac*-glycerol (*b*; 0.360 g, 2.0 mmol) was added and the mixture was heated at 60°C for 2 hr. Transformation to the thiophosphate, *1abS*, was accomplished by reaction with sulfur (0.067 g, 2.1 mmol) at the temperature indicated for 5 min. The solvent was removed under vacuum, and the compound was isolated by HPLC in a pure form. Yield of *1abS*: 1.08 g (81%); n_D^{40} = 1.5236; Rf ($CHCl_3$) = 0.56; $C_{39}H_{60}NO_4PS$ (670.1). ^{13}C NMR-{^1H} ($CDCl_3$) δ 11.9 ppm (*s*, C-18), 22.5 (*s*, C-26), 22.7 (*s*, C-27), 75.3, 75.5 (each *d*, C-3, J = 6 Hz), 112.5, 112.6 (each *s*, C-19), 117.4 (*s*, C-7), 122.5, 122.6, (each *s*, C-6), 134.1, 134.3 (each *s*, C-8), 142.2, 142.3 (each *s*, C-5), 144.5, 144.6 (each *s*, C-10), cholecalciferyl-3-*O*-fragment; 68.7 (*d*, $OCH_2\underline{C}HOP$, J = 5 Hz), 69.8, 70.2 (each *t*, $O\underline{C}H_2CHOP$, J = 5 Hz), glycero-2-*O*-fragment; 101.2 (*d*, C-7, J = 4 Hz), 126.1 (*s*, C-2, C-6), 128.2 (*s*, C-3, C-5), 129.0 (*s*, C-4), 138.0 (*m*, C-1), 1,3-benzylidene-fragment; 37.3 (*t*, CH_3N, J = 4 Hz). ^{31}P NMR-{^1H} ($CDCl_3$) δ 75.5, 75.7 ppm (each *s*, 1:1). IR (KBr, film) ν 3030 (CH=), 1640 (C=C), 1140, 1080 (COC-Aryl), 760 (P-N), 695 cm^{-1} (P=S).

1, 2-Isopropylidene- rac *-glycero-3-O-(cholecalciferyl-3-O)-(N,N-dimethyl-amido)thiophosphate,* 1acS. Using cholecalciferol (*a*; 0.769 g, 2.0 mmol) and 1,2-isopropylidene-*rac*-glycerol (*c*; 0.264 g, 2.0 mmol), the derivative *1acS* was synthesized and then purified in the same way as described for *1abS*. Yield of *1acS*: 1.05 g (85%); n_D^{40} = 1.5168; Rf ($CHCl_3$) = 0.61 $C_{35}H_{60}NO_4PS$ (622.0). ^{13}C NMR-{^1H} ($CDCl_3$) δ 11.9, 12.0 ppm (each *s*, C-18), 22.5 (*s*, C-26), 22.8 (*s*, C-27), 74.7, 75.5 (*m*, C-3), 108.2, 112.6 (each *m*, C-19), 115.8, 117.4 (each *s*, C-7), 121.1, 122.5 (each *m*, C-6), cholecalciferyl-3-*O*-fragment; 25.3, 26.7 (each *s*, *gem*-$\underline{C}H_3C$), 66.2 (*m*, $O\underline{C}H_2CHO$-), 66.4 (*m*, $OCH\underline{C}H_2OP$), 74.2 (*m*, $O\underline{C}HCH_2OP$), 109.5 (*d*, $CH_3\underline{C}$, J = 6 Hz), 1,2-isopropylidene-*rac*-glycero-3-*O*-fragment; 37.2 (*t*, CH_3N, J = 4 Hz). ^{31}P NMR-{^1H} ($CDCl_3$) δ 76.6 ppm (*m*). IR (KBr, film) ν 3030 (CH=), 1640 (C=C), 1360, 1370 (gem-CH_3), 1050, 820 (PO-C, P-OC), 750 (P-N), 720 cm^{-1} (P=S).

1, 3-Dioleoyl- rac- *glycero-2-O-(cholecalciferyl -3-O-)-(N,N-dimethylamido)thiophosphate,* 1adS. Using cholecalciferol (**a**; 0.769 g, 2.0 mmol) and 1,3-dioleoyl-*rac*-glycerol (*d*; 1.242 g, 2.0 mmol), the compound was prepared following the procedures described for **1abS**. Yield of *1adS*: 1.91 g (86 %); n_D^{40} = 1.5014; Rf ($CHCl_3$) = 0.61; $C_{68}H_{120}NO_6PS$ (1111.0). ^{13}C NMR-{^1H} ($CDCl_3$ δ 12.0 ppm (*m*,C-18), 22.6 (*s*, C-26), 22.7 (*s*, C-27), 74.7, 75.7 (each *m*, C-3), 108.2, 112.7 (each *m*, C-19), 115.8, 117.4 (each *s*, C-7), 121.1, 122.6 (each *m*, C-6), 134.0 (*m*, C-8), 142.3, 144.4 (each *m*, C-5), 144.7, 148.7 (each *m*, C-10), cholecalciferyl-3-*O*-fragment; 62.7 (*s*, $O\underline{C}H_2CHOP$), 72.5 (*m*, $OCH_2\underline{C}HOP$), glycero-2-*O*-fragment; 14.0 (*s*, C-18), 129.6, 129.9 (each *s*, C-9, C-10), 173.1 (*s*, C-1), oleoyl-fragment; 37.2 (*t*, CH_3N, J = 4 Hz). ^{31}P NMR-{^1H} ($CDCl_3$) δ 76.3 ppm (m). IR (KBr, film) ν 3030 (CH=), 1740 (C=O), 1640 (C=C), 1010, 820 (PO-C, P-OC), 750 (P-N), 720 cm^{-1} (P=S).

*1, 2-Dioleoyl-*rac -glycero-3-O-*(cholecalciferyl-3-O)-(N,N-dimethylamido)thiophosphate,* 1aeS. Compound *1aeS* was synthesized and purified as described for *1adS*. Yield of *1aeS*: 1.80 g (81%); n_D^{40} = 1.5005; Rf ($CHCl_3$) = 0.64; $C_{68}H_{120}NO_6PS$ (1111.0). ^{13}C NMR {^1H} ($CDCl_3$) δ 62.0 ppm (*s*, $O\underline{C}H_2CH(O)CH_2OP$), 64.2 (*m*, CH_2OP), 69.5 (*m*, $OCH_2\underline{C}H(O)CH_2OP$), glycero-3-*O*-fragment. The other characteristics of the spectrum were very similar to those of *1adS*. ^{31}P NMR- {^1H} ($CDCl_3$ δ 76.7 ppm (*m*). IR (KBr, film) δ 3030 (CH=), 1740 (C=O), 1640 (C=C), 1010, 820 (PO-C, P-OC), 755 (P-N), 720 cm^{-1} (P=S).

RESULTS AND DISCUSSION

Various reagents have been used for the phosphorylation of lipids containing free hydroxyl functions. Steroids, however, are known to be sensitive towards phos-

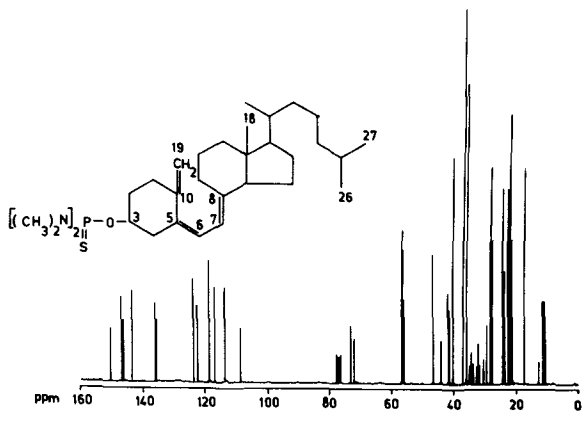


FIG. 2. The proton-decoupled 75.43-MHz ^{13}C NMR spectrum of cholecalciferyl-3-0-bis (N,N-dimethylamido)-thiophosphate.

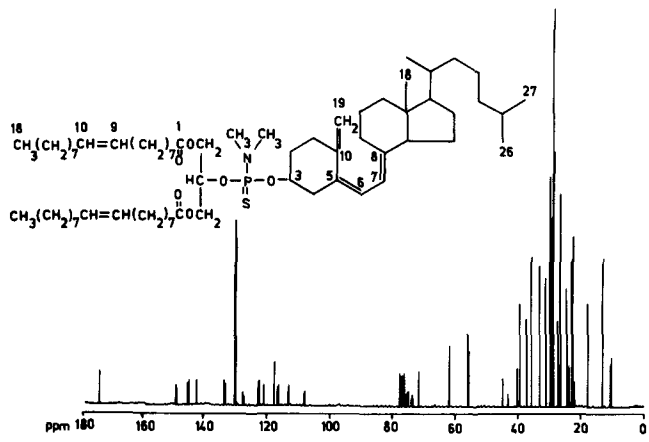
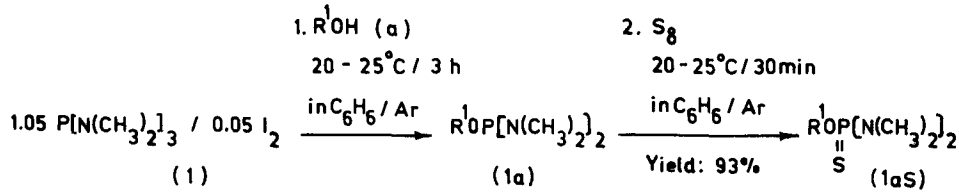
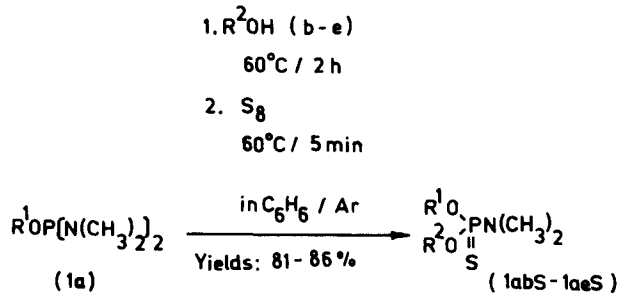


FIG. 3. The proton-decoupled 75.43-MHz ^{13}C NMR spectrum of 1,3-dioleoyl-rac-glycero-2-0- (cholecalciferyl-3-0-)-(N,N-dimethyl-amido)-thiophosphate.



SCHEME 1. For *a*, *1a*, and *1aS* R^1 = cholecalciferyl.



SCHEME 2. For *1a; 1abS — 1aeS:* R^1 = cholecalciferyl; *b* and *1abS:* R^2 = 1,3-benzylidene-*rac*-glyceryl; *c* and *1acS:* R^2 = 1,2-isopropylidene-*rac*-glyceryl; *d* and *1adS:* R^2 = 1,3-dioleoyl-*rac*-glyceryl; *e* and *1aeS:* R^2 = 1,2-dioleoyl-*rac*-glyceryl.

phorus oxychloride (15), chlorophosphates (16), hydrogen phosphates (16,17), monomeric metaphosphate (18), and phosphorus trichloride (19). Use of these conventional phosphorylating reagents is frequently accompanied by nucleophilic substitution, molecular rearrangements, elimination processes, and other side reactions (16, 18–22). The acyclic triamides of phosphorous acid which we used here afford an opportunity of avoiding these problems (23). They can be subjected to stoichiometrical alcoholysis after activation with iodine if monoester derivatives are desired. In this case, the *tris*-(*N,N*-dimethyl)amide of phosphorous acid $P[N(CH_3)_2]_3$ (**1**) is activated by the addition of iodine and an optimum molar ratio of 1.05:0.05 is used.

Cholecalciferol (*a*), 1,3-benzylidene-*rac*-glycerol (*b*) 1,2-isopropylidene-*rac*-glycerol (*c*), 1,3-dioleoyl-*rac*-glycerol (*d*), and 1,2-dioleoyl-*rac*-glycerol (*e*) were selected as lipid substrates with defined structure and potential biological activity. The phosphorylation was performed according to the method proposed recently by one of us (24). The activated phosphamide *1* was reacted with cholecalciferol *a* in stoichiometric amounts at room temperature (20–25°C) for 3 hr to give the *bis*(N,N-dimethyloamido)phosphite *1a* in close to quantitative yield. This was proven by conversion of *1a* to the corresponding thiophosphate *1aS* (Scheme 1).

The high selectivity of the reaction at the monoester stage permits the next step, *viz* the synthesis of unsymmetrical diesters, to be performed as a one-pot procedure. Thus, the consecutive treatment of the crude intermediate *1a* with equivalent quantities of *b-e* and sulfur at 60°C for 2 hr and 5 min, respectively, afforded the thiophosphate derivatives, *1abS*–1aeS, in high yields (Scheme 2).

In contrast to the first stage, the second P-O bond was introduced by moderate heating in order to avoid the extended reaction times which would be about 4 days at room temperature. An alternative route using *bis*(*N,N*-dimethylamido)glycerophosphites as intermediates was not effective because of transesterification taking place under these experimental conditions. The microanalytical and spectral data obtained for *1aS*, and *1abS* - *1aeS* confirm the structures. Some specific features detected in the ^{13}C and ^{31}P NMR spectra of the compounds synthesized are beyond the goal of the present investigation and will be discussed in detail elsewhere.

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Reaction Products of α-Tocopherol with Methyl Linoleate-Peroxyl Radicals

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α-Tocopherol was reacted with methyl linoleate-peroxyl radicals at 37°C. The peroxyl radicals were generated by the reaction of methyl linoleate with a free radical initiator, 2,2′-azo*bis*(2,4-dimethylvaleronitrile). The primary products of α-tocopherol with methyl linoleate-peroxyl radicals were isolated by reverse-phase and normal-phase high performance liquid chromatography (HPLC), and their structures were characterized by ultraviolet (UV), infrared (IR), ^{1}H and ^{13}C nuclear magnetic resonance (NMR) and mass spectrometry (MS). There were four stereoisomers of methyl 13-(8a-peroxy-α-tocopherone)-9(*Z*),11(*E*)-octadecadienoate and four stereoisomers of methyl 9-(8a-peroxy-α-tocopherone)-10(*E*),12(*Z*)-octadecadienoate.
***Lipids* 25, 152-158 (1990).**

Tocopherols are generally present in edible oils and are viewed as natural antioxidants. α-Tocopherol, which possesses the highest biological activity (vitamin E), is the most effective chain-breaking antioxidant known among the tocopherols and other phenolic antioxidants (1). α-Tocopherol inhibits autoxidation of lipids by trapping lipid-peroxyl radicals in two ways (2,3). First, lipid-peroxyl radicals are trapped by hydrogen-atom transfer, giving hydroperoxide and an α-tocopheroxyl radical. Second, the resulting α-tocopheroxyl radicals react with other lipid-peroxyl radicals or each other to form some nonradical products. To elucidate the mechanism of autoxidation inhibition by α-tocopherol, the reaction products of α-tocopherol with peroxyl radicals have been investigated (4-6). Winterle *et al.* (4) reported that α-tocopherol formed 8a-alkylperoxy-α-tocopherones via reaction of alkylperoxyl radicals with α-tocopheroxyl radicals. We have isolated and characterized 8a-alkylperoxy-α-tocopherones and some other compounds as the reaction products of α-tocopherol with an alkylperoxyl radical generated from the thermal decomposition of a radical initiator, 2,2′-azo*bis*(2,4-dimethylvaleronitrile) (AMVN) (6). However, the reaction products of α-tocopherol with lipid-peroxyl radicals formed during the autoxidation of unsaturated lipids are still unknown.

In the present study, we have isolated and characterized the reaction products of α-tocopherol with peroxyl radicals of methyl linoleate formed by molecular oxygen and initiated with AMVN.

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Abbreviations: AMVN, 2,2′-azo*bis*(2,4-dimethylvaleronitrile); CD, circular dichroism; HPLC, high performance liquid chromatography; NMR, nuclear magnetic resonance; MS, mass spectrometry; GC/MS, gas chromatography/mass spectrometry; IR, infrared; UV, ultraviolet.

MATERIALS AND METHODS

Materials. 2*R*,4′*R*,8′*R*-α-Tocopherol (Type V) was purchased from Sigma Chemical Co. (St. Louis, MO) and

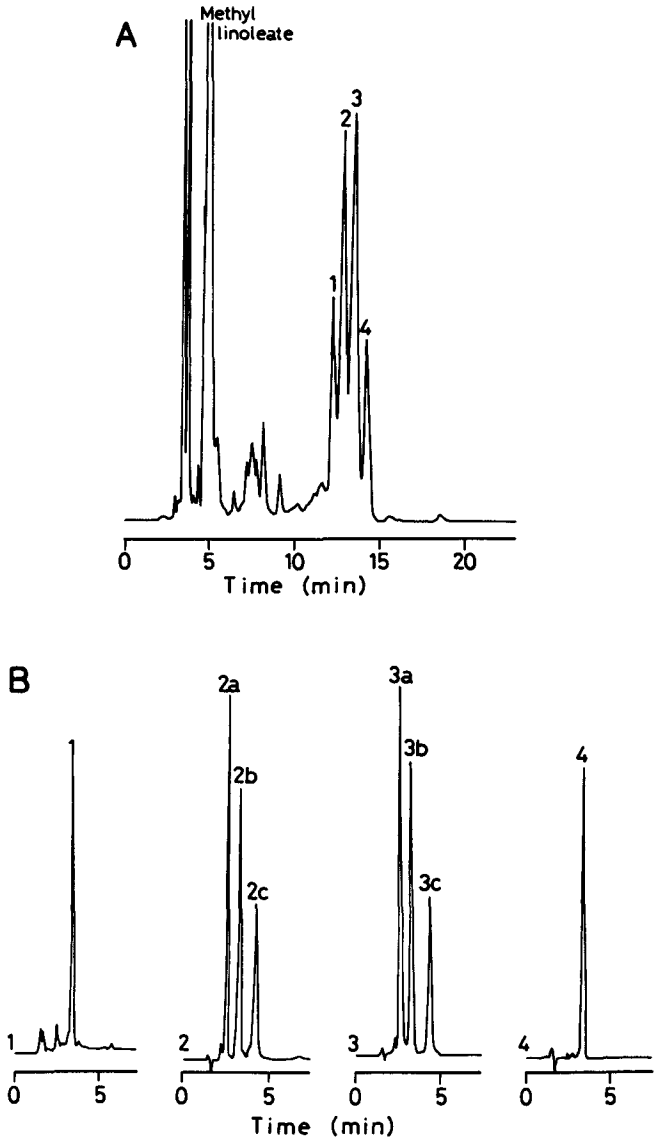


FIG. 1. Reverse-phase HPLC of the products of the AMVN-induced reaction of methyl linoleate and α-tocopherol for 23 hr (A). The products in the reaction mixture were concentrated by partitioning between hexane and methanol/water (97.5:2.5, v/v). Reverse-phase HPLC was done with a Wakosil 5C18 column developed with methanol/ethyl acetate (7:3, v/v) at a flow rate of 1.0 ml/min. The eluent was monitored by an absorbance at 260 nm. Peaks 1, 2, 3 and 4 were collected and analyzed by normal-phase HPLC (B). Normal-phase HPLC was done with a μBondasphere 5μ Si-100Å column developed with hexane/2-propanol (100:0.5, v/v) at a flow rate of 1.0 ml/min. The eluent was monitored by an absorbance at 235 nm.

purified by Sephadex LH-20 column chromatography (7). Methyl linoleate (Tokyo Kasei Co., Tokyo, Japan) was purified by silica gel column chromatography to be peroxide-free (8). Methyl linoleate monohydroperoxide was prepared from autoxidized methyl linoleate by silica gel column chromatography (8). The hydroperoxide was reduced to the corresponding hydroxy derivative by addition of a molar excess of sodium borohydride (8–10). A free radical initiator, AMVN, was purchased from Wako Pure Chemical Ind. (Osaka, Japan) and used without further purification. All solvents were distilled in an all-glass still before use.

High performance liquid chromatography (HPLC). HPLC was performed with a Jasco Trirotar V pump equipped with a Model 870-UV detector. Reverse-phase HPLC was done with a Wakosil 5C18 column (4.6 × 250 mm or 10 × 300 mm, Wako Pure Chemical Ind., developed with methanol/ethyl acetate (7:3, v/v) at a flow rate of 1.0 or 5.0 ml/min. Normal-phase HPLC was done with a μBondasphere 5μ Si-100Å column (3.9 × 150 mm, Nihon Waters Co., Tokyo, Japan) or a LiChrosorb Si 60 column (7.6 × 250 mm, Merck, Darmstadt, Federal Republic of Germany, developed with hexane/2-propanol (100:0.5 or 100:1, v/v) at a flow rate of 1.0 or 3.0 ml/min.

Reaction procedure. Methyl linoleate (5.0 g, 17 mmol), α-tocopherol (50 mg, 0.12 mmol) and AMVN (0.40 g, 1.6 mmol) were placed in a 1000 ml beaker (10 cm in diameter). To assure that the samples were well mixed, they were dissolved in diethyl ether and then dried under air and allowed to incubate at 37°C for 23 hr with mechanical shaking. To obtain a large amount of the reaction products, this reaction was repeated 20 times.

Isolation of reaction products. The reaction mixture was concentrated by partitioning between hexane (150 ml) and methanol/water (97.5:2.5, v/v; 200 ml) equilibrated with each other in a separatory funnel. The reaction mixture (ca. 5.4 g for each sample) and the solvent in the upper phase (50 ml) were poured into another separatory funnel and washed 15 times with 15 ml each of the solvent in the lower phase. The solvent of the upper phase was removed under vacuum and oily products were obtained (yield ca. 1.3 g for each sample). The concentrated products were dissolved in ethanol and analyzed by reverse-phase and normal-phase HPLC.

Spectroscopy. ^{1}H (270.05 MHz) and ^{13}C (67.8 MHz) nuclear magnetic resonance (NMR) spectra were recorded on a JEOL JNM-GX-270 FT NMR spectrometer in $CDCl_3$ with tetramethylsilane as the internal standard (ca. 27°C). Mass spectra (MS) were obtained with a Shimadzu GCMS 9020 DF gas chromatograph/mass spectrometer system. Samples were introduced via the direct inlet probe. Electron impact spectra were obtained at an ionizing energy of 70 eV. Gas chromatography/mass spectrometry (GC/MS) was performed on a Shimadzu QP-1000 quadrupole instrument oper-

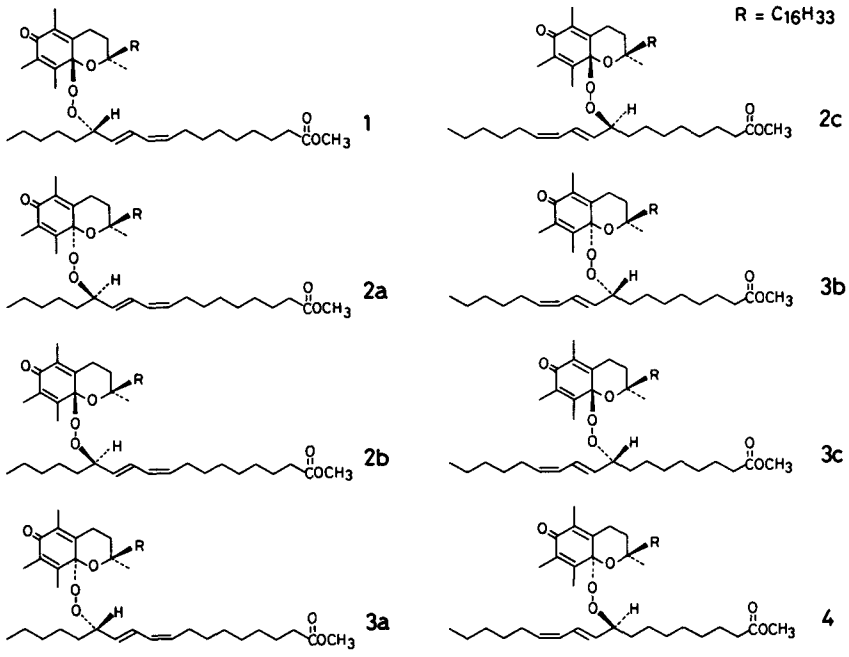
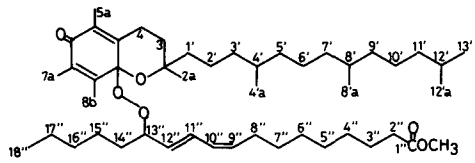


FIG. 2. Structures of compounds 1, 2a, 2b, 2c, 3a, 3b, 3c and 4 from the reactions of α-tocopherol with methyl linoleate-peroxyl radicals.

TABLE 1

^{1}H NMR Chemical Shifts of Compounds 1, 2a, 2b and 3a from the Reactions of α-Tocopherol with Methyl Linoleate-Peroxyl Radicals



1	2a	2b	3a	Proton assignment
6.36[a] (dd[b], J = 11.0, 15.3 Hz, 1H)	6.39 (dd, J = 11.6, 14.6 Hz, 1H)	6.37 (dd, J = 11.0, 15.3 Hz, 1H)	6.41 (dd, J = 11.0, 15.4 Hz, 1H)	11″
5.94 (dd, J = 10.4, 11.0 Hz, 1H)	5.95 (dd, J = 11.0, 11.0 Hz, 1H)	5.96 (dd, J = 10.4, 11.0 Hz, 1H)	5.95 (dd, J = 10.6, 11.4 Hz, 1H)	10″
5.53–5.45 (m, 2H)	5.53–5.42 (m, 2H)	5.51–5.42 (m, 2H)	5.50–5.39 (m, 2H)	9″, 12″
4.16 (q, J = 6.7 Hz, 1H)	4.16 (q, J = 6.7 Hz, 1H)	4.18 (q, J = 6.7 Hz, 1H)	4.19 (q, J = 6.6 Hz, 1H)	13″
3.66 (s, 3H)	3.66 (s, 3H)	3.66 (s, 3H)	3.66 (s, 3H)	CH_3O
2.64 (broad t, 2H)	2.63 (broad t, 2H)	2.59 (broad t, 2H)	2.58 (broad t, 2H)	4
2.30 (t, J = 7.3 Hz, 2H)	2.30 (t, J = 7.3 Hz, 2H)	2.30 (t, J = 7.3 Hz, 2H)	2.30 (t, J = 7.3 Hz, 2H)	2″
2.16 (q, J = 6.7 Hz, 2H)	2.16 (q, J = 6.7 Hz, 2H)	2.16 (q, J = 6.7 Hz, 2H)	2.17 (q, J = 7.0 Hz, 2H)	8″
1.94 (s, 3H)	1.93 (s, 3H)	1.95 (s, 3H)	1.96 (s, 3H)	8b
1.88 (s, 3H)	1.89 (s, 3H)	1.86 (s, 3H)	1.88 (s, 3H)	5a
1.86 (s, 3H)	1.87 (s, 3H)	1.86 (s, 3H)	1.87 (s, 3H)	7a
1.08 (s, 3H)	1.32 (s, 3H)	1.07 (s, 3H)	1.32 (s, 3H)	2a
2.08–1.15 (m, 41H)	2.07–1.15 (m, 41H)	2.10–1.15 (m, 41H)	2.06–1.06 (m, 41H)	3, 1′–12′, 3″–7″, 14″–17″
0.88–0.86 (m, 15H)	0.88–0.80 (m, 15H)	0.88–0.82 (m, 15H)	0.88–0.80 (m, 15H)	4′a, 8′a, 12′a, 13′, 18″

[a]Shifts in parts per million downfield relative to tetramethylsilane.
[b]Multiplicity: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet.

ated in the electron impact mode (70 eV). The gas chromatograph was equipped with a Shimadzu Hicap CBP-1 capillary column (25 m × 0.2 mm) and the temperature was programmed from 230–290°C (4°C/min). The carrier gas (helium) flow was 0.8 ml/min. Infrared (IR) spectra of samples in liquid film were measured on a Jasco A-302 IR spectrometer. Ultraviolet (UV) spectra were measured with a Hitachi Model 200-10 spectrophotometer. Specific rotations were determined with a Union PM-201 automatic digital polarimeter. Circular dichroism (CD) measurements of the optical isomers of the reduced compounds were performed with a Jasco J-6000 spectropolarimeter.

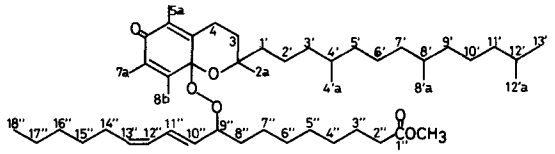
Derivatization. The isolated compounds (ca. 2 mg) were dissolved in 1 ml of methanol/ethanol (2:1, v/v) and reduced with sodium borohydride at 4°C for 2 hr, extracted with diethyl ether, converted to the corresponding trimethylsilyl ethers with *N,O-bis*(trimethylsilyl)-trifluoroacetamide (15 min at 60°C), and then analyzed by GC/MS. The positional isomers of methyl hydroxyoctadecadienoates in the reduced compounds were determined by GC/MS after hydrogenation and trimethylsilylation (8,9). Furthermore, the positional isomers were analyzed by normal-phase HPLC developed with hexane/2-propanol (100:1, v/v) (10). Peaks corresponding to methyl hydroxyoctadecadienoates were isolated and the *R* and *S* configurations of the hydroxy group were determined by CD spectra in methanol (11,12).

RESULTS

AMVN decomposes at 37°C to form alkyl radicals which, in turn, react with molecular oxygen to form alkylperoxyl radicals. The reaction mixture contains a larger amount of methyl linoleate than α-tocopherol. Therefore, most of the alkylperoxyl radicals attack methyl linoleate to generate the pentadienyl radical, which reacts rapidly with oxygen to give the methyl linoleate-peroxyl radical (3). The products of methyl linoleate-peroxyl radical with α-tocopherol in the reaction mixture could be concentrated by partitioning between hexane and methanol/water (97.5:2.5, v/v). The concentrated products were analyzed by reverse-phase HPLC (Fig. 1A). Four major peaks, **1**, **2**, **3** and **4**, that appeared to be the reaction products of α-tocopherol with methyl linoleate-peroxyl radical, were detected on the chromatogram. Peaks **1**, **2**, **3** and **4** were further analyzed by normal-phase HPLC using hexane/2-propanol (100:0.5, v/v) as the eluent (Fig. 1B). Peaks **2** and **3** could each be separated into three peaks (**2a**, **2b**

TABLE 2

^{1}H NMR Chemical Shifts of Compounds 2c, 3b, 3c and 4 from the Reactions of α-Tocopherol with Methyl Linoleate-Peroxyl Radicals



2c	3b	3c	4	Proton assignment
6.37[a] (dd[b], J = 11.0, 15.4 Hz, 1H)	6.40 (dd, J = 11.0, 15.4 Hz, 1H)	6.40 (dd, J = 11.0, 15.4 Hz, 1H)	6.42 (dd, J = 11.4, 15.0 Hz, 1H)	11″
5.94 (dd, J = 11.0, 11.0 Hz, 1H)	5.95 (dd, J = 10.3, 10.6 Hz, 1H)	5.94 (dd, J = 10.6, 11.0 Hz, 1H)	5.95 (dd, J = 10.6, 11.0 Hz, 1H)	12″
5.52–5.40 (m, 2H)	5.52–5.44 (m, 2H)	5.50–5.39 (m, 2H)	5.49–5.40 (m, 2H)	10″, 13″
4.15 (q, J = 7.0 Hz, 1H)	4.16 (q, J = 7.0 Hz, 1H)	4.17 (q, J = 6.6 Hz, 1H)	4.18 (q, J = 6.6 Hz, 1H)	9″
3.66 (s, 3H)	3.66 (s, 3H)	3.66 (s, 3H)	3.66 (s, 3H)	CH_3O
2.63 (broad t, 2H)	2.63 (broad t, 2H)	2.59 (broad t, 2H)	2.58 (broad t, 2H)	4
2.30 (t, J = 7.3 Hz, 2H)	2.30 (t, J = 7.3 Hz, 2H)	2.29 (t, J = 7.3 Hz, 2H)	2.29 (t, J = 7.3 Hz, 2H)	2″
2.16 (q, J = 6.6 Hz, 2H)	2.17 (q, J = 7.3 Hz, 2H)	2.17 (q, J = 6.8 Hz, 2H)	2.17 (q, J = 7.0 Hz, 2H)	14″
1.93 (s, 3H)	1.94 (s, 3H)	1.95 (s, 3H)	1.95 (s, 3H)	8b
1.88 (s, 3H)	1.89 (s, 3H)	1.87 (s, 3H)	1.88 (s, 3H)	5a
1.86 (s, 3H)	1.87 (s, 3H)	1.86 (s, 3H)	1.86 (s, 3H)	7a
1.07 (s, 3H)	1.32 (s, 3H)	1.06 (s, 3H)	1.31 (s, 3H)	2a
2.00–1.13 (m, 41H)	2.04–1.15 (m, 41H)	2.10–1.10 (m, 41H)	2.05–1.09 (m, 41H)	3, 1′–12′, 2″–8″, 15″–17″
0.92–0.84 (m, 15H)	0.91–0.81 (m, 15H)	0.92–0.84 (m, 15H)	0.90–0.80 (m, 15H)	4′a, 8′a, 12′a, 13′, 18″

[a]Shifts in parts per million downfield relative to tetramethylsilane.
[b]Multiplicity: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet.

and **2c** from **2** and **3a**, **3b** and **3c** from **3**, respectively).

Compounds **1–4** were obtained as colorless oils (total yields of the reaction products from 100 g of methyl linoleate and 1.0 g of α-tocopherol were as follows: **1**, 39.5 mg; **2a**, 41.6 mg; **2b**, 35.5 mg; **2c**, 34.7 mg; **3a**, 38.8 mg; **3b**, 35.3 mg; **3c**, 26.3 mg; and **4**, 37.5 mg). Each compound gave a positive peroxide test (13). The structures of **1**, **2a**, **2b**, **2c**, **3a**, **3b**, **3c** and **4** were determined as described below (Fig. 2).

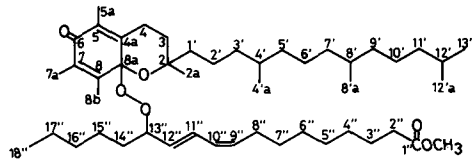
UV spectra of these compounds showed the same absorption maximum at 235.5 nm in ethanol (**1**, ε 35500; **2a**, ε 36000; **2b**, ε 35800; **2c** ε 35700; **3a** ε 35700; **3b**, ε 35300; **3c**, ε 36900; and **4**, ε 35700), indicating the presence of conjugated dienes. IR spectra of the compounds showed two absorption bands each (985–980 cm^{-1} and 945–940 cm^{-1}), and suggest that all compounds contained conjugated *cis,trans* double bonds.

Compounds **1–4** were reduced with sodium borohydride and the products were identified by GC/MS as trimethylsilyl derivatives. Compounds **1**, **2a**, **2b** and **3a** gave two major peaks which were identified to be methyl 13-hydroxy-9,11-octadecadienoate trimethylsilyl ether (14): MS *m/z* 382 (M^+, 11%), 311 (37), 225 (21), 143 (17), 130 (38), 73 (100); and α-tocopherol trimethylsilyl ether (15): MS *m/z* 502 (M^+, 100%), 277 ($[M\text{-}C_{16}H_{33}]^+$, 5), 237 (50), 73 (60); and compounds **2c**, **3b**, **3c** and **4** gave methyl 9-hydroxy-10,12-octadecadienoate trimethylsilyl ether (14): MS *m/z* 382 (M^+, 15%), 311 (11), 225 (77), 143 (16), 130 (26), 73 (100); and α-tocopherol trimethylsilyl ether (15). The results indicate that each compound consists of isomeric methyl hydroxyoctadecadienoate moiety and α-tocopherol moiety in the molecule.

The positional isomers of methyl hydroxyoctadecadienoate were confirmed by GC/MS after hydrogenation and trimethylsilylation (8,9). Compounds **1**, **2a**, **2b** and **3a** gave methyl 13-hydroxyoctadecanoate trimethylsilyl ether: MS *m/z* 371 ($[M\text{-}15]^+$, 1%), 355 (7), 339 (16), 315 (69), 173 (100), 73 (81); and compounds **2c**, **3b**, **3c** and **4** gave methyl 9-hydroxyoctadecanoate trimethylsilyl ether: MS *m/z* 371 ($[M\text{-}15]^+$, 1%), 355 (5), 339 (13), 259 (100), 229 (80), 155 (21), 73 (99), respectively. Furthermore, the methyl hydroxyoctadecadienoates were analyzed by normal-phase HPLC using hexane/2-propanol (100:1, v/v) as the eluent, and their chemical nature was confirmed by co-chromatography with authentic standards (10). Compounds **1**, **2a**, **2b** and **3a** gave a peak corresponding to methyl 13-hydroxy-9(*Z*),11(*E*)-octadecadienoate and compounds **2c**, **3b**, **3c** and **4** gave methyl 9-hydroxy-10(*E*),12(*Z*)-octadecadienoate. Peaks

TABLE 3

^{13}C NMR Chemical Shifts of Compounds 1, 2a, 2b and 3a from the Reactions of α-Tocopherol with Methyl Linoleate-Peroxyl Radicals



1	2a	2b	3a	Carbon assignment
185.4[a]	185.5	185.5	185.5	6
174.2	174.2	174.2	174.2	1″
148.9	148.8	148.7	148.7	8
145.5	145.7	146.0	146.2	4a
132.8, 132.7	132.8, 132.7	132.9, 132.8	132.8(2)[b]	5, 7
131.5, 131.3, 127.9, 127.7	131.4, 131.2, 127.9, 127.8	131.3, 131.2, 128.0, 127.6	131.4, 130.9, 127.9, 127.7	9″–12″
97.1	97.4	97.0	97.1	8a
84.9	85.0	84.5	84.5	13″
76.8	76.7	76.9	76.8	2
51.4	51.4	51.4	51.3	CH_3O
43.1	42.0	43.2	42.0	1′
39.4	39.4	39.4	39.4	11′
37.5(2), 37.3(2)	37.4(2), 37.3(2)	37.6(2), 37.4(2)	37.4(2), 37.3(2)	3′, 5′, 7′, 9′
34.1, 33.1	34.1, 33.1	34.1, 33.2	34.1, 33.1	2″, 14″
32.8(2)	32.8(2)	32.8(2)	32.8(2)	4′, 8′
32.3	32.6	32.4	32.6	3
31.7	31.7	31.7	31.7	16″
29.6, 29.1(3)	29.6, 29.1(3)	29.6, 29.1(3)	29.6, 29.1(3)	4″, 5″, 7″, 8″
28.0	28.0	28.0	28.0	12′
27.7	27.7	27.8	27.7	6″
25.7	26.8	25.7	26.9	2a
25.1, 24.9	25.1, 24.9	25.1, 25.0	25.1, 24.9	3″, 15″
24.8, 24.5	24.8, 24.4	24.8, 24.5	24.8, 24.4	6′, 10′
22.7, 22.6	22.7, 22.5	22.7, 22.6	22.7, 22.6	12′a, 13′
22.5(2)	22.5, 22.3	22.5, 22.2	22.4, 22.0	4, 17″
21.1	21.3	21.1	21.3	2′
19.7, 19.6	19.8, 19.6	19.8, 19.7	19.7, 19.5	4′a, 8′a
13.9	14.0	14.0	13.9	18″
13.5	13.5	13.6	13.6	8b
11.2	11.2	11.3	11.2	7a
10.7	10.7	10.7	10.7	5a

[a]Shifts in parts per million downfield relative to tetramethylsilane.
[b]Number of carbon in parentheses.

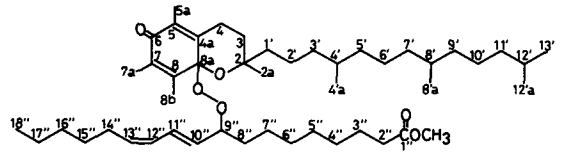
corresponding to the methyl hydroxyoctadecadienoates were isolated and their configurations were determined by CD measurement. Methyl hydroxyoctadecadienoates from compounds **1**, **2c**, **3a** and **4** showed a positive Cotton effect and those from compounds **2a**, **2b**, **3b** and **3c** showed a negative Cotton effect, respectively. Therefore, the hydroxy group of methyl hydroxyoctadecadienoates from compounds **1**, **2c**, **3a** and **4** is in the *S* configuration and that from **2a**, **2b**, **3b** and **3c** is in the *R* configuration (11,12).

The ^1H NMR spectra of the compounds **1–4** were consistent with those expected for methyl 13-(8a-peroxy-α-tocopherone)-9(*Z*),11(*E*)-octadecadienoates and methyl 9-(8a-peroxy-α-tocopherone)-10(*E*),12(*Z*)-octadecadienoates (Tables 1 and 2). In particular, the geometrical relationship between the compounds can be established by interpretation of the spectra (6). When the 8a-peroxy group of the tocopherol moiety is *trans* to the 2-methyl group, shielding of the 2-methyl group (**1**, 1.08 ppm; **2b**, 1.07 ppm; **2c**, 1.07 ppm; and **3c**, 1.06 ppm) is observed. When the 8a-peroxy group is *cis* to the 2-methyl group, deshielding of the 2-methyl group is observed (**2a**, 1.32 ppm; **3a**, 1.32 ppm; **3b**, 1.32 ppm; and **4**, 1.31 ppm). Furthermore, the geometrical configuration of the conjugated diene system in each compound can be established (16). The *cis-trans* diene systems are present in all compounds, and both *cis* (*J*, 10.3-11.6 Hz) and *trans* (*J*, 14.6-15.4 Hz) coupling constants are observed. The ^{13}C NMR spectra of the compounds show two carbonyls (174.2 ppm and 185.4–185.5 ppm), a carbon atom bearing two oxy substituents (97.0–97.4 ppm), a secondary ether (84.5–85.0 ppm), a tertiary ether (76.7–76.9 ppm) and a methoxy carbon (51.3–51.4 ppm) (Tables 3 and 4).

From these results and other spectral data, the structures of **1**, **2a**, **2b** and **3a** were identified to be stereoisomers of methyl 13-(8a-peroxy-α-tocopherone)-9(*Z*),11(*E*)-octadecadienoate. Compound **1**, methyl 13(*S*)-[8a(*S*)-peroxy-α-tocopherone]-9(*Z*), 11(*E*)-octadecadienoate: MS *m/z* 445 ([M-$C_{19}H_{33}O_3$]$^+$, 86%), 430 (82), 417 (37),

TABLE 4

^{13}C NMR Chemical Shifts of Compounds 2c, 3b, 3c and 4 from the Reactions of α-Tocopherol with Methyl Linoleate-Peroxyl Radicals



2c	3b	3c	4	Carbon assignment
185.4[a]	185.4	185.5	185.5	6
174.2	174.2	174.2	174.2	1″
148.8	148.8	148.7	148.7	8
145.4	145.7	145.9	146.1	4a
133.0, 132.6	133.0, 132.5	133.0, 132.8	133.0, 132.7	5, 7
131.5, 131.3, 127.9(2)[b]	131.4, 131.2, 127.9(2)	131.3, 131.2, 127.8, 127.6	131.4, 130.9, 127.9(2)	10″–13″
97.2	97.4	97.0	97.2	8a
85.0	85.0	84.5	84.5	9″
76.8	76.7	76.9	76.8	2
51.4	51.3	51.4	51.4	CH_3O
43.1	42.1	43.2	42.1	1′
39.4	39.4	39.4	39.4	11′
37.5(2), 37.3(2)	37.4(2), 37.3(2)	37.6(2), 37.4(2)	37.4(2), 37.3(2)	3′, 5′, 7′, 9′
34.1, 33.2	34.1, 33.5	34.1, 33.2	34.1, 33.5	2″, 8″
32.9, 32.8	32.8, 32.6	32.9, 32.8	32.8, 32.6	4′, 8′
32.3	33.1	32.4	33.1	3
31.5	31.5	31.5	31.5	16″
29.3(2), 29.0(2)	29.3(2), 29.0(2)	29.4(2), 29.1(2)	29.3(2), 29.0(2)	4″, 5″, 7″, 14″
28.0	28.0	28.0	28.0	12′
27.7	27.7	27.8	27.7	6″
25.7	26.8	25.7	26.9	2a
25.4, 24.9	25.3, 24.9	25.4, 24.9	25.4, 24.9	3″, 15″
24.8, 24.5	24.8, 24.4	24.8, 24.5	24.8, 24.4	6′, 10′
22.7, 22.6	22.7, 22.6	22.7, 22.6	22.7, 22.6	12′a, 13′
22.6, 22.1	22.6, 22.3	22.6, 22.1	22.6, 22.0	4, 17″
21.1	21.3	21.1	21.4	2′
19.8, 19.6	19.7, 19.6	19.8, 19.7	19.7, 19.6	4′a, 8′a
14.0	14.0	14.0	14.0	18″
13.5	13.5	13.6	13.6	8b
11.2	11.2	11.2	11.3	7a
10.7	10.7	10.6	10.7	5a

[a]Shifts in parts per million downfield relative to tetramethylsilane.
[b]Number of carbon in parentheses.

403 (22), 293 ($C_{19}H_{33}O_2$+, 14), 227 (11), 167 (99), 165 (86), 57 (100); IR (film) ν 1740 cm^{-1}, 1680, 1640, 985, 945; $[\alpha]_D^{25}$ +9.0 (*c* 0.60, ethanol). Compound **2a**, methyl 13(*R*)-[8a(*R*)-peroxy-α -tocopherone] - 9(*Z*),11(*E*)-octadecadienoate: MS *m/z* 445 ([M-$C_{19}H_{33}O_3$]+, 86%), 430 (90), 417 (37), 403 (18), 293 ($C_{19}H_{33}O_2$+, 19), 277 (13), 167 (81), 165 (92), 57 (100); IR (film) ν 1740 cm^{-1}, 1675, 1640, 985, 945; $[\alpha]_D^{25}$ +8.6 (*c* 0.63, ethanol). Compound **2b**, methyl 13 (*R*)-[8a(*S*)-peroxy-α-tocopherone] -9 (*Z*),11(*E*)-octadecadienoate: MS *m/z* 445 ([M-$C_{19}H_{33}O_3$]+, 72%), 430 (94), 417 (29), 403 (16), 293 ($C_{19}H_{33}O_2$+, 12), 167 (86), 165 (94), 57 (100); IR (film) ν 1745 cm^{-1}, 1680, 1640, 985, 940; $[\alpha]_D^{25}$ +29.6 (*c* 0.32, ethanol). Compound **3a**, methyl 13(*S*)-[8a(*R*)-peroxy-α-tocopherone]-9(*Z*),11(*E*)-octadecadienoate: MS *m/z* 445 ([M-$C_{19}H_{33}O_3$]+, 89%), 430 (82), 417 (34), 403 (16), 293 ($C_{19}H_{33}O_2$+, 19), 167 (88), 165 (91), 57 (100); IR (film) ν 1745 cm^{-1}, 1680, 1640, 985, 945; $[\alpha]_D^{25}$ −24.7 (*c* 0.82, ethanol). The structures of **2c**, **3b**, **3c** and **4** were identified to be stereoisomers of methyl 9-(8a-peroxy-α-tocopherone)-10(*E*),12(*Z*)-octadecadienoate. Compound **2c**, methyl 9(*S*) -[8a(*S*) - peroxy -α-tocopherone] - 10(*E*),12(*Z*) - octadecadienoate: MS *m/z* 445 ([M-$C_{19}H_{33}O_3$]+, 84%), 430 (74), 417 (29), 403 (20), 293 ($C_{19}H_{33}O_2$+, 12), 167 (100), 165 (82), 57 (74); IR (film) ν 1750 cm^{-1}, 1680, 1640, 980, 940; $[\alpha]_D^{25}$ +0.8 (*c* 0.53, ethanol). Compound **3b**, methyl 9(*R*)-[8a(*R*)-peroxy-α-tocopherone]-10(*E*),12(*Z*)-octadecadienoate: MS *m/z* 445 ([M-$C_{19}H_{33}O_3$]+, 60%), 430 (100), 417 (22), 403 (11), 293 ($C_{19}H_{33}O_2$+, 11), 167 (86), 165 (95), 57 (99); IR (film) ν 1745 cm^{-1}, 1680, 1640, 985, 940; $[\alpha]_D^{25}$ +7.6 (*c* 0.71, ethanol). Compound **3c**, methyl 9(*R*)-[8a(*S*)-peroxy-α-tocopherone]-10(*E*),12(*Z*)-octadecadienoate: MS *m/z* 445 ([M-$C_{19}H_{33}O_3$]+, 74%), 430 (100), 417 (23), 403 (14), 293 ($C_{19}H_{33}O_2$+, 9), 167 (98), 165 (94), 57 (71); IR (film) ν 1745 cm^{-1}, 1680, 1640, 985, 940; $[\alpha]_D^{25}$ +22.2 (*c* 0.53, ethanol). Compound **4**, methyl 9(*S*)-[8a(*R*)-peroxy-α-tocopherone]-10(*E*), 12(*Z*)-octadecadienoate: MS *m/z* 445 ([M-$C_{19}H_{33}O_3$]+, 62%), 430 (81), 417 (18), 403 (10), 293 ($C_{19}H_{33}O_2$+, 14), 167 (70), 165 (93), 57 (100); IR (film) ν 1745 cm^{-1}, 1680, 1640, 985, 940; $[\alpha]_D^{25}$−18.4 (*c* 0.49, ethanol).

DISCUSSION

Since α-tocopherol is known to act as an antioxidant by donating a hydrogen atom to chain-propagating

free radicals, much interest has been shown in the products of its reactions with peroxyl radicals (4-6). We have already isolated and characterized 8a-alkylperoxy-α-tocopherones and some other compounds as the reaction products of α-tocopherol with the alkylperoxyl radical generated from AMVN in ethanol (6). In the present study, we have succeeded in isolating and characterizing the primary products of α-tocopheroxyl radical with methyl linoleate-peroxyl radicals during autoxidation in bulk phase in the presence of a free radical initiator. The products are four stereoisomers of methyl 13-(8a-peroxy-α-tocopherone)-9(*Z*),11(*E*)-octadecadienoate (**1**, **2a**, **2b** and **3a**) and four stereoisomers of 9-(8a-peroxy-α-tocopherone)-10(*E*),12(*Z*)-octadecadienoate (**2c**, **3b**, **3c** and **4**) (Fig. 2). The total yield of the products 1-4 from this experiment was only about 16% of the α-tocopherol. The products are unstable for acid (6), and the chromatographic separation may result in a substantial loss of the products. However, these are not the only products. We have also detected two other products, spirodiene dimer and trimer, during autoxidation of methyl linoleate (17). However, the dimer and trimer cannot be eluted from the reverse-phase column under the present chromatographic conditions.

The autoxidation of unsaturated lipids is known to be a free radical chain process which, in homogeneous solution at sufficient oxygen pressures, proceeds by the following reaction sequence (18,19):

$$\text{initiation: LH} \rightarrow \text{L}\bullet \quad [1]$$

$$\text{propagation: L}\bullet + \text{O}_2 \rightarrow \text{LOO}\bullet \quad [2]$$

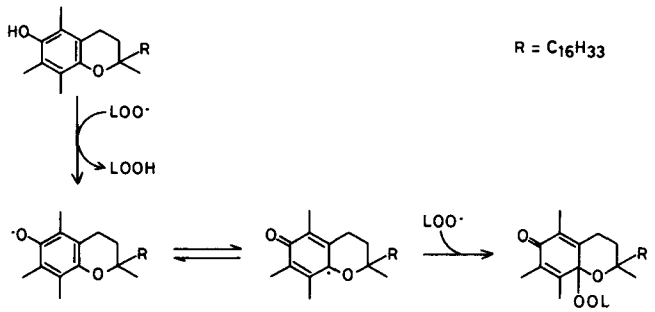
$$\text{LOO}\bullet + \text{LH} \rightarrow \text{LOOH} + \text{LOO}\bullet \quad [3]$$

$$\text{termination: 2LOO}\bullet \rightarrow \text{nonradical products} \quad [4]$$

If LH is methyl linoleate, four possible methyl linoleate-peroxyl radicals, the 9(*S*)-, 9(*R*)-, 13(*S*)- and 13(*R*)-peroxyl radicals, can be produced by oxygen addition at C-9 and C-13 of the pentadienyl radical (18,19). In the presence of α-tocopherol (TOH), reactions [3] and [4] are suppressed, and termination occurs by reaction [5]:

$$\text{LOO}\bullet + \text{TOH} \rightarrow \text{LOOH} + \text{TO}\bullet \quad [5]$$

This reaction results in the formation of hydroperoxide (LOOH) and α-tocopheroxyl radical (TO•), which reacts rapidly and irreversibly with a second peroxyl radical to form peroxide products (Scheme 1) (1-3,20). This study showed that the four methyl linoleate-peroxyl radicals attacked the tocopheroxyl radical at the 8a-position to form all eight possible 8a-peroxy-α-tocopherones (compounds 1-4). Thus, if all of the tocopheroxyl radicals react with lipid-peroxyl radicals, each α-tocopherol molecule can scavenge two lipid-peroxyl radicals during the antioxidant process (2).



SCHEME 1.

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Carotenoids of Human Colostrum

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Colostrum, the initial postpartum secretion of the breast, ordinarily has a distinct yellow color due to carotenoids of its fat globules. This pigmentation progressively diminishes as milk production increases during the first week of lactation. Identity of these carotenoids was investigated by means of thin-layer chromatography, high performance liquid chromatography and spectral analysis. α- and β-carotene, lycopene and β-cryptoxanthin were revealed as major chromogens. A component corresponding to lutein and/or zeaxanthin was also detected by both chromatographic techniques. Extracts of 23 saponified colostrum samples from 10 donors revealed considerable variation in total carotenoid concentration (0.34–7.57 μg/ml of colostrum). Multiparous mothers had greater mean colostrum carotenoid concentrations than did the primiparae, 2.18 ± 1.94 vs 1.14 ± 1.32 μg/ml, respectively. Seven of the eight primiparous donors' samples had little or no yellow color. These findings imply a difference in carotenoid transport by breasts that have lactated as compared to those that have not. The interrelation of carotenoids, lactation and breast cancer is discussed.
***Lipids 25,* 159–165 (1990).**

The characteristic yellow color of human colostrum is due to carotenoids, a class of lipid-soluble pigments of ultimate plant origin. Animals cannot synthesize carotenoids. However, as a result of ingesting plant materials, humans extract, metabolize and store these pigments (1). Meat, fish, eggs and milk (products) provide humans with secondary sources of the plant carotenoids. It is commonly observed at surgery that adipose tissue of the breast is yellow, as are other fatty depots of the adult human, but not of the newborn infant. The bright yellow pigmentation of fat globules issuing in human colostrum at parturition is evidence that carotenoids are concentrated and stored in the breast. Following a few days of breast-feeding, the color of the globules is greatly reduced (2–4).

It is known that β-carotene comprises about 85% of the total carotenoids in cow's milk, whereas the corresponding value for human milk is about 25% (1). Outside of this estimate, the carotenoids of human milk and colostrum have not been identified. The practice has been to measure light absorption at 460 nm of extracted colostrum lipids in a suitable solvent, and to express this value in terms of β-carotene or as total carotenoid. Recent studies (2–4) employing such analysis reveal mean values of about 200 μg of carotene per dl of colostrum on the first day postpartum after which the concentration falls rather quickly during the next 5 days and stabilizes at about 20 μg/dl in samples collected several weeks or more postpartum.

Some of the carotenoids are of special importance because they are precursors of vitamin A (retinol) in human and other species (5). The correlation of dietary carotenoids with reduced incidence of many forms of cancer (6), and evidence that carotenoids may have unique functions in the immune system unrelated to their provitamin A activity (7) are also arousing interest in this class of compounds. The possibility that carotenoids might play a protective role with respect to breast cancer (8), as well as health-related functions in the infant, suggested the need for further investigation of these pigments in colostrum. Thus we undertook to identify carotenoids in human colostrum, and to estimate their concentrations.

MATERIALS AND METHODS

Eleven donors provided the colostrum. All were full-term (> 37 weeks) at delivery. Five were primiparous, the other six having delivered either once or twice previously. Samples from one of the latter six were used to develop methods and were not included in quantitative studies. Collection of samples either by hand expression or electric pump (Medela Inc., Crystal Lake, IL) was made within 6 days after delivery. A few donors provided several samples throughout that period. In these instances, samples from a given donor were from the same breast. Samples ranged in volume from 0.5 to 15 ml with most between 2 and 4 ml. Samples to be shipped were packed in dry ice. All samples were stored at −70°C until analyses were undertaken.

Lycopene, α- and β-carotene, lutein and retinol were from Sigma Chemical Co., St. Louis, MO. β-Cryptoxanthin and zeaxanthin were gifts of Dr. J.C. Smith, Jr., USDA, Beltsville, MD. The carotenoids were used as references in the following TLC and HPLC analyses described in this paper. For purposes of spectral identifications and recovery experiments, the references were purified by those techniques to a single band or elution peak.

Isolation of colostrum carotenoids. In the various steps of analysis, precautions were taken to minimize the effects of light and oxygen. All manipulations were carried out in subdued light or total darkness. TLC separations were made in the dark. Samples were held in amber glass vials or containers wrapped in foil, flushed with nitrogen and tightly sealed. Butylated hydroxytoluene (BHT) was used as an antioxidant (0.1%) in solvents for extracting and storing carotenoids. Storage of extracts and solutions of reference compounds used on a day-to-day basis was at −20°C.

There are several thousand times as much glyceride (in which the carotenoids are soluble) as carotenoid in human colostrum. It is virtually impossible to separate, identify and quantify mixed carotenoids in

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Abbreviations: BHT, butylated hydroxytoluene; HPLC, high performance liquid chromatography; TG, triacylglycerol; TLC, thin-layer chromatography.

the presence of so much contaminating lipid. This complicating factor led us to develop the following saponification-extraction procedure to eliminate the glycerides:

Colostrum (2.0 ml) is placed in a 12.5 × 98 mm screw-cap-fitted tube. Ethanol (2.5 ml) and 1.5 ml of 50% (w/w) aqueous KOH are added. The tube is flushed with N_2, tightly capped and placed in a 45°C water bath for 60 min during which the tube contents are mixed occassionally by inversion. The reaction mixture is then submitted to three extractions with 1-ml quantities of hexane. The combined extracts containing the carotenoids (unsaponifiables) are evaporated under N_2, and the residue is dissolved in a suitable volume of hexane containing 0.1% BHT.

Measurement of total carotenoids. To estimate the total carotenoid content of colostrum extracts, the equation, c = D•v•f•10/2,500, was used (9). This gives the relationship between c (total carotenoids) in mg, D (the absorbance at the main absorption maximum), v (total volume of extract) in ml, f (the dilution, or concentration, factor relating extract volume to colostrum volume), and 2,500—an average extinction coefficient for carotenoids. Absorbance of extracts was measured spectrophotometrically in hexane at 460 nm.

Thin-layer chromatography (TLC) of carotenoids. The principal known carotenoids of human blood plasma, the logical source of colostrum carotenoids, are α- and β-carotene, lycopene, β-cryptoxanthin, zeaxanthin and/or lutein (10). The following TLC procedures were devised to assist in possible detection and identification of these compounds in extracts of saponified colostrum.

TLC system 1. Samples were applied at one end across the short dimension of a 10 × 20 cm silica gel-coated TLC plate (Whatman 60A). The plate was first developed to a height of 16 cm from the origin with a solvent of 1:1 (v/v) hexane benzene. On removal from the TLC chamber, the plate was allowed to dry for a minute and then developed to a height of 8 cm with a solvent of 7:3 (v/v) hexane acetone.

TLC system 2. Samples were applied along one side of a 10 × 10 cm plate coated with MgO-kieselguhr, 1:1, with $CaSO_4$ binder (Analtech, Newark, DE). The plate was developed to the top with 19:1 (v/v) hexane acetone.

Carotenoid references were applied to plates in 2-μl quantities of 0.5 mg/ml concentration. Ten to 200 μl of the colostrum extracts were used, the larger volumes being applied to analytical plates as bands. TLC chambers were lined with Whatman 1 paper and preequilibrated.

Charring of TLC plates, used to evaluate conditions for saponification of colostrum lipids, was accomplished by spraying with 50% aqueous H_2SO_4 (v/v) and heating in an oven at 150°C.

Spectral analysis. Individual bands of colostrum carotenoids resolved on TLC plates were scraped into tubes and extracted with 1–2 ml of chloroform (Fisher Scientific, spectral grade). Absorption spectra (390–550 nm) of the references and band extracts were determined with a Gilford spectrophotometer (Model 2400S).

High performance liquid chromatography (HPLC). Extracted carotenoids (≤50 μl) were injected onto a Beckman C18 Ultrasphere column equilibrated with acetonitrile tetrahydrofuran, 85:25 (v/v), containing 0.25 g/l of BHT as previously described (11). The samples were eluted isocratically with a Waters Model 510 pump at a flow rate of 2.5 ml/min. Sample components were revealed either with a Milton Roy dual wavelength detector (Model SM 4000) or a Hewlett Packard photodiode array detector (Model 1040A). Retinol content of the samples also was assayed because of its relationship to the carotenoids. It was isolated and detected at 325 nm using the same system. For quantification, peak areas of individual carotenoids and retinol were referred to standard curves. These were prepared daily using 4–6 delutions for each standard: α-carotene, β-carotene, lycopene, β-cryptoxanthin and retinol. Concentrations of the standards were determined from their absorbance values and extinction coefficients. Correlation coefficients for the standard curves were > 0.99. For identification of compounds by photodiode array, the spectrum from 200 to 500 nm was scanned. Limits of detection were 5 ng for β-carotene and 2 ng for retinol.

Control experiments. Stability of the carotenoids to the saponification procedure was evaluated as follows: The reference carotenoids (5–10 μg of each) in solution were added to the saponification tubes. The solvent was evaporated with a stream of N_2; ethanol, samples and base were added in sequence, and saponification-extraction carried out as specified. Added carotenoids were evaluated in water or colostrum as the sample material. Cryptoxanthin was used only in the colostrum experiments due to its limited supply. Extracts from the saponifications were separated on TLC-plates together with controls containing comparable amounts of the reference carotenoids. Bands of the carotenoids on the plates were scraped into tubes and eluted. Then contents of the individual components were determined from spectral absorbance as described above. Stability was estimated as the percentage of a carotenoid recovered in the relevant band from the saponified sample compared to that from the unsaponified control.

As a further check on stability of colostrum carotenoids to the saponification procedure, extracts from the saponifications were compared with lipid extracts (unsaponified) of the same samples regarding total carotenoids. Roese-Gottlieb solvent extraction (12), omitting NH_4OH, was used to obtain sample lipids including carotenoids. Solvent was removed from the extracts with a stream of N_2. The residues were taken up in defined volumes of hexane, and these solutions, together with corresponding saponification extracts, were analyzed for total carotenoid by absorbance at 460 nm.

Following collection, some of the larger colostrum samples were divided so that portions not required for analyses could be used to check stability of carotenoids during storage at −70°C. For this purpose, absorbance of saponification extracts at 460 nm was monitored.

RESULTS

The findings for total carotenoid concentrations obtained by spectral analysis of saponified colostrum

samples from 10 donors are in Table 1. Data for donors supplying multiple samples showed a downward trend with progressing time postpartum as observed in previous studies (2-4), although variations are evident. For example, the first sample donor 66 does not have the highest value, and the second sample is lower than the third for donors 52, 59 and 66. In our earlier study (3), in which colostrum carotenoids were assayed in terms of fat content, similar variations were noted. A given donor's samples did not necessarily show a smooth downward progression in carotene concentration with time postpartum, although the overall trend for each individual and the group as a whole was in that direction. Since the production of colostrum at the cellular and tissue levels is not well understood, these variations are not easily explained. It appears that the breast secretion available the first 2 or 3 days postpartum is a transcellular fluid. Its carotenoid content may be lower at times than that of the initial milk which follows.

The data of Table 1 also reveal substantial differences between individuals in carotenoid content of colostrum. Some of this may result from diet, but we find it notable that the primiparous donors had a lower mean value (1.14 μg/ml) than the multiparous (2.18 μg/ml). Actually this difference would be rendered more emphatic if the low value (0.52 μg/ml) for the sample of donor 92 was included with those of the primiparae. She was the only one among the multiparae not to nurse her previous (first) child. Visual evaluation of the primiparous samples prior to analysis indicated that they contained relatively little yellow pigment with the notable exception of sample 91. Interrogation of the primiparous donors revealed nothing unusual about their diets. All seemed to be consuming normal amounts of carotenoid-rich foods.

Identification of carotenoids. TLC on silica gel-coated plates (TLC system 1) established coincidence in mobility of major carotenoid bands from colostrum with those of the reference compounds. Reference (Rf) values for these latter were: α-carotene 0.71, β-carotene 0.71, lycopene 0.67, β-cryptoxanthin 0.23, lutein 0.14 and zeaxanthin 0.12. Thus, with this system, α- and β-carotene could not be separated, nor could lutein and zeaxanthin. Representative results for separation of the references (lane 1) and colostrum pigments (lane 2) are shown in Figure 1. A separation of the carotenoids in the same colostrum sample without benefit of saponification (lane 3) is included. It is evident, comparing lanes 2 and 3, that the large mass of triaclyglycerol in the unsaponified sample completely blocked development of the carotene and lycopene bands. There are several bands below the triacylglycerol spot (lane 3) that correspond reasonably well in density and Rf values with those from the saponified sample (lane 2). In summary, TLC system 1 yielded presumptive evidence of α- and/or β-carotene, lycopene, β-cryptoxanthin and lutein and/or zeaxanthin in colostrum.

The principal value of the MgO-kieselguhr TLC system was that it achieved a good separation of α- and β-carotene. The Rf values were 0.90 and 0.81, respectively. Using the two TLC systems in application to 10 colostrum samples from four women, spots corresponding to β-carotene were seen in all samples, and spots (faint) for α-carotene were seen only in the four samples having the highest total carotenoids. A spot corresponding to lycopene was seen in all but one of the samples, and to β-cryptoxanthin in most of the samples, although its position on some of the plates was obscured by streaking.

To provide further evidence regarding identity of carotenoids from colostrum resolved on TLC plates, bands of pigments corresponding in mobility to α- and β-carotene, lycopene and β-cryptoxanthin were scraped from plates, eluted into chloroform and analyzed spectrophotometrically between 400 and 550 nm. By this means, spectra from colostrum pigments (not shown) matching the references were obtained with maxima as follows: α-carotene—460 and 490 nm, β-carotene—465 and 494 nm, lycopene—458, 485 and 520 nm, β-

TABLE 1

Total Carotenoid Content of Colostrum Samples Collected from Various Donors

Donor	Parity	Time of sample[a]	Carotenoid (μg/ml)	Donor	Parity	Time of sample[a]	Carotenoid (μg/ml)
66	1	5	0.73	46	3	14	1.71
		24	0.25			38	1.35
		48	1.31			62	0.34
		120	0.54	52	2	3	2.22
88	1	33	0.35			34	0.50
89	1	54	1.05			54	3.00
90	1	51	0.58			108	0.78
91	1	74	4.29	59	2	7	7.57
						31	2.72
						54	4.84
						126	0.90
				72	3	26	3.41
						48	1.70
						72	1.13
				92	2	23	0.52
Mean		51	1.14 (±1.32)[b]			47	2.18 (± 1.96)[b]

[a]Hours since delivery.
[b](Standard deviation).

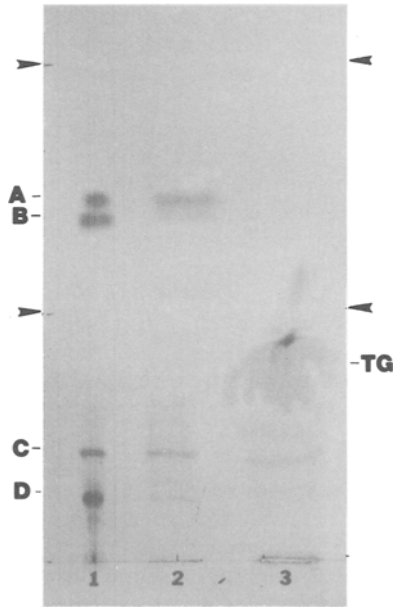

FIG. 1. TLC separation of reference carotenoids (lane 1), carotenoids extracted from saponified human colostrum (lane 2) and carotenoids extracted from the same colostrum without saponification (lane 3). The references are α- and β-carotene (A), lycopene (B), β-cryptoxanthin (C), lutein and zeaxanthin (D). They were applied at 4 to 6 μl each and at 0.5 μg/ml. The carotenoids were extracted from 0.5 ml samples of colostrum and were applied on the silica gel-coated plate in 200 μl of hexane. The plate was first developed with hexane benzene (1:1, v/v) to the position of the top arrowheads, and after brief drying, to the lower arrowheads with hexane acetone (7:3, v/v). Large spot, lane 3, is triacylglycerol(TG).

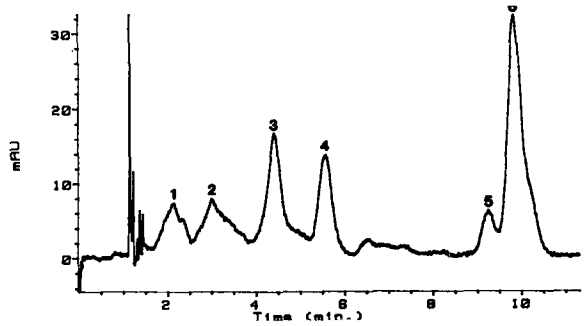

FIG. 2. A typical HPLC elution pattern of carotenoids extracted from a saponified sample of human colostrum. Absorbance of the eluate was monitored at a wavelength of 452 nm. The components were: 1. lutein-zeaxanthin, 2. unknown, 3. β-cryptoxanthin, 4. lycopene, 5. α-carotene and 6. β-carotene.

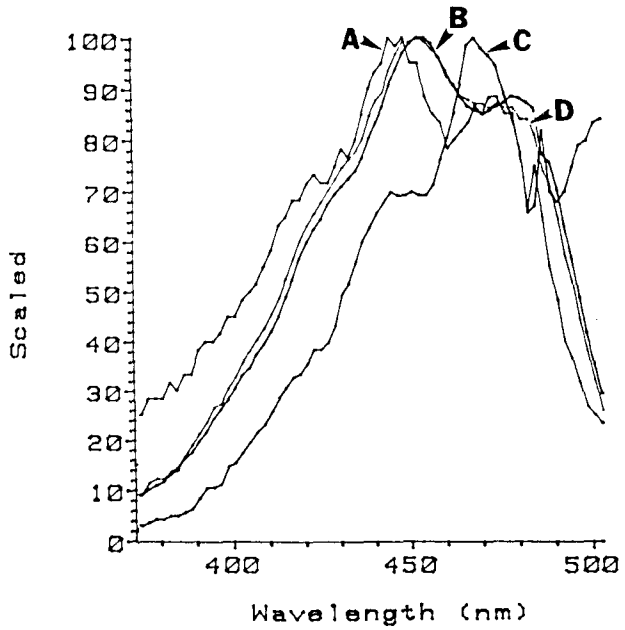

FIG. 3. Representative photodiode array spectra used in the identification of colostrum carotenoids isolated by HPLC. The spectra (and retention times) coincided with those for α-carotene (A), β-carotene (B), lycopene (C) and β-cryptoxanthin (D).

cryptoxanthin—465 and 490 nm. Thus from the TLC and spectral observations it was established that human colostrum contains carotenoids corresponding to α- and β-carotene, lycopene and β-cryptoxanthin.

Additional data confirming the presence of these four carotenoids in colostrum were obtained by HPLC. Eight samples from four donors were analyzed. All had major components with retention times corresponding to those of the four carotenoids detected by TLC. A representative elution pattern is shown in Figure 2. Spectral scanning of the pigmented components by the photodiode array revealed their carotenoid character (Fig. 3). Comparison of these spectra with those for α-and β-carotene, lycopene and β-cryptoxanthin scanned individually under the same conditions established their coidentity with the reference compounds. Representative data for wavelength maxima (nm) for standards and corresponding unknown peaks (Fig. 2) were: β-cryptoxanthin—452, 478, peak 3—452, 479; lycopene—445, 472, 500+, peak 4—446, 473, 500+; α-carotene—446, 473, peak 5—446, 472; β-carotene—453, 478, peak 6—453, 479. Peak 1 in Figure 2 exhibited a k′ value in agreement with either lutein or zeaxanthin which were not resolved by our chromatographic conditions. Its photodiode array profile form 250-500 nm tended to resemble that of lutein. This peak is followed by an unknown pigment (peak 2) also observed in the HPLC elution pattern of blood plasma carotenoids. Indeed, the overall elution pattern of colostrum carotenoids is like that seen for blood plasma carotenoids using the same type of HPLC column (10,13).

Concentrations of carotenoids and retinol in colostrum. Concentrations of the four identified carotenoids and retinol in eight of the colostrum samples were estimated from the HPLC data. The results, Table 2, show considerable variation between samples and donors presumably due to differences in fat content of samples, postpartum sampling times, and diets of donors. For example of the latter, donor 52 shows substantially more lycopene, of which tomatoes are the prominent source, than of the other three carotenoids in her colostrum. Alpha-carotene occurred in the least concentration in all the samples. Our findings confirm

TABLE 2

Concentrations of Some Major Carotenoids and Retinol in Human Colostrum and Pooled Plasma

Donor	Time of sample[a]	Colostrum				
		Cryptoxanthin	Lycopene	α-carotene	β-carotene	Retinol
				μg/ml		
46-1	62	.06	.15	.01	.04	.04
52-1	54	1.06	2.39	.12	.30	1.16
-2	108	.42	.72	.04	.11	.48
59-1	7	1.52	2.04	.48	2.09	2.09
-2	31	.73	.86	.22	.95	.87
-3	54	1.57	1.11	.32	1.45	2.28
-4	126	.25	.20	.07	.24	.55
66-1	120	.10	.21	.02	.08	.36
				Plasma[b]		
6/88		.22	1.86	.12	.29	.54
11/88		.19	.93	.11	.26	.59

[a]Time (hours) since delivery.
[b]Date of collection as indicated.

that carotenoids of human colostrum are not predominantly β-carotene, as is the case for bovine colostrum. With the exception of sample 52-1, the data of Table 2 are somewhat lower than those of Table 1 for total carotenoids, as would be expected by omission of lutein-zeaxanthin (peak 1) and the unknown (peak 2) from the HPLC data. Sample 52-1 is higher in Table 2 because of its unusually high lycopene content calculated using its extinction coefficient of 3,450 rather than the 2,500 employed for the data of Table 1. Total carotenoids (Table 2) appear to be fairly well correlated with retinol concentrations in these samples which suggests a common transportation pathway from the circulation, or that carotenoids may be converted to retinol in the mammary gland. For purposes of comparison, data we obtained on carotenoid and retinol contents of pooled human plasma are included in Table 2. With exception of findings for lycopene, it is seen that colostrum may carry many-fold the concentration of these components in plasma. However, colostrum samples obtained > 100 hr postpartum (Table 2) had values most of which were below those for plasma.

The procedure for saponifying colostrum to facilitate its analysis for carotenoids was patterned after the saponification of cow's milk used in an assay for total cholesterol (14). Exploration of the time-temperature relationship required to saponify lipids of human colostrum revealed 45°C for 60 min to be effective (Fig. 4). The major colostrum lipid, triacylglycerol, is almost totally removed by the reaction in 2 min (Fig. 4, component C). However, a second component (B), presumably fatty acid ethyl ester, rapidly forms, but is completely hydrolyzed in 60 min as are the other ester lipids. While it is possible, some minor amount of contaminating lipid could be accommodated in the TLC and HPLC analyses; by removing all the ester lipids, the saponification procedure eliminated that problematic variable. Moreover, defining the conditions of complete saponification should be useful in isolating other unsaponifiables of colostrum, which may include halogenated hydrocarbons and other environmental contaminants.

Experiments in which reference carotenoids were included in the saponifications established that all were stable. Recoveries of added carotenoids ranged from 90 to 100% when water was used in place of colostrum. Similar results were obtained with colostrum except that lutein and zeaxanthin, the most polar of the references, were not efficiently extracted by hexane. Their recoveries were 50—60% in several experiments. Comparison of total carotenoids in saponification extracts with those in lipid extracts of colostrum also indicated that the pigments are stable to saponification. Results for samples from three donors were 0.90, 3.00 and 4.29 μg/ml of colostrum by the saponification procedure and 1.02, 3.06 and 4.05 ug/ml, respectively, by the lipid extraction method. It was found that BHT should not be added to the ethanol used in the saponification because it generates a faintly detectable yellow pigment (Rf 0.50 by TLC on silica gel with 1:1 [v/v] hexane benzene). Colostrum from which lipids had been removed exhibited no extractable pigment following saponification.

Colostrum samples were checked for stability of total carotenoids to storage at −70°C. Five samples were reanalyzed after 3 or 6 months' storage. Two were evaluated first at 12 and then again at 18 months' storage. There appeared to be no change in values under these conditions. These results are reasonable in light of recent findings by Craft *et al.* (13) that there is no change in the carotenoids, retinol or tocopherol contents of human plasma during 28 months' storage at −70°C.

DISCUSSION

The main objective of this investigation was to identify major carotenoids in human colostrum. Using TLC and HPLC together with spectral analysis of isolated pigments, the presence of α- and β-carotene, lycopene

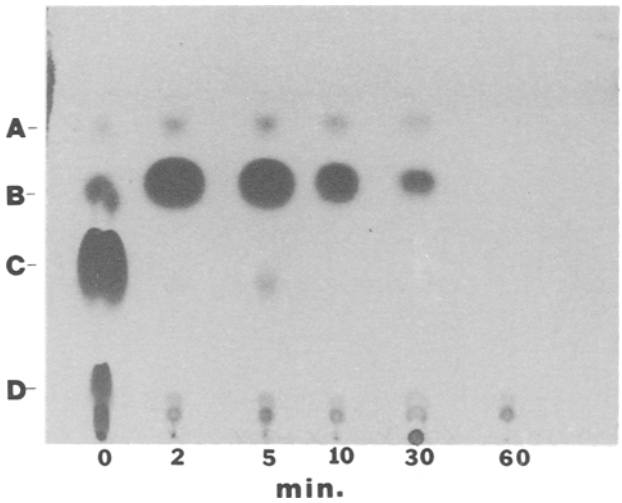

FIG. 4. TLC-separation of lipids extracted from 2-ml aliquots of a colostrum sample after saponification for varying times. Positions of components correspond to cholesteryl esters (A), fatty acid ethyl esters (B), triacylglycerols (C) and free fatty acids (D). Two-ml samples of colostrum were saponified for the time periods indicated and the reaction mixtures were immediately extracted with 1.0 ml of hexane. Ten μl of each extract was applied to a silica gel TLC plate which was then developed with hexane diethyl ether (6:4, v/v). Untreated colostrum lipid (0.4 mg) was used as the zero-time sample. Detection was by charring.

and β-cryptoxanthin was established. A component (Peak 1, Fig. 2) was tentatively identified as a mixture of lutein and zeaxanthin by comparison with authentic standards on TLC and HPLC, and by photodiode array analysis. However, the present techniques were incapable of resolving the pair. Human blood plasma has been reported to contain these same major carotenoids including lutein-zeaxanthin (10,12,13). To facilitate these identifications, it was necessary to saponify the colostrum so that overwhelming amounts of triacylglycerols (Fig. 1) would be eliminated. It was found that this did not interfere with quantitative recovery of the four identified carotenoids. However, carotenoid esters would be hydrolyzed by such treatment; thus our results do not preclude their presence. Further research will be required to identify and quantify the colostrum carotenoid tentatively designated here as lutein-zeaxanthin.

Our estimation of total (Table 1) and individual (Table 2) carotenoids in colostrum samples confirms previous results (2-4) showing decreasing concentrations of the pigments during the first week postpartum. This suggests that progressing lactation may have a flushing and diluting effect on substances stored in the non-lactating gland. Such may include halogenated hydrocarbons and other environmental contaminants (15), some of which may be carcinogenic. However, the present study indicates that this release of stored substances, as indicated by the carotenoids, is not characteristic of all women initiating a lactation. Four out of five primiparous (first lactation) donors had relatively low colostrum carotenoid concentrations (1.31 μg/ml or less, Table 1). In our previous study (3), the primiparous donor, one of five, yielded samples with the lowest values throughout the six-day sampling period. Of possible relevance is the observation that primiparous cows secrete about one-half as much insulin-like growth factor in their colostrum as do multiparous cows (16).

While the major carotenoids—α- and β-carotene and lycopene—are hydrocarbons, they are not transported into colostrum like the chlorinated hydrocarbons, DDT and PCB. The data of Rogan *et al.* (15) show that these latter, in distinction to the carotenoids (Table 1), were highest in colostrum of primiparae, and became progressively less in colostrum of succeeding lactations; the decline during a given lactation was quite gradual in contrast to the rapid drop in a few days of the carotenoids (2-4). This suggests different pathways into colostrum for these two kinds of hydrocarbon. In the lactating bovine mammary gland, carotenoids were found in high concentrations throughout cellular organelles and membranes (17). The concentrations in intracelluar fat droplets and secreted fat globules were relatively low.

Since carotenoids in the diet have been implicated in a sparing action on human cancers (6), including that of the breast (8), it is important to define any differences in the way breast tissue transports and stores these pigments. This connection is rendered the more pertinent in that lactation is known to be correlated with reduced incidence or delay in onset of breast cancer (18-20). While it is only anecdotal in our study, we find it interesting that donor 92, who did not breastfeed her first child, had one of the lowest colostrum carotenoid concentrations (0.52 μg/ml, Table 1). The implication is that the first lactation, in some manner, facilitates carotenoid transport into colostrum at the start of subsequent lactations.

An aspect of the question whether women differ in their capacity to secrete carotenoids in colostrum concerns the metabolism of lipids by mammary tissue at that time. It has been observed (3) that the fat globules of colostrum which are most yellow appear to be larger than the less pigmented globules that progressively replace them as lactation proceeds. This may indicate that these relatively large globules released at the outset of lactation had been developing in the cell for some time, and that a difference between women in carotenoids of such globules may depend upon their capacity to transport carotenoids into the epithelial cell before and during gestation. The difference between women in carotenoid content of their colostrum was a serendipitous observation in our study. It appears that a carefully controlled investigation of the phenomenon would be justified. Further knowledge in this area may better define the known sparing effect on breast cancer associated with lactation. The likelihood that colostrum carotenoids benefit the health of the newborn provides an added incentive for such an investigation.

Note added in proof: Following acceptance of our manuscript, it became possible for us to analyze total carotenoids in colostrum samples from 5 primiparous and 5 multiparous cows. These samples, a gift of Drs. C.R. Baumrucker and P.G. Campbell, were collected for a study of insulin-like growth factor-I (16). The

samples in the interim had been held at −20°C. Those we analyzed were from the first postpartum (day 1) milking of the udders; one sample (primiparous) was rejected because of blood contamination. Sample lipids were extracted (12), solvent removed, the lipids weighed and taken up in hexane. Total carotenoids were determined from the absorption of the hexane solutions at 460 nm in conjunction with the formula employed herein (Materials and Methods). Mean carotenoid contents for the primiparous samples were 0.45 μg/ml and 5.1 μg/g of lipid; and for the multiparous sample were 0.90 μg/ml and 15.4 μg/g of lipid. Thus these bovine samples showed the same tendency as those of human for carotenoid contents of colostrum from primiparae to be lower than those from multiparae.

ACKNOWLEDGMENTS

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Albumin-Bound Docosahexaenoic Acid and Collagen-Induced Human Platelet Reactivity

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An *in vitro* system designed to mimic the effect of various plasma nonesterified (polyunsaturated) fatty acids on platelet function and metabolism was employed. Human platelet aggregation induced by submaximal (1.8 μg/ml) collagen stimulation was significantly inhibited by 2 min preincubation with 20 μM albumin-bound docosahexaenoic acid (22:6n-3) (DHA), but not by the other fatty acids tested. [^{3}H]Phosphatidic acid (PA) formation, an indicator of phospholipase C activation following platelet stimulation, was moderately inhibited by eicosapentaenoic acid (20:5n-3), 11,14,17-eicosatrienoic acid (20:3n-3), dihomo-γ-linolenic acid (20:3n-6), as well as DHA, but not by arachidonic acid (20:4n-6); this inhibition of phospholipase C activation could not explain the differential effect of DHA on platelet aggregation. The decreased production of thromboxane A_2 (TxA_2), as assessed by [^{3}H]12-hydroxy-5,8,10-heptadecatrienoic acid (HHT) formation, may account for the inhibition of collagen-induced aggregation by 20 μM DHA. Surprisingly, preincubation with 40 μM albumin-bound DHA, even though resulting in greater inhibition of collagen-induced aggregation, had less impact on HHT formation. A small but significant increase in [^{3}H]prostaglandin D_2 (PGD_2) levels following 3-min collagen stimulation may have contributed to the greater antiaggregatory effect of 40 μM DHA. It is concluded that increased plasma nonesterified DHA may contribute to the dampened platelet activation and altered metabolism following fish oil supplementation of the diet.
Lipids 25, 166–169 (1990).

Epidemiological studies have suggested a protective effect of marine-based diets rich in eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) on cardiovascular disease (1–3). Many, but not all, feeding trials have demonstrated diminished platelet aggregation following fish oil supplementation of western diets (4). Although the majority of these studies employed platelet-rich plasma, the decreased platelet aggregation observed has been primarily attributed to altered platelet metabolism, in particular, reduced thromboxane A_2 (TxA_2) formation from arachidonic acid (AA, 20:4n-6) as a result of incorporation of n-3 fatty acids into platelet phospholipids (5–7). The potential impact on platelet function of alteration to the plasma (including free fatty acid) component has been studied to a very limited extent.

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Abbreviations: AA, arachidonic acid; DG, diacylglycerol; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FFA, free fatty acids; HETE, hydroxyeicosatetraenoic acid; HHT, hydroxyheptadecatrienoic acid; IP_3, inositol trisphosphate; NEFA, nonesterified fatty acid; PA, phosphatidic acid; PGD_2, prostaglandin D_2; TxA_2, thromboxane A_2; TxB_2, thromboxane B_2.

Plasma DHA, as nonesterified fatty acid (NEFA), increases dramatically within hours of consuming a large single dose of fish oil (8). Populations consuming a marine-based diet also have relatively high fasting levels of DHA in this compartment (9). Despite the fact that, in both of these cases, comparable amounts of EPA and DHA were consumed, the level of plasma nonesterified EPA was found to be much lower than that of DHA.

Collagen binding to platelets activates phosphoinositide specific phospholipase C resulting in the formation of two second messenger molecules, 1,4,5-inositol trisphosphate (IP_3) and 1,2-diacylglycerol (DG) (10,11). IP_3 has been shown to mobilize intracellular calcium (12) while DG can activate protein kinase C (13). The DG formed is rapidly phosphorylated by DG kinase to yield phosphatidic acid (PA) (14). PA has been suggested to cause mobilization of Ca^{2+} from platelet intracellular storage sites (15) as well as to mediate the selective Ca^{2+} translocation across model membranes (16). Recently PA has been suggested to act as a positive feedback signal to amplify receptor-mediated activation of PIP_2-specific phospholipase C in human platelets (17). The effect of exogenous albumin-bound DHA on PA formation has not been studied to date.

In the present investigations, it was of interest to study the effect of exogenous albumin-bound DHA on platelet aggregation as well as eicosanoid metabolism and PA formation in an *in vitro* system designed to mimic increased plasma nonesterified DHA. Other fatty acids of potential interest were used for comparative purposes.

MATERIALS AND METHODS

Materials. DHA, AA, and 11,14,17-eicosatrienoic acid were obtained from NuChek Prep (Elysian, MN). Linoleic, stearic, palmitic, palmitoleic, α-linolenic, oleic, and dihomo-γ-linolenic acids were supplied by Serdary Research Laboratories (London, ON). EPA and human serum albumin (Fraction V, essentially fatty acid free) were obtained from the Sigma Chemical Co. (St. Louis, MO). [^{3}H]AA (100 Ci/nmol), [2-^{3}H]glycerol (200 μCi/nmol), and Aquasol-2 were obtained from the New England Nuclear Corp. (Boston, MA). Collagen was purchased from Hormon-Chemie (München, FRG). All chemicals and solvents used were ACS certified.

Fatty acid additions. Stock solutions of free fatty acids (FFA) in hexane were kept at −20°C under N_2. For the preparation of the final platelet suspension buffer, albumin solutions containing FFA (molar ratio 0.7:1.0 FFA:albumin) were constituted as follows: essentially fatty acid free human serum albumin (4.375 g) was dissolved in 100 ml distilled H_2O and kept in a stoppered flask. Appropriate volumes (to yield the fatty acid composition in the final platelet suspension as indicated in Table 1) of FFA in hexane were dried

TABLE 1

Fatty Acid Composition of the Final Platelet Resuspension Buffer[a]

	μM	Mol%
16:0	101.4	30.2
16:1n-7	11.8	3.5
18:0	31.7	9.4
18:1n-9	112.5	33.5
18:2n-6	71.4	21.3
18:3n-3	7.2	2.1

[a]The molar ratio FFA/albumin = 0.7:1.0.

under nitrogen and redissolved in ethanol. Aliquots of this ethanolic solution were added slowly with stirring under N_2 to the albumin solution. Following all additions, the albumin/FFA solution was stirred for a further 4 hr. Distilled water was added to compensate for losses due to evaporation. Twenty-ml aliquots of the albumin/FFA solution were stored under nitrogen at −20°C for a maximum of two weeks. The fatty acid composition in the final platelet suspension media (Table 1) is similar to that reported for the FFA content of human plasma from healthy subjects (18,19), and represents over 90% of the albumin-bound FFA normally present in human plasma. Test fatty acid (EPA, AA, DHA, 20:3n-3 and 20:3n-6) solutions were made up in the same manner and stored in 150 μl aliquots in capped plastic Eppendorf vials. In all experiments, the final concentration of ethanol was less than 0.3% by volume, with control samples containing the same concentrations.

Platelet isolation and prelabelling. Blood samples were collected from healthy male subjects who had not taken any medication for the previous two weeks. Washed platelet suspensions were prepared according to the method of Mustard *et al.* (20) with the exception that the final suspension media contained 3.5% human serum albumin precoated with FFA as described above.

For the determination of PA formation, platelet phospholipids were prelabelled in the first wash buffer by incubation with 50 μCi of [2-^{3}H]glycerol for one hr at 37°C. [^{3}H]AA prelabelling (2.5 μCi/15 ml PRP) for two hr at 37°C was employed for the assessment of eicosanoid production following collagen stimulation of washed platelet suspensions. Platelet counts were determined using a Coulter Counter model ZM (Coulter Electronics of Canada Ltd., Burlington, ON) and adjusted to give a final concentration (after additions) of 5×10^8 platelets/ml.

Platelet aggregation. Four hundred fifty μl aliquots of platelet suspension in siliconized cuvettes were stirred at 900 rpm and 37°C in an aggregometer for one min (Payton Associates, Buffalo, NY). Test fatty acids (20 μM final concentration, molar ratio FFA/albumin, 0.7:1.0) or albumin (control) were added two min prior to the addition of 1.8 μg/ml collagen, and the light transmission was recorded for 3 min. Reactions were terminated by the addition of 3% formic acid (eicosanoids) or chloroform/methanol (1:2 v/v) (phospholipids).

Extraction and analysis of phospholipids and AA metabolites. After acidification of the platelet suspension with formic acid (pH 3.5–4.5), AA-metabolites were extracted three times with diethyl ether. Phospholipids were extracted according to the method of Bligh and Dyer (21). Two half ml aliquots of platelet suspension were pooled for the purpose of these extractions. Extracts were dried under N_2 and resuspended in 25 μl of chloroform/methanol, 2:1 (v/v). Thromboxane B_2 (TxB_2) and prostaglandin D_2 (PGD_2) were resolved by thin-layer chromatography (TLC) using the solvent system of diethyl ether/methanol/acetic acid (90:1:2, v/v/v). (22). 12-Hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE) and 12-hydroxy-5,8,10-heptadecatrienoic acid (HHT) were resolved using the solvent system diethyl ether/hexane/acetic acid (60:40:1, v/v/v) as described by Salmon and Flower (23). Phospholipids were separated using the two-dimensional TLC system of Mitchell *et al.* (24). Lipids were visualized by exposing the plates to iodine vapor, and individual bands were scraped and counted in a liquid scintillation counter (Beckman 7800).

Statistical analysis. Comparisons with control (albumin) were determined using two tailed paired Student's t-test. Statistical significance was assumed to be $P<0.05$.

RESULTS AND DISCUSSION

The concentration of collagen used in the present studies (1.8 μg/ml) resulted in a submaximal aggregation response in the majority of subjects. Of the fatty acids tested, only DHA significantly inhibited collagen-induced platelet aggregation under the conditions employed (Fig. 1). Twenty μM EPA was approximately half as potent as 20 μM DHA although this effect did not quite reach the level of significance ($P=0.07$). At much higher concentrations than were used in the present study, exogenous DHA has been reported to inhibit collagen- (25), AA- (25,26), epinephrine- (25,26), and adenosine diphosphate- (25–27) induced platelet aggregation. Since little DHA, unlike AA or EPA, is released from platelet phospholipids upon stimulation (28, 29), plasma nonesterified DHA is possibly the main source of "free" DHA which platelets are exposed to *in vivo*. Levels of plasma DHA as NEFA in the range of 14–20 μM have been reported in fasting Japanese subjects (9). Three hours following the ingestion of a large single dose of cod liver oil by western subjects, plasma DHA NEFA rose from 0.4 to approximately 13 μM (8). It appears likely, therefore, that the 20 μM DHA used in this study could be attained *in vivo* by dietary means.

The effectiveness of DHA at inhibiting collagen-induced aggregation is even more striking in light of the lack of effect of AA. As little as 0.5 μM AA has been reported to induce aggregation in platelet suspensions devoid of albumin (30). As well, it has been demonstrated that concentrations of AA too low to cause aggregation by itself enhanced the aggregation induced by submaximal collagen stimulation (31). The lack of potentiation of collagen-induced aggregation by 20 μM AA in the present study (Fig. 1) suggests that the platelets in this system were exposed to very low concentrations of "free" fatty acid (i.e., close to 100% binding to albumin was attained). This level of binding to albumin is believed to occur with plasma NEFA *in vivo* (32); however, direct determination of the extent

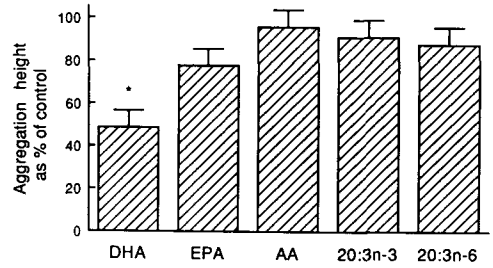

FIG. 1. Effect of albumin-bound fatty acids on collagen-induced platelet aggregation. Washed platelet suspensions (see Methods) were stirred (900 rpm) and warmed (37°C) for 1 min, exposed to 20 μM albumin-bound FFA for 2 min, and stimulated with 1.8 μg/ml collagen for 3 min. Results are expressed as the % of albumin control aggregation height at 3 min. N=4. *P<0.05.

of fatty acid binding to albumin was not performed in the present study. Hoak *et al.* (33) have demonstrated that platelets readily take up FFA from solutions containing albumin-bound fatty acids. With short-term incubation, the majority of these remain as free acids in loose association with the plasma- and surface-connected membranes.

As shown in Table 2, all fatty acids tested, with the exception of AA, exhibited a moderate inhibition of [^{3}H]PA formation, thereby suggesting a nonspecific dampening of phospholipase C activation associated with collagen stimulation. Inhibition of DG kinase, however, cannot be ruled out as a contributor to the decreased [^{3}H]PA formation in our studies. In any event, this effect did not discriminate between the fatty acids tested in regard to their antiaggregatory activity.

The effect of 20 μM and 40 μM albumin-bound DHA and 20 μM albumin-bound EPA on eicosanoid formation following collagen stimulation is reported in Table 3. Preincubation with 20 μM DHA, although not inhibiting TxB_2 formation to the level of statistical significance, did result in a significant inhibition ($P<0.05$) of HHT, an indicator of TxA_2 formation. Surprisingly, 40 μM DHA preincubation resulted in less inhibition of formation of both of these metabolites. This lack of a dose response suggests that the effect of exogenous albumin-bound DHA on eicosanoid synthesis is more complex than simple competitive inhibition of platelet cyclooxygenase (34). This result does not agree with previous reports (25,26), where platelets were exposed to a pulse of radiolabelled AA; in the present study, platelets were prelabelled with [^{3}H]AA, and eicosanoids were determined following collagen stimulation. A differential effect of the two levels of DHA on phospholipase-mediated release of AA from specific phospholipid pools, or a bimodal effect of altered membrane fluidity on cyclooxygenase activity are possible mechanisms to explain this result. Although the decreased TxA_2 formation may explain all or part of the decreased aggregation observed with 20 μM DHA, it cannot explain the greater inhibition of aggregation we observed in the [^{3}H]AA prelabelling experiment with 40 μM DHA (53.0 ± 14.5 vs 27.5 ± 8.9 mean ± S.E. expressed as percentage of control for 20 μM and 40 μM DHA respectively). Dissociation between inhibition of thromboxane formation and platelet aggregation, by various fatty acids, has been previously observed (35). Interference at the level of the TxA_2 receptor by DHA itself (36), or by lipoxygenase products of DHA (37), has also been suggested to contribute to the inhibition of platelet aggregation by exogenously added DHA. Further research is required to determine if either of these two mechanisms were operative in the present system.

Although both concentrations of DHA resulted in similar inhibition of 12-HETE formation, only 20 μM DHA reached statistical significance (Table 3). Since it has been reported that DHA does not competitively inhibit lipoxygenase in a cell-free system (34), the altered 12-HETE formation in the present study may possibly reflect a different mechanism of action of DHA.

Preincubation of platelet suspensions with 40 μM DHA led to a small but significant increase in labelled PGD_2 following collagen stimulation. Further research is required to determine the physiological significance of this effect; however, PGD_2, likely through activation of adenylate cyclase, is a known inhibitor of platelet aggregation (38,39). Recently it has been shown that increasing membrane fluidity, by means of benzyl alcohol, increases adenylate cyclase activity in platelets (40). Increased cAMP levels may, therefore, be involved in the greater inhibition of platelet aggregation observed with 40 μM as compared to 20 μM DHA.

In conclusion, a potential impact of increased plasma

TABLE 2

Effect of Various Albumin-Bound Fatty Acids (20 μM) on Phosphatidic Acid Formation Following Collagen Stimulation (1.8 μg/ml) in [^{3}H]Glycerol Prelabelled Human Platelets[a]

	Added Fatty Acids				
Albumin Control	DHA	AA	EPA	20:3n-3	20:3n-6
100	81.8[b]	91.6	73.3[c]	77.6[c]	78.7[d]
(2177 ± 609) d.p.m.	± 3.3	± 5.0	± 6.8	± 5.8	± 2.2

[a]Results are expressed as percentages ± S.E. of collagen stimulation alone. Total radioactivity in [^{3}H]glycerol prelabelled platelets (unstimulated) was 205,540 ± 72,105 dpm. Radioactivity associated with PA (unstimulated platelets) was 353 ± 74 dpm.
[b]P vs control <0.005.
[c]P vs control <0.01.
[d]P vs control <0.0001. N=4.

TABLE 3

Effect of DHA and EPA on Eicosanoid Synthesis Following Collagen Stimulation (1.8 μg/ml) in [^{3}H]Arachidonic Acid Prelabelled Platelets[a]

	Added Fatty Acids		
Eicosanoid	20 μM DHA	40 μM DHA	20 μM EPA
HHT	72.6 ± 4.9[b]	86.3 ± 10.4	81.0 ± 8.3
TxB_2	73.9 ± 16.2	85.2 ± 9.7	87.3 ± 32.2
12-HETE	67.4 ± 9.2[c]	60.2 ± 19.1	86.1 ± 18.9
PGD_2	104.1 ± 14.7	130.1 ± 9.6[c]	96.9 ± 15.0

[a]Results are expressed as percentages ± S.E. of collagen stimulation alone (100%). The average (± S.E.) in HHT, TxB_2, 12-HETE and PGD_2 in albumin control, collagen-stimulated platelets were 522 ± 85, 483 ± 158, 3553 ± 892, and 555 ± 108 dpm respectively.
[b]P vs control <0.005.
[c]P vs control <0.05. N=4.

nonesterified DHA on platelet aggregation and associated biochemical alterations has been demonstrated. *Ex vivo* experiments, following short-term ingestion of purified DHA, are planned for the future to help confirm or refute the present *in vitro* results. Further research is also required to determine the absolute concentration of plasma nonesterified DHA attainable through dietary, and/or, pharmacological manipulation.

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METHODS

Simple High Vacuum Distillation Equipment for Deodorizing Fish Oil for Human Consumption

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A simple piece of glass equipment for deodorizing fish oil (sardine oil) by high vacuum distillation was designed and constructed. The equipment has a throughput of 450–500 ml/hr working at 140°C and at a constant pressure of 2×10^{-2} mm Hg. It reduces the peroxide value and the cholesterol content of the oil and improves the flavor without affecting the EPA and DHA content. *Lipids 25,* **170–171 (1990).**

Increasing interest in the pharmacological and nutritional properties of marine fish oils rich in n-3 polyunsaturated fatty acids (1) has led to efforts to improve their chemical and organoleptic properties. As these oils are secondary products of fish meal manufacture, the procedures applied provide minimal protection against autoxidation. Therefore, in addition to any undesirable odors characteristic of the oil, high peroxide values are common because no antioxidants are added during production, although refining procedures improve the oxidative indices of the product (2).

The nutritional and pharmacological effects of marine fish oils are ascribed principally to their high content of eicosapentaenoic acid (EPA; 20:5 n-3) and docosahexaenoic acid (DHA; 22:6 n-3) (3) which have concentrations totaling 24–33% of the fatty acid content of the oil depending on the type of fish and the season of capture. In this context, any method applied to improve the chemical and organoleptic characteristics of the oil must keep the EPA and DHA composition unaltered.

Among the different procedures for deodorizing fish oil, those involving distillation for removal of impurities under high vacuum appeared as the more efficient methods (4). A deodorized fish oil should be low in free fatty acids, improved in color and odor, and containing little or no polymeric matter. Also the oil should have improved stability toward autoxidation compared to oils refined by other methods.

With the general aim of developing a method to produce (from sardine oil) high quality triglycerides suitable for human nutrition and medical research, the objectives of this research were (i) the design and construction of laboratory high vacuum distillation equipment and (ii) determination of the operating variables under which suitable deodorization is obtained with minimal alteration of EPA and DHA contents.

MATERIALS AND METHODS

The distillation equipment was fabricated of borosilicate glass Duran 3.3 (DIN ISO 3585) of high chemical and mechanical resistance and minimal thermal expansion. High vacuum (1×10^{-1} to 1×10^{-3} mm Hg) was obtained with a mechanical pump (Precision Scientific, 1057-F10, 150 l/min) in line with a Hg-glass diffusion pump (Jencons type I). Volatiles were condensed by means of a 2-l Dewar flask filled with liquid nitrogen. Heating was by recirculation of glycerol (100–200°C), and cooling of the external condenser with cold water (2–4°C).

Sardine oil (partially refined) was obtained from a local fish meal factory (Corpesca SA, Mejillones, Chile). The oil, which had no added antioxidant and had peroxide values ranging from 10 to 15 meq/kg, was winterized for 2 weeks at 15°C and one week at 4°C. The liquid phase was kept under nitrogen in sealed dark flasks until its distillation.

Analysis of fatty acids as methyl esters was performed by GLC according to Morrison and Smith (5). The peroxide values and anisidine values were measured as described (6). Cholesterol was measured spectrophotometrically (7). The effect on flavor of deodorizing was tested by a panel of 10 trained referees, and expressed as percentage of remnant odor. Results were expressed as means ± SE. All reagents were analytical grade (Merck or Baker).

RESULTS AND DISCUSSION

Figure 1 illustrates the distillation equipment. Formation of an even oil film on the central, heated column was brought about by means of a funnel-shaped Teflon ring which was located concentrically to the column, was maintained at a distance of 0.5 mm from the surface of the column, and was supported by the internal face of the condenser. This device allows the formation of a thin film of the oil. The throughput of the equipment was 450 to 500 ml/hr of recovered oil. After distillation, the deodorized oil was cooled under a stream of N_2, stabilized by the addition of D-α-tocopherol (2 g/kg oil), and kept at 4°C in a sealed, dark bottle until use. Under these conditions, the vacuum-refined oil was stable (peroxide index 2 meq/kg) for at least six months.

Figure 2 shows the variation of the EPA and DHA content, the peroxide value, the anisidine value and the cholesterol content of the distilled oil as functions of the distillation temperature at a constant pressure of 2×10^{-2} mm Hg. With the exception of the EPA and DHA content, all other parameters are improved at higher temperatures. The EPA and DHA content increase slightly until 140°C, and then show a drastic decrease as the temperature approaches 170°C. Due to this limitation, the temperature of 140°C was considered as optimal for distillation of impurities in order to maintain the conent of the higher polyunsaturated fatty acids.

Deodorization of the oil as a function of the temperature and at a constant pressure of 2×10^{-2} mm Hg is shown

*To whom correspondence is addressed.

Abbreviations: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; GLC, gas-liquid chromatography.

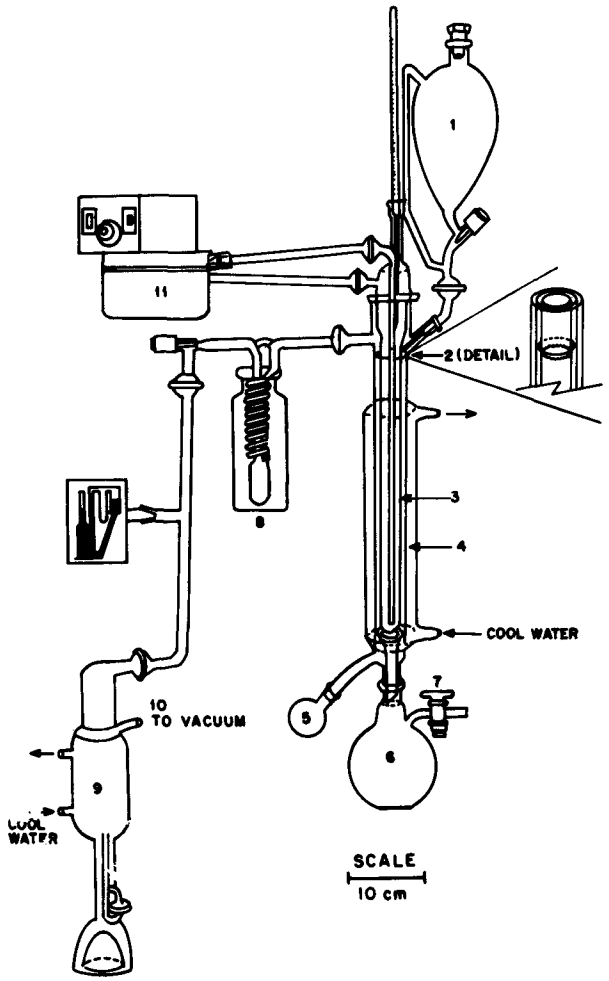

FIG. 1. (1) Storage flask for oil to be refined; (2) Teflon ring for even formation of the oil film; (3) central heated column (diameter 2.5 cm, effective length 40.0 cm); (4) condenser; (5) flask for volatiles condensed at low temperature; (6) receiver for deodorized oil; (7) valve for N_2 flushing; (8) liquid nitrogen trap for volatiles condensed at very low temperature; (9) Hg-glass diffusion pump; (10) to mechanical vacuum pump; (11) thermostated heater.

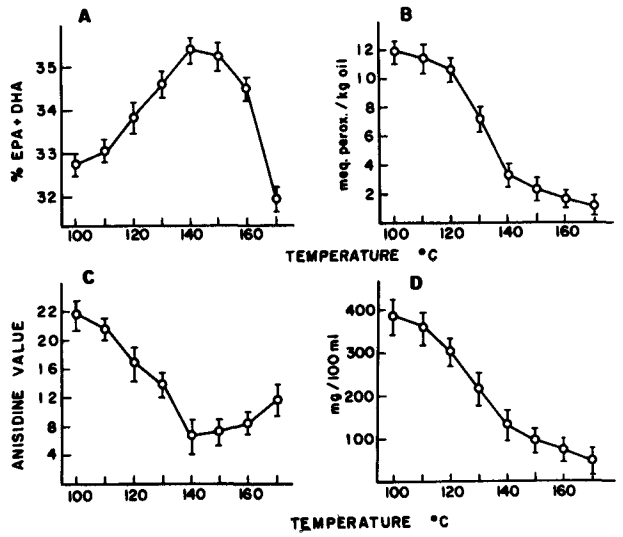

FIG. 2. Effect of distillation of sardine oil on properties: (A) EPA plus DHA content; (B) peroxide value; (C) anisidine value; (D) cholesterol content. Each point represents the mean of five experiments ± S.E. Other conditions are in the text.

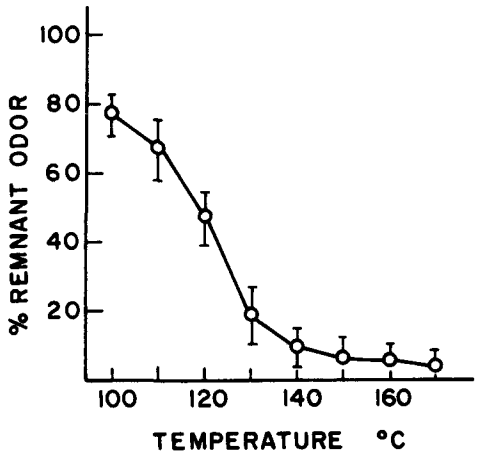

FIG. 3. Effect of distillation on the flavor of deodorized sardine oil. Each point represents the mean of six experiments ± S.E. Other conditions are in the text.

in Figure 3. It can be observed that temperatures higher than 120°C allow good deodorization. This parameter was not significantly improved at temperatures greater than 140°C.

In conclusion, we have designed a simple, low-cost, high-vacuum distillation glass unit for deodorizing fish oil for experimental studies, but operable on a scale capable of providing material for human consumption. Evaluation of the biochemical properties of this oil in human subjects affected by different types of cardiovascular diseases is currently being carried out by our group.

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COMMUNICATIONS

Oxysterol Mediated Changes in Fatty Acid Distribution and Lipid Synthesis in Cultured Bovine Aortic Smooth Muscle Cells

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We studied the actions of oxysterols on fatty acid distribution and lipid synthesis in cultured bovine aortic smooth muscle cells. Cultures were labeled with [1-^{14}C]arachidonate or [1-^{14}C]oleate. During a 24-hr incubation, 25- or 22R-hydroxycholesterol enhanced the incorporation of label into triglycerides, concomitant with a reduction in the labeling of phospholipids. Cholestantriol or 20-hydroxycholesterol had the opposite effects. They caused a higher incorporation of radiolabel into phospholipids and a reduction of labeling of triacylglycerols. Similar changes were seen in cells labeled with [1-^{14}C]acetate. Therefore, we conclude that oxysterols can promote changes in the distribution of fatty acids between neutral lipids and phospholipids through mechanisms that still need to be clarified.
Lipids 25, 172–176 (1990).

Lipid peroxidation is being increasingly implicated in human disorders, and the actions of lipid peroxides have been extensively studied, particularly in the vascular system (1). Similarly, cholesterol oxides (oxysterols) display a wide range of toxic properties (2). Their angiotoxicity was first observed in animal studies (3–5). Recent *in vitro* studies have shown that they modify various aspects of vascular physiology. They have been shown to modulate platelet aggregation (6), increase endothelial cell permeability to albumin (7) and inhibit arachidonate metabolism and prostacyclin synthesis in cultured smooth muscle cells (8). Oxysterols have long been considered sterol synthesis inhibitors (9) and they seem to be important cellular regulators of cholesterol metabolism (10). Many studies have focused on this aspect, but little is known about their possible effects on lipid metabolism. The present study was designed to investigate whether exposure to different oxysterols could modify the distribution of fatty acids among different lipid classes of bovine aortic smooth muscle cells.

MATERIALS AND METHODS

Reagents. Cholesterol oxides were purchased from Sigma Chemical Co. (St. Louis, MO). [1-^{14}C]Arachidonate (50–60 mCi/mmole), [1-^{14}C]oleate (54 mCi/mmole) and [1-^{14}C]acetate (55 mCi/mmole) were obtained from the Centre d'Etudes Atomiques (Saclay, France). Culture media and all supplements were purchased from Eurobio (Paris, France).

Cell culture. Cell culture work was carried out as described in detail elsewhere (8). Briefly, cells from explants of bovine aortic media were maintained and used until the seventh passage. Culture medium was RPMI 1640 20 mM Hepes supplemented with 10% fetal bovine serum, 50 U/ml penicillin, 50 μg/ml streptomycin, 1 μg/ml amphotericin B and L glutamin 2 mM.

Cell labeling with radioactive fatty acids and analysis of cell lipids by thin-layer chromatography (TLC). Cells were grown for three or four days after trypsinization. They were labeled for 2 hr with 0.3–0.4 μCi of [1-^{14}C]arachidonate per Petri dish. Labeled cultures were then incubated for 24 hr with the oxysterols. These two treatments were reversed in some experiments. At the end of the experiment, culture media were removed, Petri dishes were washed twice with PBS Dulbecco's, and cell lipids were extracted *in situ* by the procedure described by Hara and Radin (11) using 3 ml of hexane/isopropanol (3:2, v/v). This method gave excellent recoveries of the radioactivity associated with the cells (98%). Samples were applied to TLC plates, and plates were developed in hexane/diethyl ether/formic acid (80:20:2, v/v/v). Silica fractions were scraped off and each cm^2 was counted by liquid scintillation counting.

[1-^{14}C]*Acetate labeling of cells and lipid analysis.* After a 24-hr incubation with the oxysterols, 1 μCi of [1-^{14}C]acetate was added to each Petri dish. Twenty-four hours later, media were removed, dishes were washed twice with PBS Dulbecco's, and cultures were dissociated with 1 ml of trypsin-EDTA. Lipids were extracted from the suspension following the Folch procedure (12). Ninety-five percent of the radioactivity incorporated in the cells was recovered. Extracts were analyzed by thin-layer chromatography using the method described by Freeman and West (13). Lipid spots, revealed by iodine vapors, were scraped off and counted.

Statistics. Results are expressed in mean ± S.D. The significance of differences between groups of data was assessed by Student's *t*-test and one-way analysis of variance.

RESULTS AND DISCUSSION

[1-^{14}C]Arachidonate and [1-^{14}C]oleate distribution in cell lipids and effects of oxysterols. After 2 hr of incubation, 80% of total [1-^{14}C]arachidonate was found associated with cell lipids. Most of the label was recovered in the phospholipids (81% of total radioactivity) and in the triglyceride fraction (15% of total). Only small amounts were recovered as mono- or diglycerides, migrating in a single peak in this solvent system (1–2% of total), or as free fatty acids (1–2%). Proportions

Abbreviations: 20-Hydroxycholesterol, cholest-5-en-3β,20α-diol; 22R-hydroxycholesterol, cholest-5-en-3β,22(R)-diol; 25-hydroxycholesterol, cholest-5-en-3β,25-diol; cholestantriol, cholestan-3β,5α,6β-triol.

of cholesteryl [1-^{14}C]arachidonate were also very low (0.5%).

Figure 1 shows the results of 24 hr-incubations of [1-^{14}C]arachidonate labeled cells with different cholesterol oxides. Two different effects could be observed. 25-hydroxycholesterol or 22R-hydroxycholesterol induced a dose dependent reduction of radioactivity associated with the phospholipids. This reduction was significant at 2 μg/ml (5 μM). Concomitant with this reduction in phospholipid labeling, an increase in the radioactivity associated with triglycerides was observed (Fig. 1b). The increase in triglycerides labeling was equal to the decrease in phospholipid labeling. No reproducible changes could be seen in the mono- and diglycerides, the cholesteryl esters, or the unesterified fraction. Cholestantriol or 20-hydroxycholesterol had exactly the opposite effect. Twenty-four hr treatments led to a reduction of the radioactivity in the triacylglycerols and a higher proportion of label in the phospholipids.

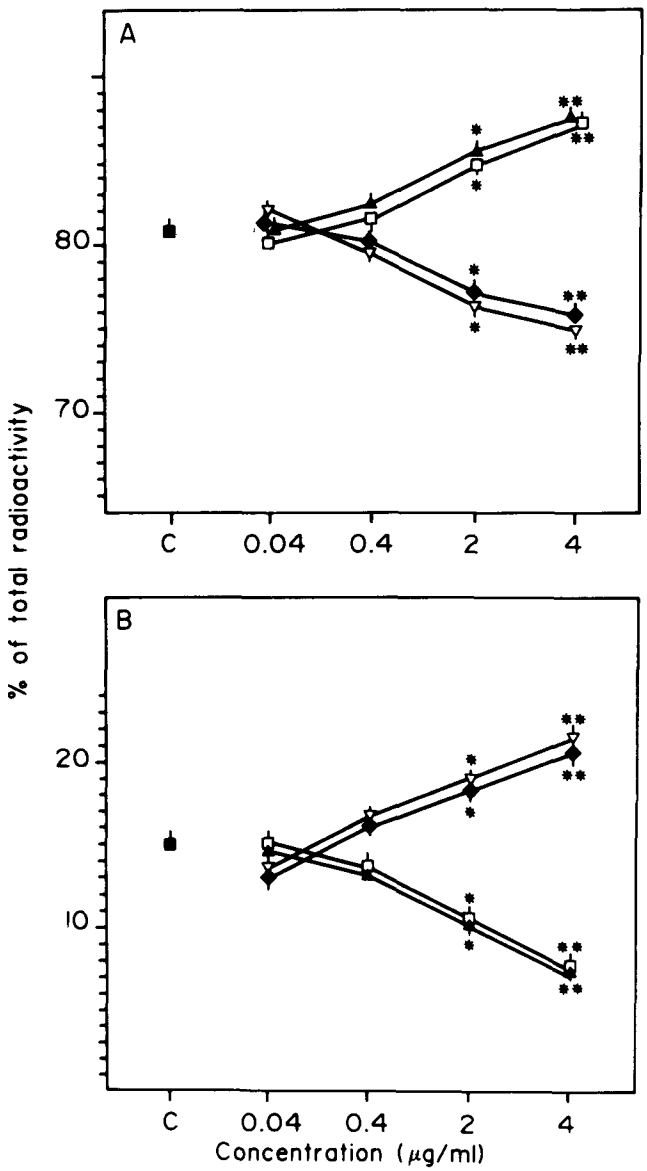

FIG. 1. Effects of oxysterols on the distribution of [1-^{14}C]-arachidonate between phospholipids and triglycerides. Cells were labeled for 2 hr with 0.3–0.4 μCi/dish of [1-^{14}C]arachidonate. Media were removed and replaced by incubation media containing ▲, 20-hydroxycholesterol; □, cholestantriol; ▽, 22R-hydroxycholesterol; ◆, 25-hydroxycholesterol. At the end of the 24-hr incubation lipids were extracted and analyzed as described under Methods. Results are expressed as percentage of the total radioactivity. Results are those of a typical experiment done in triplicates. Similar results were obtained in three separate experiments. *:P≤0.05; **:P≤0.01. Panel A:percentage of radioactivity associated with phospholipids. Panel B:percentage of radioactivity associated with triglycerides.

When the cultures were first incubated with the oxysterols for 24 hr and subsequently labeled with [1-^{14}C]arachidonate for 2 hr, similar effects were observed. Table 1 shows the results of experiments carried out with 20-hydroxycholesterol or 22R-hydroxycholesterol. Similar shifts in the incorporation of the label into the phospholipids and triglycerides were noted. In these experiments, a higher proportion of the label could be found in the unesterified form, and the oxysterols also induced opposite variations in this fraction. The pretreatment with the oxides did not modify arachidonate uptake. Cholestantriol and 25-hydroxycholesterol had effects similar to those of 20-hydroxycholesterol and 22R-hydroxycholesterol, respectively, on arachidonate distribution (not shown).

To see whether these effects were specific for arachidonate, we performed the same type of experiments using [1-^{14}C]oleate as label. Cells were preincubated for 24 hr with the oxides and labeled for 2 hr with [1-^{14}C]oleate. We found that 85% of the radioactivity was taken up by the cells and there was no difference in the uptake between control and treated cells. Figure 2 shows the results of the effects of 20- and 22R-hydroxycholesterol. Again the same shift in the distribution of the label between phospholipids and triacylglycerols was observed, and free fatty acids were modified in the same way as with arachidonate.

Effects of oxysterols on lipid synthesis. We also investigated the effects of the sterol oxides on cells labeled with [1-^{14}C]acetate. Incorporation of [1-^{14}C]acetate was low (4% of the total radioactivity added to the medium) and was not modified by either oxide. The results of such experiments are presented in Table 2. As expected, [1-^{14}C]acetate was found in every lipid class (the Table shows only the lipid classes modified by the treatment), and both 20- and 22R-hydroxycholesterol induced a dose dependent inhibition of cholesterol synthesis. This inhibition was significant at 0.4 μg/ml and maximal at 2 μg/ml. Once HMG-CoA reductase was fully inhibited, the radioactivity initially found in the free cholesterol of the controls was predominantly incorporated into the phospholipids. However, at higher concentrations, which could not promote a higher inhibition of cholesterol synthesis, the same shift as described above was observed. 20-hydroxycholesterol led to a higher incorporation of radioactivity into the phospholipids and 22R-hydroxycholesterol enhanced labeling of the triglycerides. Thus, the changes observed with radioactive fatty acids were also noted with fatty acids synthesized *de novo*.

The mechanisms underlying these changes are still

TABLE 1

Effects of Preincubations with 20-Hydroxycholesterol or 22R-Hydroxycholesterol on [1-^{14}C]Arachidonate Distribution Among Cell Lipids[a]

	Percentage of total radioactivity		
	Phospholipids	Triglycerides	Free fatty acid
Controls	82.25 ± 0.64	9.4 ± 0.71	3.5 ± 0.1
22R-hydroxycholesterol 2 μg/ml	77.70 ± 0.99**	12.65 ± 0.78**	4.6 ± 0.99*
22R-hydroxycholesterol 4 μg/ml	71.60 ± 0.99**	13.95 ± 0.21**	7.4 ± 1.27**
20-hydroxycholesterol 2 μg/ml	87.90 ± 0.80**	5.0 ± 0.65**	2.0 ± 0.09*
20-hydroxycholesterol 4 μg/ml	91.30 ± 0.75**	3.2 ± 0.30**	1.5 ± 0.02

[a]Cells were treated for 24 hr with 20-hydroxycholesterol or 22R-hydroxycholesterol and subsequently labeled for 2 hr with 0.3–0.4 μ0.4 μCi/dish of [1-^{14}C]arachidonate. Results shown are those of a typical experiment done in triplicates. Similar results were obtained in three separate experiments.

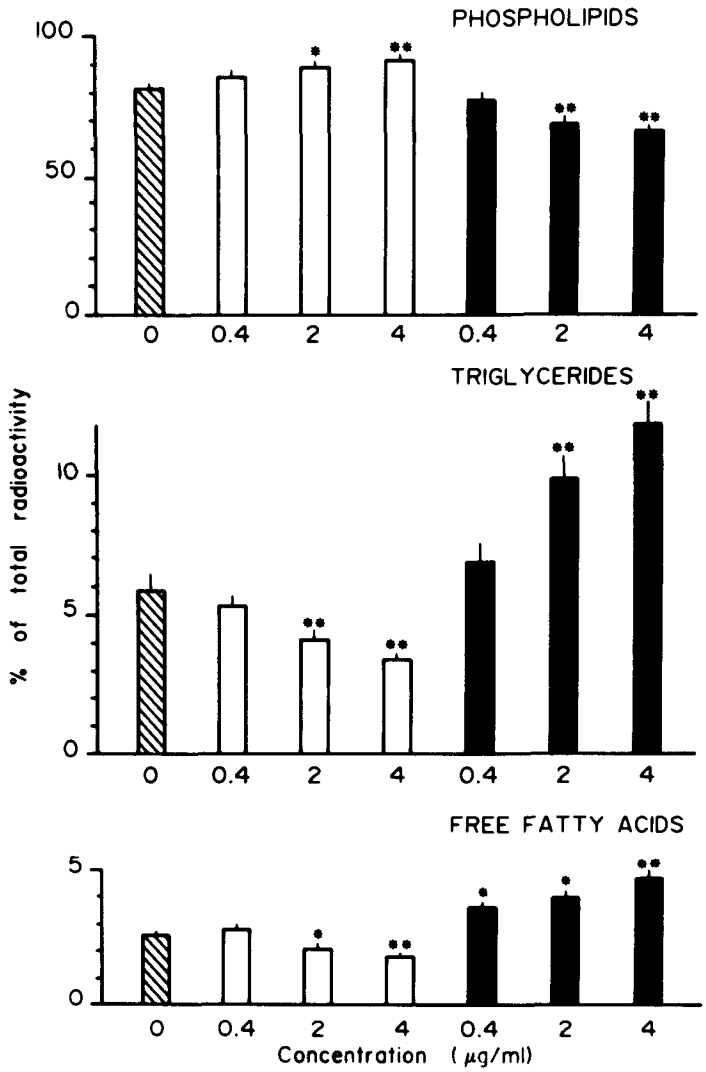

FIG. 2. Effects of 20-hydroxycholesterol and 22R-hydroxycholesterol on the distribution of [1-^{14}C]oleate. Cells were incubated for 24 hr with either medium alone (hatched bars), 20-hydroxycholesterol (open bars), or 22R-hydroxycholesterol (solid bars) at the concentrations indicated. [1-^{14}C]oleate (0.3–0.4 μCi/dish) was added into each dish and allowed to be incorporated for 2 hr. Extraction and analysis of lipids were performed as described under Methods. Results are those of a typical experiment (n=3), similar results were obtained in three separate experiments.

TABLE 2

Effects of 20-Hydroxycholesterol and 22R-Hydroxycholesterol on Distribution of [1-^{14}C]Acetate in Cell Lipids[a]

	Percentage of total radioactivity			
	Phospholipids	Cholesterol	Diglycerides	Triglycerides
Controls	78.43 ± 0.68	8.89 ± 0.52	1.60 ± 0.03	7.53 ± 0.44
22R-hydroxycholesterol 0.4 μg/ml	78.13 ± 0.78	7.92 ± 0.48[b]	1.60 ± 0.18	8.63 ± 0.61
22R-hydroxycholesterol 2 μg/ml	86.52 ± 0.73[b,c]	1.46 ± 0.07[b]	1.61 ± 0.05	8.58 ± 0.18[b]
22R-hydroxycholesterol 4 μg/ml	84.03 ± 0.50[b,d]	1.35 ± 0.09[b]	1.84 ± 0.09[b]	10.59 ± 0.39[b]
22R-hydroxycholesterol 6 μg/ml	71.43 ± 0.90[b,d]	1.34 ± 0.09[b]	2.44 ± 0.09[b]	20.72 ± 0.42[b]
20-hydroxycholesterol 0.4 μg/ml	84.70 ± 1.81[b]	4.17 ± 0.06[b]	1.65 ± 0.57	6.10 ± 1.35[b]
20-hydroxycholesterol 2 μg/ml	87.05 ± 0.76[b]	0.97 ± 0.15[b]	1.60 ± 0.32	4.97 ± 0.11[b]
20-hydroxycholesterol 4 μg/ml	88.63 ± 0.11[b]	0.99 ± 0.09[b]	1.55 ± 0.21	3.99 ± 0.15[b]
20-hydroxycholesterol 6 μg/ml	90.19 ± 0.38[b]	0.85 ± 0.10[b]	1.12 ± 0.14[b]	3.24 ± 0.21[b]

[a]Cells were incubated with 20- or 22R-hydroxycholesterol for 24 hr. 1 μCi/dish of [1-^{14}C]acetate was added. Labeling period was 24 hr. Cell lipids were extracted and analyzed as described in Methods. Results are those of a typical experiment done in triplicates. Similar results were obtained in three separate experiments.
[b]Significantly different from line 1 at $P \leq 0.05$ (at least).
[c]Significantly different from line 2 at $P \leq 0.01$.
[d]Significantly different from line 3 at $P \leq 0.01$.

to be determined. They could involve changes in the activity or synthesis of lipases. Oxysterols can act on HMG-CoA reductase synthesis and rate of degradation (14,15) and it is conceivable that they can affect the activity of other enzymes. It has also been demonstrated that oxysterol insertion into cell membranes can modify membrane fluidity (16) and, consequently, membrane-bound enzyme activities (17). Furthermore, oxysterols modify cell growth by inhibiting DNA synthesis to various extents, depending on the oxysterol (18,19). This, in turn, could result in modulation of phospholipid synthesis.

The result of these oxysterol effects appears to be an exchange of fatty acids between the membrane lipids and cytosolic lipids. One may also wonder whether long-term exposures might lead to changes in membrane composition. Prolonged exposure to oxysterols could eventually also result in a change of triacylglycerol content of the cells.

Little attention had been given to the role of neutral lipids in vascular cells. It seems that the triglyceride content of these cells is highly dependent on incubation conditions and particularly on the composition of the incubation medium (20–22). A recent study has pointed out the importance of neutral lipids in cultured endothelial cells as possible stores for arachidonate (23). Another study has shown that there are active exchanges of arachidonate between triglycerides and phospholipids in guinea pig alveolar macrophages (24). Our study shows that certain external compounds can modulate the ratio of triglycerides to phospholipids. Taken together, these observations suggest that the regulation of triglyceride content in vascular cells is a complex process and can be influenced by several factors. It would be important to understand it in light of the observations of Adelman *et al.* (25), which suggest that neutral lipids have a role in the clearance of the cholesteryl esters from cells.

Oxysterols can have various origins. They are present in the diet and are readily absorbed and incorporated into the circulating lipoproteins (26). Several investigators have also shown their presence in mammalian cells (10). It is now well established that oxidation of lipoproteins is an important event in atherogenesis (27), and it is highly conceivable that oxysterols are formed in this oxidation process. It is therefore important to better understand the biochemical actions of oxysterols on cells to which they may be exposed *in vivo*. Oxysterols have long been recognized as sterol synthesis inhibitors, but our data suggest that they can also interfere with other pathways of lipid metabolism.

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Inhibition of Cholesterol Synthesis by Lovastatin Tested on Six Human Cell Types *in Vitro*

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Lovastatin (mevinolin) caused a strong and dose-dependent inhibition of cholesterol synthesis in six types of cultured human cells. Fifty percent inhibition of cholesterol synthesis in human enterocytes was observed at a lovastatin concentration of about 0.004 ng/ml and in other cells at a lovastatin concentration of about 0.03 ng/ml. At lovastatin concentrations between 1.0 and 100.0 ng/ml, a moderate tissue selectivity of lovastatin action was noted. At optimal concentrations, lovastatin inhibited cholesterol synthesis in hepatocytes by 98%, in normal and LDL-receptor negative fibroblasts, arterial smooth muscle cells and hepatoma G-2 cells by about 90%, and in enterocytes by 75%. In rat enterocytes lovastatin inhibited cholesterol synthesis by only 60%.
***Lipids 25,* 177-179 (1990).**

A high level of serum cholesterol is considered to be an important risk factor in the development of atherosclerosis. Reducing the rate of cholesterol synthesis, which upregulates low density lipoprotein receptors and decreases plasma cholesterol concentration (1), has therefore been a promising approach to the treatment of disease. Mevinolin (2) and related compounds (3) have been found to be very effective inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which is the rate-limiting enzyme in the pathway of cholesterol biosynthesis.

The effect of mevinolin on cholesterol synthesis in cultured rabbit ileal mucosa (4), cultured rat enterocytes (5), and in various rat tissues *in vivo* (6) has recently been tested. The only human cells which have been tested in this respect have been monocyte-derived macrophages (7). Since mevinolin was found to be an effective drug in reducing serum cholesterol levels in humans (8), evaluation of its effect on cholesterol synthesis in different human cell types is of considerable importance. In the present paper, we describe the effect of mevinolin, now named lovastatin, on cholesterol synthesis in six types of human cells in culture.

MATERIALS AND METHODS

Cells. In the previous reports we have described the isolation and maintenance of isolated human enterocytes (9), cultured human hepatocytes (10) and cultured human arterial smooth muscle cells (SMC) (11). The procedure for the isolation of rat enterocytes was identical to that used for human cells. Human skin fibroblasts from healthy donors and donors affected by homozygous familiar hypercholesterolemia, as well as the human hepatoma cell line G-2, were maintained in minimum essential medium containing 10% fetal calf serum, 25 mM HEPES, 1% nonessential amino acids, 2 mM L-glutamin, 100 μg/ml kanamicin and 2.5 μg/ml fungizone (reagents were from Flow, Irvine, Scotland). Fibroblasts were used upon their fifth passage.

Cells were grown to confluency in the wells of multidishes (Nunc, Denmark). Cell protein concentrations used were 10 μg for SMC, 100 μg for fibroblasts and HEP G-2 cells, and 250 μg for hepatocytes per well. Isolated human enterocytes were used at the concentration 2×10^6 cells (about 500 μg protein) per well.

Cholesterol synthesis. The incubation mixture contained 10 μCi sodium [2-^{14}C]acetate (Amersham, Bucks, England, spec. act., 40-60 mCi/mmol), the indicated amount of lovastatin (Merck, Sharp and Dohme), and the appropriate serum-free medium containing 1 mg/ml bovine serum albumin (Sigma Chemical Co., St. Louis, MO) at a final volume of 0.5 ml. Lovastatin was added from a stock solution containing 4 mg/ml dimethylsulfoxide (DMSO). It was shown in preliminary experiments that DMSO at the concentrations used has no effect on cholesterol synthesis in any of the cell types studied.

Cells were incubated for 2 hr at 37°C in a CO_2-incubator (5% CO_2, 95% air). After incubation, cells were washed twice with phosphate buffered saline (Flow) and dissolved in 1 M NaOH. The samples were saponified by incubation for 2 hr at 100°C in a solution containing 5 M KOH in 50% (v/v) aqueous ethanol. Cholesterol was precipitated by digitonin (Serva, Heidelberg, Federal Republic of Germany) according to Sperry and Webb (12). Cell protein was determined according to Bradford (13). It was shown in preliminary experiments that the time-course of [^{14}C]acetate incorporation into digitonin-precipitated sterols was linear for at least 3 hr for all cell types studied.

Expression of results and statistics. The amount of [^{14}C]acetate incorporated into digitonin-precipitated sterols per mg cell protein was calculated. Because use of [^{14}C]acetate as precursor can lead to an underestimation of the absolute rates of cholesterol synthesis (14), the results are expressed relative to controls. Dose-dependence curves were approximated by computerized polynomial regression analysis; IC_{50} values were calculated from these curves. IC_{50} refers to the concentration of lovastatin required for inhibition of cholesterol synthesis by 50%, relative to maximal inhibition. Statistical significance was calculated with Student's *t*-test. Statistical calculations were performed with a Labtam 3003 computer (Labtam, Australia).

Each experiment was done in triplicate and reproduced 2-3 times on cells obtained from different donors. Comparison of results from different experiments gave a coefficient of variation about 8%. Representative experiments are shown in Figure 1.

RESULTS

Lovastatin at concentrations of 0.002-1000 ng/ml caused a dose-dependent inhibition of cholesterol synthesis in all

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Abbreviations: DMSO, dimethylsulfoxide; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl coenzyme A reductase; SMC, smooth muscle cells.

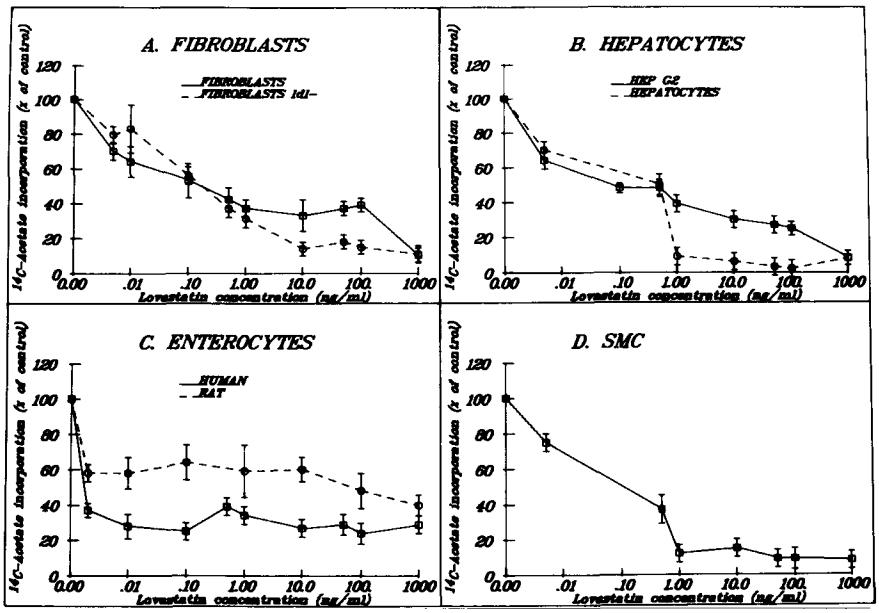

FIG. 1. Effect of lovastatin on cholesterol synthesis in normal human fibroblasts and LDL-receptor deficient human fibroblasts (A), human hepatocytes and hepatoma G2 cells (B), human and rat enterocytes (C), and human arterial intimal smooth muscle cells (D). Cells were incubated for 2 hr at 37°C in serum-free media containing sodium [^{14}C]acetate (20 μCi/ml) and indicated concentrations of lovastatin. Determination of [^{14}C]acetate incorporation into digitonin-precipitable sterols is described in the Materials and Methods section. Each data point represents the mean ± standard error of mean of triplicate determinations.

cell types tested (Fig. 1). Cholesterol synthesis was reduced by 50% at lovastatin concentrations as low as 0.003–0.06 ng/ml. The IC_{50} value for enterocytes was significantly lower than for the other cells. No statistically significant difference in IC_{50} for other cells was found using polynomial regression analysis (Table 1). A further increase in lovastatin concentration affected cholesterol synthesis in different cells in different ways. In human fibroblasts lacking the LDL receptor (Fig. 1A), human hepatocytes (Fig. 1B), human enterocytes (Fig. 1C), and human aorta intimal smooth muscle cells (Fig. 1D), cholesterol synthesis fell sharply when the lovastatin concentration was increased to 1–10 ng/ml. A further increase in lovastatin concentration had no additional effect. In normal human fibroblasts (Fig. 1A) and human hepatoma cells (Fig. 1B), a gradual decrease in the rate of cholesterol synthesis occurred when the lovastatin concentration rose to 1 μg/ml. A statistically significant difference in the effect of lovastatin on cholesterol synthesis in different cells was found at lovastatin concentrations between 1.0 and 100.0 ng/ml (Fig. 1).

The maximal inhibition of cholesterol synthesis by lovastatin was also different for different cell types (Table 1). Human hepatocytes appeared to be the most sensitive, as lovastatin caused a complete blockage of cholesterol synthesis. In fibroblasts, hepatoma cells and smooth muscle cells, lovastatin inhibited cholesterol synthesis by as much as 90%. Enterocytes appeared relatively less sensitive to lovastatin as cholesterol synthesis in these cells was inhibited at a maximum of 75%. Similar results were observed using human intestinal organ culture (unpublished observation). No correlation was found between observed sensitivity to the inhibitor and the absolute rate of cholesterol synthesis or the amount of cellular protein in the systems.

TABLE 1

Effect of Lovastatin on Cholesterol Synthesis in Human Cells[a]

Cell type	$IC_{50\%}$ (nM)	Max. effect (% of inhibition)[b]
Hepatocytes	0.16	98 ± 0.5
Hepatoma	0.06	92 ± 1
Enterocytes human	0.009[c]	75 ± 3[c]
Enterocytes rat	0.004[c]	60 ± 6[c]
SMC	0.03	91 ± 1
Fibroblasts (N)	0.06	89 ± 2
Fibroblasts (ldl-)	0.04	93 ± 1

[a] Experiments were done as described in the legend to Figure 1.
[b] Mean ± standard error of mean.
[c] $p < 0.01$ (vs. other cell types) according to Student's *t*-test.

To compare the effect of lovastatin on human cells with the effect on cells from other species, we also studied rat enterocytes. We showed that rat enterocytes are less sensitive to lovastatin than are human enterocytes (Fig. 1C, Table 1).

DISCUSSION

In the present study the effect of lovastatin (mevinolin) on cholesterol synthesis in six human cell types was tested. It was shown that lovastatin is an effective

inhibitor of cholesterol synthesis in human cells. This is consistent with the results obtained on human macrophages (7), various animal cells (4,5,6), and with enzyme preparations (2,6,15). The IC_{50} values were found to be 0.009–0.16 nM. Much higher concentrations have previously been used by others for mevinolin (4,5) and related compounds (3,6) to achieve inhibition of cholesterol synthesis in experiments with animal cells. The different sensitivities of human and rat cells was also confirmed in the present study by comparing the effect of lovastatin on the cholesterol synthesis in human and rat enterocytes (Fig. 1C). Therefore, one may conclude that human cells appear to be more sensitive to lovastatin, as compared to animal cells.

Human enterocytes were sensitive to lower doses of lovastatin than were other cells. A moderate degree of tissue specificity was also found for human cells at lovastatin concentrations of 1.0–100.0 ng/ml. In addition, in our experiments enterocytes appeared to be less sensitive, and hepatocytes more sensitive, to high concentrations of lovastatin than the other cells.The relative resistance of intestine to high concentrations of a related compound, compactin, was demonstrated by Betteridge *et al.* (16). Similar results were obtained by Tsujita *et al.* in experiments with rats *in vivo* (6). However, they found that another compound, CS-514, possesses more pronounced tissue specificity and affects cholesterol synthesis predominantly in liver and intestine. Tissue specificity cannot be explained by different rates of overproduction of HMG-CoA reductase due to the short duration of the experiments, it appears to be due to differences in permeability of the cells for lovastatin. One could assume that at a given concentration, lovastatin may preferably be used for a particular tissue to inhibit cholesterol synthesis. However, under *in vivo* conditions, a number of other factors (e.g., lactone-acid conversion, blood flow, relative barrier resistance, etc.) could influence lovastatin action in different tissues as well.

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Lipid Metabolic Interrelationships and Phospholipase Activity in Gustatory Epithelium of *Ictalurus punctatus In Vitro*

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The catfish, *Ictalurus punctatus*, is an important model for studying the biochemical mechanisms of taste at the peripheral level. The type, amount and metabolic activity of the lipids within this tissue play important roles in taste transduction by forming the matrix in which the receptors for taste stimuli are imbedded and by acting as precursors to second messengers. The metabolic interconversions that occur among the lipids on the taste organ (barbels) of this animal are reported here. When sodium [^{32}P]phosphate was incubated with minced pieces of epithelium from the taste organ of *I. punctatus*, phospholipids became labeled. Maximal incorporation occurred near 20 min for lysophosphatidylcholines (LPC), phosphatidylcholines (PC) and phosphatidylinositols (PI). The phosphatidylethanolamines (PE) and phosphatidylserines (PS) became labeled more slowly. The label in LPC and PC declined from 20 min to 120 min, while that of the other fractions increased or was stable over the 20–120 min time period. Upon addition of 1,2-di-[1′-^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine to the medium, ^{14}C was found within minutes in all of the phospholipids assayed. The amount of label incorporated increased with time, with maximum labeling for all phospholipids occurring at 15 min. However, ^{14}C appeared predominantly first (by 5 min) in a neutral lipid fraction (fraction AG, consisting of free fatty acids, mono- and diglycerides, triglycerides and methyl esters), then declined rapidly as the phospholipids gradually incorporated more label. Within minutes of addition of 1-[1′-^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine (lysophosphatidylcholine) the ^{14}C-label was detected in the neutral lipid fraction AG, then in the PC fraction, and later in the other phospholipids. The PC fraction was maximally labeled by 40 min.

Using the appropriate radiolabeled substrates, lysophosphatidylcholine phospholipase A_1 and phosphatidylcholine phospholipase D activities were detected in this tissue. Very low activity of a phosphatidylcholine phospholipase A_2 was observed. The experiments indicate that there are active and rapid exchange, degradation, synthesis and scavenger pathways of phospholipids in the taste organ of this animal, and suggest that phospholipases A_1 and D-type activities are primarily responsible for the rapid breakdown of LPC and PC.

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While the broad outlines describing the mechanisms of taste (gustatory) transduction are beginning to take shape, the details of the early events in taste reception and transduction are still incompletely described (1–3). The problems encountered in investigating molecular details of receptor-mediated taste processes in mammalian model systems are largely due to the low sensitivity of these receptors to agonists and difficulty in obtaining a sufficient amount of tissue that is dense with taste receptors.

The cutaneous taste system of the catfish (*Ictalurus punctatus*) has been exploited as a model of receptor-mediated taste transduction, since this system shows a high degree of sensitivity and specificity for certain amino acids (4–8), and a high density of taste buds on the barbel (taste organs) appendages. Because of these advantages, progress is being made toward understanding the molecular details of taste receptor processes. Purification and reconstitution of the taste receptors from ictalurid fishes are definite possibilities. In fact, reconstitution of taste plasma membranes from *I. punctatus* into phospholipids at tips of patch electrodes has been reported, and stimulus-activated cation channels were observed (3,9,10). Because of the importance of phospholipids in maintaining or stabilizing plasma membrane-associated receptors and the probable involvement of some phospholipids in signal transduction, it was of interest to examine the overall metabolism of the major phospholipids associated with this taste receptor-dense organelle.

We have reported the quantification of lipid species within taste-related epithelial tissues of steer and catfish (11–14), initial studies of lipid synthesis and turnover in catfish using [^{14}C]acetate (15) and preliminary data on the possible involvement of phosphatidylinositol metabolism in taste transduction (16,17). In general, these studies showed that the lipid composition of taste epithelial tissue was not markedly different from that of other epithelia, with the exception that both the bovine and catfish taste epithelium contained a high amount of plasmalogens. The taste epithelium from bovine lingual tissue contained a greater amount of cyclooxygenase activity than that of control epithelium devoid of taste buds. Following *in vitro* incubation of taste tissue from *I. punctatus* for 60 min with [^{14}C]acetate, over 75% of the label in the lipid fraction was detected in free fatty acids, mono- and diacylglycerols, phosphatidylcholines, phosphatidylserines and phosphatidylethanolamines, suggesting active metabolism of these fractions. In addition, L-alanine, a potent taste agonist for *I. punctatus*, stimulated production of inositoltrisphosphate from phosphatidy-

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Abbreviations: DMSO, dimethyl sulfoxide; DPM, disintegration per minute; HPLC, high performance liquid chromatography; LPC, lysophosphatidylcholine; LPC-PLA1, lysophosphatidylcholine-phospholipase A_1; PA, phosphatidic acid; PC, phosphatidylcholine(s); PC-PLD; phosphatidylcholine-phospholipase D; PE, phosphatidylethanolamine(s); PI, phosphatidylinositol(s); PS, phosphatidylserine(s); TCA, trichloroacetic acid; and TLC, thin-layer chromatography.

linositol-4,5-bisphosphate in a homogenate of taste epithelium (17).

These studies prompted us to determine the pattern of ^{32}P labeling of the lipids in taste epithelium from *I. punctatus* with respect to time. The unusually large amount of label incorporated into the lysophosphatidylcholine (LPC) and phosphatidylcholine (PC) fractions in this ^{32}P study then led us to investigate the metabolic fate of exogenously added LPC and PC labeled with ^{14}C in the acyl groups. The results of these studies here are reported here.

MATERIALS AND METHODS

Solutions. Krebs-Ringer bicarbonate was made to the following component concentrations: NaCl, 120 mM; KCl, 4.75 mM; $CaCl_2$, 1.2 mM; $MgSO_4$, 1.2 mM; KH_2PO_4, 0.12 mM; $NaHCO_3$, and 25.9 mM; pH 7.4. Lipid standards were purchased from Applied Sciences (State College, PA) and tested for purity as described below. In no instance did the level of impurities exceed 1% and in most instances, no detectable impurities were present. The radiolabeled materials were products of DuPont-NEN: The lysophosphatidylcholine used was 1-[1′-^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine (60 mCi/mmol); the phosphatidylcholine was 1,2-di-[1′-^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine (120 mCi/mmol); and disodium [^{32}P]phosphate had a specific activity of 900 mCi/mmol. All other materials were of high performance liquid chromatography (HPLC) grade purity and purchased from Sigma Chemical Co., St. Louis, MO).

Animals. Catfish, *Ictalurus punctatus*, weighing 1000–2000 g, were purchased from local commercial suppliers. They were maintained in 250 gallon aquaria at 20°C under dim light for no longer than two days. Animals were sacrificed by a sharp blow to the neurocranium (5).

Tissue collection and incorporation of labeled precursors. Epithelium from the maxillary barbels of the sacrificed animals was immediately removed by stripping it from the underlying pseudocartilage. To do this, a superficial slit was cut the length of the barbel using a scalpel and the epithelium pulled from the barbel starting at a point near the head and continuing toward the tip. The epithelial sheet that was obtained was cut into small (1–2 mm^2) pieces and placed on ice in Krebs-Ringer bicarbonate. When sufficient material was collected (from about 10 large animals), it was divided into equal aliquots and transferred to Warburg reaction vials containing 2 ml Krebs-Ringer bicarbonate. Depending on the experiment, each vial contained from 1–6 mg of protein.

Each vial was flushed with 100% O_2 for 3 seconds. Vials were incubated with labeled substrates for various time periods from 0 min to 120 min at 30°C. The three experiments reported here (using disodium [^{32}P]phosphate, [^{14}C]PC and [^{14}C]LPC) were performed on separate groups of animals on different days, but all during the summer months. For the two experiments on metabolism of exogenous PC and LPC, a total of 3 μCi of the labeled substrate was added to each vial. For the experiment on metabolism of phosphate, a total of 50 μCi of $Na_2H^{32}PO_4$ was added to each vial.

Following incubation, 8 ml of Folch reagent was added per vial. Tissues and solution were transferred to a storage vial, vigorously agitated and maintained at −15°C until the analytical procedures were begun (12 hr).

Lipid analyses. All lipids or solutions containing lipids were stored in the presence of ca. 5 μg of butylated hydroxytoluene per mg of lipid. Tissue specimens were finely chopped and homogenized in all-glass piston homogenizers; they were extracted with 100 ml of chloroform/methanol (2:1, v/v). Two extractions were required to insure that no lipid was left unextracted. Residues were transmethylated (18) and a subsequent lipid extraction performed. No additional lipid material was recovered. Repeated extractions did not yield additional lipid material.

Aliquots of each fraction were subfractionated into the major lipid classes using thin-layer chromatography (TLC) as described by Rabinowitz *et al.* (11). To quantitate the amount of ^{14}C incorporated into TLC fractions, each spot was scraped off the plate, the lipids were extracted, and the sample was divided into two equal portions. One portion was subjected to scintillation counting using a Packard Tri-Carb Scintillation Counter (Packard Instruments, Downers Grove, IL). The other portion was used for lipid analysis by charring and spectrophotometric analysis at 375 nm (11,19). Known standards were subjected to an identical analysis, and the amounts of each unknown lipid class were calculated by reference to a standard curve. Our quantitative analyses of phospholipids generally agree across multiple runs within a standard deviation of ± 7%. Data are presented as disintegrations per minute (DPM) of total radioactivity incorporated into each identified lipid fraction per mg of the total amount of that lipid fraction recovered from each vial, per mg of tissue protein in the vial (DPM/mg lipid/mg protein).

To verify the results of the TLC-charring-spectrophotometric analysis of individual lipids, total phosphorus assays were performed on some of the spots from plates of both unknown and standard samples. Total phosphorus was analyzed by the method of Chen *et al.* (20). Protein was determined by the biuret reaction (21).

Enzyme assays. Using exogenous substrates, lysophatidylcholine-phospholipase A_1 (LPC-PLA_1), phosphatidylcholine-phospholipase D (PC-PLD) and other enzymes were assayed.

Lysophosphatidylcholine-phospholipase A_1 (LPC-PLA_1). 1-[1′-^{14}C]Palmitoyl-*sn*-glycero-3-phosphocholine, dissolved in dimethylsulfoxide (DMSO), was incubated with tissue homogenate (20 μg protein) of the taste organ in an assay medium containing 50 mM Tris-maleate buffer (pH 7.1), 1 mM $MgCl_2$, 10 mM NaCl, 80 mM KCl, and 0.6 mM $CaCl_2$ + 2 mM EGTA (yielding calculated free Ca^{2+} = 53 nM). The minced pieces of barbel (collected as described above) were homogenized in a Polytron in the Tris-maleate buffer (4°C), centrifuged at 1000 × *g*, and the supernatant used in the assay. The enzyme assay was found to be linear for 5 min at 24° ± 1°C. Reactions were stopped with Dole's Reagent. The enzymatically released fatty acids were extracted and separated from the radiolabeled substrate (22). The amount of radioactivity in the fatty

acid-rich upper phase was used as a measure of LPC-PLA_1 activity (22).

Phosphatidylcholine-phospholipase D (PC-PLD). 1,2-Dipalmitoyl-*sn*-glycero-3-phospho-[*N-methyl*-^{3}H]choline, dissolved in DMSO, was incubated with catfish taste tissue homogenate (60 μg protein) in the assay medium used for the detection of LPC-PLA_1, except that the free Ca^{2+} concentration was 1 mM, and 0.1% Triton X-100 was included. After 2 min, (the linear range) reactions were stopped with trichloroacetic acid (TCA), the solutions vortexed and centrifuged at 1000 × *g*. The release of radioactivity into the aqueous phase was assumed to represent the action of phospholipase D.

Other enzymes. Using 1-palmitoyl-2-[1′-^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine as substrate (at 380 nM) in the medium described for LPC-PLA_1, in the presence of 4 mM reduced glutathione and 0.1% Triton X-100, the presence of PC-phospholipase A_2 (PC-PLA2) was evaluated. Assays were run for 1 hr. The presence of PC-phospholipase C was also evaluated using 1-palmitoyl-2-[1′-^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine as the substrate, and measuring the release of labeled diacylglycerol. Assay conditions were the same as those used for detection of PC-PLA_2.

RESULTS AND DISCUSSION

Incorporation of ^{32}P into phospholipids was observed by the first time point at 2 min, most notably in the LPC and phosphatidylinositols (PI) fractions (Fig. 1). By 5 min, the label in LPC and PI had nearly reached their maxima and the PC was rapidly incorporating label. By 15 min, LPC, PI and PC were maximally labeled, while phosphatidylethanolamines (PE) and phosphatidylserines (PS) were beginning to incorporate label. From the 30 min time point to 120 min, label in the LPC and PC fractions declined, label in the PI fraction remained stable, and label in the PE and PS fractions increased to a constant value. The decline in the amount of label in the PC and LPC fractions after 15 min incubation may indicate preferential use of these active fractions as energy sources. The incubation medium contained no exogenous metabolic substrates. At the end of the 120 min time period, all fractions contained similar amounts of ^{32}P (0.001–0.005% of original activity of ^{32}P added) and, with the exception of the LPC fraction, may have been in equilibrium with each other.

When 1,2-di-[1′-^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine was incubated with taste epithelium, the initial rapid decline of ^{14}C label in the added substrate coincided with a rapid and marked increase in incorporation of label into the free fatty acids and neutral lipid glycerides (AG) (Fig. 2). This AG fraction contained free fatty acids, methyl esters, and mono-, di-, and triglycerides, and its rapid incorporation of label was suggestive of the presence of active phospholipases A_1, A_2 and/or C. No detailed analyses of the fatty acids were made. However, throughout the incubation period, the majority of the label (>50%) was found in the free fatty acid portion of this AG fraction. The chemical amount of free fatty acid increased during the incubation from about 6% of total lipids at zero time to near 10% of total lipids at 120 min. An increase in the free fatty acid pool with time of incubation was reported previously for this tissue (15). After 10 min, the amount of label incorporated into the PC fraction was approximately 0.3% of the original amount of activity added at zero time. At this time point, the AG fraction contained the majority of the label, almost 5% of the original. Label in the AG fraction then declined with time, while label appeared and stabilized in LPC, phosphatidic acids (PA), PE, PI and PS. Appearance of label in PA is consistent with either active phospholipases D or diacylglycerol kinases.

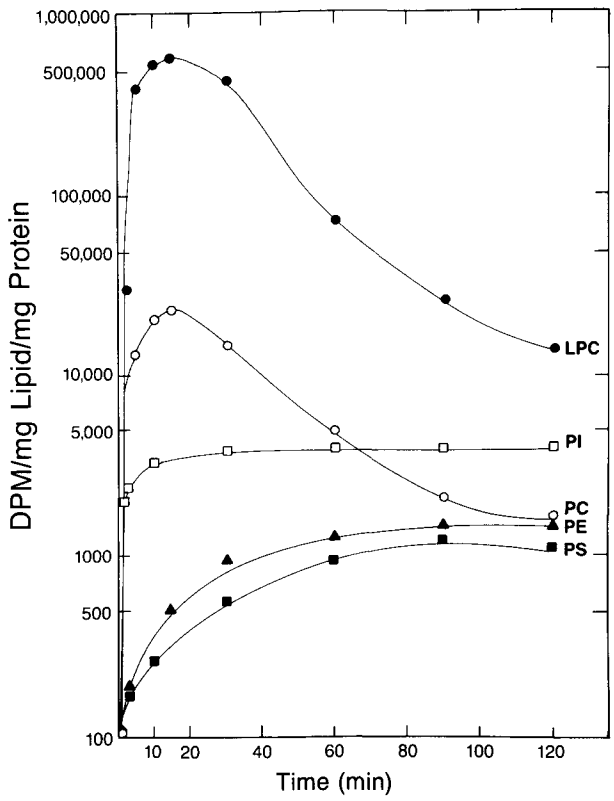

FIG. 1. Disintegrations per minute (DPM) per mg of each identified lipid fraction per mg protein in the sample vs time (min) of the incubation. Incubation at 30°C was begun at time zero with the addition of 50 μCi of ^{32}P to each vial. Each time point represents analyses of total amount of lipid and associated ^{32}P for the tissue of the vial incubated to that point. Each analysis was performed in triplicate. Triplicates agreed with each other to within ± 5%. Abbreviations are defined in the text.

Addition of 1-[1′-^{14}C] palmitoyl-*sn*-glycero-3-phosphocholine to taste epithelium resulted in a rapid accumulation of label in the AG fraction, suggesting the presence of a lysophospholipase. Later (5–20 min) label accumulated first in the PC fraction and later in the PE, PA, PI and PS fractions (Fig. 3). As with data from the study where labeled PC was utilized (Fig. 2), label was rapidly lost from the exogenously added substrate, appearing in fraction AG, then being lost from this neutral pool and apparently incorporated into phospholipids. As was found during the experiment on metabolism of ^{14}C-labeled PC, the majority of the ^{14}C of the AG fraction was found in free fatty acids

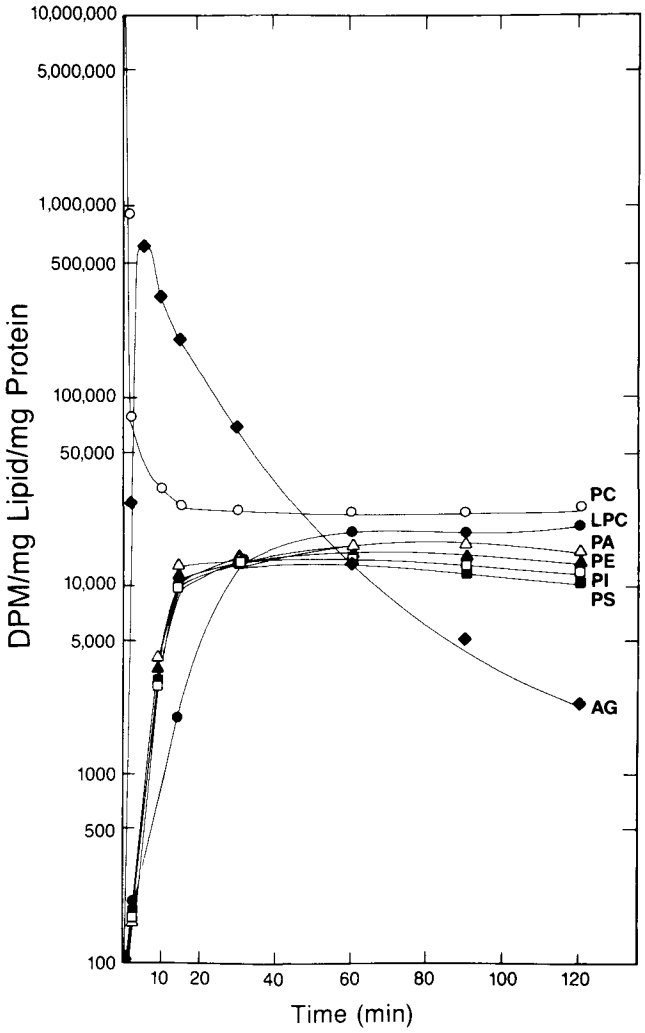

FIG. 2. Disintegrations per minute (DPM) per mg of each identified lipid fraction per mg protein in the sample vs time (min) of incubation. Incubation at 30°C was begun at time zero with the addition of 3 μCi of 1,2-di-[1′-^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine to each vial. Each time point represents analyses from a single vial incubated to that time. Lipid analyses were performed as detailed in Materials and Methods with total ^{14}C activity being assessed for each identified lipid fraction. Analyses were performed in triplicate and agreed with one another to within ± 5%.

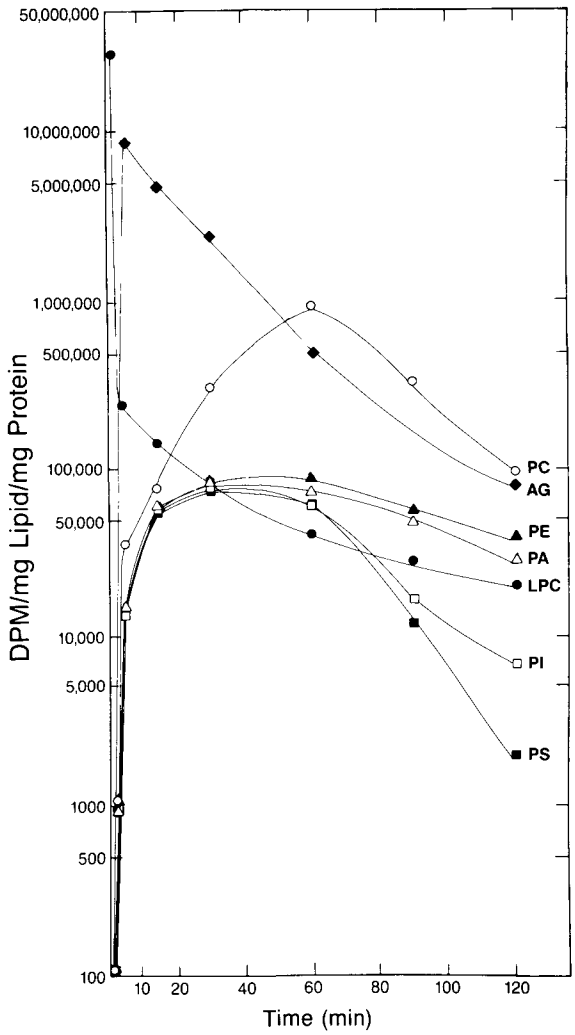

FIG. 3. Disintegrations per minute (DPM) per mg of each identified lipid fraction per mg protein in the sample vs time of incubation. Incubation at 30°C was begun at time zero with the addition of 3 μCi of 1-[1′-^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine to each vial. Each time point represents analyses from a single vial incubated to that time. Lipid analyses were performed as detailed in Materials and Methods with total ^{14}C activity being assessed for each identified lipid fraction. Analyses were performed in triplicate and agreed with one another to within ± 5%.

throughout the incubation period. At the 120 min time period, the AG fraction contained less than 0.2% of the original amount of label added at zero time, the LPC fraction contained approximately 0.06% of the original amount of label, while all the phospholipids that were assayed collectively contained approximately 0.7% of the original amount of label. In this experiment, label was continuously lost from the PC, PI and PS fractions over the time period of 60 min to 120 min, in contrast to the relatively more stable activity seen for the label in these fractions from the experiment whose data are displayed in Figure 2.

These results demonstrate that the epithelium of the taste organ (barbel) of the catfish, *I. punctatus*, contains a variety of metabolic pathways, perhaps including those for synthesis, degradation, reuse (through scavenger pathways and transesterifications), rearrangements and oxidation of precursors and products. In the experiments detailing metabolism of exogenously added PC and LPC at all time periods, continuous metabolism of the labeled acyl groups is likely. This continuous metabolism and/or turnover is consistent with the use of these phospholipids/fatty acids as metabolic precursors and with our previous observation (15) where exogenous addition of [^{14}C]acetate to this tissue resulted in liberation of $^{14}CO_2$ and production/degradation of ^{14}C-labeled phospholipid fractions.

Because of the well known metabolic interrelationships among PE, PC and PS (23-25), it is generally observed that changes in these three phospholipids are

highly correlated (25). In the current study, when the tissue of the taste organ was incubated with ^{32}P, label was first incorporated into the LPC, PC and PI fractions; only later, after about 80 min, did label in the three fractions, PC, PE and PS, remain proportional. A separate total lipid analysis (15) had previously shown that the relative amounts of these three phospholipids were nearly identical (i.e., approximately 12—13% of total lipid). The striking feature of the ^{32}P incorporation studies was the rapid incorporation and high degree of activity in the LPC and PC fractions (Fig. 1).

The incorporation of ^{32}P into the LPC fraction (Fig. 1) contrasts with the low relative amounts of this fraction (~2.5% of total lipids) in the taste epithelium of catfish (15). The data of Figure 1 indicate that by 20 min, ^{32}P label in fractions LPC and PC accounted for nearly 99% of the total label in the phospholipids analyzed, yet previous analyses have found that these two fractions accounted for only 58% of the five phospholipids surveyed here (15). The activity of the LPC fraction is, therefore, dramatically out of proportion compared to its relative amount. These results suggested that the LPC/PC pair are highly metabolically active fractions in this tissue, and we therefore carried out additional studies to directly determine the metabolic fate of the acyl fatty acid groups on both PC and LPC when these are added to the tissue.

In agreement with the observations of Seidner *et al.* (26), ^{14}C from radiolabeled LPC was rapidly (20 min) incorporated into the PC fraction, with label then appearing in other phospholipids. With the taste organ epithelium, however, an intervening metabolic step was apparently detected, indicating the possibility that the ^{14}C of the acyl group of radiolabeled LPC (Fig. 3) and the ^{14}C of at least one of the acyl groups of the radiolabeled PC (Fig. 2) were initially transferred to the neutral glycerides and free fatty acids, then re-esterified into or exchanges made with phospholipids. This rapid movement of ^{14}C from exogenous phospholipid (PC and LPC) to neutral glycerides and free fatty acids (AG of Figs. 2 and 3) is consistent with the existence of potent lysophospholipases as well as phospholipases A_1, A_2 and/or phospholipases D and C.

LPC-PLA_1 activity was observed which had a linear time course of basal activity up to 5 min. At an LPC concentration of 712 nM, the activity of the enzyme was 24 pmol/min/mg protein. Similar activity was also detected at 1 mM Ca^{2+}. PC-PLD activity was also readily detected under the conditions of the assay. The time course of basal activity was linear for 2 min. At a PC concentration of 110 nM, the activity of the enzyme was 0.5 pmol/min/mg protein and at a PC concentration of 132 μM, the activity was 926 pmol/min/mg protein. No detectable activity was observed in the absence of Triton X-100 at a Ca^{2+} concentration of 53 nM. Under the conditions of assay described in Methods, a low level of basal PC-PLA_2 was detected—less than 1.6 pmol/hr/mg protein. In the absence of glutathione, the activity was barely detectable. By measuring labeled diacylglycerol, and under the conditions of this assay, no detectable phospholipase C specific for 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine was observed at either a Ca^{2+} concentration of 53 nM or 1 mM. A phospholipase A_2 capable of using PE (1-palmitoyl-2-linoleoyl) as substrate has been detected in this taste tissue (Huque, unpublished observations). Also, under the conditions used here, exogenously added snake venom (*Crotalus atrox*) resulted in rapid release of labeled fatty acid from L-1-palmitoyl-2-[1′-^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine. It is known that this tissue also contains phospholipases C capable of using PI, PIP and PIP2 as substrates (16) and PE as substrate (Huque, unpublished observations).

It is likely, therefore, that the rapid appearance of label in the AG fraction (Figs. 2 and 3) is due to active PLA_1's. The free fatty acids released could be then oxidized, re-esterified, degraded and/or reused through a variety of pathways to obtain the labeling patterns observed in Figs. 2 and 3.

These studies have shown that *in vitro*, the major phospholipids of the taste organ of *I. punctatus* are metabolically active. Exogenous addition of ^{32}P allowed observation of rapid labeling of and thus, by implication, turnover of PC, LPC and PI. PS and PE incorporated label more slowly. Activity of the PI fraction is at least partially explicable by the observation that taste agonists to this tissue enhance production of inositol triphosphates (16,17). The metabolic profile of PC and LPC *in vitro*, particularly their apparent rapid interchange (Figs. 2 and 3), is not without precedent (26). Taste receptors in this animal respond to a wide concentration range of agonists across a broad temperature range (3,5–9). The metabolic activity observed in the phospholipid fractions of this tissue may reflect a need to stabilize these macromolecular receptor proteins and their coupled transductive components toward these ranges of concentration and temperature. Recent reports have emphasized the roles of choline phospholipids and arachidonic acid in calcium regulation and signal transduction (27–30). It is possible that, in light of the current observations on the active metabolism of LPC and PC, the taste system also uses choline phospholipids for this purpose. Given our previous observation on prostaglandin production in taste tissue (13), it is also possible that the rapid interchanges between PC and LPC may represent a mechanism of incorporating unsaturated fatty acids into the 2-position of a previously saturated phospholipid (31).

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Lipids

Notices
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81st Annual Meeting of the American Oil Chemists' Society, Baltimore, MD, April 22–25, 1990. The technical program will include over 400 papers and posters. A tentative list of speakers and titles is printed in the January 1990 issue of the new AOCS journal *INFORM*. Major symposia will discuss the biochemistry of phospholipids, the relationship of dietary proteins and health, milk lipids in infant nutrition, dietary aspects of blood cholesterol and of cancer, plant and fungal sterols, anaytical methods, and related topics. For more information, call Jean Bremer at AOCS. Phone: (217) 359-2344.

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Effect of Dietary Palm Oil and Its Fractions on Rat Plasma and High Density Lipoprotein Lipids

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Male Sprague Dawley rats were fed semipurified diets containing 20% fat for 15 weeks. The dietary fats were corn oil, soybean oil, palm oil, palm olein and palm stearin. No differences in the body and organ weights of rats fed the various diets were evident. Plasma cholesterol levels of rats fed soybean oil were significantly lower than those of rats fed corn oil, palm oil, palm olein or palm stearin. Significant differences between the plasma cholesterol content of rats fed corn oil and rats fed the three palm oils were not evident. HDL cholesterol was raised in rats fed the three palm oil diets compared to the rats fed either corn oil or soybean oil. The cholesterol-phospholipid molar ratio of rat platelets was not influenced by the dietary fat type. The formation of 6-keto-$PGF_{1\alpha}$ was significantly enhanced in palm oil-fed rats compared to all other dietary treatments. Fatty acid compositional changes in the plasma cholesterol esters and plasma triglycerides were diet regulated with significant differences between rats fed the polyunsaturated corn and soybean oil compared to the three palm oils.
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It has previously been demonstrated that polyunsaturated oils have a plasma lipid lowering effect as compared to saturated fats (1-3). The results of such studies have helped to fuel the perception that saturated fats are indeed a risk factor in coronary heart disease (4). Recent studies (5,6) have also indicated that monounsaturated fats, long thought to be neutral in their effects, have a lipid lowering quality equal to or better than the polyunsaturated fats. The monounsaturated oils used included olive oil and high oleic safflowerseed oil. Although consumption of palm oil has increased significantly, there is still a lack of balanced evidence on its effects on various lipid-related parameters. Palm oil has a fatty acid composition in which the saturated and unsaturated fatty acids are distributed in almost equal ratios.

In human studies (5,7,8), it has been reported that subjects on a palm oil diet, defined by the authors as the saturated fat period, had plasma and LDL cholesterol levels that were elevated as compared to the polyunsaturated or monounsaturated regimes. However, a closer examination of these and other data has revealed that plasma cholesterol levels after the palm oil period were actually lower than those of subjects on normal diets (9). Hornstra (10) has further demonstrated that in his arterial thrombosis model, palm oil was actually antithrombotic.

Depending on the extent of processing and its final intended usage, palm oil used for human consumption can take several forms. Hence the food industry uses a variety of palm products including palm oil, palm olein and palm stearin in their formulations (11). These products differ not only in their fatty acid compositions but also in their actual physico-chemical characteristics. Palm olein and palm stearin are obtained by fractionating palm oil. As a result of fractionation, a more liquid oil, palm olein, is obtained in which palmitic acid is reduced and linoleic acid is slightly increased. Palm stearin, on the other hand, is the solid fraction of palm oil with a much increased content of palmitic acid and reduced levels of oleic and linoleic acids (12).

Nutritional data on these products are limited. The present study was designed to systematically evaluate the effect of a high fat diet containing palm oil, palm olein and palm stearin on plasma lipid and HDL lipoproteins in the rat model.

MATERIALS AND METHODS

Male Sprague Dawley rats, 35 days old, were obtained from the animal breeding unit of University Malaya, Kuala Lumpur, Malaysia, and randomly assigned to 5 different dietary groups. To each group was assigned a total of 18 rats, and dietary feeding was commenced at 40 days of age. The rats were fed *ad libitum* for a total of 15 weeks a 20% (w/w) high fat diet containing either corn oil, soybean oil, palm oil, palm olein or palm stearin. Rats were kept on a semipurified diet (ICN Nutritional Biochemicals, Cleveland, Ohio) containing by weight: vitamin free casein 22%, dextrose 45%, alphacel 7%, vitamin mixture (AIN-76) 1%, salt mixture (AIN-76) 4.5%, dl-methionine 0.3%, choline bitartrate 0.2%, and dietary fat 20% (either corn oil, soybean oil, palm oil, palm olein or palm stearin). All rats were housed in polypropylene cages in a single air-conditioned room maintained at 22°C with a dark cycle from 18.00-07.00 hr. Rats were weighed once a week throughout the experiment, and fresh diet was provided on alternate days.

Measurement of plasma lipids and high-density lipoproteins. At the end of 15 weeks, rats were fasted overnight and blood was collected by abdominal aorta puncture from ether anesthetized rats. Blood from 12 rats per group was collected in tubes containing sodium EDTA (1 mg/ml blood), and plasma was prepared for lipid and lipoprotein studies.

Three ml of plasma was pipetted into a Beckman 50.3 Ti ultraclear centrifuge tube and overlayered with 2 ml of NaCl solution ϱ_{20} = 1.006 g/ml to separate the very low density lipoproteins (VLDL) by preparative ultracentrifugation (13) for 18 hr at 115,000 × *g* using

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Abbreviations: CO, corn oil; HDL, high density lipoprotein; HMG-CoA, hydroxymethylglutaryl coenzyme A; PO, palm oil; POL, palm olein; PGI_2, prostacyclin I_2; PS, palm stearin; PUFA, polyunsaturated fatty acid; SBO, soybean oil.

a Beckman 50.3 Ti rotor in a Beckman LM8-70 ultracentrifuge. At the end of the run, 2 ml of the top fraction containing VLDL was aspirated. The remaining fraction was readjusted with normal saline to an exact volume of 3 ml and selectively precipitated with dextran sulphate and $MgCl_2$ (14). The HDL components remaining in the supernatant were used for the determination of HDL cholesterol, triglycerides and phospholipids.

Determination of plasma and HDL lipids. Plasma cholesterol and HDL cholesterol in the above supernatant were determined using a commercial enzyme kit (Biomerieux, France). Plasma and HDL lipids were extracted with chloroform/methanol (2:1, v/v) and partitioned with dilute salt solution according to Folch *et al.* (15). The lower chloroform layer was dried under nitrogen and quantified gravimetrically as described (16). Aliquots of lipid extracts in chloroform were spotted on thin-layer plates coated with silica gel G, and the lipid components were separated using hexane/diethyl ether/formic acid (80:20:2, v/v/v) as solvent system in a TLC tank that was saturated with the vapors of the solvent mixture. Lipid spots were visualized by transient exposure to iodine vapors, identified against authentic lipid standards, and scraped for analysis. Cholesterol esters and triglycerides were quantified by the method of Marsh and Weinstein (17), and phospholipids were quantified by phosphorus estimation according to Rouser *et al.* (18). Free cholesterol is reported as the difference between total and esterified cholesterol. Plasma lipids intended for fatty acid analysis were similarly separated by thin-layer chromatography, sprayed with 7,12-dichlorofluoresceine dye in ethanol, and visualized under ultraviolet light. The lipid bands were scraped off and stored at −20°C until methylation.

Platelet preparation. Blood obtained by abdominal puncture of 6 rats per group under ether anesthesia was used for preparing platelets. Nine ml of blood was collected in centrifuge tubes containing 1.5 ml acid-citrate-dextrose anticoagulant (19), and platelets were prepared by differential centrifugation and washed free from plasma according to Rand *et al.* (20). The washed platelets were resuspended in 0.5 ml of NaCl solution (139 mM). An aliquot of this suspension was used for the determination of protein content (21). Platelet lipids were extracted with chloroform/methanol (2:1, v/v), and cholesterol and phospholipid contents of platelets were determined after TLC separation as described for plasma and HDL lipids.

Measurements of 6-keto-PGF$_{1\alpha}$. From 8 rats per group, 0.9 ml of blood was drawn into a prewarmed syringe (40°C) containing 0.1 ml sodium citrate solution (3.8%, w/v, pH 7.4). The blood was carefully mixed and transferred into an Eppendorf tube containing indomethacin (final concentration of 10 μg/ml blood), and placed in a water bath at 37°C. Collagen (Sigma Chemical Co., St. Louis, MO) at a final concentration of 5.0 μg/ml was added to the tube while agitating. After 10 min, incubation was stopped and the sample centrifuged for 3 min at 10,000 × *g* in an Eppendorf centrifuge. The plasma was removed, frozen immediately in liquid nitrogen, and stored at −20°C for the measurement of 6-keto-PGF$_{1\alpha}$ the stable breakdown product of prostacyclin PGI_2 using a radioimmunoassay kit supplied by New England Nuclear (NEK 008, NEN).

Fatty acid analysis. Fatty acid compositional analyses of the dietary oils and of plasma lipids were performed following transmethylation of the sample using methanolic HCl under reflux at 120°C for two hr (22). The fatty acid methyl esters were extracted and purified on Florisil columns as described (23). Fatty acids were then analyzed by gas chromatography (Shimadzu GC 8A, Shimadzu Seisakusho Co., Kyoto, Japan) using a glass column (2 mm × 2 m) packed with 10% SP 2300 (Supelco, Inc., Bellefonte, PA) and temperature programmed from 200°C to 230°C at 2°C per min. Peak areas were quantified by a Shimadzu CR 3A electronic data processor (Shimadzu Seisakusho Co., Kyoto, Japan) and results were expressed as a percentage of the total fatty acids.

Analysis of tocopherols and tocotrienols in the dietary oils was performed by HPLC (24) and the results are expressed as parts per million.

Analysis of data. All data were checked for their frequency distribution using Rankitts plots. Analysis of variance and the Bonferroni Inequality test were used to test the differences between dietary treatments. Two tailed test was performed and treatments were considered significant when $p<0.05$.

RESULTS

The fatty acid compositions of the different dietary oils used in this study are shown in Table 1.

Corn oil and soybean oil are highly polyunsaturated in comparison to palm oil, palm olein and palm stearin. The three palm oils contained about 10% linoleic acid which was considered adequate nutritionally and hence no enrichment of linoleic acid was provided to overcome essential fatty acid deficiency. The tocopherol content and the tocotrienol content of corn and soybean oils also differ significantly from palm oil and its fractions (Table 2). Corn and soybean oils are high in tocopherols but are completely devoid of tocotrienols. Palm oil, olein and stearin have a comparatively

TABLE 1

Fatty Acid Composition (%) of Dietary Oils[a]

Fatty acid	Corn oil	Soybean oil	Palm oil	Palm olein	Palm stearin
12:0	0.1	0.1	0.3	0.2	0.1
14:0	trace	trace	0.8	0.8	1.2
16:0	9.7	8.9	39.5	37.2	50.3
16:1 (n-7)	0.3	0.3	0.3	0.4	0.3
18:0	2.7	3.6	4.3	4.2	4.7
18:1 (n-9)	37.0	20.6	43.1	43.6	34.1
18:2 (n-6)	48.7	57.2	10.5	11.7	7.9
18:3 (n-3)	1.1	8.4	0.5	0.3	0.5
20:0	0.4	0.3	0.5	0.3	0.5
20:1 (n-9)	trace	trace	0.2	0.2	0.2
22:1 (n-9)	n.d.	0.6	n.d.	n.d.	n.d.
Saturates	12.5	12.9	45.4	42.7	56.8
Monoenes	37.3	20.9	43.6	44.2	34.6
Polyenes	49.8	65.6	11.0	12.0	8.4

[a]Traces <0.1% occur and n.d. denotes "not detected."

TABLE 2

Tocopherol and Tocotrienol Content (ppm) of Dietary Oils[a]

	Corn oil	Soybean oil	Palm oil	Palm olein	Palm stearin
Alpha-T	126	28	97	81	19
Gamma-T	446	235	n.d.	n.d.	n.d.
Delta-T	25	145	n.d.	n.d.	n.d.
Total-T	597	408	97	81	19
Alpha-T_3	n.d.	n.d.	161	92	30
Gamma-T_3	n.d.	n.d.	203	135	38
Delta-T_3	n.d.	n.d.	51	39	16
Total-T_3	NIL	NIL	415	266	84

[a]T=tocopherols, T_3=tocotrienols and n.d.=not detected.

lower level of tocopherols than corn and soybean oils but are significantly richer in their tocotrienol content.

Rats fed the experimental high fat diets demonstrated normal growth throughout the experimental duration. At autopsy, a diet-induced difference in body weight nor in the various organ weights was not generally evident (Table 3). However, spleen weight of the soybean oil-fed rats was significantly lower than the rats fed a palm olein diet, and consequently a significantly lower spleen/body weight ratio (%) between these groups was also observed.

Plasma lipids (Table 4) were generally modulated by the dietary fat type. Plasma cholesterol was measured as free and esterified cholesterol. Free cholesterol content was lowest in the soybean group and significant only in comparison to the palm oil group. Cholesterol esters were however more responsive to the dietary fat manipulations and were decreased significantly on soybean oil feeding in comparison to all other dietary treatments. Total plasma cholesterol was significantly higher in all dietary treatments compared to the soybean oil-fed rats.

Plasma triglycerides and phospholipids were generally elevated in the three palm oil-fed groups compared to corn and soybean oil groups. Triglycerides were significantly higher on palm stearin feeding than in corn- and soybean oil-fed rats. Between the three palm oil diets however, plasma triglyceride content did not differ significantly. Plasma phospholipids were significantly enhanced in the palm stearin group compared to all other dietary treatments. Similarly, in the palm oil- and palm olein-fed rats, significantly higher plasma phospholipid levels were detected in comparison to corn and soybean oil feeding.

HDL-cholesterol was elevated in all palm oil diets (palm oil = 29.4 ± 2.8 mg/dl; palm olein = 34.7 ± 2.0 mg/dl; palm stearin 36.2 ± 2.7 mg/dl) compared to both corn oil (27.4 ± 3.3 mg/dl) and soybean oil (24.1 ± 2.0 mg/dl). However statistical significance was apparent only between the palm stearin- and soybean oil-fed rats. In all dietary treatments 19–22% of the HDL-cholesterol was transported as unesterified cholesterol. A diet-induced difference in the distribution of HDL-triglycerides was not apparent in this study. HDL-phospholipid concentrations were however, diet responsive. Corn oil feeding resulted in an HDL-phospholipid content of 22.1 ± 2.4 mg/dl which was significantly lower than that of rats fed either palm olein (31.9 ± 2.5 mg/dl) or palm stearin (31.5 ± 2.6 mg/dl). Although soybean feeding resulted in lower levels of HDL-phospholipids (27.7 ± 2.2 mg/dl) than both palm olein and palm stearin, these differences were not statistically significant.

The cholesterol-phospholipid (C/P) molar ratio of the platelets was monitored as a possible indicator of the dietary fat effect on membrane fluidity (Table 5). Platelet cholesterol due to corn oil feeding was elevated in comparison to all other dietary treatments and this elevation was significant. Platelet phospholipid was highest in the corn oil-fed rats and reduced on palm stearin feeding. This reduction in the palm stearin-fed rats was significant when compared to corn- and palm olein-fed rats. Although differences in the platelet cholesterol and phospholipids were evident, the calculated cholesterol-phospholipid molar ratio was essentially identical and not modified by the dietary fat type.

Prostacyclin formation in activated whole blood was also affected by the dietary fat. The formation of 6-keto-$PGF_{1\alpha}$ was enhanced significantly in palm oil-fed rats compared to all other dietary treatments (Fig. 1). Prostacyclin formation in soybean oil-fed rats was also lower than in all other dietary treatments. Differences between the three palm oil diets in their ability to regulate prostacyclin formation in activated whole blood was evident with palm olein being the lowest and palm stearin was intermediate between palm oil and palm olein.

TABLE 3

Influence of Dietary Fats on Body and Organ Weights of Rats*

Weight	Corn oil	Soybean oil	Palm oil	Palm olein	Palm stearin
Body (g)	387 ± 13	385 ± 7	388 ± 10	379 ± 14	387 ± 8
Liver (g)	13.5 ± 0.7	12.9 ± 0.7	13.4 ± 0.3	14.7 ± 1.0	14.1 ± 0.3
Liver/body (%)	3.5 ± 0.2	3.4 ± 0.1	3.5 ± 0.1	3.9 ± 0.2	3.7 ± 0.1
Heart (g)	1.4 ± 0.06	1.5 ± 0.10	1.3 ± 0.04	1.4 ± 0.07	1.5 ± 0.10
Heart/body (%)	0.37 ± 0.02	0.40 ± 0.01	0.35 ± 0.01	0.38 ± 0.01	0.39 ± 0.02
Spleen (g)	0.9 ± 0.09	0.8 ± 0.04[a]	0.9 ± 0.02	1.1 ± 0.07[b]	0.9 ± 0.04
Spleen/body (%)	0.24 ± 0.02	0.22 ± 0.02[a]	0.24 ± 0.01	0.28 ± 0.02[b]	0.24 ± 0.01
Kidney (g)	2.9 ± 0.1	2.6 ± 0.1	2.7 ± 0.1	2.9 ± 0.2	2.9 ± 0.1
Kidney/body (%)	0.75 ± 0.02	0.70 ± 0.03	0.71 ± 0.03	0.76 ± 0.024	0.75 ± 0.03

*Values are means ± SEM, n=18. Means across a horizontal column with superscript "a" are significantly different from "b" ($p<0.05$).

TABLE 4

Plasma Lipid Content (mg/dl) in Rats Fed High Fat Diets*

Dietary oil	Free cholesterol	Esterified cholesterol	Total cholesterol	Triglycerides	Phospholipids
Corn oil	11.8 ± 0.7	58.0 ± 3.0^b	70.4 ± 4.0^b	46.3 ± 5.4^a	61.3 ± 2.7^{b,d}
Soybean oil	10.2 ± 0.5^a	46.1 ± 1.4^a	56.3 ± 1.7^a	47.9 ± 2.9^a	60.7 ± 1.7^{b,d}
Palm oil	12.9 ± 1.0^b	60.2 ± 2.6^b	70.8 ± 3.2^b	57.6 ± 3.6	70.9 ± 1.7^{b,c}
Palm olein	12.1 ± 1.0	55.4 ± 2.2^b	65.8 ± 2.0^{b,c}	58.5 ± 5.4	73.8 ± 1.2^{b,c}
Palm stearin	11.7 ± 0.5	61.1 ± 2.3^b	76.5 ± 2.7^{b,d}	66.7 ± 3.5^b	92.2 ± 2.7^a

*Values are means ± SEM; n=10. Means in vertical columns with superscript "a" are significantly different from "b", and "c" is significantly different from "d" ($p<0.05$).

TABLE 5

Effect of Dietary Fats on the Cholesterol Phospholipid Molar Ratio (C/P) of Rat Platelet Total Lipids*

Dietary group	Platelet cholesterol (μmol mg protein^{-1})	Platelet phospholipid (μmol mg protein^{-1})	C/P ratio
Corn oil	0.260 ± 0.010^a	0.448 ± 0.030^a	0.590 ± 0.027
Soybean oil	0.199 ± 0.009^b	0.375 ± 0.033	0.538 ± 0.047
Palm oil	0.196 ± 0.018^b	0.351 ± 0.026	0.553 ± 0.027
Palm olein	0.204 ± 0.012^b	0.377 ± 0.011^c	0.549 ± 0.030
Palm stearin	0.170 ± 0.010^b	0.298 ± 0.013^{b,d}	0.585 ± 0.018

*Values are means ± SEM; n=6. Means in vertical columns with superscript "a" are significantly different from "b", and "c" is significantly different from "d" ($p<0.05$).

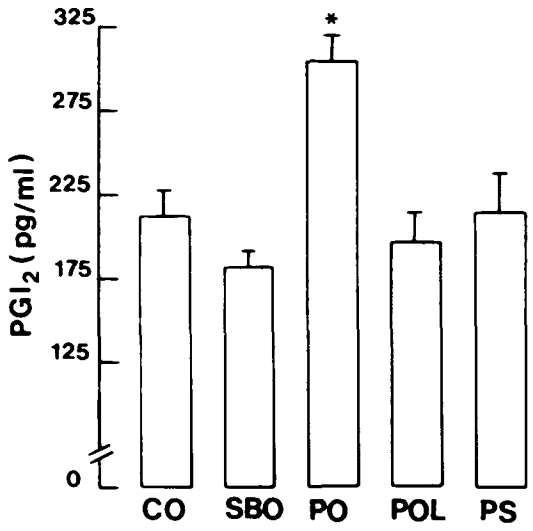

FIG. 1. Effect of dietary fat on PGI_2 production in collagen-activated whole blood. Values are means from 8 animals per group and asterisk (*) indicates significantly different from other groups ($p<0.05$).

Changes in the fatty acid compositions of plasma cholesterol esters and triglycerides were apparently diet regulated. Changes were especially evident in the major fatty acids of the cholesterol esters (Table 6). Both palmitic (16:0) and oleic (18:1) acids were significantly elevated in all palm oil diets compared to the corn and soybean oil diets. On the other hand, a significant reduction in the linoleic acid content in the palm oil-fed rats was also evident.

Plasma triglyceride fatty acid composition (Table 7) was similarly altered by the dietary fat type. Palmitic acid was significantly elevated and linoleic acid was significantly reduced in all three palm oil diets compared to the polyunsaturated oils. However triglyceride oleic acid did not differ significantly among the different dietary treatments. Plasma phospholipid fatty acids were far less responsive to the dietary fat type and only minor differences were detected (data not shown).

DISCUSSION

The results of this study demonstrate an effect of dietary fat composition on plasma and HDL lipoprotein lipids. Compared to corn oil, palm oil, palm olein and palm stearin, soybean oil was hypocholesterolemic. This was expected based on the higher polyunsaturated content of the soybean oil used in this study. Palm olein-fed rats had lower plasma cholesterol content than the corn oil-fed rats. Kris-Etherton *et al.* (25) could not establish a significant cholesterol elevation due to palm oil feeding as compared to corn oil. Similarly Sugano *et al.* (26) were unable to establish significant differences in plasma cholesterol for rats fed mold oil or palm olein using diets in which the protein was contributed as casein. Our results are in agreement with these observations in which significant differences in rats fed corn oil and the three palm oils were not evident.

The fatty acid composition of the palm oils rich in palmitate and oleate does not warrant this finding, and we therefore examined the minor components in all our dietary oils. The three palm oils used in this study were rich in tocotrienols whereas corn and soybean oils were completely devoid of these vitamin E isomers.

TABLE 6

Major Fatty Acids (%) in Rat Plasma Cholesterol Esters*

Fatty acid	Corn oil	Soybean oil	Palm oil	Palm olein	Palm stearin
16:0	9.7 ± 1.4^{a}	12.8 ± 1.9^{c}	18.1 ± 2.7^{b}	18.6 ± 3.2^{b}	19.6 ± 0.7^{b,d}
16:1 (n-7)	2.9 ± 0.2^{a}	2.8 ± 0.1^{a}	5.3 ± 1.7	4.9 ± 0.6^{b}	5.9 ± 0.7^{b}
18:0	1.5 ± 0.5^{b}	3.4 ± 0.7^{a}	1.2 ± 0.6^{b}	1.6 ± 0.3^{b}	1.7 ± 0.4^{b}
18:1 (n-9)	43.9 ± 0.5^{a}	26.0 ± 2.4^{b,c}	56.8 ± 3.6^{b,d}	57.2 ± 3.9^{b,d}	54.0 ± 5.8^{b,d}
18:2 (n-6)	28.8 ± 3.4^{a}	34.4 ± 2.7^{a}	5.7 ± 0.8^{b}	6.2 ± 2.2^{b}	5.6 ± 0.8^{b}
18:3 (n-3)	0.4 ± 0.1	1.1 ± 0.4	0.2 ± 0.1	0.5 ± 0.2	0.7 ± 0.5
20:1 (n-9)	1.3 ± 0.6	1.2 ± 0.3	1.2 ± 0.3	1.2 ± 0.3	0.4 ± 0.1
20:4 (n-6)	4.7 ± 1.9	2.3 ± 0.7	2.8 ± 0.5	2.0 ± 0.7	4.2 ± 0.6
20:5 (n-3)	0.5 ± 0.1^{b}	1.2 ± 0.2	n.d.	0.4 ± 0.2^{b}	0.5 ± 0.2^{b}
22:5 (n-6)	0.4 ± 0.1	2.8 ± 0.9	n.d.	0.3 ± 0.1	n.d.
22:6 (n-3)	Trace	3.1 ± 1.0^{a}	0.5 ± 0.2^{b}	Trace	0.5 ± 0.1^{b}
24:0	n.d.	0.7 ± 0.4	1.0 ± 0.7	1.2 ± 0.4	Trace

*Values are means ± SEM; n=8. Means across a horizontal column with superscript "a" are significantly different from "b", and "c" is significantly different from "d" ($p<0.05$). Trace indicates that the mean value was not significantly different from zero and n.d. indicates "not detected in sample."

TABLE 7

Major Fatty Acids (%) in Rat Plasma Triglycerides*

Fatty acid	Corn oil	Soybean oil	Palm oil	Palm olein	Palm stearin
12:0	1.4 ± 0.3	1.2 ± 0.2	1.2 ± 0.6	0.8 ± 0.3	0.6 ± 0.2
16:0	16.6 ± 1.4^{a}	12.9 ± 1.3^{a}	31.1 ± 3.9^{b}	26.0 ± 1.7^{b}	27.4 ± 2.9^{b}
16:1 (n-7)	2.9 ± 0.6	2.5 ± 0.5	2.2 ± 0.9	1.9 ± 0.6	2.0 ± 0.2
18:0	3.2 ± 1.4	2.7 ± 1.0	4.4 ± 1.8	3.4 ± 0.8	2.9 ± 0.2
18:1 (n-9)	31.4 ± 4.4	35.0 ± 4.2	41.7 ± 5.7	37.9 ± 4.6	35.6 ± 5.1
18:2 (n-6)	25.2 ± 5.3^{a}	21.5 ± 3.0^{a}	8.4 ± 1.4^{b}	10.3 ± 1.5^{b}	6.1 ± 2.6^{b}
18:3 (n-3)	0.6 ± 0.1^{b}	2.2 ± 0.4^{a}	0.5 ± 0.3^{b}	0.7 ± 0.3^{b}	0.6 ± 0.2^{b}
20:1 (n-9)	4.7 ± 0.5^{a}	0.9 ± 0.2^{b,c}	1.7 ± 0.5^{b}	2.1 ± 0.3^{b,d}	2.4 ± 0.9
20:4 (n-6)	1.8 ± 0.2	1.7 ± 0.5	1.5 ± 0.5	1.1 ± 0.2	0.8 ± 0.5
22:0	n.d.	n.d.	n.d.	n.d.	1.5 ± 0.3
20:5 (n-3)	0.7 ± 0.4^{a}	3.1 ± 0.4^{b,d}	1.9 ± 0.2^{b,c}	2.6 ± 0.4^{b}	3.6 ± 0.3^{b,d}
22:4 (n-6)	Trace	5.7 ± 1.3	3.6 ± 1.5	2.9 ± 1.1	2.7 ± 0.5
22:5 (n-6)	0.5 ± 0.1^{b}	0.6 ± 0.1^{b}	1.4 ± 0.3^{a}	0.7 ± 0.2	0.8 ± 0.3
22:6 (n-3)	3.0 ± 0.5^{a}	3.5 ± 0.6^{a}	1.8 ± 0.4^{c}	1.5 ± 0.2^{b,c}	0.6 ± 0.3^{b,d}
24:0	3.0 ± 0.4^{a}	2.4 ± 0.4^{a}	0.5 ± 0.1^{b}	1.0 ± 0.4^{b}	2.8 ± 0.2^{a}

*Values are means ± SEM; n=8. Means across a horizontal column with superscript "a" are significantly different from "b", and "c" significantly different from "d" ($p<0.05$). Trace indicates that the mean value was not significantly different from zero and n.d. indicates "not detected in sample."

Qureshi (27) reported that d-α-tocotrienol, isolated from barley, suppressed HMG-CoA reductase activity, the first rate limiting enzyme of cholesterol synthesis. The palm oil tocotrienols could have similarly suppressed HMG-CoA reductase activity, and this suppression may have been significant enough to overcome the higher saturation of the palm oils. Hence, in rats, a significant increase in the plasma cholesterol levels between the corn oil and the palm oils expected on the basis of differences in the fatty acid composition was not observed.

Palm oil upon fractionation into olein and stearin was observed to have a differential effect on plasma cholesterol in the rat model. Palm stearin had a higher melting point (50.3°C) and was more saturated than palm olein. The increased saturation in palm stearin was a result of about 35% elevation in the palmitic acid content and a 21% lowering of the monounsaturated oleic acid content in comparison to palm olein. The shifts in these physiologically important fatty acids were sufficient to produce significant changes in the lipid profiles of rats fed a palm stearin diet. Plasma cholesterol and phospholipids were significantly elevated in rats fed a palm stearin diet compared to rats receiving the palm olein diet. Increased triglyceride synthesis was also evident. Furthermore fractionating palm oil into palm olein and stearin produced appreciable shifts in the tocopherol and tocotrienol content of these oils. The resulting tocotrienol content in palm stearin (84 ppm) was much lower than that of palm olein (266 ppm). This lower availability of the palm stearin tocotrienols may not have effectively suppressed HMG-CoA reductase activity, and hence, significantly higher plasma cholesterol than that of a palm olein diet may have resulted.

HDL-cholesterol, on the other hand, was highest in rats fed palm stearin; this elevation was significant only in comparison to soybean oil. A similar reduction in HDL-cholesterol on feeding polyunsaturated oils has also been previously reported (28). The calculated HDL/

total cholesterol ratio was highest for palm oil (0.53) and lowest for corn oil (0.39). However, this calculated ratio most probably is a direct reflection of the higher VLDL/LDL cholesterol in the rats fed the palm oils. In rats, cholesterol esters produced in HDL by the action of cholesterol acyltransferase are accumulated in HDL (29,30). Thus exchange of these esters with other lipoproteins is minimized. This also explains the higher percentage of cholesterol carried in the rat HDL fraction.

A hypolipidemic effect of polyunsaturated fatty acids has been well documented in man and in a variety of animal models. In rats, a polyunsaturated diet has been shown to lower (31) plasma cholesterol which is normally accompanied by a decrease in HDL-cholesterol (32,33). Our observations with soybean oil and corn oil also indicate a reduction in HDL-cholesterol in comparison with the palm oils. Nevertheless, this phenomenon was most pronounced in soybean oil-fed rats.

In this study, the platelet cholesterol-phospholipid (C/P) molar ratio was unaltered despite the striking differences in the fatty acid composition of the corn oil, soybean oil and the three palm oils. This observation is in agreement with previous studies (34) which reported that the platelet C/P molar ratio was almost identical despite the dietary fat type. The C/P molar ratio is known to modulate membrane fluidity, and, on the basis of our findings, differences in membrane fluidity between the palm oil diets and the polyunsaturated diets should not occur. Using the fluorescence polarization technique with a hydrophobic probe, Rand *et al.* (35) demonstrated that the fluidity of membrane platelets was significantly enhanced in rats fed a diet rich in polyunsaturated sunflowerseed oil whereas palm oil failed to modify the overall fluidity of platelets as compared with the low fat control. However, they too failed to establish significant differences among the C/P molar ratios of platelets from rats fed these diets. Hence a more direct estimate of membrane fluidity due to our dietary oils can possibly only be obtained by a more sophisticated technique, such as steady state fluorescence anisotropy measurements.

The production of 6-keto-$PGF_{1\alpha}$ measured in collagen-activated whole blood is most likely a reflection of the prostacyclin produced by monocytes (36). This may be due to the ability of the monocytes to utilize endogenous arachidonic acid or to "scavenge" arachidonic acid from activated platelets. Palm oil feeding resulted in a significantly enhanced production of prostacyclin in collagen-activated whole blood. This finding agrees with an earlier observation (10) according to which palm oil feeding increased the prostacyclin content and reduced the formation of thromboxane in comparison to rats fed a sunflowerseed oil diet. Consequently a reduced thromboxane-prostacyclin ratio was reported. We did not measure thromboxane in the same activated whole blood and are therefore unable to draw further conclusions.

Galli *et al.* (37) observed a lowering of arterial PGI_2 production in rats fed high dietary levels of corn oil suggesting that platelets in the corn oil group stimulate to a lower extent arterial PGI_2 formation. In contrast to oleic acid, linoleic acid significantly depressed PGI_2 formation in studies with endothelial cell cultures (38,39). Linoleic acid content of both corn oil and soybean oil being higher than palm oil may have played a role in effectively suppressing PGI_2 formation in collagen-activated blood as well. However, the different levels of prostacyclin production due to palm oil and palm olein feeding cannot be explained on this basis since linoleic acid content was almost identical. Whether significant differences in platelet phospholipid arachidonate content were brought about by the palm oil and palm olein diets causing these shifts in prostacyclin production needs to be evaluated.

The marked changes in the fatty acid composition of the plasma cholesterol esters and triglycerides are not unexpected and clearly show how these compositions are affected by diet. The increased palmitic and oleic acid contents in the cholesterol esters of palm oil-, palm olein- and palm stearin-fed rats clearly arise from a greater availability of these fatty acids through the dietary oils. Similarly the linoleic acid content is significantly reduced in comparison to both corn and soybean oil diets. A significantly higher level of fatty acids of the n-3 family, i.e. 18:3, 20:5 and 22:6, in cholesterol esters of the soybean oil-fed rats signifies the chain elongation products of 18:3 (n-3) available from this dietary oil. These fatty acids are virtually negligible in the cholesterol esters of other dietary groups. Palmitic acid in plasma triglycerides of the rats fed the three palm oil diets was almost 50% higher than that of rats fed either corn or soybean oil diets. Triglyceride linoleic acid showed a reverse trend in that the PUFAs elevated this fatty acid by an average of 2.5 times that of the palm oils. Linoleic acid was lowest in triglycerides of palm stearin-fed rats reflecting a sequential lowering in the incorporation of this fatty acid into the triglycerides due to a lower availability brought about by the increased saturation in this dietary fat.

This study demonstrates the effects of palm oil, palm olein and palm stearin on plasma lipids and HDL lipoproteins in the rat model. The observed effects of palm olein were not clearly predictable on the basis of its fatty acid composition alone. Measurement of routine indicators of nutritional adequacy such as body and organ weights do not show any deleterious effects while plasma fatty acid compositions are as expected. Further studies of the effects of these oils on the lipoprotein classes will be important.

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Effect of Dietary Menhaden Oil and Vitamin E on *In Vivo* Lipid Peroxidation Induced by Iron

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Weanling rats were fed diets containing 10% menhaden oil (MO) or 10% corn oil-lard (1:1, COL) with low (≤5 IU/kg) or supplementary (35 IU/kg) vitamin E for six weeks. The rats were killed 30 min after injection with 24 mg iron/kg as ferrous chloride because thiobarbituric acid-reactive substances (TBARS) in liver homogenates were highest at 30 min after injection of iron into rats fed a standard diet. Tissue homogenates were used either without incubation (zero-time) or after incubation at 37°C for 1 hr. In addition to TBARS and conjugated dienes, headspace hexanal and total volatiles (TOV) determined by capillary gas chromatography were useful indices of lipid peroxidation since they were decreased by vitamin E supplementation and were increased with increasing iron dose. Regardless of the dietary lipid used, vitamin E supplementation decreased headspace hexanal, TOV, TBARS and conjugated dienes in both zero-time and incubated homogenates of liver and kidney. Dietary MO increased TBARS in both zero-time and incubated homogenates of tissue from rats injected with iron. In contrast, dietary MO decreased hexanal and TOV in incubated tissue homogenates. The study demonstrated the usefulness and limitations of using hexanal and TOV as indices of lipid peroxidation.
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Iron is an important component of lipid peroxidation both *in vitro* and *in vivo* (1,2). The primary mechanism of action for iron involves catalysis of either the decomposition of lipid hydroperoxide to generate alkoxyl or peroxyl radicals, or the decomposition of hydrogen peroxide to generate hydroxyl radicals (2,3). The involvement of iron in lipid peroxidation *in vivo* has been demonstrated by the increased production of breath ethane (4), pentane (5,6) or urinary malonaldehyde (7) in vitamin E-deficient animals. In addition to ethane and pentane, many carbonyl compounds, including alkanals, alkenals and hydroxyalkenals, are formed upon decomposition of lipid hydroperoxides in biological samples (8,9). Some of these products may be useful indices of lipid peroxidation. A rapid headspace capillary gas chromatographic method has been developed for determination of hexanal and total volatiles (TOV) as measures of lipid peroxidation in biological samples (10).

Lipid peroxidation is a potential problem of fish oil consumption (11–13). A recent study (14) showed that tissues from rats fed menhaden oil (MO) were more susceptible to lipid peroxidation *in vitro* than those from rats fed corn oil-lard (COL). Using iron injection to induce lipid peroxidation *in vivo,* the present study compared dietary MO and COL and examined the usefulness of headspace hexanal and TOV as indices of lipid peroxidation.

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Abbreviations: ANOVA, analysis of variance; COL, corn oil-lard; GSH, reduced glutathione; MO, menhaden oil; PUFA, polyunsaturated fatty acids; TBARS, thiobarbituric acid-reactive substances; TOV, total volatiles.

MATERIALS AND METHODS

Preparation of tissue homogenates. Liver and kidney were homogenized in 19 volumes of 1.15% KCl/0.01 M phosphate buffer, pH 7.4. A 4-ml aliquot of each homogenate was placed in a 10-ml serum bottle, sealed with a rubber septum and incubated at 37°C for 1 hr before analyses (incubated). A portion of homogenates was used without incubation (zero-time) for analyses.

Iron injection of rats fed a nonpurified diet. In order to select an appropriate time for killing the rats following iron injection, male Sprague-Dawley rats (260–280 g) fed a nonpurified laboratory diet containing 4.5% fat (Purina Rodent Chow, Purina Mills, Inc., St. Louis, MO) were injected i.p. with 30 mg iron as ferrous chloride/kg body wt and were killed by decapitation at 30, 60 and 90 min following injection. Livers were excised, homogenized and incubated as described above. Rats receiving no injection were used to obtain baseline values of thiobarbituric acid-reactive substances (TBARS) and reduced glutathione (GSH).

Iron injection of rats fed corn oil-based diets low in or supplemented with vitamin E. An experiment was done to select a suitable dose of iron and to test the usefulness of the headspace method for measurement of hexanal and TOV. Weanling male Sprague-Dawley rats were fed a low-vitamin E (≤ 2 IU/kg) diet containing 10% corn oil (TD 77068, mineral mix #170911, Teklad Test Diets, Madison, WI) and 0.2 ppm selenium (as sodium selenite) without or with addition of *d-α*-tocopheryl succinate to a total of 38 IU vitamin E/kg. After 9–10 weeks, the rats were injected i.p. with 15 or 30 mg iron as ferrous chloride/kg body wt 30 min before killing.

Iron injection of rats fed diets containing MO or COL with low or supplemental vitamin E. A fat-free, vitamin E-deficient diet containing 0.2 ppm selenium as sodium selenite was used as the basal diet (14). Either 10% MO (Zapata Haynie Corp., Reedville, VA) that contained approximately 50 IU vitamin E/kg oil, or a 10% mixture of tocopherol-stripped corn oil-lard (1:1) (COL) that contained ≤ 20 IU vitamin E/kg oil was added to this diet. Weanling male Sprague-Dawley rats were fed these low vitamin E (≤ 5 IU) diets or the same diets supplemented with *d-α*-tocopheryl succinate to a total of 35 IU vitamin E/kg for six weeks. Because of the occasional death of rats injected with 30 mg iron/kg, the rats were injected i.p. with 24 mg iron as ferrous chloride/kg body wt and were killed 30 min following injection.

Measurements of headspace hexanal and TOV. The capped serum bottles containing 4 ml of zero-time or

incubated tissue homogenates were quickly frozen in dry ice/ethanol. Headspace hexanal and TOV were assayed by gas chromatography (10). TOV, which consisted of mainly propanal, pentane and hexanal, were defined as the total peak areas minus the peak area of a blank obtained by injecting 1 ml of laboratory air. For convenience, TOV were expressed as hexanal equivalents (nanomoles hexanal) based on the peak areas of hexanal standards.

Measurements of TBARS, conjugated dienes, fatty acids and reduced glutathione. TBARS and conjugated dienes were determined as described previously (14). Absorption spectra were routinely recorded to confirm the presence of a primary peak at 532 nm for malonaldehyde, a major component of TBARS. GSH was determined in zero-time liver as nonprotein sulfhydryls by the method of Sedlak and Lindsay (15).

Statistical analysis. Data were analyzed by analysis of variance (ANOVA) using a Minitab program (16). The least significant difference test with equal or unequal replication (17) was used to compare individual means when significant F ratios were found. Values of p less than 0.05 were considered significant.

RESULTS

Changes in TBARS and GSH levels in liver with postinjection time. TBARS in zero-time or incubated homogenates of liver from rats fed a standard laboratory diet and injected with 30 mg iron/kg were highest when rats were killed at 30 min following injection, and then decreased at 60 and 120 min (Fig. 1). Injection of rats with 0.9% NaCl (solvent control for ferrous chloride) did not affect levels of TBARS in liver, indicating that the increase in TBARS was caused by iron. In subsequent experiments, rats were killed 30 min after injection since TBARS decreased thereafter.

The rise in TBARS at 30 min following injection was accompanied by a small decrease (18%) in GSH in zero-time homogenates of liver (Fig. 2). There was not an inverse relationship between TBARS and GSH, as both were decreased 1 and 2 hr after injection. GSH was 37% lower at 2 hr than in noninjected controls.

Effect of iron dose and dietary vitamin E on hexanal and TOV in liver from rats fed a corn oil-based diet. Hexanal was detected only in incubated liver homogenates. A high iron dose (30 mg/kg) significantly increased hexanal in liver homogenates from rats fed low vitamin E but not supplemental vitamin E (Table 1). Vitamin E supplementation decreased hexanal when rats were injected with 30 mg but not with 15 mg iron/kg.

An increase in iron dose from 15 to 30 mg/kg increased both TOV and TBARS in zero-time, and incubated liver homogenates at both dietary vitamin E levels. In general, vitamin E supplementation decreased hexanal, TOV, TBARS and conjugated dienes in liver homogenates from iron-injected rats.

Effect of dietary MO and vitamin E on lipid peroxidation in liver and kidney homogenates of iron-injected rats. The fatty acid composition of MO and COL was the same as that reported previously (14). Linoleic acid was the only polyunsaturated fatty acid (PUFA) present in a significant amount (36.4%) in COL, whereas MO contained 1.2% linoleic acid, 16.2% eicosapentaenoic acid and 9.6% docosahexaenoic acid. Diets that contained COL or MO had comparable PUFA contents, 3.7% for COL diets and 3.3% for MO diets, but a greatly different ratio of n-3/n-6 PUFA (COL:MO = 0.02:12.8).

In zero-time homogenates of tissues from rats injected with 24 mg iron/kg, hexanal was not detected in significant amounts in either liver or kidney, regardless of dietary lipid and vitamin E (Table 2). Dietary vitamin E decreased liver and kidney TBARS from rats fed either lipid, and decreased liver and kidney conjugated dienes from rats fed COL but not MO. Except for kidney from rats fed COL, vitamin E had little effect on TOV. Dietary MO significantly increased TBARS in both liver and kidney, but had no consistent effect on conjugated dienes or TOV.

As compared to zero-time homogenates, incubated

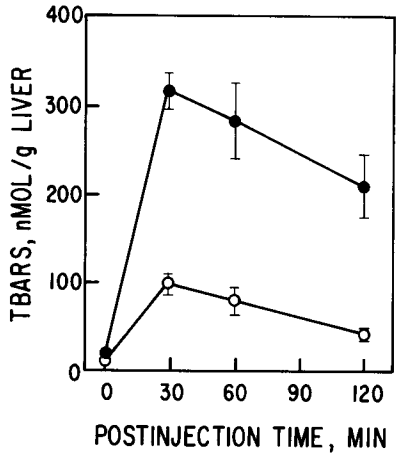

FIG. 1. TBARS in homogenates of liver as a function of time following injection of iron (30 mg iron as ferrous chloride/kg body wt) into rats fed a standard laboratory diet. Each data point is the mean ± SEM for 4–5 rats. (○), zero-time; and (●), incubated at 37°C for 1 hr.

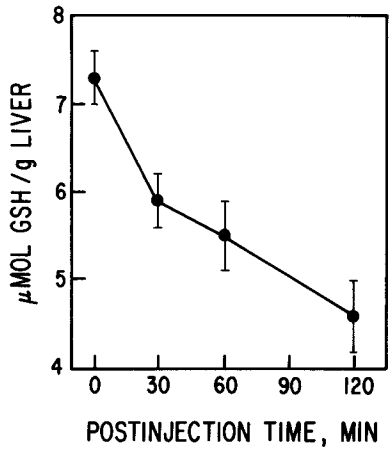

FIG. 2. GSH in zero-time homogenates of liver as a function of time following injection of iron (30 mg iron as ferrous chloride/kg body wt) into rats fed a standard laboratory diet. Each data point is the mean ± SEM for three rats.

TABLE 1

Effect of Ferrous Chloride Injection on Lipid Peroxidation in Homogenates of Liver from Rats Fed Low or Supplemental Vitamin E[a]

Measurement[b]	Iron injected (mg iron/kg)	Dietary vitamin E		p^c
		Low	Supplemented	
Zero-time (nonincubated)				
Hexanal	15	Trace	ND[d]	
	30	Trace	ND	
TOV	15	25 ± 6	11 ± 7	NS
	30	98 ± 7	47 ± 7	<0.001
p^e		<0.001	<0.01	
TBARS	15	59 ± 2	39 ± 3	<0.01
	30	100 ± 4	101 ± 5	NS
p		<0.001	<0.001	
Conjugated dienes	15	704 ± 18	557 ± 36	<0.01
Incubated (37°C, 1 hr)				
Hexanal	15	108 ± 19	115 ± 25	NS
	30	205 ± 21	103 ± 47	<0.05
p		<0.05	NS	
TOV	15	580 ± 32	388 ± 86	NS
	30	1322 ± 58	840 ± 99	<0.001
p		<0.001	<0.001	
TBARS	15	334 ± 17	191 ± 30	<0.001
	30	528 ± 21	410 ± 11	<0.01
p		<0.001	<0.001	
Conjugated dienes	15	907 ± 35	731 ± 99	NS

[a]Weanling rats were fed a corn oil-based diet containing ⩽ 2 IU (low) or 38 IU (supplemental) vitamin E/kg for 9–10 week.
[b]Values are means ± SEM for five rats fed the low vitamin E diet and three rats fed vitamin E-supplemented diet. All measurements are expressed as nmol/g liver.
[c]Significance of difference by ANOVA and the least significant difference test for values within a row; NS, not significant ($p>0.05$).
[d]Not detected.
[e]Significance of difference for values in the same column.

homogenates of liver and kidney had much higher levels of hexanal, TOV and TBARS (Table 3). Vitamin E supplementation consistently decreased hexanal, TOV and TBARS in both liver and kidney from rats fed either dietary lipid. Dietary MO consistently increased TBARS in liver and kidney, but decreased hexanal and TOV in both liver and kidney, regardless of dietary vitamin E level.

DISCUSSION

A recent study (14) demonstrated an enhanced susceptibility to *in vitro* lipid peroxidation induced by iron of tissues from rats fed MO as compared to rats fed COL. The present study investigated whether dietary MO enhances lipid peroxidation *in vivo*, and examined the usefulness of headspace hexanal and TOV as indices of lipid peroxidation. Like TBARS and conjugated dienes, hexanal and TOV were useful indices of lipid peroxidation since they were decreased by vitamin E supplementation and were increased when the iron level was increased from 15 to 30 mg/kg.

In agreement with results (14) obtained from incubation with ferrous sulfate of tissues from rats fed MO or COL, the results of the present study showed that lipid peroxidation induced *in vivo* by iron was decreased by vitamin E supplementation. In addition, TBARS were higher and hexanal and TOV were lower in homogenates of tissues from iron-injected rats fed MO than rats fed COL when dietary vitamin E was maintained at comparable levels. Dietary MO increased n-3 PUFA but decreased n-6 PUFA in liver and kidney from rats fed MO (14). This could account for the increased production of TBARS (mainly malonaldehyde) and for the decreased production of hexanal, which is only produced by n-6 PUFA (18). Lipid peroxidation measured as TBARS occurred mainly in n-3 PUFA containing five or six double bonds in cellular membranes exposed to oxidants (19). Several studies (12,14,20,21) demonstrated enhanced susceptibility to lipid peroxidation of tissues from rats fed diets high in n-3 PUFA. The main difference between the present study and other studies is that lipid peroxidation was induced *in vivo* by iron in the present study.

That hexanal was not detectable in zero-time tissue homogenates indicates that the headspace method of hexanal measurement as a lipid peroxidation index is not as sensitive as is the method of TBARS. One explanation is that hexanal is more rapidly metabolized by aldehyde dehydrogenase than is malonaldehyde. Indeed, substrate specificity of hepatic microsomal aldehyde dehydrogenases increases with increasing carbon chain length (22–25). Nonetheless, hexanal can be a specific index of lipid peroxidation *in vitro* when pure lipids or tissue membrane containing n-6 PUFA are peroxidized. The method of headspace hexanal for lipid peroxidation measurement is specific and simple (10). Future research directed toward co-determination of propanal, a specific oxidation product of n-3 PUFA, and hexanal will enable simultaneous determination of lipid peroxidation products of both membrane n-3 and n-6 PUFA.

TABLE 2

Effect of Dietary Lipids and Vitamin E on Lipid Peroxidation in Zero-Time Homogenates of Tissues from Rats Injected with Iron[a]

Measurement[b]	Dietary vitamin E		
	Low	Supplemented	p[c]
Hexanal			
Liver			
COL	Trace	ND[d]	
MO	ND	ND	
Kidney			
COL	8 ± 2	ND	
MO	Trace	Trace	
TOV			
Liver			
COL	89 ± 11	76 ± 10	NS
MO	87 ± 10	74 ± 9	NS
p[e]	NS	NS	
Kidney			
COL	138 ± 11	83 ± 7	<0.01
MO	102 ± 8	106 ± 11	NS
p	<0.05	NS	
TBARS			
Liver			
COL	46 ± 2	23 ± 2	<0.01
MO	71 ± 8	50 ± 4	<0.05
p	<0.01	<0.01	
Kidney			
COL	40 ± 2	28 ± 3	<0.001
MO	53 ± 2	38 ± 2	<0.001
p	<0.001	<0.01	
Conjugated dienes			
Liver			
COL	911 ± 29	718 ± 10	<0.01
MO	876 ± 34	847 ± 60	NS
p	NS	<0.05	
Kidney			
COL	404 ± 21	347 ± 12	<0.05
MO	359 ± 10	387 ± 13	NS
p	NS	NS	

[a]Rats fed corn oil-lard or menhaden oil diets with ⩽ 5 IU (low) or 35 IU (supplemental) vitamin E/kg for six weeks were injected with 24 mg iron/kg as ferrous chloride.
[b]Values are means ± SEM for five low vitamin E and four vitamin E-supplemented rats. All measurements are expressed as nmol/g tissue.
[c]Significance of difference by ANOVA and the least significant difference test for values within a row; NS, not significant ($p>0.05$).
[d]Not detected.
[e]Significance of difference for values in the same column.

TABLE 3

Effect of Dietary Lipids and Vitamin E on Lipid Peroxidation in Incubated Homogenates of Tissues from Rats Injected with Iron[a]

Measurement[b]	Dietary vitamin E		
	Low	Supplemented	p[c]
Hexanal			
Liver			
COL	233 ± 30	141 ± 50	<0.05
MO	74 ± 8	46 ± 5	NS
p[d]	<0.01	<0.05	
Kidney			
COL	289 ± 11	171 ± 28	<0.001
MO	112 ± 16	87 ± 9	NS
p	<0.001	<0.01	
TOV			
Liver			
COL	1115 ± 103	590 ± 97	<0.001
MO	840 ± 50	474 ± 52	<0.01
p	<0.05	NS	
Kidney			
COL	1495 ± 53	1031 ± 138	<0.05
MO	1169 ± 144	960 ± 99	NS
p	NS	NS	
TBARS			
Liver			
COL	378 ± 12	175 ± 14	<0.001
MO	467 ± 21	293 ± 29	<0.001
p	<0.01	<0.01	
Kidney			
COL	313 ± 12	244 ± 11	<0.001
MO	330 ± 9	286 ± 9	<0.05
p	NS	<0.05	

[a]Rats fed corn oil-lard or menhaden oil diets with ⩽5 IU (low) or 35 IU (supplemental) vitamin E/kg for six weeks were injected with 24 mg iron/kg as ferrous chloride. Liver and kidney homogenates were incubated at 37°C for 1 hr.
[b]Values are means ± SEM for five low vitamin E and four vitamin E-supplemented rats. All measurements are expressed as nmol/g tissue.
[c]Significance of difference by ANOVA and the least significant difference test for data within a row; NS, not significant ($p>0.05$).
[d]Significance of difference for data in the same column.

The GSH redox cycle plays a vital role in the cellular response to various classes of foreign compounds (26). One mechanism for oxidation of GSH during iron-induced lipid peroxidation is its use as a cofactor by GSH peroxidase in scavenging lipid peroxides and hydrogen peroxide. The relatively small decrease in GSH, 18% and 37% at 30 min and 2 hr, respectively, following iron injection, may indicate a relatively minor role of GSH oxidation during iron-induced lipid peroxidation. The rapid decrease in TBARS, possibly by aldehyde dehydrogenase, concurrent with the decrease in GSH following iron injection shows the lack of a simple inverse relationship between GSH and TBARS in liver of iron-injected rats.

In conclusion, dietary vitamin E decreased iron-induced lipid peroxidation *in vivo* as evidenced by decreased levels of hexanal, TOV, TBARS and conjugated dienes in homogenates of liver and kidney from rats fed MO or COL. Dietary MO increased production of TBARS in both zero-time and incubated tissue homogenates. However, conjugated dienes assayed in zero-time tissue homogenates as an *in vivo* lipid peroxidation index were not affected by dietary MO. Thus, whether MO feeding enhanced lipid peroxidation *in vivo* awaits further investigation.

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Biokinetics of Dietary *RRR*-α-Tocopherol in the Male Guinea Pig at Three Dietary Levels of Vitamin C and Two Levels of Vitamin E. Evidence that Vitamin C Does Not "Spare" Vitamin E *in Vivo*[1]

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The net rates of uptake of "new" and loss of "old" *2R,4'R,8'R*-α-tocopherol (*RRR*-α-TOH, which is natural vitamin E) have been measured in the blood and in nine tissues of male guinea pigs over an eight week period by feeding diets containing deuterium-labelled α-tocopheryl acetate (d_6-*RRR*-α-TOAc). There was an initial two week "lead-in" period during which 24 animals [the "high" vitamin E (HE) group] received diets containing 36 mg of unlabelled (d_0) *RRR*-α-TOAc and 250 mg of ascorbic acid per kg diet, while another 24 animals [the "low" vitamin E (LE) group] received diets containing 5 mg d_0-*RRR*-α-TOAc and 250 mg ascorbic acid per kg diet. The HE group was then divided into three equal subgroups, which were fed diets containing 36 mg d_6-*RRR*-α-TOAc and 5000 mg [the "high" vitamin C (HEHC) subgroup], 250 mg [the "normal" vitamin C (HENC) subgroup] and 50 mg [the "low" vitamin C (HELC) subgroup] ascorbic acid per kg diet. One animal from each group was sacrificed each week and the blood and tissues were analyzed for d_0- and d_6-*RRR*-α-TOH by gas chromatography-mass spectrometry. The LE group was similarly divided into three equal subgroups with animals receiving diets containing 5 mg d_6-*RRR*-α-TOAc and 5,000 mg (LEHC), 250 mg (LENC) and 50 mg (LELC) ascorbic acid per kg diet with a similar protocol being followed for sacrifice and analyses. In the HE group the total (d_0- + d_6-) *RRR*-α-TOH concentrations in blood and tissues remained essentially constant over the eight week experiment, whereas in the LE group the total *RRR*-α-TOH concentrations declined noticeably (except in the brain, an organ with a particularly slow turnover of vitamin E). There were no significant differences in the concentrations of "old" d_0-*RRR*-α-TOH nor in the concentrations of "new" d_6-*RRR*-α-TOH found in any tissue at a particular time between the HEHC, HENC and HELC subgroups, nor between the LEHC, LENC and LELC subgroups. We conclude that the long-postulated "sparing" action of vitamin C on vitamin E, which is well documented *in vitro*, is of negligible importance *in vivo* in guinea pigs that are not oxidatively stressed in comparison with the normal metabolic processes which consume vitamin E (e.g., by oxidizing it irreversibly) or eliminate it from the body. This is true both for guinea pigs with an adequate, well-maintained vitamin E status and for guinea pigs which are receiving insufficient vitamin E to maintain their body stores.

The biokinetics of vitamin E uptake and loss in the HE guinea pigs are compared with analogous data for rats reported previously (*Lipids 22*, 163–172, 1987). For most guinea pig tissues the uptake of vitamin E under "steady-state" conditions was faster than for the comparable rat tissues. However, the brain was an exception with the turnover of vitamin E occurring at only one-third of the rate for the rat.

Lipids 25, 199–210 (1990).

We have recently employed *2R,4'R,8'R*-α-tocopheryl acetate substituted in a metabolically inactive position with three atoms of deuterium (d_3-*RRR*-α-TOAc) to make the first measurements of the net, long-term uptake of (deuterated) natural vitamin E, d_3-*2R,4'R,8'R*-α-tocopheryl (d_3-*RRR*-α-TOH) in the male rat under normal laboratory dietary conditions, using a diet in which the d_3-*RRR*-α-TOAc (36 mg/kg diet) was the only source of vitamin E (1). We discovered that there were dramatic differences in uptake kinetics between tissues. For example, the equalization time, $t_{1:1}$, which is the time required for the new (deuterium-labelled) α-TOH concentration to become equal to that of the old (unlabelled) α-TOH, was estimated to be ca. 9, 18, 40 and 72 days in the lung, heart, brain and spinal cord, respectively (K.U. Ingold, G.W. Burton, and W. Siebrand, 1990, unpublished results).

Since analogous, tissue-sampling experiments on man are almost inconceivable, we decided to examine the biokinetics of natural vitamin E in a different laboratory animal in order to see if the same general pattern of fast-uptake tissues and slow-uptake tissues obtained. We chose the guinea pig as our experimental animal and carried out the biokinetics under conditions similar to those employed in the earlier study on rats. However, in order to increase the sensitivity of the measurement of small amounts of deuterated tocopherol in the presence of a large amount of the unlabelled compound, we employed *2R,4'R,8'R*-α-tocopheryl acetate labelled in metabolically inactive positions with six atoms of deuterium (d_6-*RRR*-α-TOAc). As will be reported elsewhere (K.U. Ingold, G.W. Burton, and W. Siebrand, 1990, unpublished results), guinea pigs and rats have a somewhat similar pattern of fast-uptake and slow-uptake tissues. That is, corresponding tissues could be classified either as "fast" or "slow." However, the fast-uptake tissues of the guinea pig were somewhat "faster" than those of the rat and, while some of the slow-uptake tissues of the guinea pig were "faster" than for the rat, others were dramatically slower, e.g., for the brain, $t_{1:1}$ was 40 and 107 days in the rat and guinea pig, respectively (K.U. Ingold, G.W. Burton, and W. Siebrand, 1990, unpublished results).

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Abbreviations: AH_2, vitamin C; α-TOH, α-tocopherol; *RRR*-α-TOH, *2R,4'R,8'R*-α-tocopherol; *RRR*-α-TOAc, *RRR*-α-tocopheryl acetate; d_0-*RRR*-α-TOH, unlabelled *RRR*-α-tocopherol; d_3-*RRR*-α-TOH, d_3-*RRR*-α-(5-CD_3)tocopherol; d_3-*RRR*-α-TOAc, *RRR*-α-(5-CD_3)tocopheryl acetate; d_6-*RRR*-α-TOH, *RRR*-α-(5,7-$(CD_3)_2$)tocopherol; d_9-*ambo*-α-TOH, *2RS,4'R,8'R*-α-(5,7,8-$(CD_3)_3$)tocopherol; $t_{1:1}$, equalization time; LE, low vitamin E; HE, high vitamin E; LELC, low E, low (vitamin) C; LENC, low E, normal C; LEHC, low E, high C; HELC, high E, low C; HENC, high E, normal C; HEHC, high E, high C; α-TO•, α-tocopheroxyl radical; RBC, red blood cells; ROO•, peroxyl radical; ROOH, hydroperoxide.

The guinea pig was chosen for our second whole animal biokinetic experiment with vitamin E because, unlike a rat but like man, the guinea pig cannot synthesize ascorbic acid (vitamin C, AH_2). The availability of vitamin C depends, therefore, on the dietary level of this compound, which can be manipulated over a wide range. This is important because both vitamin E (2-5) and vitamin C (6-8) are chain-breaking antioxidants and there is a considerable body of evidence which indicates that there is a synergistic antioxidant interaction between these vitamins in a wide variety of *in vitro* model systems (9-39). There is sound experimental evidence that this synergism is due to the "regeneration" of α-TOH by reduction of its initial oxidation product, the tocopheroxyl radical, α-TO$^{\bullet}$, by the ascorbate anion, AH^-. That is, the lipid-soluble α-TOH traps lipid peroxyl radicals, ROO$^{\bullet}$, forming α-TO$^{\bullet}$ and lipid hydroperoxide, ROOH, and the α-TO$^{\bullet}$ is then reduced by the water-soluble ascorbate rather than being irreversibly oxidized by reaction with a second peroxyl radical. The overall synergistic interaction between these two radical-trapping antioxidants *in vitro* can be represented by reactions 1-3.

$$ROO^{\bullet} + \alpha\text{-TOH} \rightarrow ROOH + \alpha\text{-TO}^{\bullet} \quad [1]$$

$$\alpha\text{-TO}^{\bullet} + AH^- \rightarrow \alpha\text{-TOH} + A^{\bullet -} \quad [2]$$

$$A^{\bullet -} + A^{\bullet -} \xrightarrow{H^+} A + AH^- \quad [3]$$

There is some much less compelling evidence for an interaction between vitamin E and vitamin C *in vivo* for rats (40-47), guinea pigs (48-57), and premature infants (58). Synergistic E/C interactions have generally been reported, a result which lends support to the hypothesis, drawn from *in vitro* experiments, that vitamin C can "regenerate" vitamin E *in vivo*, or at least "spare" vitamin E by some other *in vivo* mechanism. For example, dietary vitamin C has been reported to enhance plasma levels of vitamin E (40,45,47,50,58), to enhance vitamin E levels in other tissues (52,53,56), and to partially reverse effects due to vitamin E deficiency (47). Antagonistic E/C interactions have also been reported (41-43,48,51). Thus, dietary vitamin C has been reported to lower plasma levels of vitamin E (42,51) and to enhance erythrocyte hemolysis (42,43,51). The difficulties involved in determining whether there is any E/C interaction *in vivo* and, if so, whether the interaction is synergistic or antagonistic can be further illustrated by the pioneering measurements of expired pentane as a measure of lipid peroxidation as described by Tappel and co-workers (41,44,54). These studies demonstrated that the level of lipid peroxidation induced in rats by methyl ethyl ketone peroxide is uninfluenced by vitamin C if the rats have an adequate vitamin E status, but is enhanced by vitamin C when the animals are vitamin E deficient (41), while for iron-loaded rats (44) and CCl_4-intoxicated guinea pigs (54), dosing with vitamin C reduced *in vivo* lipid peroxidation. Overall, the literature indicates that a prooxidant effect of vitamin C has generally, though not always (51), been observed in severely vitamin E deficient animals and might be attributed to a prooxidant (chain-initiating) effect on lipid peroxidation by ascorbate, particularly in the presence of iron (59,60), as well as to the reduced molar effectiveness of ascorbate as a chain-breaking antioxidant at high concentrations (7). With both synergistic and antagonistic E/C interactions being reported it is not surprising to find that slight modifications of the experimental conditions or the use of some different measure of antioxidant status can lead to a failure to detect any kind of E/C interaction *in vivo* (40-42,44-48,50-52,55,56).

Guinea pigs on a diet containing d_6-*RRR*-α-TOAc provide a unique opportunity to search for any *in vivo* protective or destructive effect of vitamin C on vitamin E. An experimental protocol carefully designed to measure vitamin E turnover would be very much more sensitive than any of the earlier whole animal studies in detecting the existence, or otherwise, of an *in vivo* interaction between vitamins E and C. It would therefore provide a far more definitive answer to the important question: Does vitamin C "spare" vitamin E *in vivo*?

We chose essentially the same experimental protocol for the present study of the biokinetics of vitamin E uptake and loss in the guinea pig that we had previously employed with rats (1). However, because far more guinea pigs were employed than in the earlier study on rats (48 vs 9 animals) we limited the number of biological tissues and fluids examined to 11 (vs 23 for the rats). Another difference was that the guinea pigs were given the diet containing d_6-*RRR*-α-TOAc after a two week "lead-in" period, during which the animals were fed a diet containing the same concentration of the unlabelled material, d_0-*RRR*-α-TOAc, whereas for the rats the corresponding "lead-in" time was four weeks. The maximum length of time the animals were on deuterated vitamin E was similar (56 days for guinea pigs vs 65 days for rats), but the guinea pigs were sacrificed for tissue analyses at regular seven day intervals whereas the rats had been sacrificed on days 1, 2, 4, 8, 16, 31 and 65 after being switched to the deuterated tocopherol diet.

The rationale behind the protocol (see Methods section and Fig. 1) is that if vitamin C really does "spare" vitamin E *in vivo* then, at high dietary levels of vitamin C, one would expect a slower than normal loss of "old" vitamin E (d_0-*RRR*-α-TOH) from a tissue and a corresponding slower than normal uptake of "new" vitamin E (d_6-*RRR*-α-TOH). Similarly, at low dietary levels of vitamin C one would expect a faster than normal loss of "old" and a faster than normal uptake of "new" vitamin E.

MATERIALS AND METHODS

Materials. *2R,4′R,8′R*-α-(5,7-$(CD_3)_2$)tocopherol (d_6-*RRR*-α-TOH) was prepared by deuteriomethylation of δ-tocopherol (61) and was then converted to the acetate as previously described (1). *2RS,4′R,8′R*-α-(5,7,8-$(CD_3)_3$)tocopherol (d_9-*ambo*-α-TOH) was prepared for use as an internal standard by condensation of hydroquinone with phytol followed by deuteriomethylation.

Methods. Forty-eight male, two-week-old, specific pathogen free guinea pigs (170-222 g) obtained from Charles River Canada, Inc., P.Q. (St. Constant, Quebec, Canada) were divided into two main groups, a high vitamin E group of 24 animals, HE, and a low vitamin E group of 24 animals, LE. The animals were housed four per plastic cage (1720 cm^2 floor area) with ground corn cob bedding and were fed a Reid-Briggs Guinea Pig diet modified as follows: corn oil (which contains α-TOH) was omitted as were vitamins E and C; tocopherol-stripped corn oil (7.3%

by weight) containing d_0-*RRR*-α-TOAc or d_6-*RRR*-α-TOAc (5 or 36 mg/kg diet) was added together with ascorbic acid (50, 250 or 5000 mg/kg diet). During these experiments the guinea pigs consumed ca. 15–20 g of diet per day.

All the animals in the HE group were fed a diet containing 36 mg d_0-*RRR*-α-TOAc/kg diet [the same level as for the rats in the earlier study (1)] and 250 mg ascorbic acid/kg diet for two weeks. After this "lead-in" period, i.e., on day 0 of the actual experiment, the HE group of animals were divided into three equal subgroups, all of which received a diet containing 36 mg d_6-*RRR*-α-TOAc but different levels of vitamin C (Fig. 1). The high vitamin C subgroup, HEHC, were fed the megadose (62) level of 5,000 mg ascorbic acid/kg diet; the normal vitamin C subgroup, HENC, continued with 250 mg ascorbic acid/kg diet; and the low vitamin C subgroup, HELC, received the barely antiscorbutic (49–51,53,55,56,63–68) level of 50 mg ascorbic acid/kg diet.

A similar protocol was followed with the LE group, but for these guinea pigs the levels of d_0- and d_6-*RRR*-α-TOAc wre only 5 mg/kg diet. A two week "lead-in" with d_0-α-TOAc and 250 mg ascorbic acid/kg diet was followed by the d_6-*RRR*-α-TOAc and the same three levels of vitamin C as for the HE subgroups. That is, the three LE subgroups, LEHC, LENC, and LELC, received 5,000 mg, 250 mg, and 50 mg ascorbic acid/kg diet, respectively (Fig. 1).

One guinea pig from each of the six sub-groups was sacrificed weekly on days 7, 14, 21, 28, 35, 42, 50 and 56.

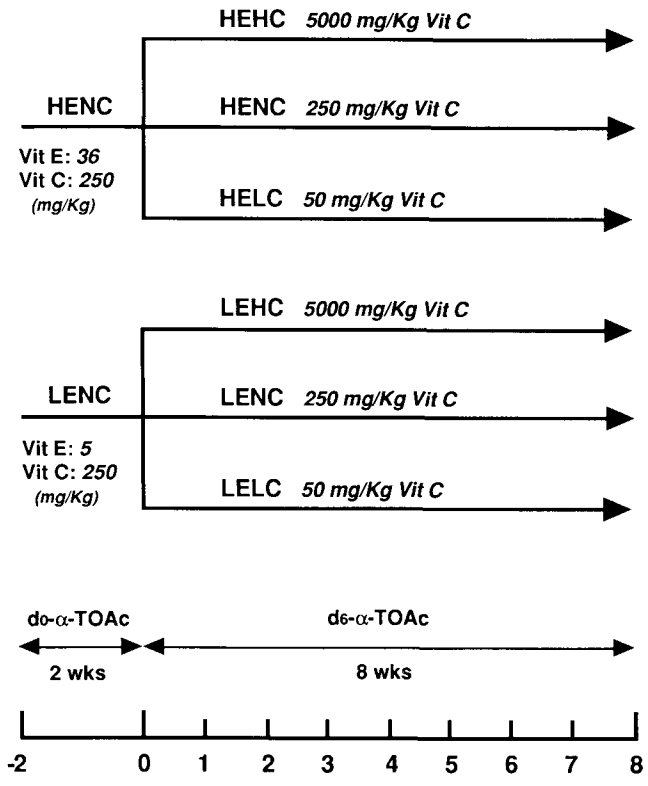

FIG. 1. Dietary regimes for the guinea pigs employed in this study.

Blood samples were obtained by heart puncture with the animals anesthetized with Innovar-Vet™ (Pitman-Moore Ltd., Don Mills, Ontario). The animals were then sacrificed by arterial perfusion with isotonic NaCl. Blood was separated into plasma and red blood cell (RBC) fractions by centrifugation, and the RBC were washed as described previously (1). The plasma, RBC, and nine weighed tissues (adrenal, brain, heart, kidney, liver, lung, muscle [biceps femoris, b.f.], spleen, and testis) were stored at −80°C prior to analysis. The frozen tissue samples were thawed and 7.95 nmol d_9-*ambo*-α-TOH in 50 μl heptane was immediately added to them; this was followed by tissue homogenization and extraction of α-TOH into heptane, as described previously. The entire brain, the two adrenals, one kidney, and one testis were utilized while weighed portions (0.5–1.0 g) of the heart, spleen, b.f. muscle, liver, and lung were employed. The plasma (500 μl) and RBC (in phosphate buffered saline, 1.3 ml, hematocrit 45%) were mixed with 3.98 nmol and 7.95 nmol d_9-*ambo*-α-TOH in heptane, respectively, as soon as they thawed and the α-TOH was extracted immediately.

The heptane extracts were assayed using a Varian model 5000 high performance liquid chromatography (HPLC) (Varian Associates, Palo Alto, CA) equipped with a 250 × 4 mm Lichrosorb Si 60 (5μ particle size) using 90% hexane/10% butyl methyl ether as the eluent, and the α-TOH fraction in each sample was collected automatically. The relative proportions of d_0-*RRR*-α-TOH, d_6-*RRR*-α-TOH, and d_9-*ambo*-α-TOH in each sample were determined as described previously (1) by gas chromatography-mass spectrometry (GC-MS) analysis following conversion of these tocopherols to their trimethylsilyl ethers. Since the absolute amount of d_9-*ambo*-α-TOH added to each sample was known, the absolute concentrations of "old" d_0-*RRR*-α-TOH and "new" d_6-*RRR*-α-TOH could be readily calculated.

RESULTS

Our complete results are presented in Table 1 which lists the absolute concentrations of d_0-*RRR*-α-TOH and d_6-*RRR*-α-TOH as a function of time in the plasma, RBC, and nine tissues for the six dietary regimes employed in these experiments.

In accordance with our experience with rats (1), the total concentration of vitamin E (i.e., d_0- + d_6-*RRR*-α-TOH) in the tissues of the HE group of guinea pigs remained essentially constant during the eight week experimental period, as is best seen in Figure 2. However, for the LE group of guinea pigs the total concentration of vitamin E declined significantly during the eight weeks in all tissues examined except the brain (where turnover is extremely slow).

DISCUSSION

Does vitamin C "spare" vitamin E in vivo? The rationale for having both the HE and the LE groups of guinea pigs is that at high dietary levels of vitamin E any "sparing" action by vitamin C might be masked from experimental observation because adequate "new" vitamin E would always be available to replace any used, "old" vitamin E. However, with sufficiently low dietary levels of vitamin E the rate of depletion of "old" vitamin E in a tissue

TABLE 1

Time-Dependence of the Absolute Concentrations of d_0- and d_6-*RRR*-α-TOH in Blood and Tissue[a]

Tissue	Day	HEHC		HENC		HELC		LEHC		LENC		LELC	
		d_0	d_6	d_0	d_6	d_0	d_6	d_0	d_6	d_0	d_6	d_0	d_6
Adrenal	7	437	458	401	358	689	383	217	52	217	56	341	54
	14	238	467	117	410	145	518	74	46	152	86	106	44
	21	196	800	108	525	163	726	66	52	58	41	44	36
	28	104	786	61	521	49	561	58	64	129	128	40	34
	35	114	790	64	520	93	617	39	35	22	17	31	59
	42	57	779	41	544	85	755	16	23	28	35	14	14
	49	79	840	—	—	33	437	14	23	20	31	10	16
	56	20	364	33	384	44	672	7.8	22	15	24	27	45
Brain	7	38	2.0	31	1.5	35	1.5	34	0.3	29	0.4	34	0.3
	14	18	1.4	21	1.5	21	2.2	30	0.8	35	1.0	18	0.8
	21	31	4.1	30	5.8	31	6.0	35	1.2	49	1.3	46	1.4
	28	18	4.6	17	4.0	15	3.3	42	1.5	40	1.8	46	1.9
	35	66	18	26	6.0	33	7.4	41	2.3	43	1.3	41	2.6
	42	20	8.1	13	4.2	16	5.8	36	2.3	37	2.4	32	1.4
	49	39	17	—	—	26	10	31	2.5	35	2.4	36	2.1
	56	15	4.9	14	4.8	16	5.8	26	1.4	26	2.2	32	3.8
Heart	7	19	13	21	12	24	10	17	2.5	17	2.5	17	1.9
	14	18	18	11	19	14	23	5.2	2.5	11	5.9	14	7.1
	21	13	27	10	30	10	27	11	6.4	13	7.2	7.4	5.6
	28	7.0	35	6.7	27	3.6	19	7.6	6.3	11	8.3	8.1	6.0
	35	7.2	40	5.7	31	7.7	35	6.9	6.9	3.7	3.2	5.0	10
	42	3.6	34	3.3	28	4.7	33	2.7	3.9	4.7	5.7	4.0	4.0
	49	4.4	44	—	—	3.9	43	3.4	5.3	3.4	4.9	3.1	5.0
	56	1.7	27	2.7	26	2.4	32	0.9	2.5	2.3	3.8	5.0	8.5
Kidney	7	13	11	12	9	18	9	12	1.6	13	1.7	12	1.5
	14	8.0	8.8	8.8	12	10	16	4.5	1.9	9.1	3.6	5.6	2.4
	21	5.7	14	5.7	17	8.6	23	5.1	3.2	7.3	3.8	4.3	2.8
	28	6.8	22	3.7	16	3.6	17	5.0	3.8	8.3	5.9	5.8	4.1
	35	6.6	30	3.9	21	3.8	18	5.0	3.9	2.1	1.7	2.3	4.1
	42	3.1	22	2.1	16	3.7	20	2.3	2.7	3.6	3.8	2.3	2.1
	49	3.5	29	—	—	2.0	20	1.9	2.7	2.1	2.7	2.6	3.7
	56	0.9	12	1.7	14	1.5	16	0.8	1.9	1.5	2.2	2.9	4.6
Liver	7	16	41	17	41	34	55	22	7.0	14	5.5	22	5.6
	14	13	33	11	46	13	67	8.4	5.1	17	13	16	9.0
	21	6.7	42	7.4	41	8.8	53	7.9	7.4	11	9.5	5.3	5.2
	28	8.0	43	4.5	31	6.4	59	7.3	7.5	8.2	7.8	14	12
	35	8.7	67	6.6	54	6.1	48	5.9	6.3	2.1	1.8	4.3	9.4
	42	3.7	55	3.7	43	5.8	51	2.4	3.8	5.4	7.3	4.4	5.0
	49	4.9	55	—	—	3.8	45	3.5	6.7	5.2	8.8	3.9	7.1
	56	1.5	22	2.7	23	2.5	34	0.9	2.6	3.4	5.8	4.6	7.4
Lung	7	25	21	17	12	20	10	20	2.8	28	4.8	34	4.1
	14	11	15	9.5	19	17	39	18	7.3	13	7.2	20	5.0
	21	10	28	14	43	14	49	12	7.3	28	14	11	8.0
	28	11	50	8.6	44	4.5	25	9.3	9.4	15	13	18	13
	35	6.8	40	7.6	47	11	55	12	11	5.7	5.3	6.7	13
	42	4.1	42	6.0	55	9.3	64	5.3	7.1	6.9	8.7	4.7	5.5
	49	5.7	59	—	—	2.9	35	3.4	5.3	4.8	7.3	5.9	10
	56	2.9	44	4.2	44	4.1	53	1.1	3.5	4.0	7.0	8.3	14
Biceps femoris	7	12	3.9	8.9	2.3	12	2.6	9.9	0.5	10	0.6	10	0.4
	14	10	4.5	8.2	4.3	10	5.2	7.2	1.1	7.1	1.2	7.8	1.2
	21	7.3	5.8	7.9	6.3	6.5	4.7	6.8	1.5	6.8	1.6	5.3	1.2
	28	—	—	6.3	7.2	4.2	7.4	5.6	1.5	6.4	2.1	6.5	1.7
	35	8.2	14	5.6	11	5.2	8.0	5.9	2.0	2.5	1.0	2.2	2.0
	42	9.1	17	3.4	9.2	6.2	12	2.8	1.4	4.3	2.1	1.9	1.0
	49	4.2	13	—	—	2.8	9.5	1.7	1.2	2.4	1.4	1.8	1.3
	56	2.3	9.4	2.6	8.7	2.6	12	0.7	0.8	1.5	1.0	2.8	2.4
Plasma	7	2.2	5.1	1.7	4.0	4.8	8.5	2.9	0.7	4.2	1.4	4.3	1.0
	14	1.5	4.1	1.4	3.8	1.4	6.2	1.2	1.1	1.9	2.0	—	—
	21	1.0	7.1	1.5	7.0	1.5	6.9	2.2	1.8	4.3	2.3	1.5	1.3
	28	1.2	7.6	0.5	3.7	0.4	3.9	1.3	3.7	1.5	1.4	1.4	1.1
	35	1.4	10	0.7	4.9	0.9	6.7	1.9	1.7	1.2	0.7	1.5	2.1
	42	0.6	8.6	0.5	5.6	0.7	5.4	1.5	1.8	2.5	1.9	1.0	0.9
	49	0.9	9.7	—	—	0.4	4.9	1.7	1.2	0.8	1.5	0.6	1.1
	56	0.4	3.4	0.6	4.9	0.3	4.6	0.4	1.2	0.5	0.9	0.6	1.2

(Continued next page)

TABLE 1 *(Continued)*

Tissue	Day	HEHC		HENC		HELC		LEHC		LENC		LELC	
		d_0	d_6	d_0	d_6	d_0	d_6	d_0	d_6	d_0	d_6	d_0	d_6
Red cells	7	2.5	5.5	2.1	4.1	3.3	4.8	3.1	1.2	3.9	1.2	4.1	0.9
	14	1.4	3.3	0.3	1.2	1.0	4.3	1.2	1.1	1.3	1.2	—	—
	21	1.1	5.0	2.7	6.0	1.1	6.2	2.5	1.5	1.2	0.6	1.9	1.1
	28	1.2	7.5	0.7	4.8	0.5	4.0	1.8	1.4	1.7	1.5	1.8	1.6
	35	1.1	7.6	0.8	4.3	0.8	6.1	2.3	1.2	1.0	0.5	1.4	1.9
	42	0.5	5.8	0.4	3.9	0.6	4.6	1.3	1.2	1.6	1.3	1.2	0.8
	49	0.6	6.5	—	—	0.5	5.3	0.7	1.2	0.8	1.1	0.8	1.4
	56	0.0	4.8	1.1	4.7	0.0	6.7	0.2	0.7	0.6	1.0	0.8	1.3
Spleen	7	17	38	14	28	22	32	15	6.9	17	6.4	17	4.5
	14	—	—	8.8	40	7.7	40	6.3	4.7	11	8.7	12	7.4
	21	12	45	7.3	44	7.2	46	10	8.5	11	8.2	6.3	6.1
	28	6.9	56	4.3	39	3.3	39	6.3	6.9	11	11	11	10
	35	6.7	56	5.0	40	4.7	38	6.6	7.0	4.4	3.6	3.4	7.6
	42	3.1	47	2.5	34	4.9	43	2.1	3.6	4.6	7.0	3.4	4.2
	49	4.8	56	—	—	3.1	41	3.3	6.2	3.9	7.2	3.5	6.7
	56	1.7	26	2.4	24	2.2	32	0.9	2.8	2.8	5.0	5.5	9.2
Testis	7	14	7.7	15	6.0	17	5.0	14	1.2	12	1.4	16	1.3
	14	14	11	5	5.5	9.5	9.2	13	5.1	15	4.9	13	2.6
	21	4.9	9.2	10	16	31	42	7.1	3.3	5.9	2.9	6.4	2.8
	28	11	29	6.2	16	5.6	15	4.7	2.7	6.6	3.7	6.8	3.0
	35	6.4	18	3.6	11	4.8	13	4.2	2.5	2.5	1.5	2.5	4.0
	42	4.0	22	3.7	18	3.1	18	3.3	2.8	3.5	2.9	2.6	1.9
	49	3.6	24	—	—	2.1	15	2.3	2.7	2.5	2.8	2.2	2.4
	56	3.2	16	2.4	13	2.3	16	0.6	1.2	1.4	1.6	2.5	3.5

[a]Concentrations are in nmol/ml of plasma or packed red cells or nmol/g of tissue. HE and LE, 36 and 5 mg α-TOAc/kg diet; HC, NC and LC, 5,000, 250 and 50 mg ascorbic acid/kg diet, respectively.

should exceed its rate of replacement by "new" vitamin E. Under such dietary conditions, any *in vivo* "sparing" action of vitamin C on vitamin E should become obvious as a fairly dramatic difference in the rates of loss of "old" vitamin E (and, indeed, of total vitamin E) between the LEHC, LENC, and LELC subgroups of animals, with this rate being least for the LEHC subgroup and greatest for the LELC subgroup. Thus, the LEHC, LENC, and LELC subgroups should provide an even more sensitive probe for any *in vivo* vitamin C/vitamin E interaction than would the three HE subgroups.

We have recently analyzed the biokinetics of vitamin E in rats, guinea pigs and man under "steady-state" conditions (K. U. Ingold, G. W. Burton, and W. Siebrand, 1990, unpublished results), i.e., under conditions where the total concentration of "new" (deuterium-labelled) and "old" (unlabelled) vitamin E in a tissue remained essentially constant during the experiment. In Figure 2, we show the total concentration of *RRR*-α-TOH (i.e., d_0 + d_6) for plasma and eight tissues from the HEHC, HENC, and HELC subgroups of guinea pigs at each time point. Not surprisingly, the data show considerable scatter because these comparisons of the total tocopherol concentration in a tissue at any particular time are based on samples obtained from three different animals. Furthermore, "identical" guinea pigs unfortunately show more individual variations in vitamin E levels than do "identical" rats in similar experiments. Nevertheless, despite the scatter two things are immediately obvious: First, in each of the subgroups the total concentration of vitamin E in a given tissue remained approximately constant for the eight week experiment. Second, the total concentration of vitamin E (given as its eight week average in Table 2) does not differ significantly in any tissue between the HEHC, HENC, and HELC subgroups. Hence, it appears that any "sparing" or "regenerating" action by vitamin C on the rate at which vitamin E is consumed (by all metabolic routes) is negligible in comparison with the normal turn-over of vitamin E in guinea pigs under the conditions of the HE experiment.

This conclusion was somewhat unexpected in view of the extensive and conclusive evidence that vitamin C "spares" and/or "regenerates" vitamin E in a wide variety of *in vitro* model systems (9–37) and the extensive, though less conclusive, evidence for the same phenomenon *in vivo* (40,45–47,50,52,53,56,58). We have therefore confirmed this conclusion by reanalyzing the data in Table 1 to demonstrate that the rate of loss of "old" vitamin E from a particular tissue is not significantly different between the HEHC, HENC, and HELC subgroups of guinea pigs. Figure 3 shows plots for plasma and eight tissues of the ratio of "old" vitamin E/total vitamin E, i.e., d_0-*RRR*-α-TOH/[d_0- + d_6-*RRR*-α-TOH]. We use "old" vitamin E/total vitamin E ratios in order to minimize the effect of differences between individual animals and temporal fluctuations within an animal. Figure 3 provides convincing proof that the rate of loss of "old" vitamin E from a tissue is quite uninfluenced by the level of vitamin C in the diet of the HE group of guinea pigs. Indeed, plots of log[d_0-*RRR*-α-TOH] vs time, which were found to be approximately linear (vide infra), showed no statistically significant differences between the HEHC, HENC, and HELC slopes for each tissue.

Confirmation that increased levels of vitamin C in the

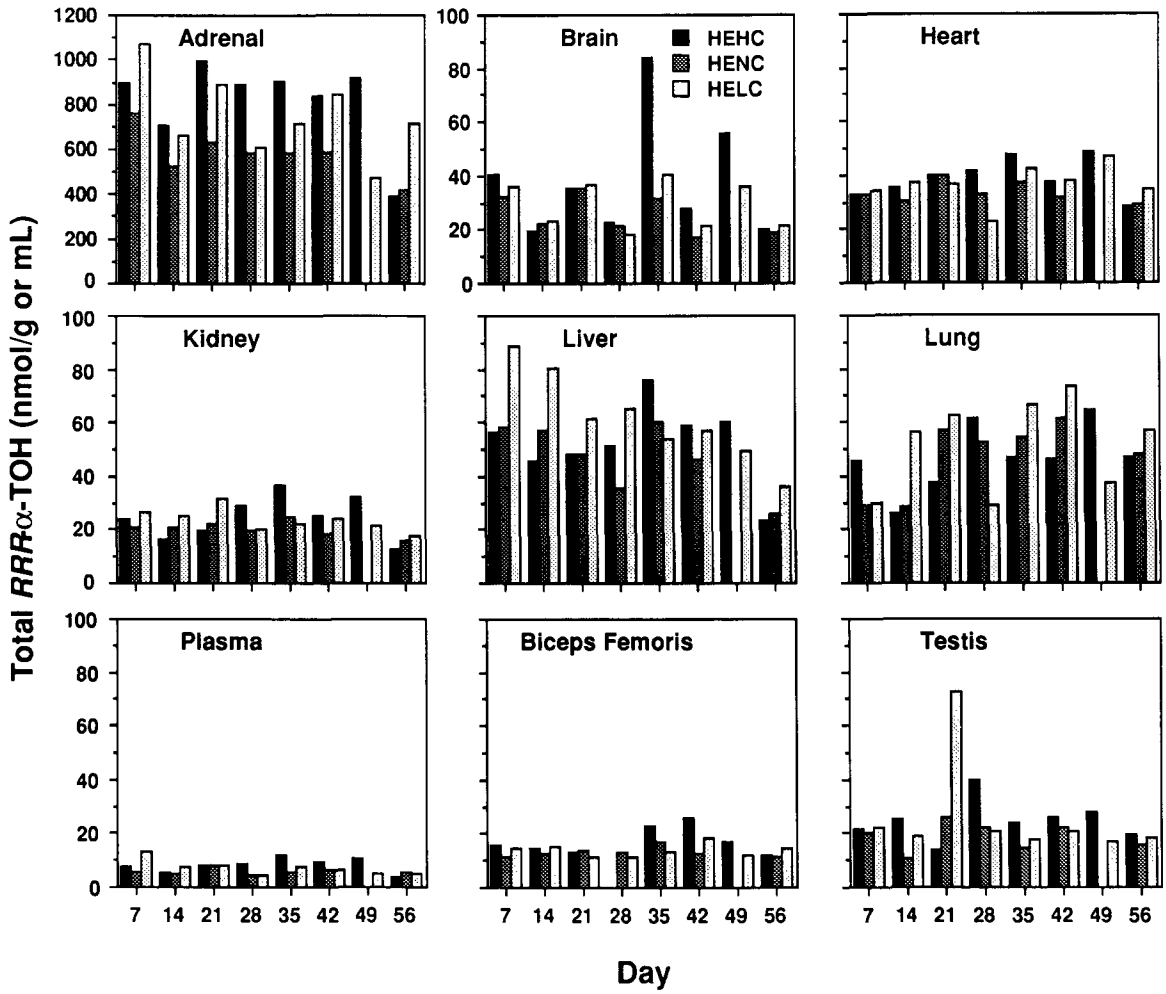

FIG. 2. Total (i.e., d_0- + d_6-) *RRR*-α-TOH concentrations for plasma and eight tissues from the HEHC, HENC, and HELC subgroups of guinea pigs have been plotted at weekly intervals. Note the different vertical scale for adrenal.

TABLE 2

Effect of Vitamin C on Total α-Tocopherol Concentrations in Blood and Tissues of Guinea Pigs[a]

	HEHC	HENC	HELC
Adrenal	816 ± 194	584 ± 103	746 ± 185
Brain	38 ± 22	26 ± 7	29 ± 9
Heart	39 ± 7	33 ± 4	37 ± 7
Kidney	25 ± 8	20 ± 3	24 ± 4
Liver	53 ± 15	47 ± 13	62 ± 17
Lung	47 ± 12	47 ± 13	51 ± 17
Biceps femoris	17 ± 5	13 ± 2	14 ± 2
Plasma	8 ± 3	6 ± 1	7 ± 3
Red cells	7 ± 2	5 ± 2	6 ± 1
Spleen	47 ± 22	42 ± 8	46 ± 7
Testis	25 ± 7	19 ± 5	26 ± 19

[a]Guinea pigs were maintained on high vitamin E (HE) diets (36 mg acetate/kg diet) containing vitamin C at high (HEHC, 5000 mg/kg), normal (HENC, 250 mg/kg) or low (HELC, 50 mg/kg) levels. Concentrations (nmol/g or ml), are the mean and standard deviation of values obtained from eight animals killed at the rate of one per week over an eight week period. The only statistically significant difference found was between adrenal HEHC and HENC ($p < 0.05$).

diet did, indeed, lead to increased levels in the animals was obtained by measuring, using an HPLC method (69), the ascorbate levels in extracts of homogenized samples of a selection of frozen tissues from animals in the HE group. The concentrations of ascorbate in the livers of each of the LC, NC, and HC animals were found to be 42, 237, and 750 nmol/g, respectively, at two weeks, and 100, 179 and 788 nmol/g, respectively, at five weeks. Values obtained for lung in the LC and HC groups at two weeks were 47 and 199 nmol/g, respectively, and the corresponding values for testis were 156 and 749 nmol/g, respectively.

In the LE group of guinea pigs the total concentration of vitamin E decreased substantially over eight weeks in all tissues except the brain (Fig. 4). [The absence of a noticeable decline in the brain is due to the very slow loss of "old (and gain of "new") vitamin E by this organ (Table 1). The literature suggests that had we examined adipose tissue it also would have shown a very slow turnover of vitamin E (70).] The LE group certainly does not have a "steady-state" vitamin E status but is instead progressing towards deficiency or a very much lower steady-

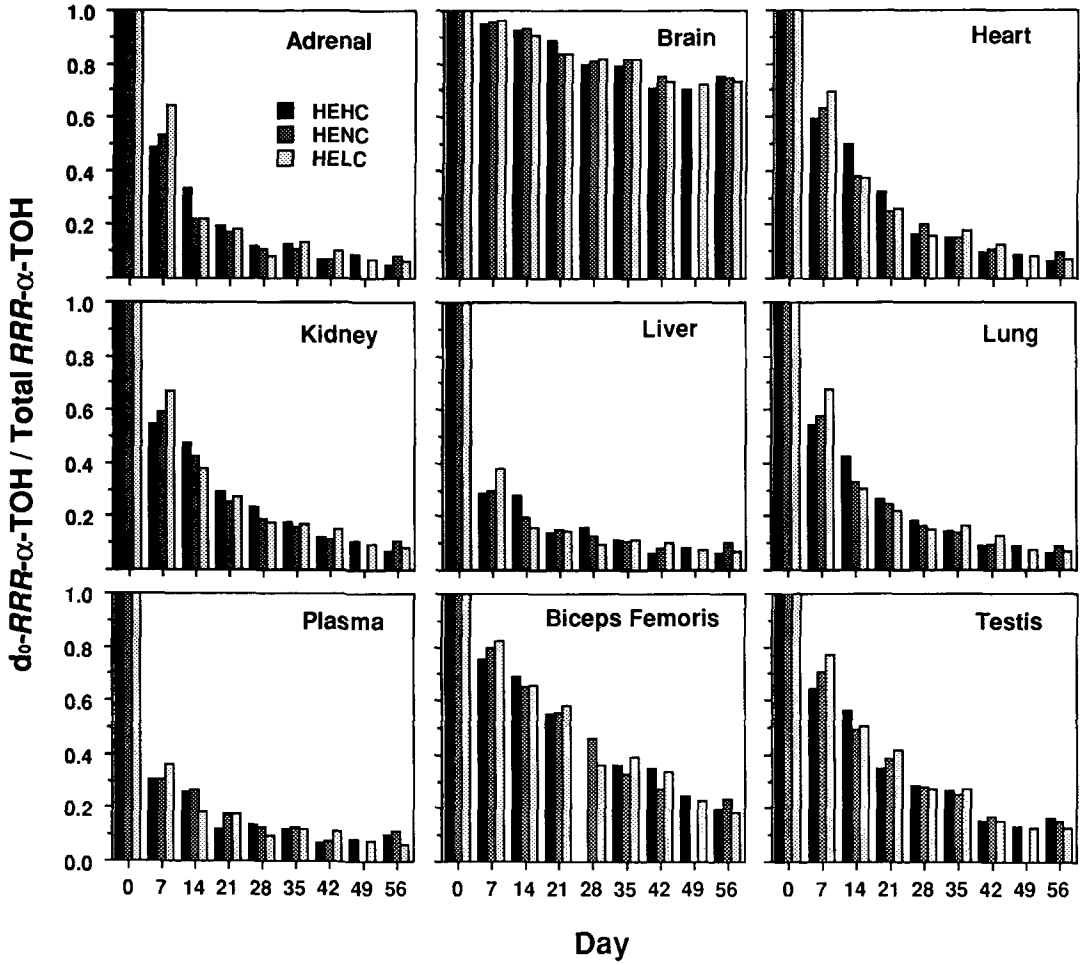

FIG. 3. "Old" d_0-*RRR*-α-TOH/total (d_0- + d_6-) *RRR*-α-TOH ratios for plasma and eight tissues from the HEHC, HENC, and HELC subgroups of guinea pigs have been plotted at weekly intervals.

state level of vitamin E, during the eight week experiment. Nevertheless, the data shown in Figure 4 demonstrate that there is no statistically significant difference in the total concentration of vitamin E in a particular tissue between the LEHC, LENC, and LELC subgroups of guinea pigs over the eight week course of this experiment. We conclude that any "sparing" or "regenerating" of vitamin E by vitamin C is negligible in comparison with those metabolic processes which consume vitamin E or eliminate it from the body, even in a guinea pig which is receiving insufficient vitamin E in its diet to maintain its body stores.

This conclusion was also unexpected. Therefore, in Figure 5 we present plots of the ratio of "old" vitamin E/total vitamin E for plasma and eight tissues. Again, differences between the slopes of plots of log[d_0-*RRR*-α-TOH] vs time for the LEHC, LENC, and LELC subgroups for each tissue were not statistically different. Obviously, the rate of loss of "old" vitamin E from a tissue was quite uninfluenced by the level of vitamin C in the diet of the LE group just as was the case for the HE group of guinea pigs.

In summary, we can find no evidence for an interaction between vitamin C and vitamin E *in vivo* despite a careful, sensitive, and sophisticated search using an appropriate animal model, i.e., an animal which does not synthesize ascorbic acid. We conclude that any synergistic (i.e., "sparing") or antagonistic interaction between these two vitamins *in vivo* in animals not subject to enhanced oxidative stress is negligible in comparison to other metabolic processes. Strictly speaking, this conclusion applies only to guinea pigs. However, we can see no reason why it should not also apply to other animals, including man.

To conclude this section we note that our present results demonstrate that even the most carefully modeled *in vitro* system may fail to reproduce the *in vivo* reality. In this case, the most careful models have involved α-tocopherol dissolved in dilinoleoylphosphatidylcholine (26) or soybean phosphatidylcholine (27) multilamellar liposomes dispersed in water containing ascorbic acid which was subjected to attack at 37°C by thermally-generated, water- or lipid-soluble peroxyl radicals (71). On addition of vitamin C, the water-soluble peroxyl radicals were efficiently trapped which prevented them from attacking the phospholipid bilayer and hence "spared" the vitamin E until all the vitamin C had been consumed (26,27). This result has been confirmed not only

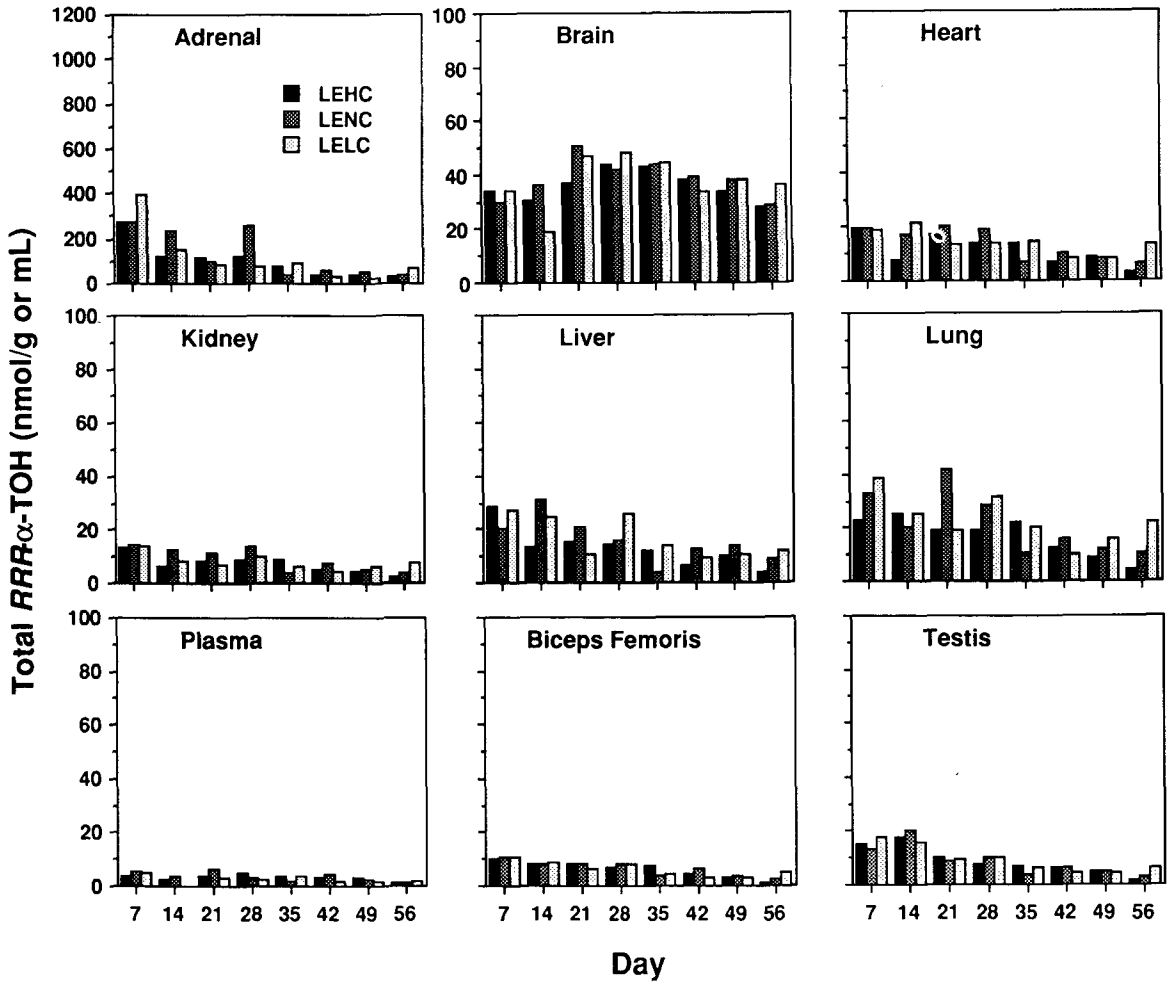

FIG. 4. Total (i.e., d_0- + d_6-) *RRR*-α-TOH concentrations for plasma and eight tissues from the LEHC, LENC and LELC subgroups of guinea pigs have been plotted at weekly intervals. Note that the vertical scales in this figure (including that for adrenal) have been made the same as those in Figure 2 for comparative purposes.

in analogous liposomal and micellar model systems (24,25,28,33) but also in plasma when the plasma was subjected to attack by water-soluble peroxyl radicals (69,72, 73). However, vitamin C is not unique in such systems since other water-soluble, radical-trapping antioxidants (e.g., plasma proteins, glutathione, cysteine, urate, bilirubin, etc.)—both in plasma (69,72,73) and in liposomal model systems (74–77)—also "spare" vitamin E from attack by water-soluble peroxyl radicals. More interesting are the liposomal systems in which lipid-soluble peroxyl radicals are generated from a lipid-soluble initiator (71). In such systems ascorbate by itself was ineffective at protecting the phospholipid from peroxidation (26,27,78). The same is true for most other potentially available water-soluble physiological antioxidants [except for conjugated bilirubin and biliverdin (78)] both by themselves and [except for cysteine (77)] in the presence of vitamin E (74,76,78). Ascorbate is virtually unique in that it regained its antioxidant capabilities in the presence of vitamin E (26,27). That is, when oxidation is initiated in the lipid phase, vitamin C is the only water-soluble antioxidant which becomes active when vitamin E is present. Presumably this "sparing" by vitamin C of vitamin E occurs via the "regenerating" reaction [2], as was suggested over 40 years ago by Golumbic (79).

The question as to why water- and lipid-soluble peroxyl radicals in the presence of phospholipid bilayers and water-soluble peroxyl radicals in plasma do not reflect the situation in guinea pigs with steady or declining vitamin E status is intriguing. The simplest answer (Occam's answer) would be that under normal conditions the flux of peroxyl radicals which enters the lipids of a healthy animal is not nearly as high as has frequently been supposed. This answer receives support from Tappel's finding that expired pentane levels from animals are extremely low, even for animals that are receiving inadequate or no vitamin E, relative to the levels reached for animals that are oxidatively stressed in various ways (41,44,54,80) (A. L. Tappel, 1989, private communication). This would mean that for oxidatively unstressed animals, only a very small fraction of the available vitamin E is actually destroyed by trapping the peroxyl radicals. A much more interesting possibility is that the tocopheroxyl radical is formed extensively even in a healthy animal, but is reduced *in vivo* not by ascorbate but by some other, possibly enzymic, process. There is, in fact, considerable

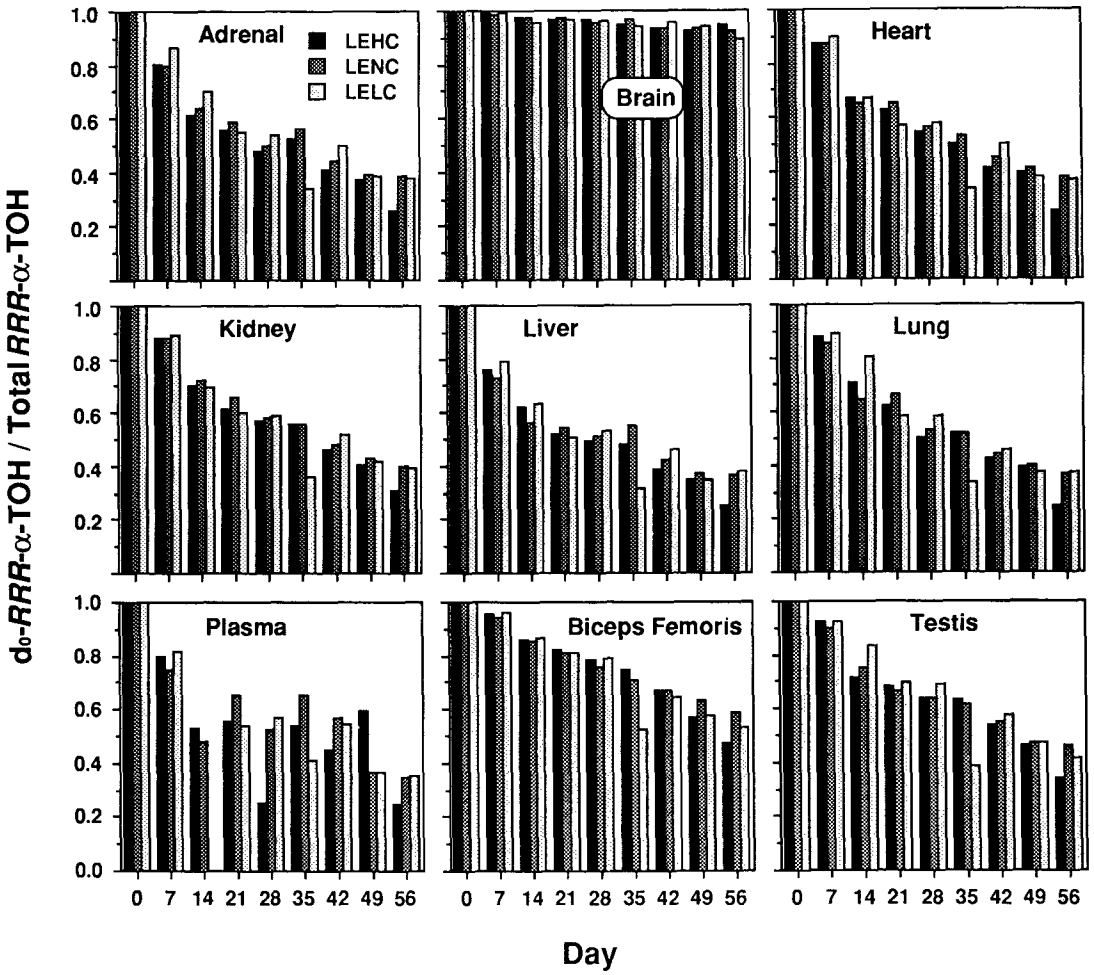

FIG. 5. "Old" d_0-*RRR*-α-TOH/total (d_0- + d_6-) *RRR*-α-TOH ratios for plasma and eight tissues from the LEHC, LENC, and LELC subgroups of guinea pigs have been plotted at weekly intervals.

evidence that rat liver microsomes and other organelles and tissues contain a membrane-bound, heat-labile, glutathione-dependent, free-radical reductase which probably acts by converting the tocopheroxyl radical to tocopherol (38,81-90) and which therefore participates in the *in vivo* protective system against lipid peroxidation. Free radical reductase activity in microsomes may also be NADPH dependent (91). A quantitative and unequivocal determination of the peroxyl radical flux in the lipids of healthy animals would add enormously to our understanding of free radical biology.

Biokinetics of vitamin E in guinea pigs. Comparison with rats. There are no significant differences in the biokinetics of vitamin E between the HEHC, HENC, and HELC subgroups of animals and between the LEHC, LENC, and LELC subgroups. This allows us to combine the biokinetic data for all the HE and all the LE guinea pigs, which simplifies biokinetic comparisons between these groups. Moreover, the HE biokinetic data can be compared with the analogous data for the same tissues obtained from the HE rats (1).

Under "steady-state" conditions of vitamin E the tissues of an animal can be divided broadly into two kinetic groups (K. U. Ingold, G. W. Burton, and W. Siebrand, 1990, unpublished results). The first group, which includes brain, heart, muscle, and testes, shows slow, first order (i.e., exponential) loss of "old" and gain of "new" vitamin E. The second group, which includes plasma, liver, lung, adrenal gland, and kidneys, shows non-exponential behavior with an initial, rapid change in vitamin E concentration compared with later stages. Formally, the behavior of the fast tissues can be represented as the sum of two (or more) first-order processes (K. U. Ingold, G. W. Burton, and W. Siebrand, 1990, unpublished results).

Since this detailed kinetic analysis of the HE ("steady-state") group of guinea pigs will be reported elsewhere (K. U. Ingold, G. W. Burton, and W. Siebrand, 1990, unpublished results), though without the supporting raw data which is given in Table 1, we will not repeat it here. For present purposes it is sufficient to give the time required under "steady-state" conditions for the concentration of deuterium-labelled, "new" vitamin E in a tissue to become equal to the concentration of unlabelled, "old" vitamin E. These equalization times, $t_{1:1}$, give a simple measure of the speed with which vitamin E turns over in different tissues under "steady-state" conditions. Equalization times can be estimated by inspection of the

TABLE 3

Comparison of Tissue Equalization Times ($t_{1:1}$; days) in Young Guinea Pigs and Rats[a]

Tissue	Guinea pig	Rat
Plasma	3.7	6.2
Liver	3.0	6.9
Lung	9.9	8.8
Kidney	9.8	13
Heart	14	18
Testis	17	40
Biceps femoris	24	23
Brain	107	40

[a]Values for all tissues, except guinea pig brain, were obtained by interpolation of concentration data plotted in single- or multiexponential form vs time. Because of a very slow rate of turnover, the value for guinea pig brain was estimated by extrapolation.

raw data but can be determined more reliably from exponential (slow tissues) or multiexponential (fast tissues) plots (K. U. Ingold, G. W. Burton, and W. Siebrand, 1990, unpublished results). Values of $t_{1:1}$ for the HE guinea pigs' tissues, obtained in this way after combining the HEHC, HENC, and HELC data, are given in Table 3. For comparison this table also includes the $t_{1:1}$ values for the same classes of tissue obtained from HE rats, i.e., from rats fed a diet containing 36 mg d_3-*RRR*-α-TOAc/kg diet and having essentially constant concentrations of vitamin E in their tissues. Some of the rat $t_{1:1}$ values differ from those previously reported (1) because of our current application of a more sophisticated kinetic treatment (K. U. Ingold, G. W. Burton, and W. Siebrand, 1990, unpublished results).

Inspection of Table 3 reveals that all but one of the fast (multiexponential kinetics) tissues from guinea pigs exchange vitamin E more rapidly than the corresponding rat tissues, whereas the slow (monoexponential kinetics) tissues from the guinea pig may exchange vitamin E more, or less, rapidly than the corresponding rat tissues. The most striking difference in the tissue biokinetics of vitamin E between guinea pigs and rats lies in the brain [and probably in adipose tissue (cf., 1,70)]; under "steady-state" conditions the transport of vitamin E into or out of the guinea pigs' brain occurs at only ca. 40% of the rate for the rat. It would be extremely interesting to have analogous data for humans.

There appears to be only one earlier "comparative" study of vitamin E uptake by rats and guinea pigs and only blood, adrenals, heart, and liver were examined (92). On diets containing 30 mg *all-racemic*-α-TOAc per kg of diet the rats after 46 weeks had from 1.4 to 2.0 times as much α-TOH (per ml or per g tissue) as did the guinea pigs in the same tissues after 32 weeks (92). The relevance of this observation to our own measurements is not obvious.

Equalization times for the LE group of guinea pigs have not been calculated because the vitamin E status of these animals is not at a "steady-state," but declines in all tissues except the brain (Table 1 and Fig. 4). This fact is especially evident in plots of the logarithm of total tissue vitamin E vs time (Fig. 6).

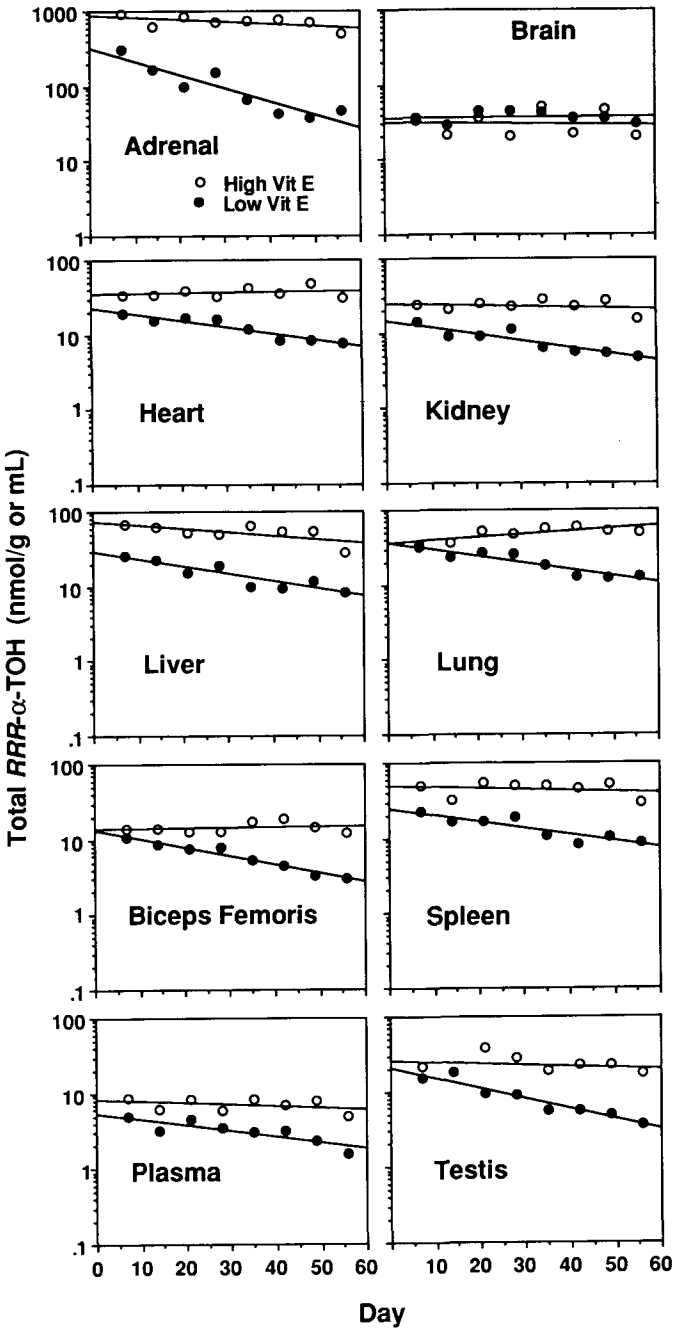

FIG. 6. Semi-logarithmic plots of total (d_0- + d_6-) *RRR*-α-TOH concentrations vs time for plasma and nine tissues from the HE (○) and LE (●) groups of guinea pigs. The individual points which are displayed are the averaged values found for the HC, NC and LC subroups of animals. However each line is the least squares fit to all of the HC, NC and LC data points. Note the different vertical scale for adrenal and brain.

Interestingly, the rates of loss of "old" vitamin E from the tissues of the LE animals were not different from the corresponding rates of the HE animals. This is shown in Figure 7 in which we have plotted log[d_0-*RRR*-α-TOH] vs time for both fast and slow tissues. Although, under "steady-state" conditions, the biokinetics for the fast tissues can be somewhat better described in terms of a

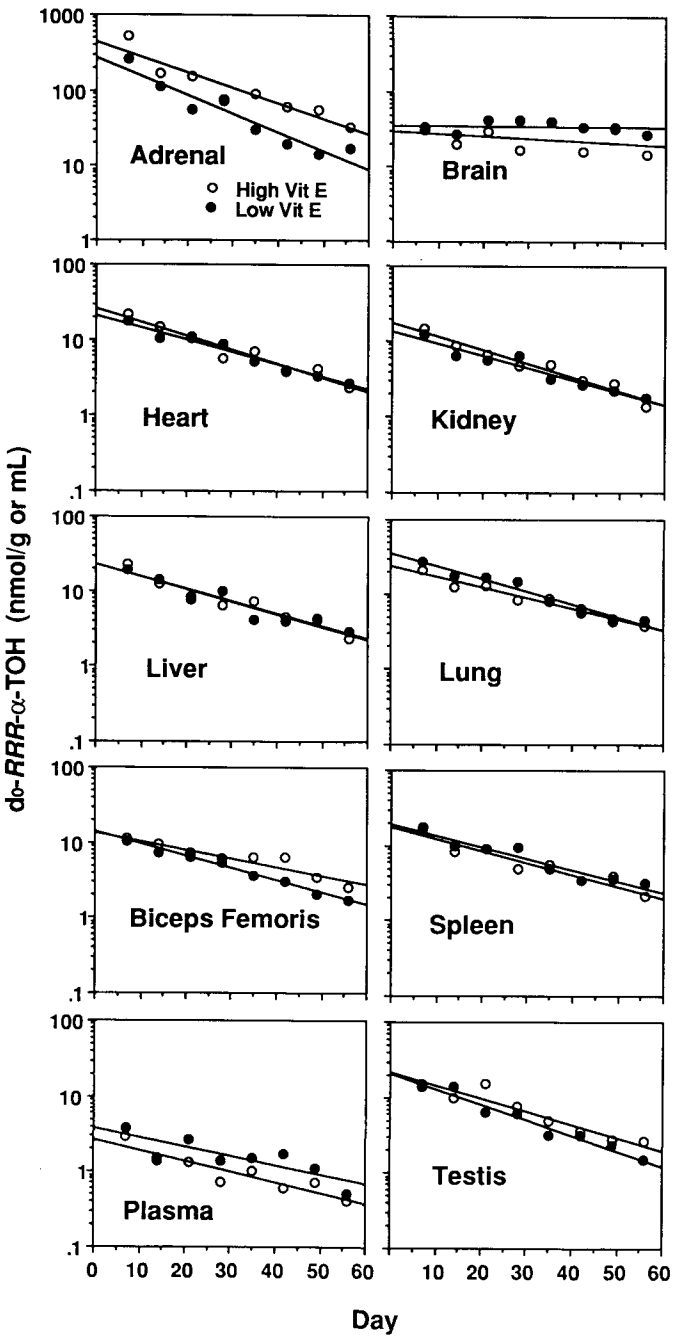

FIG. 7. Semi-logarithmic plots of "old" d_0-*RRR*-α-TOH concentrations vs time for plasma and nine tissues from the HE (○) and LE (●) groups of guinea pigs. The individual points which are displayed are the averaged values found for the HC, NC and LC subgroups of animals. However each line is the least squares fit to all of the HC, NC and LC data points. Note the different vertical scale for adrenal and brain.

multiexponential (K. U. Ingold, G. W. Burton, and W. Siebrand, 1990, unpublished results), it is reasonable to use a single exponential because the data in Figure 7 do not include the initial rapid decline that occurs in the first few days.

It is not unreasonable that "old" vitamin E should be lost from a specific tissue of the LE and HE groups of animals at the same rate, since different rates would imply the existence of some physiological "feed-back" mechanism which could "sense" the vitamin E status of the animal. We hypothesize that such a physiological "feed-back" process may be present in certain "critical" tissues which have very slow rates of uptake and loss of vitamin E. The brain would appear to be a prime candidate for possession of a control mechanism which would ensure that the rate at which it lost vitamin E would depend on the animal's overall vitamin E status, i.e., the rate of loss would be lower when the animal was put on a diet containing an inadequate (or no) vitamin E compared with an animal on a normal diet. Unfortunately, our eight-week-long study with the guinea pigs was of too short a duration for any such effect (or lack of such effect) to demonstrate itself. We therefore plan to carry out further experiments to determine whether certain critical tissues do or do not possess a "feed-back" mechanism.

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Chain Elongation of Polyunsaturated Fatty Acids by Vascular Endothelial Cells: Studies with Arachidonate Analogues

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This study has utilized radiolabeled analogues of arachidonic acid to study the substrate specificity of elongation of long-chain polyunsaturated fatty acids. Human umbilical vein endothelial cells were incubated for 2–72 hr in medium supplemented with 0.9–2.6 μM [^{14}C]fatty acid, and cellular glycerolipids were analyzed by gas-liquid chromatography with radioactivity detection. Elongation of naturally occurring C_{20} polyunsaturated fatty acids occurred with eicosapentaenoate (20:5(n-3)) > Mead acid (20:3(n-9)) > arachidonate (20:4(n-6)). Chain length markedly influenced the extent of elongation of 5,8,11,14-tetraenoates (18:4 > 19:4 > 20:4 > 21:4); effects of initial double bond position were also observed (6,9,12,15-20:4 > 4,7,10,13-20:4. Neither 5,8,14- nor 5,11,14-20:3 was elongated to the extent of 5,8,11-20:3. Differences between polyunsaturated fatty acids were observed both in the initial rates and in the maximal percentages of elongation, suggesting that the content of cellular C_{20} and C_{22} fatty acids may represent a balance between chain elongation and retroconversion.

Umbilical vein endothelial cells do not exhibit significant desaturation of either 22:4(n-6) or 22:5(n-3). By contrast, incubation with 5,8,11,14-[^{14}C]18:4(n-4) resulted in formation of both [^{14}C]20:5(n-4) and [^{14}C]22:5(n-4). The respective time courses for the appearances of [^{14}C]22:5-(n-4) and [^{14}C]20:5(n-5) suggests Δ6 desaturation of [^{14}C]-22:4(n-4) rather than Δ4 desaturation of [^{14}C]20:4(n-4).
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The phospholipids of mammalian membranes usually contain C_{22} polyunsaturated fatty acids including docosatetraenoate (22:4(n-6)), docosapentaenoates (both 22:5(n-6) and 22:5(n-3)) and docosahexaenoate (22:6(n-3)) (1–3). Brain and retina are particularly enriched with docosahexaenoate (22:6(n-3)) (4,5); development of the nervous system, optimal synaptic function and photoreceptor membrane biogenesis are among the processes requiring 22:6 (6). Although the C_{20} fatty acids—arachidonic acid (20:4(n-6)) and eicosapentaenoic acid (20:5(n-3))—serve as the major precursors for eicosanoid synthesis, both 22:4(n-6) and 22:6(n-3) have also been shown to be substrates for cyclooxygenation and lipoxygenation in several tissues (7–10). C_{22} Polyunsaturated fatty acids are also competitive inhibitors of cyclooxygenase and thus modulators of prostaglandin synthesis (11). The presence of C_{22} polyunsaturated fatty acids in membrane phospholipids may also be necessary for function of some membrane enzymes (12,13).

Synthesis of C_{22} polyunsaturated fatty acids from their corresponding C_{20} fatty acids has been demonstrated in many cell types including fibroblasts (14), endothelial cells (15,16), smooth muscle cells (17), and neuroblastoma (18). Elongation of eicosapentaenoate is usually much greater than that of arachidonate. High concentrations of arachidonate appear to promote increased synthesis of 22:4(n-6) (14). The reverse pathway, chain shortening or retroconversion of 22:4(n-6) to arachidonate has been demonstrated in endothelial (19) and smooth muscle cells (17); endothelial cells also retroconvert 22:6(n-3) to eicosapentaenoate (20).

This present study has sought to investigate the substrate specificity of the chain elongation of C_{20} polyunsaturated fatty acids in vascular endothelial cells. In this communication we present the results of experiments using nonphysiological arachidonic acid analogues differing in chain length, position of the double bond and degree of unsaturation.

MATERIALS AND METHODS

Materials. [1-^{14}C]Arachidonic acid (57 mCi/mmol) and [1-^{14}C]eicosapentaenoic acid (56–59 mCi/mmol) were obtained from New England Nuclear (Boston, MA). All other labeled fatty acids were prepared by organic synthesis (21,22). Specific activities were 50–60 mCi/mmol except for 4,7,10,13-[1-^{14}C]20:4(n-7) (26 mCi/mmol), 5,8,11,14-[1-^{14}C]18:4(n-4) (19 mCi/mmol) and 5,8,11-[1-^{14}C]20:3(n-9) (33 mCi/mmol). Prior to these experiments, each [^{14}C]fatty acid was purified by high performance liquid chromatography; single peaks were obtained upon subsequent analysis by both thin layer and gas-liquid chromatography. [^{14}C]Fatty acids were stored in methanol or ethanol under a nitrogen atmosphere at −20°C.

Culture medium 199, Hepes buffer and trypsin were obtained from GIBCO (Grand Island, NY). Fetal bovine serum was obtained from Hyclone Laboratories (Logan, UT), Endothelial Cell Growth Supplement (ECGS) from Collaborative Research (Waltham, MA), and gelatin from J.T. Baker (Phillipsburg, NJ). Bovine serum albumin, fraction V (fatty acid-free) was obtained from ICN InmunoBiologicals (Lisle, IL). Type 1A collagenase, heparin (sodium salt), and lipid standards were purchased from Sigma (St. Louis, MO).

Cell culture. Human endothelial cells obtained from umbilical veins by collagenase digestion were seeded in gelatin-coated 25-cm^2 flasks (23). Culture medium 199 was supplemented with 10 mM Hepes buffer, 10% fetal bovine serum, 30 μg/ml Endothelial Cell Growth Supplement, and 90 μg/ml heparin. The cells from primary cultures, confluent within 5–8 days, were removed from the culture surface with trypsin and passaged with a 1:3 split. All experiments used replicate cultures of second passage endothelial cells (24).

[^{14}C]Fatty acid incorporation. Aliquots of the [^{14}C]-fatty acid solutions were gently evaporated to dryness under nitrogen and redissolved in 95% ethanol. These solutions were added to fetal bovine serum and then diluted into the culture medium. Flasks (25 cm^2) of

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Abbreviations: ECGS, Endothelial Cell Growth Supplement; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

endothelial cells were labeled by incubation at 37°C for up to 72 hr with 0.5 μCi [^{14}C]fatty acid in 4 ml of complete culture medium (24). Final concentrations of [^{14}C]fatty acids were 2.3–6.6 μM; the final concentration of ethanol was <0.08%.

Lipid extraction and analysis. Cells were harvested by trypsinization and cellular lipids were extracted with ethyl acetate and acetone (24). [^{14}C]Fatty acid methyl esters were prepared from cellular phospholipids using methanolic base and separated by gas-liquid chromatography using 10% SP-2330 on 100/120 Chromasorb W AW (Supelco, Bellefonte, PA) (15,16). Radioactivity in the respective peaks was detected with an on-line Packard Gas Flow Proportional Counter. Peaks were identified on the basis of the retention times compared with known standards and the substrate [^{14}C]fatty acids.

RESULTS

Elongation of arachidonate, eicosapentaenoate, and 5,8,11-eicosatrienoate. Figure 1 shows the time dependence of the utilization of exogenous [^{14}C]arachidonate (20:4(n-6)), [^{14}C]eicosapentaenoate (20:5(n-3)), and [^{14}C]eicosatrienoate (20:3(n-9)) by human umbilical vein endothelial cells. Although total incorporation of [^{14}C]arachidonate and [^{14}C]eicosapentaenoate was quite similar (Fig. 1A), the extent of the metabolic modification of these two C_{20} polyunsaturated fatty acids was markedly different (Fig. 1B). Elongation of incorporated [^{14}C]eicosapentaenoate was 37% by 2 hr, and reached 70% by 24 hr. As in previous studies (16), Δ4 desaturation of the resultant 22:5(n-3) was not observed. By contrast, only 16% of the incorporated [^{14}C]arachidonate was elongated by 2 hr; after that time, no further increase in percentage of elongation was observed. It should be noted that, in these studies, the [^{14}C]arachidonate was diluted by arachidonate moieties in both fetal bovine serum and cellular glycerolipids. It is thus likely, however, that the total mass of arachidonate elongated exceeded that of eicosapentaenoate.

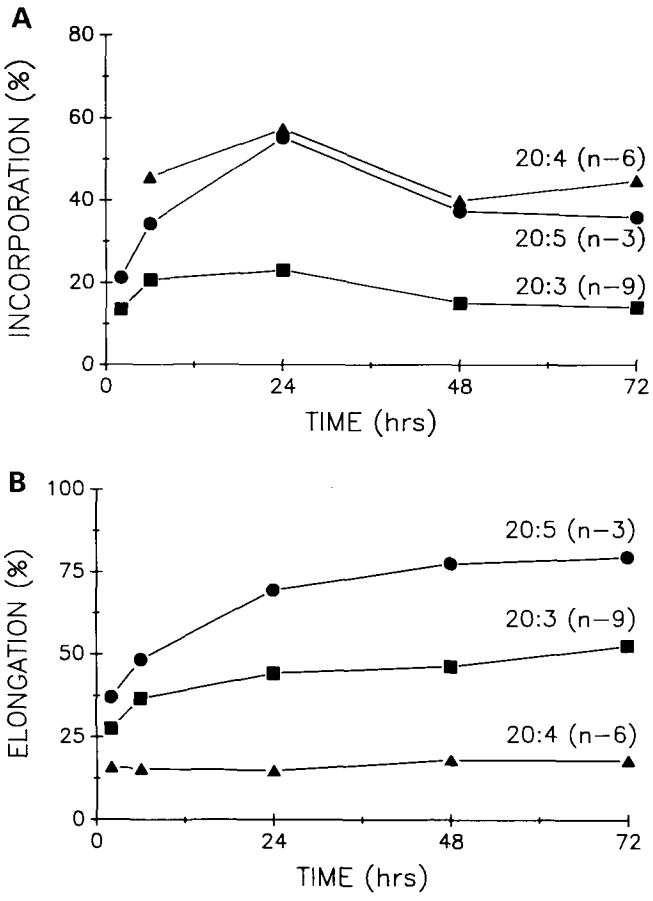

FIG. 1. Incorporation and elongation of [^{14}C]arachidonate, [^{14}C]-eicosapentaenoate and 5,8,11-[^{14}C]eicosatrienoate by human umbilical vein endothelial cells. Confluent endothelial monolayers were incubated for 24 hr in culture medium plus 10% fetal bovine serum supplemented with 0.5 μCi [^{14}C]fatty acid/4 ml. This provided 16 nmoles of [^{14}C]20:3(n-9) and 9.2 nmoles of [^{14}C]20:4(n-6) and [^{14}C]20:5(n-3). (A) Total incorporation into cellular glycerolipids. (B) Elongation of incorporated [^{14}C]fatty acid as a percentage of total incorporation. Each point is the mean of duplicate determinations on each of two flasks.

Incorporation of 5,8,11-[^{14}C]eicosatrienoate was less extensive than that of either arachidonate or eicosapentaenoate. There was, however, substantial elongation of the incorporated eicosatrienoate. At all time points, the extent of elongation of incorporated eicosatrienoate was intermediate between that of eicosapentaenoate and arachidonate.

Elongation of an isomeric series of eicosatetraenoic fatty acids. All the physiological C_{20} polyunsaturated fatty acids have an initial double bond at carbon 5. To determine the effects of double bond position on the extent of elongation, we used isomers of arachidonate which differed in double bond position. As seen in Table 1, both nonphysiological isomers were extensively incorporated; incorporation of 6,9,12,15-[^{14}C]eicosatetraenoate was similar to that of arachidonate. Furthermore both 4,7,10,14- and 6,9,12,15-[^{14}C]eicosatetraenoate were elongated to C_{22} fatty acids. As with [^{14}C]arachidonate, there was also a small quantity of radioactivity associated with 24:4. The total percentage of radiolabeled substrate which was converted into elongated products was 28% for 6,9,12,15-20:4, 23% for 5,8,11,14-20:4 and 18% for 4,7,10,13-20:4. Although these differences are far smaller than those between arachidonate and eicosapentaenoate, there does appear to be some specificity among isomeric eicosatetraenoates based on double bond position.

The influence of chain length on the elongation of 5,8,11,14-tetraenoic fatty acids. The next experiments utilized radiolabeled fatty acids with *cis* 5,8,11,14 bonds and 18–21 carbon atoms. There was an inverse correlation between chain length and extent of elongation (Table 2). By 24 hr, only 31% of the [^{14}C]18:4 remained unmodified as compared to 88% of the [^{14}C]21:4. Although the incorporation of [^{14}C]arachidonate was greater than that of the other 5,8,11,14-tetraenoic fatty acids, the extent of elongation of the incorporated [^{14}C]arachidonate was intermediate between that of [^{14}C]19:4 and [^{14}C]21:4. A greater variety of metabolites was produced from [^{14}C]18:4(n-4) than the longer tetraenoates; there was both significant further elongation of the [^{14}C]20:4(n-4) to [^{14}C]22:4(n-4), and desaturation of the elongation products to pentaenoic fatty acids.

Effect of double bond position on the elongation of eicosatrienoic fatty acids. As seen in Figure 1b, 5,8,11–20:3 which lacks the *cis* 14 double bond is elongated more

TABLE 1

Elongation and Desaturation of Isomeric Eicosatetraenoic Acids[a]

Substrate [^{14}C]fatty acid	Incorporation (nmol/flask)	Distribution of cellular [^{14}C]fatty acids (%)			
		Substrate	Products		
		20:4	22:4	22:5	24:4
6,9,12,15-20:4(n-5)	3.5	71.7 ± 0.2	26.0 ± 0.3	2.3 ± 0.4	—
5,8,11,14-20:4(n-6)	4.4	76.7 ± 0.7	21.4 ± 0.6	0.4	1.5 ± 0.3
4,7,10,13-20:4(n-7)	4.9	82.0 ± 0.9	16.3 ± 0.4	—	1.7 ± 0.2

[a]Confluent endothelial monolayers were incubated for 24 hr in culture medium plus 10% fetal bovine serum supplemented with 0.5 μCi [^{14}C]fatty acid/4 ml. This provided 20 nmoles of 4,7,10,13-[^{14}C]20:4 and 9.2 nmoles of 6,9,12,15-[^{14}C]20:4 and 5,8,11,14-[^{14}C]20:4. Values are means ± S.E., n = 3 from one of two similar experiments.

TABLE 2

Elongation and Desaturation of C_{18}-C_{21} Tetraenoic Fatty Acids[a]

Exogenous [^{14}C]fatty acid	Incorp. (nmoles/flask)	Distribution of cellular ^{14}C fatty acid (%)					
		Substrate	Elongation 1[b] tetraene	Elongation 1[b] pentaene	Elongation 2[c] tetraene	Elongation 2[c] pentaene	% Elong.[d]
5,8,11,14-18:4	7.8	31.3	33.8	9.6	17.4	8.0	68.8
5,8,11,14-19:4	2.8	50.2	47.4	—	2.2	—	49.6
5,8,11,14-20:4	4.4	76.7	21.4	—	1.5	—	22.9
5,8,11,14-21:4	2.2	88.1	11.9	—	—	—	11.9

[a]Confluent endothelial monolayers wre incubated for 24 hr in culture medium plus 10% fetal bovine serum supplemented with 0.5 μCi [^{14}C]fatty acid/4 ml. This provided 26.4 nmoles of 5,8,11,14-[^{14}C]18:4 and 9.2 nmoles of the other [^{14}C]tetraenoic fatty acids. Values are means (n = 3) from one of two similar experiments.

[b][^{14}C]Fatty acids which are two carbons longer than the [^{14}C]fatty acid substrate.

[c][^{14}C]Fatty acids which are four carbons longer than the [^{14}C]fatty acid substrate.

[d]Total products of elongation, including both tetraenoic and pentaenoic [^{14}C]fatty acids.

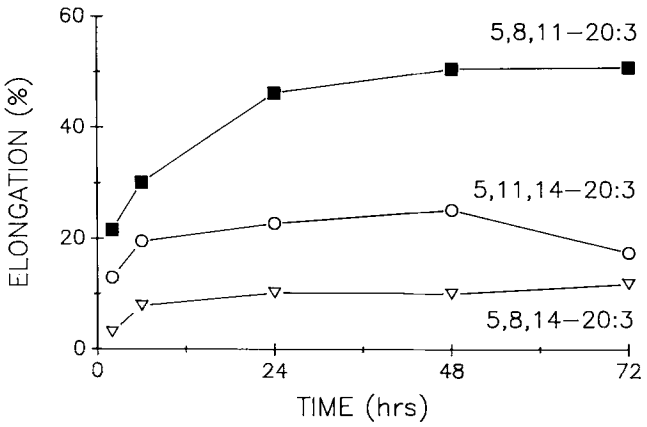

FIG. 2. Elongation of isomeric [^{14}C]eicosatrienoates by human endothelial cells. Confluent endothelial monolayers were incubated in medium with 0.5 μCi [^{14}C]fatty acid/4 ml as in Figure 1. This provided 16 nmoles of 5,8,11-[^{14}C]20:3 and 8.7 nmoles of 5,11,14-[^{14}C]20:3 and 5,8,14-[^{14}C]20:3. Each point is the mean of duplicate determinations on each of two flasks, and is expressed as a percentage of total cellular radioactivity.

extensively than arachidonate (5,8,11,14-20:4). The next experiments compared the elongation of the isomeric eicosatrienoates 5,11,14-20:3 and 5,8,14-20:3 with that of 5,8,11-20:3. These [^{14}C]eicosatrienoates were incorporated into cellular glycerolipids to a similar extent [data not shown; (24)]. As seen in Figure 2, elongation of both 5,11,14- and 5,8,14-20:3 was far less extensive than that of 5,8,11-20:3.

Elongation and desaturation of [1-^{14}C]octadecatetraenoic acid (5,8,11,14-18:4). Of all the polyunsaturated fatty acids used in these experiments, significant amounts of desaturated metabolites were observed only with [^{14}C]-18:4(n-4). The time course of conversion of incorporated [^{14}C]18:4 to elongated and desaturated products is shown in Table 3. The initial elongation to [^{14}C]20:4 was quite rapid, reaching nearly 40% in 2 hr. By contrast accumulation of desaturated products was quite slow until 48 hr. At 12 and 24 hr, the major desaturated product was [^{14}C]22:5. At later time points, however, there was more [^{14}C]20:5 than [^{14}C]22:5. Indeed, by 72 hr, the largest peak of radioactivity was associated with [^{14}C]20:5.

DISCUSSION

The present study has confirmed and extended previous observations which indicated marked specificity in the extent of elongation of long-chain polyunsaturated fatty acids by cells in culture. As in previous studies with both human skin fibroblasts (14) and vascular endothelial cells (16,25), the extent of elongation of incorporated eicosapentaenoate (20:5(n-3)) greatly exceeds that of arachidonate (20:4(n-6)), while 20:3(n-9) is elongated to an intermediate

TABLE 3

Endothelial Metabolism of 5,8,11,14-[^{14}C]Octadecatetraenoic Acid

Time (hr)[a]	Distribution of cellular [^{14}C]fatty acid (%)							
	5,8,11,14-18:4	7,10,13,16-20:4	4,7,10,13,16-20:5[c]	9,12,15,18-22:4	6,9,12,15,18-22:5[c]	24:4	% Elongation[d]	% Desaturation[e]
2	54.3[b]	39.1	0.5	6.1	—	—	45.7	0.5
6	44.0	46.7	trace	8.0	1.3	trace	56.0	1.3
12	33.4	50.6	1.7	11.2	3.1	trace	66.6	4.8
24	35.4	38.7	4.7	15.9	5.3	trace	64.6	10.0
48	22.1	31.0	24.7	11.8	8.4	2.0	77.9	33.1
72	12.4	29.7	38.0	10.7	9.1	—	87.5	47.1

[a] Confluent endothelial monolayers were incubated for the indicated times in culture medium plus 10% fetal bovine serum supplemented with 0.5 μCi [^{14}C]18:4(n-4)/4 ml as in Table 2.

[b] Values are Means (n = 3) from one of two similar experiments.

[c] Tentative assignment of double bonds, assuming additional desaturation results in typical methylene-interrupted pattern.

[d] Total products of elongation, including both tetraenoic and pentaenoic [^{14}C]fatty acids.

[e] Products of desaturation (20:5 + 22:5) as a percentage of total elongated (C_{20} + C_{22}) [^{14}C]fatty acids.

extent. The availability of radiolabeled analogues of arachidonic acid has permitted us to further elucidate the specificity of long-chain fatty acid elongation in intact cells. Recent studies indicate that endothelial cells readily utilize these nonphysiological fatty acids; they are rapidly incorporated, and, like arachidonate, esterified into the major classes of cellular phospholipids (24).

The results indicate that the extent of elongation is markedly dependent upon chain length: both 5,8,11,14-18:4 and 5,8,11,14-19:4 are extensively elongated while there is little elongation of 5,8,11,14-21:4. By contrast, the position of the double bonds has only a small effect, with elongation of 6,9,12,15-20:4 being greater than that of 4,7,10,13-20:4. The extent of elongation of incorporated [^{14}C]arachidonate (5,8,11,14-20:4) is consistent with both its chain length and the position of its double bonds.

In these experiments, the [^{14}C]arachidonate was diluted by arachidonate moieties both in fetal bovine serum and in cellular glycerolipids. Thus, although the percentage elongation of [^{14}C]arachidonate was similar to that of 4,7,10,13-[^{14}C]20:4 and 6,9,12,15-[^{14}C]20:4, the total mass of arachidonate elongated was substantially greater than that of its isomers. Studies with fibroblasts (14) have demonstrated that the percentage of a particular C_{20} polyunsaturated fatty acid which is elongated is relatively independent of its concentration. Although increasing the concentrations of exogenous [^{14}C]arachidonate or [^{14}C]-eicosapentaenoate from 0.5-40 μM substantially increased the nmoles of the respective [^{14}C]fatty acid elongated, the percentage of incorporated [^{14}C]eicosapentaenoate elongated remained 3-4 fold that of [^{14}C]arachidonate. The present study has not attempted to correct for dilution with nonlabeled fatty acids, but has instead focused on the relative extent of elongation of the various arachidonate analogues. It should be noted, however, that, for the nonphysiological fatty acids, radioactivity provides a direct measure of mass of each fatty acid in cellular glycerolipids.

Elongation of fatty acids occurs primarily in the microsomal fraction and involves an initial, rate-limiting condensation of the acyl-CoA with malonyl-CoA followed by a sequence of reduction reactions (26,27). Chain elongation appears to have less substrate specificity than desaturation, as demonstrated by elongation of a wide variety of polyunsaturated fatty acids (28). Studies using isomeric C_{18} polyunsaturated fatty acids have demonstrated that chain elongation is regulated primarily by the rate of the initial condensation step, and influenced by chain length as well as both the number and position of double bonds (1,29). C_{18} fatty acids were elongated more extensively than C_{20} fatty acids with the same double bond positions. For both octadecadienoates (18:2) and octadecatrienoates (18:3), the rates of elongation increased with the position of the initial double bond (*cis* 4 < *cis* 5 < *cis* 6) (29); these differences were, however, more pronounced than those observed in the present study. Although the present studies utilize whole cells rather than microsomes, the patterns of rates of elongation observed for tetraenoic fatty acids are similar to those for dienoic and trienoic fatty acids.

In endothelial cells, as in fibroblasts (14), differences in extent of elongation of incorporated polyunsaturated fatty acids are evident both in early time points (2-6 hr), and at the maximal levels observed after 24-48 hr. Since membrane lipids are continuously turned over and remodelled (30), it is unlikely that cessation of net elongation of fatty acids such as arachidonate is due to complete sequestration of fatty acids in inaccessible pools. Instead, the lack of further increase in percentage of elongation may represent a balance between rates of chain elongation and retroconversion.

Although studies with isolated liver cells indicate that retroconversion or chain shortening of both 22:4(n-6) and 22:6(n-3) involves peroxisomal β-oxidation (31), the process and its substrate specificity are not well characterized. In this context, it is interesting to examine the data on isomeric eicosatrienoates. The extent of net elongation of 5,8,11-20:3(n-9) is over two-fold greater than that of either 5,11,14-20:3(n-6) or 5,8,14-20:3(n-6). These differences could be due to higher retroconversion rates for n-6 fatty acids. Indeed, studies in progress in our laboratory (data not shown) suggest that retroconversion of [^{14}C]22:4(n-6) is substantially greater than that of

[^{14}C]22:5(n-3).

Despite the fact that there was at least some elongation of all of the [^{14}C]fatty acids utilized in the present study, only the elongation products of 5,8,11,14-[^{14}C]18:4 were desaturated. As in previous studies, essentially no Δ4 desaturation of 22:4(n-6) or 22:5(n-3) was observed (16, 32,33). It is thus possible that endothelial cells do have significantly higher Δ4 desaturase activity for 7,10,13,16-20:4 than for 7,10,13,16-22:4. Alternatively, the accumulation of both [^{14}C]20:5(n-4) and [^{14}C]22:5(n-4) in cells incubated with [^{14}C]18:4(n-4) may reflect Δ6 desaturation of 9,12,15,18-22:4 followed, in part, by retroconversion. The time course study would appear to support this latter alternative in that the initial desaturation product was primarily [^{14}C]22:5(n-4); with increased time, [^{14}C]20:5-(n-4) was the major metabolite produced. The findings that endothelial cells produce small amounts of 24:5(n-6) and 26:5(n-6) from [^{14}C]20:4(n-6) are also consistent with Δ6 desaturation of very long-chain polyunsaturated fatty acids (e.g. 9,12,15,18-24:4(n-6)) (15).

In summary, the present study has demonstrated that human endothelial cells elongate a variety of arachidonic acid analogues. The extent of elongation is determined both by chain length, and by the number and position of the double bonds. Further understanding of chain elongation will be required to elucidate its role in modulating the effects of exogenous n-6 and n-3 fatty acids on membrane composition and thus on cellular functions.

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Cholesteryl Ester Analogs Inhibit Cholesteryl Ester but not Triglyceride Transfer Catalyzed by the Plasma Cholesteryl Ester-Triglyceride Transfer Protein

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A lipid transfer protein complex (LTC), purified from human plasma by immunoaffinity chromatography, catalyzed the interlipoprotein transfer of cholesteryl esters (CE) and triglycerides (TG). The CE transfer activity of LTC was governed by the strucutre of the CE. Incubation of LTC with long chain CE both activated and stabilized LTC. Short chain CE also enhanced the CE and TG transfer activity of LTC during the initial time of incubation. However, LTC's incubation with short chain CE induced a subsequent and time-dependent loss of CE transfer activity without concomitant loss of TG transfer activity. The data indicate that the CE and TG transfer activity of LTC can be reglated independently.
***Lipids 25,* 216-220 (1990).**

Neutral and polar lipids are transported in the circulation as soluble macromolecules known as lipoproteins (Lp), specifically chylomicrons, very low density (VLDL), low density (LDL) and high density (HDL) lipoproteins (1). Lipoprotein lipids are in a state of constant interparticle flux despite their low solubilities in the plasma. Cholesteryl esters (CE), triglycerides (TG) and diacylphospholipids, such as phosphatidylcholine (PC), are transported between lipoproteins by a transfer catalyst denoted the lipid transfer protein complex (LTC) (2). Lipid transfer catalyzed by LTC is achieved by a random sequential addition of substrate-carrying lipoproteins to LTC, resulting in the formation of a ternary complex in which lipid transfer occurs. The ternary complex model is inferred from results of kinetic studies by Ihm *et al.* (3) which indicate that LTC mediates a productive collision between the lipoproteins involved in exchange of lipids. For LTC-catalyzed lipid movement, collision results in the formation of an exchange complex, Lp1-LTC-Lp2. Evidence which supports a collisional interaction of LTC with lipoproteins was provided by Pattniak and Zilversmit (4), who showed that cholesteryl ester transfer activity can associate with HDL and with multilamellar vesicles containing acidic phospholipids. LTC can also catalyze the transfer of lipids between synthetic liposomes and lipoproteins (5) and between liposomes and mitochondria (6), and can remove neutral lipids from neutral lipid-phospholipid monolayers (7). This latter result indicates that LTC could act as a lipid carrier between physically separated lipid interfaces, and Barter and Jones (8) have proposed a ping-pong mechanism for the cholesteryl ester transfer protein (CETP), which is a component of LTC. The role of a direct interaction between the lipid molecule undergoing transfer and the catalytic protein has not been established.

Recently, we developed a method by which LTC can be isolated rapidly and intact from plasma by immunoaffinity chromatography (9). The major protein present in immunoaffinity purified LTC, a lipoprotein of hydrated density 1.14-1.18 g/ml (10), had a M_r of 67 kDa, as determined by SDS-PAGE. The association of lipid with this protein was demonstrated by Ihm *et al.* (2). Other investigators have provided evidence to suggest that specific interactions can occur between LTC and the lipid molecules undergoing transfer and that binding is required for lipid transfer. Harmony *et al.* (7) demonstrated that LTC removes cholesteryl oleate and triolein from monolayers composed predominately of phosphatidylcholine and 2-6 mol% of CE or TG. This transfer occurred in the absence of acceptor lipoproteins, suggesting that LTC can bind and remove lipids by direct interaction with the lipid. Swenson *et al.* (11) demonstrated that CE, bound to CETP, could be transferred to an acceptor lipoprotein. In this report, we demonstrate that the exchange of CE and TG between LDL and HDL can be dissociated by the uptake of short chain cholesteryl esters by LTC. Binding of short chain CE results in the specific inhibition of facilitated exchange of CE without inhibition of facilitated exchange of TG.

MATERIALS AND METHODS

Materials. Benzene and pyridine, obtained from Fisher Scientific (Cincinnati, OH), were distilled over calcium chloride prior to use. Bromoacetyl chloride, purchased from Aldrich Chemical Co. (Milwaukee, WI), was used without further purification. Cholesteryl acetate, purchased from Sigma Chemical Co. (St. Louis, MO), was judged to be pure by thin-layer chromatography (TLC) analysis.

Synthesis of a bromoacetyl ester of cholesterol (CAB). Bromoacetylchloride ($BrCH_2COCl$; 0.41 g, 2.6 mmol) in 10 mL of dry benzene was added with constant stirring to pyridine (0.26 mL, 3.12 mmol) in 80 mL of dry benzene; the temperature was maintained at 10°C. Ten mL of cholesterol (100 mg/mL, 2.6 mmol) in dry benzene was added to the reaction mixture; the reaction temperature was maintained near 5°C. In some preparations, tracer amounts of [^{3}H]cholesterol were included in the starting material. The solution was allowed to warm to room temperature with constant stirring over a period of 2-4 hr. The resulting precipitate (HCl salt of pyridine) was removed by filtration, and the filtrate containing the

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Abbreviations: BSA, bovine serum albumin; CA, cholesteryl acetate; CAB, cholesteryl acetylbromide; CE, cholesteryl ester; CETP, cholesteryl ester transfer protein; CL, cholesteryl linoleate; CO, cholesteryl oleate; HDL, high density lipoproteins; HPLC, high performance liquid chromatography; LDL, low density lipoproteins; Lp, lipoproteins; LTC, lipid transfer protein complex; NMR, nuclear magnetic resonance; TG, triglyceride; TLC, thin-layer chromatography; TMS, trimethyl silane; VLDL, very low density lipoproteins.

bromoester product of cholesterol was dried by flash evaporation. To enhance recovery of the ester, the precipitate was washed twice with a small volume of benzene; the wash solutions were combined with the filtrate. The dried product was solubilized in 50 mL of benzene. Unreacted $BrCH_2COCl$ and remaining HCl were removed by extracting the solution three times with water and once with sodium bicarbonate (1 M). The benzene was again flash evaporated to yield a white crystalline product. This material was recrystallized from methanol, taking care not to overheat the mixture which caused solvolysis of the product, or from a ternary solution of petroleum ether/diethyl ether/acetic acid (85:14:1, by vol) by placing the solubilized sample at −20°C for 12 hr.

Structure analysis of CAB. Reverse phase high performance liquid chromatography (RP-HPLC) and TLC were used to assess the purity of the product. For the HPLC analysis, a RP-C18 column (DuPont, Boston, MA) was used and the samples were eluted with a linear gradient of 1-propanol and water over 60 min. The peak areas were integrated automatically and converted to percent to determine the proportion of bromoester to free cholesterol in the product. Greater than 96% of the product eluted as a single peak. TLC analysis of the HPLC-purified product was performed, using silica gel-IB2 strips in a developing solution of petroleum ether/diethyl ether/acetic acid (84:14:1, by vol). Relative migrations (R_f) were determined and compared to those of cholesteryl acetate (CA), cholesterol, and cholesteryl linoleate and oleate (CL/CO) mixtures. The product migrated with an R_f value similar to that of CA rather than cholesterol. The structure of the product was confirmed by proton nuclear magnetic resonance (NMR) spectroscopy, using a Varian CFT-20 Pulse Fourier Transform NMR Spectrometer. Corrected NMR assignments were recorded in ppm relative to trimethyl silane (TMS), and the spectrum was compared to that of cholesterol and cholesteryl oleate.

Assay for lipid transfer activity. LTC-facilitated transfer of CE and TG was assayed, using prelabeled LDL (density 1.019–1.063 g/mL) as lipid donor and unlabeled HDL (density 1.063–1.21 g/mL) as acceptor, as previously described (2,3,9). Inhibition assays were performed the same way with an additional preincubation of LTC with CL/CO, CAB or CA as described. CL/CO, CAB and CA were examined for their ability to affect both LTC-mediated CE and TG exchange. To introduce these compounds into LTC, 10 mg of CAB, CA or a 50:50 mixture of CL/CO were solubilized in 500 μL of chloroform and plated on the bottom of individual, 50-mL, round bottom flasks by evaporation of the solvent under a stream of nitrogen. Plated esters were then placed under house vacuum overnight. Individual 25 mL samples of LTC (0.2 mg/mL), purified as described by Busch *et al.* (9), or plasma density buffer (PDB; 10 mM Tris-HCl, pH 7.4, containing 150 mM NaCl and 0.02% NaN_3) were placed over each of the plated cholesteryl esters or in an empty, round bottom flask as a control. All reaction vessels were flushed with N_2, the mixtures were incubated at 27°C, and aliquots were removed from each incubation and dialyzed overnight at 4°C against PDB. The amount of CAB solubilized was assessed by UV spectroscopy and quantitated based on the cpm of radiolabeled CAB (spec. act. 1,500 cpm/nmol). Each sample was assessed for protein recovery and assayed for lipid transfer activity. The data were compared with results from control solubility assays in which PDB or non-LTC protein in PDB was incubated over CAB. The data represent individual experiments performed at least twice, and usually three times. The experimental error is given in each figure legend.

RESULTS

Solubilization of CAB by LTC. Initial experiments were performed to determine the effect of temperature on the stability of the lipid transfer activity. The temperature was varied between 25–42°C, and the transfer activity of LTC, which had been incubated in glass, round bottom flasks, was assessed. Glass-incubated LTC lost up to 70% of its lipid transfer activity in 72 hr at temperatures of 37°C or above. At 25°C, CE and TG transfer activities decreased by less than 30%. The incubation temperature of 25°C was used for all subsequent experiments.

UV spectroscopy was utilized to determine if solubilization of CAB occurred in solutions of LTC vs non-LTC protein or buffer alone. CAB had an absorbance maximum at ~236 nm in hexane (Fig. 1A). The LTC solutions were compared after incubation in the absence (Fig. 1C) and presence (Fig. 1D) of CAB by difference spectroscopy (Fig. 1E). As determined by the increase in absorbance at 236 nm (Fig. 1E), CAB was solubilized by the solution containing LTC during a 72 hr incubation period. Using radiolabeled CAB in an identical experiment, LTC to CAB molar ratios in these solutions ranged from 1:1 to 1.6:1 (LTC M_r, 67 kDa), as determined after prolonged dialysis in three separate experiments. The recovery of LTC protein was >98%, based on protein analysis. CAB could also be solubilized by bovine serum albumin (BSA). Under the same conditions, 1.49 nmol of BSA solubilized 6 nmol of CAB, suggesting that CAB can associate with other lipid-binding proteins. In contrast, CAB was not readily soluble in PDB, as is evident by the absence of a peak at 236 nm (Fig. 1B). Based on the UV absorbance properties of the solution and on the level of solution radioactivity when radiolabeled CAB was used, less than 17 pmol of CAB were solubilized in 25 mL of PDB in 72 hr. Similar results were obtained for CA and CL/CO (1.9 and 0.68 pmol/mL, respectively) in buffer only.

The effect of CAB on LTC-mediated CE transfer. At 25°C, incubation of LTC in the presence of mixed long chain cholesteryl esters resulted in stimulated CE transfer activity (Fig. 2) which remained elevated for the duration of the experiment. Initially, LTC activation also occurred when the catalyst was incubated over CAB. The rate of CAB solubilization, as determined by radioisotope techniques, approached equilibrium in a continuous fashion, exhibiting an initial rapid solubilization phase followed by a slower solubilization phase that plateaued by 72 hr. The time period in which activation of LTC's CE transfer activity occurred corresponded to the rapid phase of CAB solubilization.

The slower CAB solubilization phase was characterized by a time-dependent loss of LTC's CE transfer activity to levels well below that of LTC incubated with CO/CL or over glass. After 72 hr, the CE transfer activity of LTC incubated over CO/CL decreased by only 21% compared to the maximum activity at 20 hr. However, the activity of LTC at this point was still significantly higher than

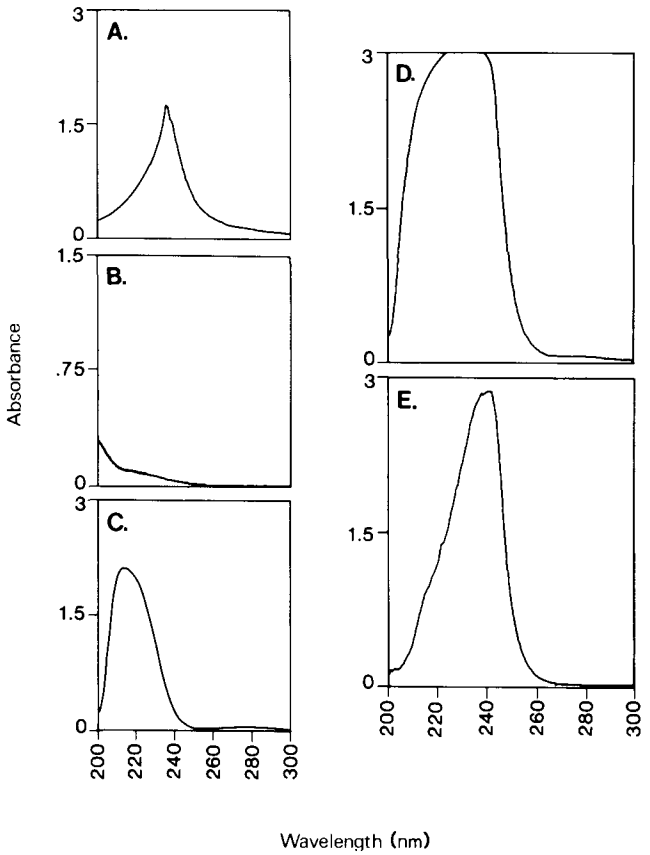

FIG. 1. CAB was solubilized by LTC. In panels A–E, ultraviolet (UV) absorption spectroscopic analysis showed that the solubilization of CAB required protein. For A, CAB was solubilized in hexane, and a UV scan was recorded over the range of 200–300 nm to determine the absorption maximum (~237–240 nm). For B, 10 mg of CAB was plated on the bottom of a 50 mL round bottom flask, as described in Methods, and incubated at 25°C for 168 hr under 25 mL of PDB. One mL of this solution was then analyzed by absorption spectroscopy. Panel C shows the absorption spectrum of a solution of LTC (200 μg/mL) in PDB. For D, the spectrum of the same solution is shown following its incubation over plated CAB for 72 hr at 25°C. Panel E shows the difference spectrum of D vs C.

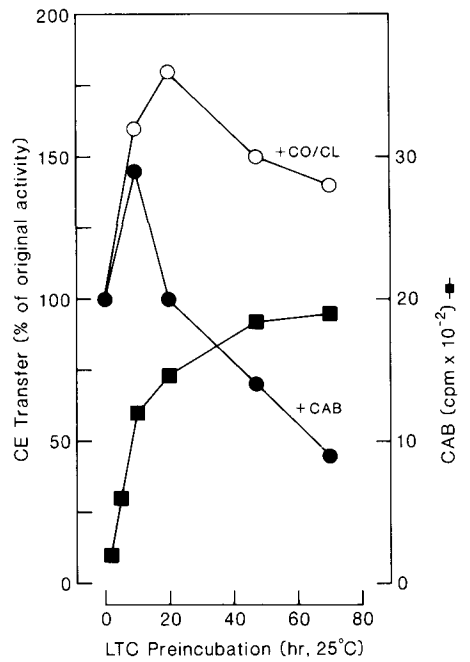

FIG. 2. CE transfer activity of LTC was inhibited by CAB but not by CL/CO. Immunoaffinity-purified LTC was assayed as described in Methods. Samples of 10 mg of a 50:50 mixture of CL and CO or CAB alone were plated on the wall of a round bottom flask under a stream of nitrogen. Twenty-five mL samples of LTC (0.2 mg/mL) were incubated over each lipid for a defined period of time at 25°C. Aliquots of 80 μg (0.4 mL) of LTC were removed from each incubation at the indicated time, dialyzed at 4°C for 18 hr and subsequently assayed for CAB incorporation by scintillation counting. Each aliquot was then assayed in duplicate for CE transfer activity, using 80 nmol of [^{3}H]CE-LDL and 40 nmol of HDL in a final volume of 1 mL of PDB. The error did not exceed 6% of the mean. Control assays were performed in the absence of LTC.

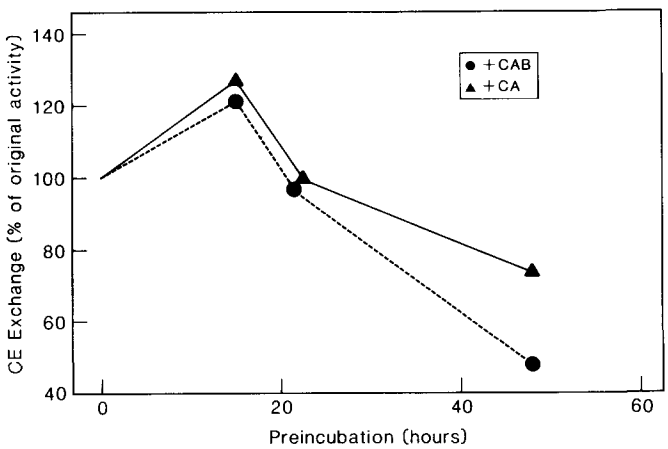

FIG. 3. Cholesteryl acetate inhibited CE transfer but not as effectively as CAB. LTC was assayed as described in the legend for Figure 2. Aliquots of 10 mL of a 0.18 mg/mL solution of LTC in PDB were incubated at 27°C over a 50:50 mixture of CL/CO (control), CAB (-●-) or CA (-▲-) for 0, 15, 22, and 48 hr. At each time point 90 μg (0.5 mL) of LTC were removed in duplicate and stored at 4°C until the final time point was reached. The samples were dialyzed for 18 hr at 4°C against PDB and assayed for CE transfer activity. At the final time point, protein recovery was determined to be greater than 94%. Assays were performed in a final volume of 1 mL, using 112 nmol (CE) of [^{3}H]CE-LDL (666 cpm/nmol CE) and 58 nmol (CE) of HDL. Duplicates differed by ≤5%.

the original value. The loss of CE transfer activity of LTC incubated from 24–72 hr with CO/CL was less than the loss of activity of control CE incubated over glass only (21% vs 30%). In contrast, LTC incubated with CAB lost 75% of its maximum CE transfer activity.

When corrected for activity lost from glass-incubated controls, CE transfer activity at 72 hr was inhibited by 45% by CAB. The LTC/CAB ratio was determined to be 0.3 μg (0.63 nmol) of CAB to 80 μg of LTC (1.2 nmol of 67 kDa protein), or 0.53:1. This result suggests that LTC activity is inhibited in proportion to the amount of associated CAB. Cholesterol oxidation has been reported recently (12) to inhibit CE transfer activity. Since LTC incubated with CO/CL lost very little activity over 72 hr, the oxidation of CE is unlikely to account for the selective inhibition of CE transfer activity by the short chain CE, CAB.

Cholesteryl acetate (CA) also inhibited LTC-facilitated CE transfer. To test the importance of the bromo group of the inhibitory cholesteryl ester analog, LTC was

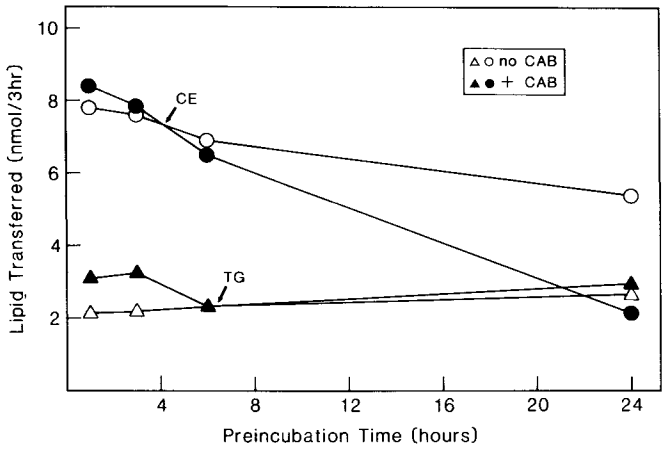

FIG. 4. CAB did not inhibit the TG transfer activity of LTC. LTC was incubated over CAB or glass for 24 hr and assayed for CE and TG transfer activity. Ten mL of LTC (0.18 mg/mL in PDB) was placed over CAB or glass and incubated at 27°C for 1, 3, 6, and 25 hr prior to analysis. Aliquots were removed in duplicate at the respective times and stored at 4°C until the final time point. Aliquots of 0.5 mL (90 μg) were dialyzed at 4°C for 18 hr and assayed for CE (-○-), TG (-△-) transfer activity: 64 nmol (CE) of [^{3}H]CE-LDL or 64 nmol (CE) of [^{14}C]TG-LDL were added to 16 nmol (CE) of HDL and 0.5 mL of LTC, and the volume was adjusted to 1 mL with PDB. Control assays included PDB without added LTC, and LTC incubated for the same period of time over glass. The error did not exceed 6% of the mean. The ratio of TG transfer activity to CE transfer activity has not been corrected for the TG and CE content of the donor lipoproteins which is 1:4 (3).

incubated in the same experiment with CA or CAB. The CE transfer activity of LTC incubated with either CA or CAB increased initially (24 or 18% for CA or CAB, respectively) during the incubation (Fig. 3). After 48 hr, CE transfer activity in the presence of CA had decreased by 46%, whereas CE transfer activity in the presence of CAB was decreased by 60%. These data suggest that short chain esters of cholesterol can inhibit LTC-catalyzed transfer of CE, and that the more polar CAB exhibits a slightly greater inhibitory potency than CA.

CAB inhibited LTC-facilitated CE transfer, but not TG transfer. LTC catalyzed the transfer of CE and TG (Fig. 4). CAB caused a measurable increase in both CE and TG transfer within the first 4 hr of incubation. This increase in CE transfer activity was not as large as that reported in Figures 2 and 3, and did not persist for as long, perhaps due to the variability in LTC preparations isolated from different donors. Over a period of 24 hr, control LTC incubated over glass lost 30% of its initial CE activity (from 7.9 to 5.5 nmol CE transferred in 3 hr) without significant loss of protein; TG activity increased by 24% (from 2.1 to 2.6 nmol of TG transferred in 3 hr), suggesting that TG transfer activity is more stable under these conditions than CE transfer activity. The CE transfer activity of LTC incubated with CAB decreased by 74% (8.4 to 2.1 nmol of CE transferred in 3 hr), a difference of 62% relative to the control at 24 hr. The level of TG transfer activity was essentially unaffected (3.0 vs 2.9 nmol of TG transferred in 3 hr). CAB binding data indicated that 0.65 μg (1.3 nmol) of CAB was associated with 100 μg (1.5 nmol at 67 kDa) of LTC, or an LTC/CAB ratio of 0.86.

DISCUSSION

Immunoaffinity-purified LTC bound CE, with subsequent alteration of LTC-catalyzed transfer of CE, but not of TG. Whether the association of LTC with CE resulted in activation or inhibition of transfer activity depended on the chain length of the fatty acyl constituent of the CE. Long chain CE activated and stabilized CE transfer activity. Short chain CE analogs, specifically CA and the more polar CAB, activated CE and TG transfer activities initially, but subsequently induced a time-dependent inhibition of CE transfer without concomitant inhibition of TG transfer activity. The loss of CE transfer activity was not due to selective loss of the active transfer protein from the complex as evidenced by the absence of detectable loss of protein and the lack of change of TG transfer activity. CAB inhibited LTC-mediated CE transfer activity with a LTC/CAB stoichiometry of approximately 1:1. Recently, Swenson *et al.* (11) demonstrated that CETP, a major component of LTC, directly binds CE and can, after reisolation, transfer the bound lipid to lipid acceptors. Our results not only substantiate their finding but demonstrate for the first time that the structure of the CE can modulate the catalytic activity of the transfer protein.

The mechanism by which CEs influence CETP's catalytic potential remains to be determined. The fact that LTC's CE transfer activity was significantly activated and modestly stabilized by CO/CL suggests that substrates influence the structure of the CETP. Activation of transfer capacity also occurred during the initial incubation of LTC with CA or CAB. Lipid binding to LTC may effect the polymeric state of the catalyst (13), as in the case of pancreatic cholesterol esterase (14), or the conformation of the active subunit so as to promote CE transfer. The initial and rapid activation of both TG and CE transfer activities by CE argues for such a general mechanism that effects both activities of the complex. Inhibition of LTC by short chain CE occurred slowly as compared to the rate at which they were bound, consistent with a time-dependent secondary (e.g., reorganizational) process.

Our results document, for the first time, that CEs can modulate CE transfer activity independently of TG transfer activity and revive the postulate, originally proposed by Morton and Zilversmit (15), of separate sites for CE and TG transfer on the transfer protein. The data are consistent with two lipid-binding domains, one specific for CE and one specific for TG, or with one CE/TG site which can be remodeled to favor CE or TG. A monoclonal antibody specific for CETP can remove both CE and TG, transfer activity from solution (12), suggesting that both activities reside within the same protein. The sequence of a CE transfer protein, isolated from human plasma, was deduced from its cDNA (16). It will be important to determine whether this cDNA, when expressed in cells, encodes a protein that facilitates the movement of both CE and TG. Support for independent modulation of CE and TG transfer is also provided by the fact that TG, but not CE, transfer activity can be inhibited by organomercurial compounds which are specific thiolaffinity reagents (15,17,18). Other compounds, such as β-mercaptoethanol, a strong reductant, can stimulate and stabilize TG transfer activity (17) without altering CE and PC transfer

activities. Such data suggest that a free sulfhydryl group is important in the TG, but not the CE, transfer mechanism. Separate modulation of CE and TG activity is further implicated by the monolayer studies of Harmony *et al.* (7), who found that PC was required in the lipid monolayer for LTC-facilitated removal of CE, but not of TG. The additional information provided by this study supports the model of independent CE and TG sites. Confirmation of such a model awaits site-specific affinity labeling or mutageneis studies.

In sum, the inhibition of CE transfer but not TG transfer by short chain CE indicates that the two transfer activities can be modulated independently by CE structure, a fact of obvious physiological relevance. Combined with inhibitors of TG transfer, the short chain CEs provide us with an opportunity to examine the interrelationship of LTC-mediated CE and TG movement between lipoproteins.

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Separation and Differential Activation of Rat Liver Cytosolic Cholesteryl Ester Hydrolase, Triglyceride Lipase and Retinyl Palmitate Hydrolase by Cholestyramine and Protein Kinases

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Cholesteryl ester hydrolase (CEH), triacylglycerol lipase (TGL) and retinyl palmitate hydrolase (RPH) were measured in 104,000 x *g* supernatants from rat liver under optimal conditions for measurement of cytosolic CEH. Similar levels of hydrolytic activity were seen with oil droplet dispersions of cholesteryl oleate, trioleoylglycerol and retinyl palmitate. No cytosolic TGL activity was seen with substrate presented in the triton-albumin emulsion used for measurement of lipoprotein lipase-like TGL associated with hepatic plasma membrane. Cytosolic CEH, TGL and RPH were differentially partially purified by both ammonium sulfate precipitation and anion exchange fast protein liquid chromatography (FPLC). Of the three activities, only CEH was stimulated by cholestyramine feeding and by activators of protein kinases A and C. All three activities were inhibited by alkaline phosphatase treatment, although to different degrees. It is concluded that these activities are catalyzed by at least three differentially regulated enzymes with a high degree of specificity for their respective substrates.
Lipids 25, 221–225 (1990).

Cholesteryl ester hydrolase (EC 3.1.1.13) (CEH), triacylglycerol lipase (EC 3.1.1.3) (TGL) and retinyl palmitate hydrolase (ED 3.1.1.21) (RPH) are hepatic enzyme activities with similar properties, substrates, intracellular distributions and functions (1,2). Specifically, they have both cytosolic and membrane-bound forms with similar p-H optima, exhibit similar activation in the presence of bile salts and hydrolyze very hydrophobic long chain fatty acyl esters, presumably from lipoproteins or intracellular stores of cholesteryl, glyceryl or retinyl esters (2). Moreover, under similar assay conditions, the activities of these enzymes parallel each other in liver homogenates of widely varying activity and during extensive partial purification (2,3), although differential sensitivity to inhibitors and differential solubility at pH 5 have suggested three different active centers and at least two different enzymes (3,4). In contrast, in several extrahepatic tissues, hydrolysis of both cholesteryl ester and triacylglycerol is reported to be catalyzed by a single protein kinase activated enzyme (5). We have recently reported evidence for activation of cytosolic liver CEH by cAMP-dependent protein kinase and protein kinase C (6). Although Prystowsky *et al.* (2) stated that RPH was not activated in the presence of cAMP and Mg^{2+}-ATP, they did not give details of their experiments or examine the effects of protein kinase activators on hepatic TGL.

Using different assay conditions for CEH and TGL, respectively, Chen *et al.*, (1) demonstrated a releasable extracellular TGL—apparently lipoprotein lipase (EC 3.1.1.34)—which had no CEH activity, and an intracellular CEH, which had no measurable TGL activity (1). Whereas this study suggests that the extrahepatic TGL is lacking in CEH activity, the method of substrate presentation used for the TGL assay (triton-albumin emulsion) was not that shown to be optimal for the intracellular CEH described first by Deykin and Goodman (7), and further characterized by others (2-4,6,8). Thus, it is unclear that the TGL activity described by Chen *et al.* (1) is the same activity measured by Prystowsky et al. (2), who added substrates in a very small volume of ethanol or isopropanol, essentially as a dispersion of oil droplets. Severson and Fletcher (9) and Brecher et al. (10) have stressed the importance of the mode of substrate presentation in studying the properties of lipid metabolizing enzymes. These authors demonstrated CEH activity with a broad pH optimum between 7 and 8 in chicken adipose tissue and rabbit aorta when cholesteryl oleate was presented as an acetone dispersion and this substrate was not hydrolyzed by acid CEH. Regulation of these lipolytic enzymes may also depend on the physical form in which the substrate is presented to the enzyme (11,12).

In the current study, we reexamine the relationships between cytosolic CEH, TGL and RPH activities which hydrolyze oil droplet dispersions of their respective substrates; and present evidence that they comprise at least three distinct enzymes with different substrate specificities and independent mechanisms for regulation.

MATERIALS AND METHODS

[1-^{14}C]Cholesteryl oleate (56.6 mCi/mmol), glyceryl [1-^{14}C] trioleate (109 mCi/mmol) and [1-^{14}C]palmitic acid (53 mCi/mmol) were purchased from New England Nuclear (Boston, MA). High performance liquid chromatography (HPLC) grade solvents were purchased from Fisher Scientific (Columbia, MD). ATP disodium salt, adenosine 3′,5′-cyclic monophosphate (sodium salt), alkaline phosphatase (Type IX from bovine liver), phorbol 12-myristate 13-acetate, phosphatidylserine, diolein, all-*trans* retinol, retinyl palmitate and cholestyramine were obtained from Sigma Chemical Co (St. Louis, MO). All other chemical used were of analytical grade

Preparation of rat liver cytosol. Adult, male, Sprague-Dawley rats (Flow Laboratories, Dublin, VA) were used for the experiments. They had free access to food and

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Abbreviations: Alk. Pase, alkaline phosphatase; AS, ammonium sulfate; ASP, ammonium sulfate precipitation; CEH, cholesteryl ester hydrolase; cAMP, adenosine 3′, 5′-cyclic monophosphate; FPLC, fast protein liquid chromatography; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; RPH, retinyl palmitate hydrolase; S104, 104,000 x g supernatant; SEM, standard error of the mean; TCA, trichloroacetic acid; TGL, triglyceride lipase.

water and were kept at 24°C on a cycle of 12 hr light/12 hr darkness. Rat liver was processed as described previously (13), with minor modifications. Rats weighing 200-300 g were sacrificed by decapitation and the liver removed and washed in 20 mM Tris HCl buffer pH 7.5, containing 5mM 2-mercaptoethanol, 100 mM sucrose and 80 mM KCl. The tissue was homogenized with a loose Teflon pestle in 2 ml buffer per gram of tissue. The homogenate was centrifuged at 2000 × *g* for 30 min, 10000 × *g* for 30 min and then at 104000 × *g* for 90 min to yield a high speed supernatant (S104). Endogenous substrate (cholesteryl oleate, trioleoylglycerol, retinyl palmitate) was undetectable by thin-layer chromatography in aliquots containing three times the protein used in any single assay. S104 was also devoid of CEH activity at pH 4.8, optimal for rat liver lysosomal acid CEH (14).

Preparation of hepatocytes. Rats were fed on a 5% cholestyramine diet for five days. Hepatocytes were prepared by the procedure outlined by Bissel and Guzelian (15). Cell suspension was centrifuged for 5 min at 500 × *g*. Cells were resuspended in 20 mM Tris HCl buffer, pH 7.5, containing 5 mM 2-mercaptoethanol and 100 mM sucrose and sonicated on ice for three cycles (30 sec on/30 sec off), using Heat Systems Ultrasonics Inc. (Farmingdale, NY) model W-380 sonicator. This homogenate was centrifuged at 104,000 × *g* to obtain S104.

Preparation of retinyl palmitate. The symmetric anhydride of [1-^{14}C]palmitic acid was prepared as described by Selinger and Lapidot (16). Retinol was allowed to react with the anhydride to form retinyl [1-^{14}C]palmitate (17) and the product purified by preparative reverse phase HPLC on a 25 cm Supelcosil (Supelco Bellefonte, PA) LC-18 column eluted with MeOH toluene (80:20, v/v). Retinyl palmitate was detected and quantitated by monitoring optical density of effluent at 325 nm, using authentic retinyl palmitate as a standard.

Enzyme assays. CEH and TGL were assayed as described by Chen *et al.* (1), and RPH was assayed by a modification of the method described by Prystowsky et al. (2). Cholesteryl [1-^{14}C]oleate, glyceryl [1-^{14}C]trioleate and retinyl [1-^{14}C]palmitate were dissolved in acetone (7) and used as substrates for CEH, TGL and RPH, respectively. In brief, the assay mixture consisted of 20 mM Tris HCl buffer, pH 7.5, containing 5 mM 2-mercaptoethanol, 80 mM KCl and 100-400 μg (S104 or ASP) or 5-30 μg [fast protein liquid chromatography (FPLC) fractions] protein in a final volume of 500 μl. The reaction was started by addition of 70 μM substrate (30,000-40,000 dpm) and the mixture was incubated for 30 min at 37°C. The reaction was terminated by addition of 3.25 ml of methanol/chloroform/heptane (3.85:3.42:2.73, v/v/v) and 50 μl of 1 M NaOH. Each tube was vortexed immediately and the phases were separated by centrifugation for 10 min at 2000 rpm. One ml of the upper phase was removed into scintillation vials. Five ml of Aquasol-2 was added and the associated radioactivity determined. The reaction rate was linear with respect to protein concentration and within the time of incubation employed.

Activation by protein kinases. Activition of CEH, TGL and RPH by protein kinases was carried out as described by Ghosh and Grogan (6). Activation by cAMP-dependent protein kinase (PKA) was determined using the same reaction mixture described above with the addition of 1 mM $MgCl_2$, 5mM ATP and 10 μM cAMP. Similarly, activation by protein kinase C (PKC) was determined by including 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM ATP, 20 μg/ml phosphatidylserine and 4 μg/ml diolein in the reaction mixture. Phosphatidylserine and diolein solution was prepared as described by Beg et al. (18).

Protein estimation. Since the homogenizing buffer contained 2-mercaptoethanol, protein was determined by the BCA procedure following TCA precipitation (19).

RESULTS AND DISCUSSION

Cytosolic and extracellular TGL as a function of assay conditions. Prystowsky *et al.* (2) reported close correlation between CEH, TGL and RPH in liver homogenates in which activity varied as much as 50-fold from animal to animal. They also found copurification of the three activities during partial purification of solubilized preparations of the same homogenates. This led them to suggest that all three activities may be catalyzed by the same protein or regulated by a common mechanism. Later studies from the same laboratory also showed extensive copurification of activities but differential sensitivities to inhibitors and differential solubility in 10 mM sodium acetate at pH 5.0, suggesting different active centers or enzymes (3,4). Chen *et al.* (1) later differentiated CEH from an extracellular TGL on the basis of selective metal ion effects and selective release of TGL from hepatocytes. They were also able to selectively inhibit TGL activity with a specific antibody and to separate the two activities by preparative gel electrophoresis. However, the TGL assay used by the latter investigators presented substrate as a triton-albumin emulsion. In contrast, the earlier investigators presented all substrates for CEH, TGL and RPH in ethanol or isopropanol, which produces an oil droplet dispersion of the highly insoluble substrates (2-4). In the current studies, no cytosolic TGL activity was detected using the triton-albumin emulsion as substrate (data not shown). This is not surprising in view of the observation by Chen *et al.* (1) that only 3% of TGL activity remained in hepatocytes following heparin-stimulated release of the extracellular membrane-associated activity, suggesting that they were measuring very little, if any, cytosolic activity. However, in the current study, when we presented substrate in a small volume of acetone, i.e., as an oil droplet dispersion, cytosolic TGL activity was similar to that of CEH and RPH (Fig. 1-4). Clearly, the extracellular lipoprotein lipase-like TGL reported by Chen *et al.* (1) differs from the intracellular activity closely associated with CEH and RPH, and these enzymes can be distinguished by their preferences for the physical state of the substrate as well as by cellular location. Thus, for all subsequent analyses, we measured all three enzyme activities under conditions found by Deykin and Goodman (7) and by us (6) to be optimal for CEH, varying only the substrate. These conditions are well within the range found by other investigators to be optimal for TGL and RPH (3,4).

Differential ammonium sulfate precipitation. As shown in Figure 1, cytosolic CEH and TGL activities can be substantially separated by ammonium sulfate precipitation (ASP). Recovery of each enzyme activity was 90-100% of that measured in the S104, with a two-fold increase in specific activity. Whereas more than half of CEH is precipitated by 30% ammonium sulfate (AS), 77% of RPH precipitates in a bimodal distribution over the range 0-50% AS,

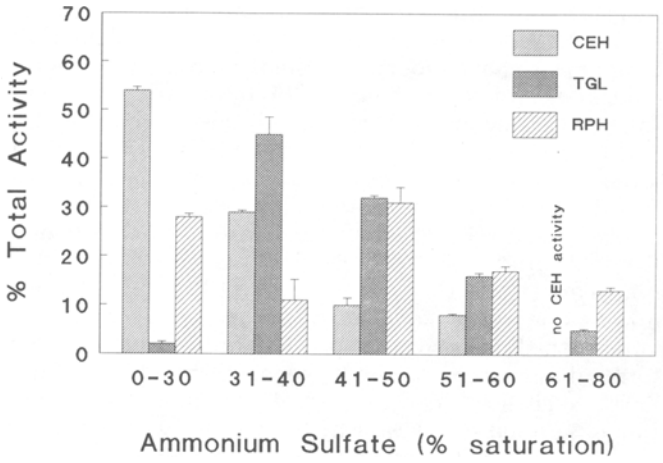

FIG. 1. Differential precipitation of CEH, TGL, and RPH by ammonium sulfate. S104 was subjected to ammonium sulfate fractionation as indicated. Each fraction was assayed for CEH, TGL, and RPH activity as described in Methods. The data given are % total activity ± SEM for quadruplicates. The experiment was repeated with similar results.

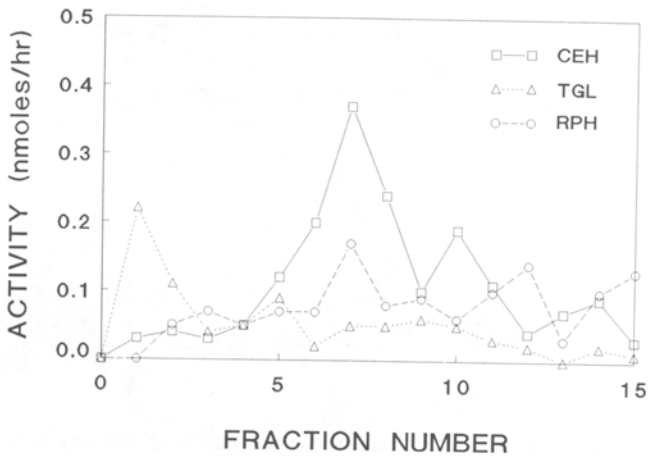

FIG. 2. Separation of CEH, TGL, and RPH by anion exchange FPLC. S104 (5 mg protein) was loaded on an FPLC Mono Q (anion exchange) column and washed with 20 mM Tris HCl buffer pH 7.5 containing 80 mM KCl and 5 mM 2-mercaptoethanol. Proteins were eluted with a linear NaCl gradient (0-1 M). One-ml fractions were collected and duplicates of 200 μl aliquots (5-30 μg protein) were assayed for CEH, TGL, and RPH activity. Experiments were repeated with similar results.

with 61% of the activity occurring in fractions which are not associated with the peak activities of either CEH or TGL (40% AS). Thus, the pattern of ASP is not consistent with the presence of all three activities on a single enzyme.

Differential precipitation of cytosolic CEH and TGL by ammonium sulfate has also been reported by Pittman and Steinberg in rat adrenal cortex (20). Based on this partial separation and differential inhibition by an organophosphate, these authors concluded that the two activities were largely due to separate enzymes.

Separation by anion exchange chromatography. Fractionation of hepatic 104,000 × g supernatant by anion exchange FPLC (Fig. 2) resulted in complete separation of CEH and TGL activities with ~100-fold increase in specific activity and recovery of 70-80% of activity measured in S104. Most of the TGL did not bind to the column and was recovered in the flow through volume, whereas CEH eluted with peak activity at 250 mM NaCl and a secondary peak at 430 mM NaCl. This bimodal distribution of CEH may reflect monomeric and tertrameric forms of the enzyme reported by other investigators (8). The activities of CEH and TGL were each negligible in the fractions of peak activity of the other. RPH, on the other hand, was much more broadly distributed, suggesting multiple enzymes or aggregation, but showed two low peaks of activity, one coinciding with the peak of CEH activity, the other occurring at 550 mM NaCl, well removed from peak CEH or TGL activity. No RPH activity was detectable at the peak of TGL. Thus, CEH and TGL are clearly associated with different proteins. The major TGL activity is not associated with RPH, but a significant fraction of the RPH activity coelutes with, and might possibly be associated with, CEH. However, occurrence of at least one peak of RPH activity not associated with either of the other enzymes strongly suggests that at least a significant fraction of this activity is catalyzed by a unique enzyme or enzymes. This view is further supported by the bimodal distribution of RPH in ASP fractions (Fig. 1). Similarly, Cooper and Olson reported that pig liver RPH also precipitates from AS in a bimodal distribution and suggested that there may be three different RPH enzymes in that species (21). As noted earlier, most of the RPH activity did not coprecipitate with the peak activities of either CEH or TGL. Earlier, Prystowsky *et al.* (2) noted a tendency of RPH to aggregate, which resulted in a diffuse elution pattern from gel permeation chromatography, not unlike that which we observe from anion exchange FPLC (Fig. 2). RPH is clearly different from the other two enzymes in this respect. Further efforts to purify CEH by gel permeation or chromatofocusing FPLC resulted in loss of 90-95% of activity measured in S104 (data not shown).

Differential effects of cholestyramine feeding. Whereas Prystowsky *et al.* (2) reported that CEH, TGL and RPH tended to correlate over a broad range of activities, we have examined two different mechanisms for regulation of CEH for their effects on TGL and RPH. In the first instance, cholestyramine, an anion exchange resin which lowers cholesterol levels by sequestering bile acids and indirectly inducing bile acid synthesis (22), was fed to rats to induce a compensatory increase in CEH (Fig. 3.). As can be seen, cytosolic CEH activity was more than threefold higher than control values in hepatocytes from cholestyramine-fed rats. In contrast, TGL and RPH were not significantly changed (P = .05) by cholestyramine.

Differential effects of protein kinase activators and alkaline phosphatase. In the second instance, CEH, TGL and RPH activities were measured in the presence of cofactors known to stimulate cAMP-dependent protein kinase or protein kinase C, which we have previously shown to activate hepatic CEH, or, following treatment with alkaline phosphatase, to inactivate CEH (6). As shown in Figure 4, cofactors for both kinases resulted in increases in CEH activity within the range which we have previously observed. Neither TGL nor RPH was activated under identical conditions. In contrast, all three enzymes were strongly inactivated by alkaline phosphatase. Although

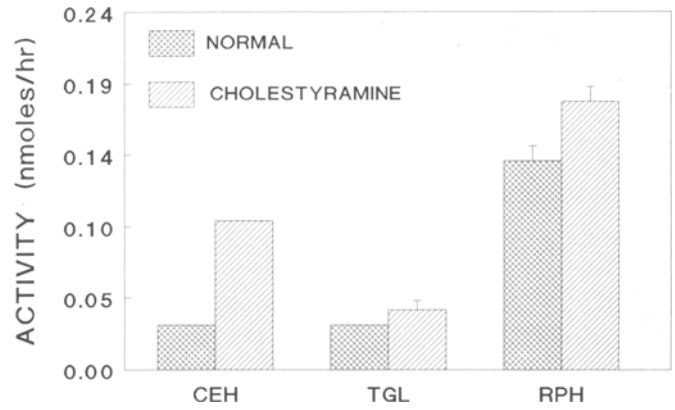

FIG. 3. Effect of cholestyramine feeding on CEH, TGL, and RPH in hepatocytes. Rats were fed a 5% cholestyramine diet for five days and hepatocytes were prepared from both normal and cholestyramine-fed rats. CEH, TGL, and RPH activities were measured in S104 obtained from hepatocytes. Values given are mean ± SEM for triplicates. Where SEM is not seen, value was below limits of resolution of the graph. Experiments were repeated with similar results.

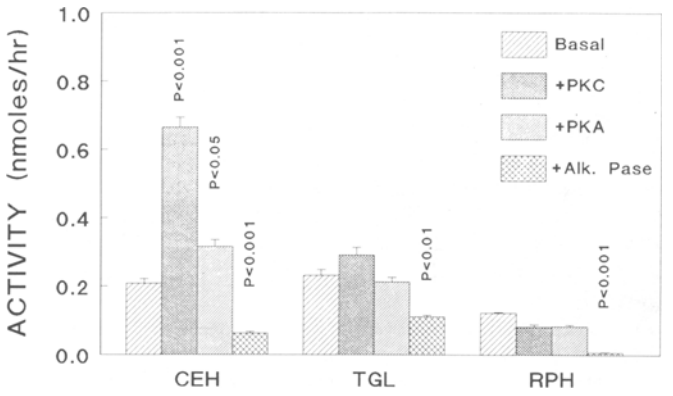

FIG. 4. Activation of CEH, TGL, and RPH by cofactors for protein kinases and inactivation by alkaline phosphatase. CEH, TGL, and RPH were assayed in the presence and absence of cofactors for PKC or PKA as described in Methods. To evaluate the effect of alkaline phosphate, S104 was preincubated with 0.1 units of alkaline phosphatase for 30 min prior to the addition of the substrate. The values shown are mean ± SEM (n = 8). P values are given for the differences which were significant in comparison to untreated controls.

these results suggest that all three enzymes are more active in a phosphorylated form, only CEH was activated by protein kinase cofactors under identical assay conditions. Moreover, in simultaneous experiments, each of the three enzymes was inactivated to different degrees by phosphatase. Of the three, RPH was much more susceptible to inactivation and was not significantly different from blank (assay buffer) controls after phosphatase treatment.

In view of these very large changes in activity induced in all three enzymes by protein kinase cofactors or phosphatase, it is possible that relative activities of endogenous kinases and phosphatases may mediate the interanimal variability in activity of CEH, TGL and RPH previously observed by Prystowsky *et al.* and others reported to be as high as 50-fold (2,23). Naghshineh *et al.* (24) reported similar variability in baseline activity for the sterol ester hydrolase of bovine adrenal cortex, which is also activated by cAMP-dependent protein kinase. Inasmuch as Cooper *et al.* have reported optimal conditions which reduce interanimal variability in RPH by an order of magnitude (23), they may be using conditions which stabilize endogenous kinase or phosphatase activities at optimal levels. As is apparent from the data in Figures 1-4, we also found relatively little interanimal variation in the current study. Similarly, in earlier studies, we found no more than five-fold variation in CEH specific activity over a period of two years (6). In the case of TGL and RPH (Fig. 4), this may be due to the fact that these enzymes were consistently in the fully activated (presumably phosphorylated) form under our conditions of preparation and assay. Similarly, Naghshineh *et al.* reported protein kinase activation of adrenal sterol ester hydrolase only when baseline activity was low [i.e., when the enzyme was substantially less than fully phosphorylated (24)]. These observations underscore the importance of stabilizing and determining the degree of activation of these enzymes whenever activities are being compared.

Conclusions. Whereas we find that the cytosolic TGL is not active under conditions used by Chen *et al.* (1) in differentiating the lipoprotein lipase-like extracellular hepatic TGL from CEH, we have shown that the cytosolic TGL is also a separate enzyme from the cytosolic CEH originally characterized by Deykin and Goodman (7). This conclusion is supported by separation of the activities by both ASP and anion exchange chromatography and by demonstration of differential regulation of CEH in hepatocytes from cholestryamine-fed rats and in 104,000 × *g* supernatants treated with protein kinase activators or alkaline phosphatase. Although activity of both enzymes may be affected by protein phosphorylation, neither is analogous to the hormone sensitive lipase of other tissues which has both CEH and TGL activity (5), in that neither of the hepatic enzymes shows significant hydrolytic activity with the substrate of the other. It is also clear that TGL and RPH are associated with different enzymes, on the basis of their complete separation by anion exchange chromatography and differential inhibition of TGL and RPH by alkaline phosphatase. The major contributions to CEH and RPH activities, respectively, also appear to come from different proteins, as these activities are differentially affected by cholestyramine, protein kinase activators and alkaline phosphatase and show quite different ASP and anion exchange elution profiles. However, it is not yet clear whether the diffuse anion exchange elution profile of RPH reflects multiple enzymes, as Cooper and Olson have reported in pig liver (21), or the previously reported strong tendency of this enzyme to aggregate (8). Inhibition of RPH by phosphatase represents the first reported evidence for regulation of this enzyme by phosphorylation.

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METHODS

Measurement of Malondialdehyde by High Performance Liquid Chromatography with Fluorescence Detection

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A method has been developed to measure malondialdehyde (MDA) in biological systems. MDA was reacted with 2-thiobarbituric acid (TBA) in the presence of butylated hydroxytoluene (BHT) to minimize formation of artifacts. Initial separation of the TBA—MDA adduct was accomplished by isobutanol extraction. Further elimination and separation of interfering substances was achieved by high performance liquid chromatography. The mobile phase consisted of a 1:1 (v/v) mixture of methanol and water with 0.05% (w/v) tetrabutyl ammonium dihydrogen phosphate added as an ion pairing reagent. At a flow rate of 1 ml/min, the TBA—MDA adduct was eluted from a 15-cm, c-18, reversed phase column in approximately 4.9 min. The TBA—MDA adduct was quantitated with a fluorescence detector set at 515 nm excitation and 550 nm emission. Using this method, picomole quantities of MDA can be easily detected in plasma and liver samples.
Lipids 25, 226-229 (1990).

Free radical induced-lipid peroxidative tissue damage has been implicated in the toxic action of many chemicals and environmental agents, and in the pathogenesis of a number of degenerative diseases (1-3). However, due to the presence of various antioxidant defense systems (3) and the lack of a sensitive and reliable method for measuring the products of peroxidation in biological systems, the precise role of lipid peroxidation in tissue damage or disease processes has yet to be delineated.

Many methods have been suggested or employed to assess the degree of lipid peroxidation (4,5). Measurement of the adduct formed by reacting 2-thiobarbituric acid (TBA) with malondialdehyde (MDA), a major secondary product of lipid hydroperoxides, is one method that has often been used to quantitate the extent of lipid peroxidation. Spectrophotometric measurement of the TBA-MDA adduct is simple and sensitive, but it lacks specificity (4-6). Modifications of the method designed to improve specificity often lose simplicity and can introduce undesirable TBA reactive substances formed during sample processing (5). In addition, many methods for MDA measurement, while useful for model systems such as free fatty acids or isolated membranes, do not give satisfactory results when applied to biological samples. In this paper, we describe a method, applicable to a wide range of sample types, for measuring the TBA-MDA adduct using high performance liquid chromatography (HPLC) and fluorescence detection following isobutanol extraction. The procedure is highly sensitive and specific, and yet retains the simplicity of the basic TBA method.

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Abbreviations: BHT, butylated hydroxytoluene; HCl, hydrochloric acid; HPLC, high performance liquid chromatography; MDA, malondialdehyde; TBA, 2-thiobarbituric acid; TMP, 1,1,3,3-tetramethoxypropane.

MATERIALS AND METHODS

Chemicals and reagents. HPLC grade methanol and hydrochloric acid (HCl) were purchased from Fisher Scientific (Fair Lawn, NJ). 1,1,3,3-tetramethoxypropane (TMP), 2-thiobarbituric acid, butylated hydroxytoluene (BHT), linolenic acid, and soybean lipoxidase Type I came from Sigma Chemical Company (St. Louis, MO). Boric acid and isobutyl alcohol were obatained from Mallinckrodt Chemicals (St. Louis, MO). Tetrabutyl ammonium dihydrogen phosphate was purchased from Aldrich Chemicals (Milwaukee, WI). Potassium hydroxide came from Pfaltz and Bauer, Inc, (Stamford, CT). Ethanol (95%) came from Midwest Grain Products of Illinois (Pekin, IL).

MDA standard. MDA was prepared by acid hydrolysis of 1,1,3,3,-tetramethoxypropane (TMP). A stock solution of 100 nM TMP in 0.01 N HCl was made up and a standard curve was obtained by adding appropriate amounts of stock solution to test tubes containing 1 ml of TBA solution. This TBA solution was prepared by mixing two parts 0.4% TBA in 0.2 N HCl with one part distilled water. Standards were heated for 45 min in a 90°C water bath. After incubation, the TBA-MDA complex was extracted with isobutanol. This extract was mixed (2:1) with methanol prior to being injected into the HPLC system.

Oxidation of linolenic acid. Linolenic acid was oxidized enzymatically according to the modified procedure of Aoshima *et al.* (7). A 2mM solution of linolenic acid in 0.1 M K_3BO_3 buffer (pH 8.0) was oxidized with soybean lipoxidase (1500 units/ml oxidation mixture). At various time points, 0.1 ml samples of this reaction mixture were removed and mixed with 0.5 ml of TBA solution (two parts 0.4% TBA in 0.2 N HCl and one part distilled water) and 0.07 ml of 0.2% BHT in 95% ethanol. Samples were then placed in a 90°C water bath for 45 min. After incubation, the TBA-MDA adduct was extracted with isobutanol. The isobutanol extract was mixed with methanol (2:1) prior to injection into the HPLC system.

Effect of BHT on MDA production. Several studies have shown that BHT or other antioxidants are required to prevent oxidation from occurring under the conditons of the TBA test (8). The effect of BHT on MDA production was determined by including BHT in the TBA reaction mixture at concentrations ranging from 0-0.35%. A solution of 1% BHT in ethanol was prepared and appropriate quantities were added to enzymatically oxidized linolenic acid and MDA standards, which were than processed as described above.

HPLC analysis. Samples were injected into the HPLC system by a TosoHaas TSK-6080 Sample Processor (TosoHaas, Philadelphia, PA) equipped with a 50 μl loop. A Beckman 112 Solvent Delivery Module (Beckman Instruments, Fullerton, CA) was used to deliver the mobile phase, which was a 1:1 (v/v) mixture of HPLC grade methanol and double distilled water. Tetrabutyl ammonium dihydrogen phosphate (0.05%, w/v) was added to the mobile phase as an ion pairing reagent. The TBA-MDA adduct was detected using a Kratos FS 970 L.C. Fluo-

rometer (Kratos Analytical Instruments, Romsey, NJ) set at an excitation wavelength of 515 and an emission wavelength of 550. The detector range was set at 0.05 uA. At a flow rate of 1.0 ml/min, the retention time of TBA-MDA was 4.9 min. An Alltech 5μ C-18 column (Alltech Assoc., Deerfield, IL), 4.6 mm I.D., length 15 cm, was used along with a guard column. Peaks were recorded and quantitated using a Shimadzu C-R3A Chromatopac (Shimadzu Scientific Instruments, Inc., Columbia, MD).

Analysis of plasma and liver samples. In order to evaluate the applicability of the procedure, plasma and liver samples were analyzed for MDA content. One-half ml of plasma, or 20% liver homogenate was mixed with 1.0 ml of 0.4% TBA in 0.2N HCl and 0.15 ml of 0.2% BHT in 95% ethanol. The samples were then incubated in a 90°C water bath for 45 min. The pH of the reaction mixture (1.5-1.8) changed very little during the incubation period. After incubation, the TBA-MDA adduct was extracted with isobutanol. The isobutanol extract was mixed with methanol 2:1 prior to injection into the HPLC system. In order to compare the HPLC method with direct spectrophotometric measurement, the MDA content of the liver isobutanol extracts was also determined by measuring the absorbance at 532 nm. Known amountts of MDA and MDA-TBA (0.5-2 nmoles) were added to tissue samples in order to determine the recovery rate of the procedure.

RESULTS

Standard curve. A typical MDA standard curve is shown in Figure 1. For standards ranging from 8.3-66.7 picomoles, the response was linear (correlation coefficient 0.99). As is shown in Table 1, detection, even at low levels, was accomplished with relatively small variation. The coefficient of variation at 8.3 picomoles, for example, was 11%, and 3.6% for 66.7 picomoles. With a flow rate of 1ml/min, the retention time for the TBA-MDA adduct was 4.9 min.

Linolenic acid oxidation. Figure 2 illustrates the typical course of MDA development during enzymatic oxidation of linolenic acid under the described experimental conditions. Prior to the addition of lipoxidase, there was no detectable MDA in the reaction mixture. Five min after addition of the enzyme, MDA levels reached 4.2 nmoles/ml reaction mixture and at the end of 30 min, MDA levels had risen to 7.7 nmoles/ml reaction mixture. After 30 min, MDA levels remained fairly constant; after 60 min, the reaction mixture contained 7.9 nmoles MDA/ml.

Effect of BHT on linolenic acid oxidation. As is shown in Figure 3 in the absence of BHT, as much as 6.0 nm MDA/ml oxidation mixture was detected 5 min following the addition of lipoxidase. The value was reduced to 3.1

TABLE 1

Analysis of MDA Standards Using High Performance Liquid Chromatography

MDA (picomoles)	Relative fluorescence intensity[a]	Coefficient of variation (%)
8.3	11408 ± 1256	11.0
16.7	20297 ± 2504	12.3
33.3	41302 ± 3499	8.5
66.7	71227 ± 2597	3.6

[a]Mean ± standard deviation, n = 5.

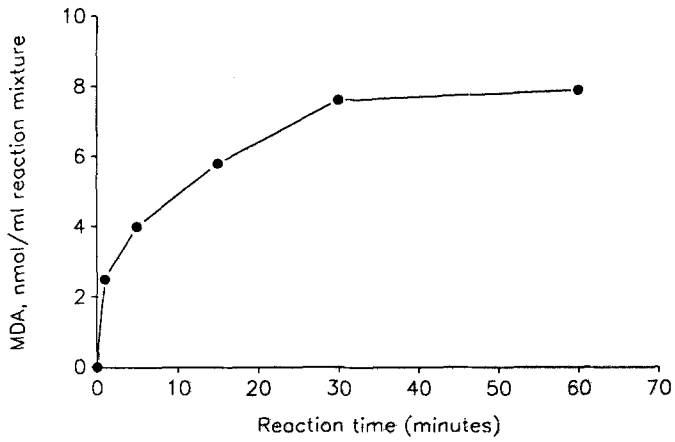

FIG. 2. Lipoxidase catalyzed oxidation of linolenic acid. Reaction mixture 2.0 mM linolenic acid in 0.1 M K_3BO_3 buffer.

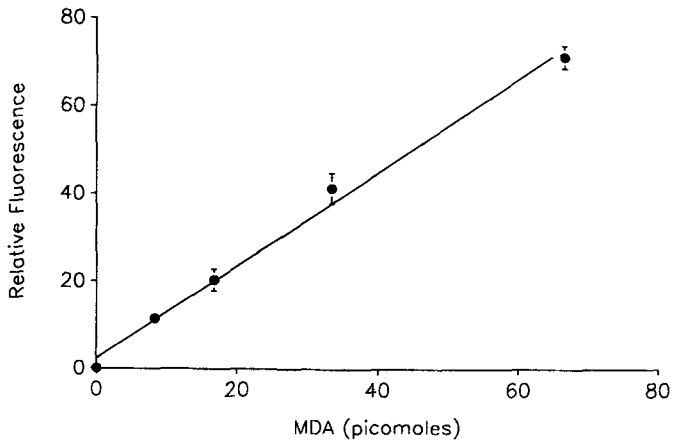

FIG. 1. MDA Standard Curve. Relative fluorescence intensity of TBA-MDA adduct prepared from known quanitites of MDA. Data graphed as mean ± SD (n= 5).

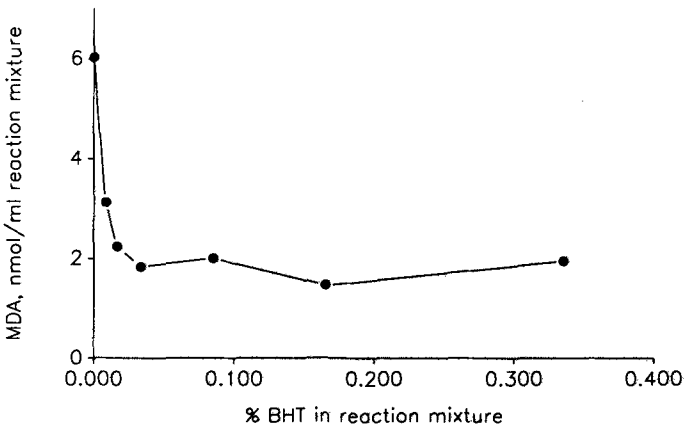

FIG. 3. Effect of BHT on MDA formation. Reaction mixture 2.0 mM linolenic acid in 0.1 M K_3BO_3 buffer. Samples were taken 5 min after lipoxidase addition.

nm/ml by addition of 0.009% BHT to the TBA reaction mixture. Increasing BHT levels to 0.0165% further reduced MDA levels to 2.2 nm/ml. BHT levels as high as 0.335% or above did not interfere with MDA measurement. However, higher concentrations did not provide additional benefit. Therefore, a level of 0.02% was selected for routine use. BHT added to MDA standards had no effect on formation or detection of the TBA-MDA adduct.

MDA values in tissue samples. MDA levels in samples of human plasma, rat plasma, and rat liver were determined using the HPLC method and are summarized in Table 2. The MDA content of rat liver averaged 10.6 nm/gm tissue and the plasma values averaged 0.67 nm/ml for rats and 0.83 nm/ml for humans. The range of values obtained in human plasma (0.25-1.58 nm/ml) was relatively wider than the range of values obtained in rat plasma (0.49-0.86 nm/ml). Of the added MDA and MDA-TBA, 90-104% were recovered from the tissue samples.

TABLE 2

MDA Levels in Plasma and Liver

Tissue	MDA[a]	n
Human plasma (nmoles/ml)	0.83 ± 0.4 (0.25- 1.58)	7
Rat plasma (nmoles/ml)	0.67 ± 0.11 (0.49- 0.86)	20
Rat liver (nmoles/gm)	10.62 ± 1.95 (5.10-14.35)	22

[a]Mean ±standard deviation. The range of values is shown in parenthesis.

TABLE 3

MDA Content of Rat Liver Determined by Spectrophotometric and HPLC Analysis.

Sample number	nmoles MDA/gm tissue	
	Spectrophotometric analysis	HPLC analysis
1	23.7	12.1
2	27.4	12.1
3	26.1	14.4
4	19.6	7.8
5	38.9	14.2
6	23.8	9.8
7	34.1	12.2
8	33.2	10.5
9	29.7	11.7
10	33.4	9.6
11	28.9	9.9
12	32.3	10.9
13	36.1	11.5
14	33.2	10.2
15	34.7	9.1
16	31.9	9.7
17	28.1	10.6
18	27.6	12.0
19	25.5	9.5
20	30.3	10.3
21	19.7	5.1
22	27.3	11.7
	29.3 ± 5.1[a]	10.6 ± 2.0

[a]Mean ± standard deviation.

Comparison of rat liver MDA values obtained by using the HPLC procedure and the spectrophotometric method is shown in Table 3. Using the HPLC procedure, the samples were found to contain an average of 10.6 nmoles MDA/gm tissue with a range of 5.1-14.4 nm/gm. In contrast, the value averaged 29.3 nmoles MDA/gm tissue with a range of 19.5-36.1 nm/gm, with direct spectrophotometric measurement.

DISCUSSION

Despite the lack of specificity, measurement of the TBA-MDA adduct, due to its simplicity and sensitivity, has been widely employed to assess the degree of lipid peroxidation in biological samples (4). Through this research, a more specific and sensitive method for measuring the TBA-MDA adduct has been developed by minimizing artifact formation and by separating interfering substances and quantitating the adduct by HPLC with fluorescence detection.

As is shown in Figure 3, it is essential to include a sufficient amount of antioxidant to prevent autoxidation of lipids and subsequent formation of MDA during the development of the TBA-MDA adduct. Significant quantities of MDA, and thus the TBA-MDA adduct, can result from the autoxidation of lipids during the heating process when BHT is absent or added in insufficient amounts.

Following the development of the TBA-MDA adduct, a preliminary separation was achieved by extracting the adduct with isobutanol. While the TBA-MDA adduct was easily extractable, many interfering substances were not. The extraction step also facilitates subsequent HPLC analysis by removing proteins and other compounds which reduce HPLC effectiveness. Isobutanol was found to be a more desirable solvent than n-butanol for this purpose. Methanol was mixed with isobutanol extract prior to HPLC analysis in order to increase sample miscibility with the mobile phase. However, while quantitative extraction of added MDA and MDA-TBA can be achieved under the experimental conditions, it is possible that a portion of a MDA-TBA may remain unextracted, and thus cause the value to be underestimated.

Increased specificity or selectivity of the HPLC analysis of the TBA-MDA adduct over the traditional spectrophotometric method is evidenced by a reduction of 63% in the quantity of adduct detected in liver samples (Table 3). Plasma and liver TBA-MDA values obtained with this method are either comparable to (9), or lower than, published values (9-11). A comparison of the MDA levels of human and rat plasma (Table 2) shows a greater range of values for humans than for rats. This is not unexpected considering the degree of heterogeneity in human diets vs the high degree of homogeneity in the standard laboratory rat diet.

The use of HPLC with fluorescence detection also increases the sensitivity of TBA-MDA detection over the spectrophotometric method. Using this method, quantities as small as 8.3 picomoles of MDA were measured accurately. Spectrophotometric measurement, however, required a much larger quantity of MDA for a reliable quantitation.

Measurement of the TBA-MDA adduct by HPLC with UV-visible detection at 532 nm (9-11) and direct measurement of MDA by HPLC with UV detection at 267 nm (12-15) have also been reported. While no direct compar-

ison has been made, it is conceivable that fluorescence detection of the TBA-MDA adduct may be more specific and sensitive than that of UV-visible detection. In addition to the TBA-MDA adduct, other MDA derivatives have been used to measure MDA levels in an attempt to assess the extent of lipid peroxidation. For example, Kikugawa *et al.* (16) use a Hantzsh fluorimetric method based on the formation of 1,4-dimethyl-1,4-dihydropyridine-3,5-dicarbaldehyde by the reaction of MDA, methylamine and acetaldehyde, and Esterbauer and Zollner (17) have measured MDA as its 2,4-dinitrophenylhydrazine derivative.

As a major secondary oxidation product of polyunsaturated fatty acids, measurement of MDA has often been regarded as a measurement of lipid hydroperoxides (9,18,19). While MDA does derive from peroxidized polyunsaturated fatty acids, the quantitative conversion of lipid hydroperoxides to MDA is highly variable and is dependent on a number of factors, including pH, incubation time and temperature, and the presence of metal ions (8,14). Furthermore, a number of compounds present in biological systems can directly or indirectly react with TBA to form TBA-MDA or TBA-MDA-like substances during the course of adduct development. In addition to inclusion of TBA-MDA-like substances and MDA formed during assay procedures, the relatively high values of MDA reported by Yamamoto *et al.*(20) and Miyazawa *et al.* (21) compared to the measured peroxide values may be due to the accumulation of MDA from the breakdown of lipid hydroperoxides and from the prostaglandin synthesis pathway over a period of time. An investigaiton is under way in our laboratory to determine the effectiveness of separation of TBA-MDA from various TBA-MDA-like substances by the HPLC method developed.

In the present study, a more sensitive and specific method has been developed for measuring MDA in a variety of materials. The key features of the method include the prevention of artifact formation by adding sufficient antioxidant to the assay system, separation or elimination of interfering substances by isobutanol extraction and HPLC, and quantitation of the adduct with fluorescence detection. The procedure is simple and can be easily applied to biological samples to detect picomolar quantities of MDA.

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COMMUNICATIONS

Sterol Composition of the Phytolaccaceae and Closely Related Families

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Total sterols were analyzed from 28 species of Phytolaccaceae and from 29 species of closely related families—Basellaceae, Portulaccaceae, Molluginaceae, and Stegnospermataceae. Eighteen of twenty-eight species of Phytolaccaceae contained dominant Δ^7-sterols while six species had dominant Δ^5-sterols. Three species had dominant Δ^0-sterols. Sterol composition strongly reflected taxonomic position. Nineteen of twenty-nine species from Basellaceae, Portulaccaceae, Molluginaceae, and Stegnospermataceae contained dominant Δ^7-sterols while ten species contained dominant Δ^5-sterols. Until recently Δ^7-sterols were considered rare in higher plants. It appears that a large number of species in the order Caryophyllales contain primarily Δ^7-sterols.
Lipids 25, 230–234 (1990).

Although algae and fungi have been recognized as containing a wide variety of sterols (1,2), higher plants have been assumed to contain primarily Δ^5-24α-alkyl sterols since such data were first available (3,4). Occasional reports of Δ^7-sterols in higher plants were considered unusual exceptions to the rule (3). The dominance of sitosterol in higher plants and cholesterol in mammals led Bergmann to comment that these sterols appeared to represent "survival of the fittest" sterols in plant and animal evolution, respectively (5).

During the last decade, however, data have accumulated which indicate that sitosterol and related Δ^5-sterols may not be so dominant in higher plants as was previously thought. Alfalfa and spinach contain primarily Δ^7-sterols as do all species of the Cucurbitaceae (3,4,6,7). *Aesculus hippocastanum* (Hippocastanaceae) (8) and *Hacquetia epipactis* (Apiaceae) (9) have also been reported to contain Δ^7-sterols. It is in the order Caryophyllales, however, that Δ^7-sterols appear to be more common than the ubiquitous Δ^5-sterols. *Lophocereus schottii* (10), *Phytolacca esculenta* (11), *Beta vulgaris* (12), and numerous species in the families Amaranthaceae (13), Caryophyllaceae (14), and Chenopodiaceae (15,16) were shown to contain primarily Δ^7-sterols. As it is now constructed (17), the order Caryophyllales is composed of 12 families. The Phytolaccaceae is considered to be the most primitive family of the order and all other families of the order are thought to be derived from it, directly or indirectly (17). Members of Phytolaccaceae are widespread in tropical and subtropical regions. The family consists of 28 genera and about 125 species worldwide (17). An analysis of sterol composition in species of Phytolaccaceae and its closely related families should reveal affinities between families since other families of the order Caryophyllales are thought to have originated from Phytolaccaceae.

EXPERIMENTAL

All samples analyzed were herbarium specimens except *Anredera coridifolia* and *Phytolacca dioica,* collected fresh from the Royal Botanic Gardens at Kew near London, *Mollugo verticellata, Phytolacca americana* and *Claytonia virginica,* collected fresh from College Park, Maryland, and *Lewisia rediviva* which was provided fresh by J. Strachan. The remaining samples were obtained from specimens deposited in the U.S. National Herbarium at the Smithsonian Institution in Washington, D.C. Only mature photosynthetic tissue was used for analysis. Dry tissue (0.3 g) was ground in a knife mill, and sterols were extracted and purified as described previously (16). Sterols were identified and quantitated using capillary GLC on 30 m SE-30, SPB-1, and SP-2330 columns and capillary GC-MS on a DB-1 column with a Finnigan-MAT Model 4500 Spectrometer equipped with an Incos Data System (16).

RESULTS AND DISCUSSION

After careful comparison with most current taxonomic classifications, it was determined that 28 of the 57 species examined are currently classified as Phytolaccaceae (17–20). The remaining species are classified as either Basellaceae, Portulaccaceae, Stegnospermataceae, or Molluginaceae—all families included in or regarded as closely related to Phytolaccaceae. Results from analyses of the sterol composition revealed that the great majority of the Phytolaccaceae examined had Δ^7-sterols as their principal sterols. In Table 1, sterol composition is viewed in relation to current taxonomic position of the species (20). Ten of the fourteen sterols in Tables 1 and 3 are asymmetric at C-24 with two isomers possible. The methods used in this work do not differentiate between isomers at C-24. The trivial names used are for the predominant isomer in most higher plants which is almost always the 24α isomer (4) (except in 24S-7,25-stigmastadienol which is expected to be 24β). In all members examined in subfamilies Gisekioideae (2 species) and Phytolaccoideae (5 species), Δ^7-sterols predominate. Subfamily Rivinoideae is composed of three tribes. In tribes Microteeae and Seguierieae, Δ^7-sterols are predominant (compared to Δ^5). In tribe Rivineae 6 species were "Δ^7-species" (Δ^7-sterols predominate), 3 species were "Δ^5-species" and 3 species were "Δ^0-species" (A Δ^0 sterol here refers to one with no nuclear double bond, although it may have a side chain double bond). Stanols are common as minor constituents of the sterol mixture of plants, but to the authors' knowledge this is the first report of

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Abbreviations: GC-MS, gas chromatography-mass spectrometry; GLC, gas-liquid chromatography.

TABLE 1

Desmethylsterol Composition of Phytolaccaceae[a]

Phytolaccaceae	Sterols[b]													
	A	B	C	D	E	F	G	H	I	J	K	L	M	N
Subfamily: Gisekioideae														
Gisekia pharnaceoides	3	—	3	5	—	—	8	36	22	—	7	—	14	2
G. miltus	6	—	6	5	9	2	5	23	14	5	2	7	17	—
Subfamily: Phytolaccoideae														
Phytolacca americana	—	—	t	—	t	—	—	86	13	—	—	—	—	—
P. dioica	—	—	—	4	5	—	7	27	33	5	2	—	19	—
Anisomeria littoralis	2	1	5	4	—	—	2	35	34	5	3	4	5	1
A. coriacca	2	—	—	2	—	—	—	28	55	5	3	—	5	1
Ercilla spicata	—	—	2	1	9	—	—	34	45	—	1	—	10	—
Subfamily: Rivinoideae														
Tribe: Microteeae														
Microtea paniculata	—	2	8	2	27	—	—	37	21	—	—	—	3	—
M. maypurensis	—	—	—	—	37	—	—	39	24	—	—	—	—	—
M. debilis	4	3	7	6	3	—	—	58	8	—	—	—	10	—
Tribe: Seguierieae														
Seguieria langsdorfii	2	—	1	1	—	—	—	21	1	—	1	1	15	58
S. americana	6	—	—	5	—	—	—	49	31	—	2	—	8	—
S. aculeata	3	—	—	1	7	—	—	57	28	3	—	—	1	—
Gallesia integrifolia	1	—	3	15	22	2	—	2	5	4	1	3	30	9
Tribe: Rivineae														
Hilleria latifolia	2	—	1	4	4	—	—	15	12	—	—	—	46	16
H. secunda	4	—	—	—	7	—	—	18	7	—	—	—	45	19
Ledenbergia macrantha	3	1	1	1	—	—	—	37	9	2	—	2	26	19
L. peruviana	2	—	1	3	10	—	13	55	4	2	—	—	10	—
L. sequirerioides	5	—	2	2	4	—	—	56	28	2	—	2	1	—
Monococcus echinophorus	3	—	—	2	5	—	—	45	9	—	—	3	17	17
Petiveria alliaceae	3	—	1	4	2	—	—	15	4	—	—	1	33	35
Psammotropha myariantha	4	—	7	6	10	—	1	59	3	—	—	3	6	—
Schindleria racemosa	3	—	2	25	27	—	—	2	3	—	—	1	23	15
S. densiflora	1	—	7	29	39	—	—	9	4	—	1	—	10	—
Trichostigma octandium	2	—	2	1	2	—	—	68	3	1	—	3	4	15
T. polyandrum	5	—	4	27	46	—	—	2	1	—	—	1	9	5
Subfamily: Agdestidoideae														
Agdestis clemeatides	5	—	7	18	56	—	—	—	9	1	—	—	3	1
Subfamily: Lophiocarpoideae														
Lophiocarpus polystachyus	5	1	13	11	55	3	6	3	—	—	—	—	2	—

[a]As % of total desmethyl sterols.

[b]A = cholesterol
B = 24-methylenecholesterol
C = campesterol
D = stigmasterol
E = sitosterol
F = isofucosterol
G = 7-ergostenol
H = spinasterol
I = 7-stigmastenol
J = (24S)7,25-stigmastadienol
K = 7,(Z)24(28)-stigmastadienol
L = campestanol
M = stigmastanol
N = 22-stigmastenol

TABLE 2

Relative Quantities of Δ^7, Δ^5, and Δ^0 Sterols in Phytolaccaceae

Phytolaccaceae	Sterols[a] Δ^7	Δ^5	Δ^0
Subfamily: Gisekioideae			
Giseka pharnaceoides	73	11	16
G. miltus	50	27	23
Subfamily: Phytolaccoideae			
Phytolacca americana	99	1	—
P. dioica	72	9	19
Anisomeria littoralis	78	12	10
A. coriacca	91	4	5
Ercilla spicata	80	10	10
Subfamily: Rivinoideae			
Tribe: Microteeae			
Microtea paniculata	58	39	3
M. maypurensis	63	37	—
M. debilis	67	23	10
Tribe: Seguierieae			
Seguieria langsdorfsii	22	4	74
S. americana	90	6	4
S. aculeata	87	12	1
Gallesia integrifolia	13	45	42
Tribe: Rivineae			
Hilleria latifolia	27	11	62
H. secunda	25	11	64
Ledenbergia macrantha	85	12	3
L. peruviana	75	15	10
L. seguirerioides	48	6	45
Monococcus echinophorus	53	10	37
Petiveria alliaceae	19	11	70
Psammotropha myariantha	63	28	9
Schindleria racemosa	5	56	39
S. densiflora	14	76	10
Trichostigma octandium	72	7	21
T. polyandra	4	81	15
Subfamily: Agdestidoideae			
Agdestis clemeatides	11	85	4
Subfamily: Lophiocarpoideae			
Lophiocarus polystachyus	9	89	2

[a]As % of total desmethylsterol.

a saturated sterol being the principal sterol in a plant (stigmastanol in *Hilleria latifolia* and *H. secunda*). In subfamilies Agdestidoideae and Lophiocarpoideae (1 species examined for each), Δ^5-sterols predominate. The data indicate that sterol composition is strongly correlated to taxonomic placement. That some exceptions occur (one species of Trichostigma is a Δ^5-species) points to the need for care in both chemical and taxonomic identifications, and, in particular, for more work in certain groups. Table 2 shows relative amounts of Δ^7, Δ^5 and Δ^0-sterols in the plants examined. In an overwhelming number of species, the Δ^7-sterols are dominant. Previous work in the order Caryophyllales (12–16) has shown the dominance of Δ^7-sterols in several other families of the order Caryophyllales (12–16) but the quantity of Δ^0-sterols found in this study, especially in tribe Rivineae, was unexpected.

Twenty-nine species in other families closely related to Phytolaccaceae were also examined for sterol composition (Table 3). Each of the four species of Basellaceae and the three assigned to Stegnospermataceae primarily contained Δ^7-sterols. Five species of Portulaccaceae were Δ^7-species while five were Δ^5-species. In Molluginaceae, seven samples were Δ^7-species and five samples were Δ^5-species, although the quantities of Δ^7- and Δ^5-sterols in several species were nearly equal (Table 4).

In the order Caryophyllales, Δ^7-sterols appear much more frequently (12–16) than would be expected on the basis of reports from other plant taxa (4). The basal family of Caryophyllales (Phytolaccaceae), from which other

TABLE 3

Desmethyl Sterol Composition of Species in Some Families Closely Related to Phytolaccaceae

Family	Sterols[a]													
	A	B	C	D	E	F	G	H	I	J	K	L	M	N
Basellaceae														
Anredera cordifolia	1	—	4	7	5	—	8	31	26	—	—	5	4	—
A. vesicaria	3	2	1	14	15	2	2	23	33	—	1	2	—	2
Roussingaltia remosa	2	—	1	9	14	1	1	21	44	—	1	1	—	5
Basella rubra	—	—	3	18	11	—	2	24	40	—	3	—	—	—
Portulaccaceae														
Portulaca eliatior	7	1	13	30	47	—	—	—	2	—	—	—	—	—
P. oleracea	—	—	14	14	72	—	—	—	—	—	—	—	—	—
P. halimoides	6	—	13	7	42	—	2	—	2	—	—	—	5	23
P. grandiflora	—	—	20	11	69	—	—	—	—	—	—	—	—	—
Montia parvifolia	5	—	8	3	36	13	—	—	—	—	2	1	—	31
Grahamia bracteata	—	—	2	12	19	5	—	21	24	—	—	—	7	9
Calandrinia gayana	5	4	2	1	5	1	3	58	7	—	—	4	5	6
C. caulescena	6	—	13	—	8	—	2	31	18	—	6	2	10	3
Clayton virginica	3	—	1	1	8	—	6	11	61	3	—	2	5	—
Lewisia rediviva	4	—	2	6	14	6	2	35	20	—	4	—	6	—
Molluginaceae														
Limeum fenestrata	3	—	6	7	79	—	—	—	4	—	—	—	—	—
L. dinteri	2	—	3	28	38	—	—	4	2	6	1	—	15	—
L. aethiopicum	2	—	7	15	71	—	—	—	5	—	—	—	—	—
Adenogramma glomerata	3	—	3	4	16	—	2	47	7	—	—	1	17	—
A. galioides	5	—	2	6	9	2	5	44	14	—	1	—	9	—
Mollugo verticellata	1	—	14	37	39	—	1	—	4	—	—	1	3	—
M. pinosia	2	4	10	3	29	10	5	25	5	—	1	3	3	—
M. nudicaulis	3	8	5	9	13	2	4	28	8	—	6	4	7	3
Pharnaceum serpyllifolium	3	7	5	12	12	3	3	29	6	—	7	3	5	4
Glinus orygiodes	8	—	1	9	9	—	3	57	11	—	—	1	—	—
Macarthuria apetala	3	—	8	21	7	—	4	31	9	—	7	—	5	5
Stegnospermataceae														
Stegnosperma watsonii	4	—	3	6	6	3	5	35	34	4	—	—	—	—
S. halimifolia	4	—	—	—	3	—	2	39	50	2	—	—	—	—
S. cubense	6	—	3	11	14	—	2	43	18	—	—	2	—	2

[a]A = cholesterol
B = 24-methylenecholestertol
C = campesterol
D = stigmasterol
E = sitosterol
F = isofucosterol
G = 7-ergostenol
H = spinasterol
I = 7-stigmastenol
J = 7,25-stigmastadienol
K = 7,(Z)24(28)0-stigmastadienol
L = campestanol
M = stigmastanol
N = 22-stigmastenol

families are thought to originate, contains primarily Δ^7-species (18 of 28 species). Of the species which were examined from families closely related to Phytolaccaceae, 19 of 29 species were Δ^7-species. Recent reports (12–16) have already established Δ^7-sterols as dominant in three other families related to Phytolaccaceae. Since most higher plants are Δ^5-species, it would appear that as one goes farther away from Phytolaccaceae, the frequency of encountering Δ^5-sterols increases. The sterol composition of families of the order Caryophyllales, phylogenetically more advanced than those studied here, will be the subject of a future study.

TABLE 4

The Relationship Between Δ^7, Δ^5, and Δ^0 Sterols in Some Families Closely Related to Phytolaccaceae

Family	Sterols[a]		
	Δ^7	Δ^5	Δ^0
Basellaceae			
Anredera cordifolia	74	17	9
A. vesicaria	59	37	4
Boussingaltia ramosa	67	27	6
Basella rubra	69	31	—
Portulaccaceae			
Portulaca eliatior	2	88	—
P. oleraceae	—	100	—
P. halimoides	4	68	28
P. grandiflora	—	100	—
Montia parvifolia	2	66	32
Grahamia bracteata	45	38	16
Calandrinia gayana	68	18	15
C. caulescena	58	27	15
Claytonia virginica	80	13	7
Lewisia rediviva	6	33	6
Molluginaceae			
Limeum fenestrata	5	95	—
L. dinteri	12	73	15
L. aethiopicum	5	95	—
Adenogramma glomerata	56	25	19
A. galioides	69	22	10
Mollugo verticellata	5	91	4
M. pinosia	36	58	6
M. pentaphylla	48	45	7
M. nudicaulis	46	40	14
Pharnaceum serpyllifolium	45	43	13
Glinus orygiodes	71	28	1
Macarthuria apetala	52	39	10
Stegnospermataceae			
Stegnosperma watsonii	78	22	—
S. halimifolia	92	8	—
S. cubense	62	33	—

[a]As % of total desmethylsterol.

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Identification of the New 18-Hexacosenoic Acid in the Sponge *Thalysias juniperina*

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The phospholipid fatty acids from the sponge *Spheciospongia cuspidifera* were studied revealing the presence of the rare 10-octadecenoic acid (10-18:1) and a new 2-methoxyhexadecenoic acid. The phospholipid fatty acids from *Thalysias juniperina* were also studied revealing the presence of the hitherto unreported 18-hexacosenoic acid (18-26:1). These results tend to indicate that the biosynthetic pathway from 10-18:1 to 18-26:1 may be operative in nature. The phospholipid mixture from the sponges was also analyzed by ^{31}P-NMR and shown to mainly consist of phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, and phosphatidylglycerol. Phosphatidylcholine was not found in the sponges analyzed in this work.
Lipids 25, 235–237 (1990).

Sponges have been the target of marine lipid chemists in recent years for the plethora of intriguing new fatty acids and biosynthetic pathways that can be found in these invertebrates (1). Two rather interesting fatty acids are the 17- and 19-hexacosenoic acids. The 17-hexacosenoic acid has been of interest since it has been found to be present in several sponges such as *Microciona prolifera* and *Xestospongia halichondroides* (2,3) as well as in Baltic herring lipids and *Tropaeolum speciosum* seed fat (4,5). The 19-hexacosenoic acid has also been isolated from several sponges, including *M. prolifera* and *X. halichondroides* (3). The biosynthetic origin of these hexacosenoic acids can be explained in terms of an eight-carbon chain elongation of the common *cis*-vaccenic and oleic acids, 11-18:1 and 9-18:1, respectively.

Recently, we reported on the isolation of the not-so-common 12-icosenoic acid (12-20:2) from the sponge *Euryspongia rosea,* and indicated that the biosynthetic pathway 10-18:1-12-20:1 can be operative in sponges, despite the fact that 10-octadecenoic acid has never been encountered before in sponges (6). The phospholipid ester-linked fatty acid, 10-octadecenoic (10-18:1), has been recognized to exist in several methanotropic soil samples, including the obligate methanotroph *Methylosinus trichosporium,* and in human plasma lipids (7–9). On the other hand, an eight-carbon chain elongation of 10-octadecenoic acid (10-18:1) or a six-carbon chain elongation of the recently reported 12-icosenoic acid (12-20:1) could afford the hitherto unknown 18-hexacosenoic acid (18-26:1). Therefore, the likelihood of finding the hitherto unknown biosynthetic route, 10-18:1-18-26:1, in sponges is very high.

In the present manuscript we wish to report the isolation of the rare 10-octadecenoic acid from the sponge *Spheciospongia cuspidifera* (order Hadromerida) and the isolation of the new 18-hexacosenoic acid from the sponge *Thalysias juniperina* (family Microcionidae, order Poecilosclerida), indicating that the biosynthetic pathway, 10-18:1-18-26:1, is probably active in these invertebrates. We also wish to report the isolation of a new 2-methoxyhexadecenoic acid from *S. cuspidifera.*

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Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; TLC, thin-layer chromatography.

EXPERIMENTAL PROCEDURES

Spheciospongia cuspidifera and *Thalysias juniperina* were collected June 8, 1988, at Ahogado Reef, La Parguera, Puerto Rico, at a depth of 10 ft. The sponges (400–500 g) were washed in sea water, carefully cleaned of all non-sponge debris and cut into small pieces. Immediate extraction with 600–800 mL of chloroform/methanol (1:1, v/v) yielded the total lipids. The neutral lipids, glycolipids and phospholipids (100–200 mg), were separated by column chromatography on silica gel (60–200 mesh) by using a similar procedure to that of Privett *et al.* (10). The phospholipid classes were investigated by preparative thin-layer chromatography (TLC) by 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 (11) followed by purification by column chromatography, eluting with hexane/diethyl ether (9:1, v/v). The resulting methyl esters were analyzed by gas chromatography-mass spectrometry by using a Hewlett Packard 5995 A gas chromatograph-mass spectrometer equipped with a 30 m × 0.25 mm nonpolar fused silica column coated with DB-1. For the location of double bonds, *N*-acylpyrrolidide derivatives were prepared by direct treatment of the methyl esters with pyrrolidine/acetic acid (10:1, v/v) in a capped vial (2 hr at 100°C) followed by ethereal extraction from the acidified solution and purification by preparative TLC. Hydrogenations were carried out in 10 mL of absolute methanol using catalytic amounts of platinum oxide (PtO_2). Mass spectral data for the key fatty acids is presented below.

9-Octadecenoic acid pyrrolidide. MS *m/z* (rel intensity) 335 (M^+, 9.6), 292 (1.5), 278 (2), 264 (2), 250 (3.4), 236 (3.4), 222 (2.3), 210 (1.3), 208 (2.1), 196 (2), 194 (1.5), 182 (6.8), 180 (2.5), 168 (6), 154 (3), 141 (2.8), 140 (9), 126 (70), 113 (100), 98 (21), 85 (8), 72 (11), 70 (17), 55 (29).

10-Octadecenoic acid pyrrolidide. MS *m/z* (rel intensity) 335 (M^+, 4.6), 306 (0.4), 292 (0.7), 278 (1), 265 (0.4), 264 (1.4), 250 (1.6), 238 (0.4), 237 (0.4), 236 (1.7), 224 (0.5), 222 (1), 210 (1.1), 208 (0.9), 196 (1.6), 194 (0.8), 183 (0.8), 182 (4.6), 180 (1.4), 169 (1.1), 168 (4.8), 166 (0.9), 155 (1.4), 154 (3), 152 (1), 141 (2.4), 140 (7), 138 (0.6), 127 (12), 126 (56), 113 (100), 98 (24), 85 (11), 81 (6), 72 (17), 70 (28), 55 (66).

2-Methoxyhexadecenoic acid methyl ester. MS *m/z* (rel intensity) 298 (M^+, 1), 267 (1.2), 266 (M^+ − MeOH, 6), 240 (3), 239 ($M^+ - COOCH_3$), 234 (3), 207 (M^+ − 91, 6), 206 (7), 180 (6.5), 153 (4.5), 152 (6), 150 (12), 140 (6), 138 (10), 136 (12), 126 (6), 125 (12), 123 (16), 121 (12), 117 (8), 111 (19), 109 (30), 104 (100), 97 (22), 95 (71), 94 (19), 93

(14), 83 (31), 81 (56), 79 (28), 75 (24), 71 (52), 69 (33), 67 (60), 57 (23), 55 (57).

18-Hexacosenoic acid pyrrolidide. MS *m/z* (rel intensity) 447 (M^+, 4), 446 (0.5), 418 (1), 376 (1.4), 362 (2), 348 (2), 334 (1.6), 322 (1.6), 308 (2), 294 (0.5), 280 (1), 266 (0.7), 252 (2), 238 (3), 224 (2), 210 (1), 206 (0.2), 196 (1), 183 (1.4), 182 (4), 169 (1), 168 (1.6), 154 (0.5), 150 (1), 140 (2), 137 (8), 127 (7), 126 (26), 113 (100), 98 (6), 96 (5), 91 (4), 85 (4), 83 (5), 72 (22), 55 (36).

RESULTS

The phospholipid fatty acid composition of *Spheciospongia cuspidifera* is presented in Table 1. The principal fatty acids were tetradecanoic (14:0), 4,8,12-trimethyltridecanoic (16:0), hexadecanoic (16:0), octadecanoic (18:0) and a series of Δ5,9 very long-chain fatty acids, 5,9-hexacosadienoic acid (26:2) being the most abundant (18%).

TABLE 1

The Phospholipid Fatty Acids from *Spheciospongia cuspidifera* and *Thalysias juniperina*

Fatty acid	Abundance (%)	
	S. cuspidifera	*T. juniperina*
Dodecanoic (12:0)	1	—
Tetradecanoic (14:0)	7	6
4,8,12-Trimethyltridecanoic (16:0)	11	—
13-Methyltetradecanoic (*i*-15:0)	—	1
12-Methyltetradecanoic (*a*-15:0)	—	2
Pentadecanoic (15:0)	1	—
14-Methylpentadecanoic (*i*-16:0)	—	1
9-Hexadecenoic (16:1)	—	2
Hexadecanoic (16:0)	10	12
15-Methylhexadecanoic (*i*-17:0)	1	4
14-Methylhexadecanoic (*a*-17:0)	1	1
Heptadecanoic (17:0)	1	2
2-Methoxyhexadecenoic (2-OMe-16:1)	5	—
9-Octadecenoic (18:1)	3	—
10-Octadecenoic (18:1)	2	—
11-Octadecenoic (18:1)	—	4
Octadecanoic (18:0)	5	6
11-Methyloctadecanoic (19:0)	—	7
17-Methyoctadecanoic (*i*-19:0)	2	—
16-Methyloctadecanoic (*a*-19:0)	1	—
Nonadecanoic (19:0)	3	1
Methylnonadecanoic (20:0)	4	2
11-Icosenoic (20:1)	6	—
Icosanoic (20:0)[a]	1	3
19-Methylicosanoic (21:0)	4	1
18-Methylicosanoic (21:0)	2	1
Heneicosanoic (21:0)	2	1
Methylheneicosanoic (22:0)	1	1
15-Docosenoic (22:1)	—	3
Docosanoic (22:0)	1	2
Tricosanoic (23:0)	1	—
5,9-Tetracosadienoic (24:2)	1	1
Tetracosanoic (24:0)	—	9
5,9-Pentacosadienoic (25:2)	4	10
Pentacosanoic (25:0)	—	2
5,9-Hexacosadienoic (26:2)	18	13
18-Hexacosenoic (26:1)	—	1
5,9-Heptacosadienoic (27:2)	1	1

[a]Formerly "eicosa-" (changed by IUPAC Commission on Nomenclature of Organic Chemistry, 1975).

Many of the acids were simply identified by comparing their ECL values with those of known esters and by coinjection with standards. A capillary GC analysis of a hydrogenated aliquot of the methyl esters provided more information for identification as well as GC-MS analysis of the whole mixture. Three rather interesting fatty acids were identified in the fatty-acid-methyl-ester mixture. The mass spectrum of two of the methyl-ester derivatives presented a molecular ion peak at *m/z* 296, indicative of isomeric 18:1 acids. Upon catalytic hydrogenation with PtO_2 both fatty acid methyl esters were converted to the straight chain C_{18} saturated ester (*m/z* 298), thus excluding the possibility of any branching. The corresponding pyrrolidide derivatives were then prepared to determine the double-bond position in each isomer (12). In the mass spectrum of the pyrrolidide of one of the acids an interval of 12 amu was encountered between fragments at *m/z* 196 (C_8) and *m/z* 208 (C_9) corresponding to unsaturation between carbons 9 and 10, indicating the presence of 9-octadecenoic acid, better known as oleic acid. The pyrrolidide spectrum of the second acid presented a difference of 12 amu between the fragments at *m/z* 210 (C_9) and *m/z* 222 (C_{10}) indicating unsaturation between carbons 10 and 11 (Fig. 1). The experimental data indicate that we have identified the rare 10-octadecenoic acid, which to the best of our knowledge has never been encountered before in any sponge.

Another interesting acid in *S. cuspidifera,* occurring in 5% abundance, showed an ECL value of 17.22. The mass spectrum of this fatty acid methyl ester showed a molecular weight at *m/z* 298 with a base peak at *m/z* 104. This base peak could only be explained by a McLafferty rearrangement, and the additional 30 amu could only be provided by a methoxy group at the 2-position. Important for this confirmation was the presence of a strong $M^+ - 59$ peak at *m/z* 239 (loss of $COOCH_3$) and another $M^+ - 91$ peak at *m/z* 207 (loss of $COOCH_3$ plus CH_3OH). In fact, comparison of this mass spectrum with similar spectra for other 2-methoxy acids (13) confirmed that we have identified the new 2-methoxyhexadecenoic acid. We could not determine the position of the double bond because we were unsuccessful in preparing the corresponding pyrrolidide derivative, in part due to the small amounts of acid available. However, Ayanoglu *et al.* (13) reported that the African sponge *Higginsia tethyoides* contained the acids 2-methoxy-17-tetracosenoic (17-24:1), 2-methoxy-19-hexacosenoic (19-26:1), and 2-methoxy-21-octacosenoic

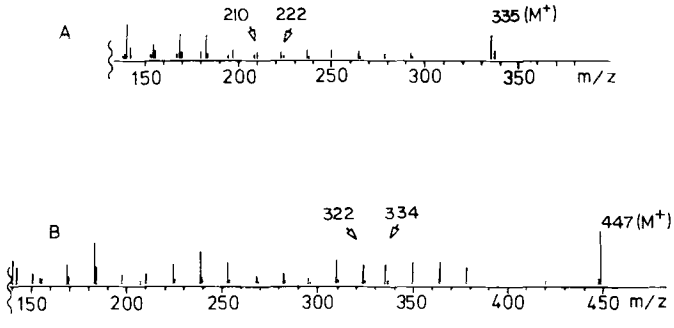

FIG. 1. Partial spectra of 10-octadecenoic acid pyrrolidide (A) and 18-hexacosenoic acid pyrrolidide (B) indicating the 12 amu separations critical for the double-bond location. The spectra are expanded 4 times.

(21-28:1). The latter acids could all arise from chain elongation of 2-methoxy-9-hexadecenoic acid, a likely alternative for our acid. Therefore, on biosynthetic grounds, we tentatively assign the double bond to position 9.

The phospholipid mixture from *Spheciospongia cuspidifera* was analyzed by means of TLC and shown to consist, when compared with known standards, mainly of phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI). A ^{31}P NMR (121.6 MHz) spectrum confirmed the absence of phosphatidylcholine (PC) in the mixture.

The phospholipid fatty acid composition of *Thalysias juniperina* is also presented in Table 1. The principal fatty acids in this case are tetradecanoic (14:0), hexadecanoic (16:0), and a series of Δ5,9 very long-chain fatty acids with 5,9-pentacosadienoic (25:2) and 5,9-hexacosadienoic (26:2) being the most abundant (ca. 23% of the total fatty acid composition). The most interesting fatty acid methyl ester in the mixture presented a molecular ion at *m/z* 408, corresponding to a 26:1 fatty acid methyl ester. Upon catalytic hydrogenation (PtO_2), this fatty acid methyl ester was converted to the corresponding saturated hexacosanoic acid methyl ester (26:0), thus excluding the possibility of any branching. The corresponding pyrrolidide derivative was instrumental in its characterization because it showed a difference of 12 amu between fragments at *m/z* 322 (C_{17}) and 334 (C_{18}) indicating unsaturation at Δ18 (Fig. 1). From the available experimental data we have to conclude that the identified acid in this case is 18-hexacosenoic acid (18-26:1), an acid that has not been recognized before to exist in nature. FT-IR spectra of this compound, and other identified mono- and diolefinic acids in this work, gave no prominent absorption at 968–980 cm^{-1}, indicating *cis* rather than *trans* unsaturation in each case.

The phospholipid mixture from *Thalysias juniperina* was also analyzed by means of TLC and, when compared with known standards, was shown to consist of mainly PE, PS, PI, and phosphatidylglycerol (PG). A ^{31}P NMR (121.6 MHz) spectrum also confirmed the absence of PC in the mixture.

DISCUSSION

Of the few hexacosenoic acids known to exist in nature, the most ubiquitous in sponges are 17-hexacosenoic acid (17-26:1) and 19-hexacosenoic acid (19-26:1). These acids are just the end products of an eight-carbon chain elongation of oleic acid (9-18:1) and *cis*-vaccenic acid (11-18:1), respectively (14). From the sponge *Euryspongia rosea* our recent report of the rare acid 12-icosanoic (12-20:1) suggested that the biosynthetic pathway, 10-18:1–12-20:1, could be operative in these invertebrates. In this paper we identified the acid 10-octadecenoic (10-18:1) in *S. cuspidifera* and the new acid 18-hexacosenoic in *T. juniperina*. Our findings tend to suggest that in addition to the other two rather interesting biosynthetic pathways recognized before in sponges, namely the routes 9-18:1 to 17-26:1 and 11-18:1 to 19-26:1, the biosynthetic pathway 10-18:1 to 18-26:1 appears also viable. *T. juniperina* is the first organism from which the new fatty acid 18-hexacosenoic has been isolated, and we predict that other sponges might also possess this acid. *S. cuspidifera* is also unique because it possesses the acid 10-octadecenoic as well as a new 2-methoxyhexadecenoic acid. Alpha-methoxy acids are very interesting acids since they have just been recently encountered in the phospholipids of sponges, and they seem to possess a unique biosynthetic origin in these invertebrates. To our knowledge, a biosynthetic route to these alpha-methoxy-substituted phospholipid acids is not known (13).

The presence of unusually long-chain unsaturated acids in the phospholipids of a primitive marine organism is unprecedented, and raises interesting questions about their biological function in the membranes in addition to the above discussed biosynthetic pathways. One interesting finding with the sponges analyzed in this work is the fact that both of them had practically no phosphatidylcholine in their membranes, a fact that we have observed before in other sponges. This is certainly worthy of investigation because PC is the main phospholipid in the majority of marine invertebrates and vertebrates. Work is in progress in our laboratory where we are trying to elucidate the complex biosynthetic routes which are operative in these sponges.

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(^{3}H/^{14}C)Beta-Methylheptadecanoic Acid Subcellular Distribution and Lipid Incorporation in Mouse Heart

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Beta-methylfatty acids are transported into myocardial cells as other fatty acids would be, but are incapable, for example, of undergoing complete catabolism. Our previous studies have established the structure-location relationship and the imaging characteristics of these analogs. In the present study in mouse myocardium, microautoradiography and electron microscopy were used to show the distribution of [7,8-^{3}H] beta-methylheptadecanoic acid ([^{3}H]BMHA) in mitochondria and lipid droplets. Thin-layer chromatography demonstrated the presence of [1-^{14}C] beta-methylheptadecanoic acid ([^{14}C]BMHA) and its metabolites in various lipid pools. These studies complement our earlier findings which showed that similarities exist in the initial metabolic fate of BMHA and physiological fatty acids.

Lipids 25, 238–240 (1990).

Both at rest and during exercise, nonesterified fatty acids (NEFA) supply about 65% of the energy requirements of myocardial metabolism (1). Because of their high rate of utilization, NEFA labeled with positron emitting radionuclides in conjunction with tomographic techniques would provide an excellent means of measuring *in vivo* regional myocardial metabolism.

Two approaches can be used to quantify *in vivo* the utilization of substrates. The first approach involves the administration of radiolabeled "physiological" substrates. This approach has been used to assess glucose metabolism in monkey brain with carbon-11 labeled glucose (2) and whole heart fatty acid metabolism in dogs and humans with carbon-11 labeled palmitate ([^{11}C[16:0) (3). Recent studies in dogs using [^{11}C]16:0, however, have shown that during ischemia, because of the compound's complex metabolic fate, quantitation is difficult (4). The second approach involves the administration of tracers that enter known metabolic pathways and, because of their unique chemical structure, are metabolized only to a certain extent thus leaving the radiolabel trapped in the cell. This principle of metabolic trapping has been used successfully with 2-[^{18}F]fluoro-2-deoxy-D-glucose (2FDG) to measure regional glucose metabolic rates in human brain *in vivo* (5).

Fatty acids having a methyl group at the beta position are potential agents for studying fatty acid trapping in the myocardium. Because the methyl group inhibits beta-oxidation of these compounds, they are expected to be trapped in the myocardium as beta-hydroxy-beta-methyl acyl CoA. Our preliminary evaluation of [1-^{11}C]beta-methylheptadecanoic acid ([^{11}C]BMHA) in rats and imaging studies in dogs demonstrated the retention of activity in the heart over a long period of time and the excellent imaging properties of the compound (6). However, before [^{11}C]BMHA can be used for quantitation of myocardial metabolism, it is necessary to show that BMHA enters the metabolic pathway of native fatty acids. In the present study we have investigated in mouse myocardium the subcellular distribution of [7,8-^{3}H]beta-methylheptadecanoic acid (The synthesis of [^{3}H]BMHA will appear elsewhere.) ([^{3}H]BMHA) using microautoradiography and electron microscopy (EM), and have followed the appearance of [1-^{14}C]beta-methylheptadecanoic acid ([^{14}C]BMHA) (7) in various lipid pools using thin-layer chromatography.

MATERIALS AND METHODS

For microautoradiography with EM, two Balb C mice were injected through the tail vein with 185 MBq (5 mCi) of [^{3}H]BMHA. The mice were sacrificed 10 min later, their hearts removed immediately, and 1.5-to-2-mm slices of the left ventricle were placed in a solution of formalin-glutaraldehyde-picric acid. The tissue samples were embedded in epon-araldite, cut into 80-to-100 μm sections and coated with ILFORD L4 emulsion. After exposure for 14 days, the autoradiographs were developed in Kodak D19, cleaned in hypo, stained with lead citrate, and examined by electron microscopy (8).

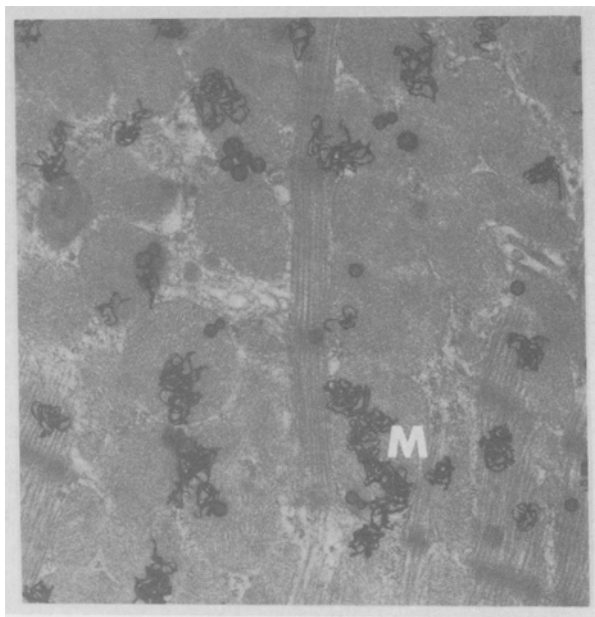

FIG. 1. Microautoradiograph of seciton of left ventricle from mouse injected with [^{3}H]BMHA. Radioactivity is located in mitochondria (M).

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Abbreviations: NEFA, Nonesterified fatty acids; BMHA, beta-methylheptadecanoic acid; EM, electron microscopy.

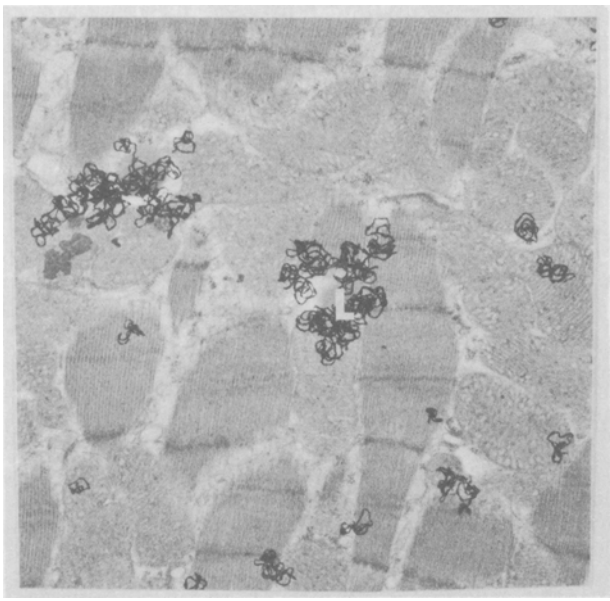

FIG. 2. Microautoradiograph of section of left ventricle from mouse injected with [^{3}H]BMHA. Radioactivity is located in lipid droplets (L).

The incorporation of [^{14}C]BMHA into the various lipid pools in mouse myocardium was determined and compared with that of a normal chain fatty acid, [1-^{14}C]palmitic acid ([^{14}C]16:0) (New England Nuclear Corporation, Bedford, MA). Mice were injected intravenously with 0.185 to 0.37 MBq (5 to 10 μCi) of either fatty acid, and animals were sacrificed after 5, 15 or 30 min. Lipids were extracted according to Folch et al. (9). The lipids were analyzed by thin-layer chromatography on silica gel-coated aluminum plates using hexanes/diethyl ether/glacial acetic acid (50:50:1, v/v/v) as developing solvent. The sheets were cut into 1-cm sections and the adsorbent was scraped into vials. Liquid scintillation fluid was added, and the samples were counted.

RESULTS AND DISCUSSION

The autoradiographs (Fig. 1, 2) show silver grains distinctly localized over lipid droplets (L) and mitochondria (M). The lipid droplets, which are known to consist largely of triglycerides, are contained in the microsomal fraction. Since the mitochondria are considered the intracellular site for the beta-oxidation process, the concentration of mitochondrial radioactivity was attributed to [^{3}H]BMHA and/or its products in the metabolic pathway up to beta-keto acyl CoA. Harris *et al.* (10) have demonstrated that perfusion of rat heart with tritiated oleic acid leads to localization of activity mainly in lipid droplets and mitochondria. In their study, the mitochondrial activity decreased significantly two min after perfusion with the tritiated oleic acid, whereas in our study with tritiated BMHA the mitochondrial radioactivity was persistent ten min after i.v. injection showing that BMHA enters the beta oxidative pathway and is trapped in the mitochondria of the myocardial cell.

Table 1 describes the results of the lipid extraction and thin-layer chromatographic analysis. The data show that [^{14}C]BMHA is incorporated into the same lipid pools as is [^{14}C]16:0, except for the following differences: (i) the triglyceride pool is slightly higher for [^{14}C]BMHA between 5 and 30 min; and (ii) the free fatty acid pool is higher at all times for [^{14}C] 16:0. Similar data have been reported by others using 3-methyl branched radioiodinated fatty acids (11). Studies with [^{14}C]BMHA in isolated, perfused rat heart at different substrate levels showed that 0.06 $\pm$ 0.003% of [^{14}C]BMHA was metabolized to $^{14}CO_2$ during a 110-minute period (Brunengraber, H., personal communication). Moreover, imaging studies in dogs showed a constant level of heart tissue activity for over one hour (starting 10 to 15 min after injection) which would not be expected if substantial oxidation were taking place (6). In contrast, Abendschein et al. (12) observed the formation of $^{11}CO_2$ in dogs after [^{11}C]BMHA infusion; their studies indicated that for the 5-to-20-min period 16.7$\pm$15.2% of the activity was attributed to CO_2 production in the control group, as compared to 3.7$\pm$3.9% in the ischemic group.

We have previously examined the relationship between flow and fatty acid utilization in normotensive and hypertensive rats of the Dahl strain using dual-tracer autoradiography with ^{201}Tl and [^{14}C]BMHA, respectively. Visual and quantitative autoradiography showed that severe hypertension was associated with a homogeneous pattern of regional perfusion, but that fatty acid metabolism was focally decreased in the free wall of the left ventricle. The decrease in uptake of [^{14}C]BMHA was associated with a concomitant increase in glucose utilization (measured with 2-[^{18}F]FDG in the same rats) (13). In another study with dogs, the regional myocardial uptake of [^{11}C]BMHA and flow measured with gamma-labeled microspheres were correlated in the same segments of the myocardium before and after changing myocardial blood flow by LAD occlusion and dipyridimole. A mean reduction in flow of 53.0$\pm$10.5% was accompanied by a smaller decrease in the regional myocardial uptake of [^{11}C]BMHA (18.7$\pm$12.3%) (14). These earlier studies had indicated that BMHA is not a blood flow tracer.

TABLE 1

Distribution of [^{14}C]BMHA and [^{14}C]16:0 in Lipid Pools of Mouse Heart

Lipids	5 min		15 min		30 min	
	[^{14}C]BMHA	[^{14}C]16:0	[^{14}C]BMHA	[^{14}C]16:0	[^{14}C]BMHA	[^{14}C]16:0
Free fatty acids	4.82±1.17	12.14±3.94	7.40± 3.37	12.42± 9.38	5.91±3.87	7.18±3.92
Phospholipids plus monoglycerides	8.30±1.52	15.15±9.85	6.97±0.57	17.25±2.35	9.56±2.82	9.03±1.86
Diglycerides	4.2±1.27	4.72±2.68	11.56±7.90	4.51±1.90	4.71±2.05	2.88±0.24
Triglycerides	81.86±2.62	63.63±7.34	69.41±16.80	61.03±15.48	76.75±6.64	69.76±6.36

In conclusion, the observed microsomal and mitochondrial activity of [^{3}H]BMHA in combination with the metabolic behavior and biodistribution of [^{14}C] BMHA (7) show that the transport of this fatty acid analog and its initial metabolic behavior mimic those of physiological fatty acids. Together with our earlier results which indicated the retention of BMHA activity in the heart over extended time periods and because of the excellent imaging properties of the compound, our findings suggest that [^{11}C]BMHA might be used for the quantification of regional myocardial fatty acid metabolism by imaging techniques. The potential use of this fatty acid analog for quantitating initial fatty acid metabolism in the myocardium based on a mathematical model is being explored in the canine heart.

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The Effect of Glutathione on the Vitamin E Requirement for Inhibition of Liver Microsomal Lipid Peroxidation

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Vitamin E dependent inhibition of rat liver microsomal lipid peroxidation in an NADPH and ADP-Fe^{+3} containing system occurred at lower vitamin E concentrations in the presence of glutathione (GSH). Using microsomes from rats fed a vitamin E deficient diet, vitamin E was shown to be required for inhibition. Inhibition also required the presence of a storage labile microsomal component, since no inhibition was observed when using microsomes that had been stored for one month. This observation provides evidence that direct reduction of reversibly oxidized vitamin E by GSH does not appear to contribute significantly to inhibition of peroxidation. During GSH and vitamin E dependent inhibition of lipid peroxidation, vitamin E (reduced form) concentrations remained constant, indicating that GSH maintained vitamin E concentrations. Without GSH, vitamin E concentrations dropped rapidly. By adding vitamin E to microsomes, it was found that inhibition of lipid peroxidation in the presence of GSH occurred at about fivefold less vitamin E than in the absence of GSH. Inhibition at these lower levels of vitamin E was 85–90% complete. Results indicate that GSH can be used to maintain vitamin E (reduced form) concentrations, thereby lowering the concentration of vitamin E necessary to inhibit microsomal lipid peroxidation.
***Lipids* 25, 241–245 (1990).**

Vitamin E is believed to inhibit lipid peroxidation via donation of a hydrogen atom to a lipid peroxyl radical forming a lipid hydroperoxide (LOOH) and reversibly oxidized vitamin E (I•) (1). Vitamin E dependent inhibition of peroxidation is directly affected by the relative reaction rates of lipid peroxyl radicals (LOO•) with polyunsaturated fatty acids (LH) (reaction 1), and with vitamin E (IH) (reaction 2) (2). In these reactions, LOO•, produced by an initiation event, abstracts a hydrogen atom either from LH to propagate lipid peroxidation (reaction 1), or from IH to inhibit it (reaction 2).

$$LOO^{\bullet} + LH \rightarrow LOOH + L^{\bullet} \quad [1]$$

$$LOO^{\bullet} + IH \rightarrow LOOH + I^{\bullet} \quad [2]$$

Because reactions [1] and [2] both compete for LOO•, the relative concentration of vitamin E and polyunsaturated fatty acids also affects whether propagation or inhibition reactions ultimately predominate in the system. During inhibition, vitamin E (IH) is consumed and hence, in *in vitro* incubations, the concentration of vitamin E used must be artifactually high, partly to supply the amount of vitamin E oxidized plus that required so that inhibition reactions predominate. In contrast, *in vivo* cellular processes presumably maintain vitamin E in the reduced form (IH), thereby decreasing the importance of its loss through oxidation. Thus, vitamin E dependent inhibition of *in vivo* lipid peroxidation probably occurs at vitamin E concentrations lower than those necessary *in vitro*. This hypothesis is supported by data showing that when glutathione (GSH) is added to an *in vitro* microsomal system, it effectively lowers the concentration of vitamin E required to inhibit lipid peroxidation (3–6). Reddy *et al.* (3) suggested that low vitamin E levels were maintained by GSH donating a hydrogen atom to reversibly oxidized vitamin E as shown in reaction [3].

$$I^{\bullet} + GSH \rightarrow IH + \tfrac{1}{2}\, GSSG \quad [3]$$

The inhibitory effect of the vitamin E and GSH interaction has since been shown to be proportional to the GSH concentration (4,6), although the vitamin E concentration necessary to maintain the inhibition was not determined. Reddy *et al.* (3) also provided preliminary evidence for the involvement of a membrane protein in this mechanism. The membrane protein was shown to be specific for GSH and relatively unstable (3–5). It will be referred to as GSH-dependent vitamin E reductase. In this report we extend these findings to demonstrate that inhibition of peroxidation in the presence of GSH in an *in vitro* rat liver microsomal system is dependent on the vitamin E concentration.

MATERIALS AND METHODS

ADP (Type III), *dl*-α-tocopherol, 2-thiobarbituric acid and GSH were purchased from Sigma Chemical Company (St. Louis, MO). Ferric chloride was purchased from Baker Chemical Company (Phillipsburg, NJ). *L*-ascorbic acid was purchased from Fisher Scientific Company (Fairlawn, NJ). NADPH and the vitamin E deficient rat diet (Vitamin E Test Diet) were obtained from U.S. Biochemical Company (Cleveland, OH). The standard laboratory ration (Wayne Rodent Blox) was purchased from Wayne Pet Foods (Chicago, IL).

The Vitamin E Test Diet was fed *ad libitum* to weanling rats for at least eight weeks before they were killed and their liver microsomes isolated.

Rat liver microsomes wre isolated from Sprague-Dawley rats by the method of Pederson *et al.* (7) and stored in argon-saturated 50 mM Tris HCl, pH 7.4, containing 50% glycerol (v/v). Microsomes could be stored up to one week without loss of GSH-dependent vitamin E reductase activity. Microsomal lipid peroxidation reaction mixtures contained microsomes (0.6 mg protein/ml), ADP-Fe^{+3} (500 μM ADP, 100 μM $FeCl_3$), and NADPH (0.4 mM) in 50 mM NaCl that had been passed through Chelex 100 ion exchange resin (Bio-Rad Laboratories,

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Abbreviations: GSH, reduced glutathione; GSSG, oxidized glutathione dimer; I•, reversibly oxidized vitamin E; IH, reduced vitamin E; L•, lipid alkyl radical; LH, polyunsaturated fatty acid; LOO•, lipid peroxyl radical; LOOH, lipid hydroperoxide; MDA, malondialdehyde.

Richmond, CA) to minimize contaminating transition metal ions. Reactions were carried out at pH 7.0 and 37°C in a shaking water bath and initiated by addition of NADPH. When included, GSH (freshly prepared, pH 7.0 solution in 50 mM NaCl) was added at the same time as NADPH to a final concentration of 5 mM. The amount of lipid peroxidation was determined by taking samples of incubations at various times and assaying for thiobarbituric acid reactive material. Then the molar extinction value for malondialdehyde ($1.56 \times 10^5\ M^{-1}\ cm^{-1}$) (8) was used and the data expressed as nmol malondialdehyde (MDA)/mg protein.

Vitamin E was added to microsomes as follows: An aliquot of *dl*-α-tocopherol in argon-saturated $CHCl_3$/CH_3OH (2:1, v/v) was evaporated to dryness under a stream of argon. Microsomes in Tris-glycerol buffer were added. The tubes were stoppered, inverted several times, wrapped with aluminum foil, left at room temperature for 10 min, and then stored overnight at 4°C. Any vitamin E not associated with the microsomal membranes was removed by diluting the microsomes five-fold with argon-saturated Tris-glycerol buffer followed by microsomal resedimentation. Nearly 100% of the added vitamin E was found associated with the microsomes.

The total (reduced plus reversibly oxidized) vitamin E content of the microsomes was assayed by the method of Desai (9), except that saponification was not done. Saponification has no effect on microsomal vitamin E assays (data not shown). In this method, sample aliquots were placed into ethanol/saturated NaCl (1:1, v/v) containing ascorbate prior to hexane extraction. Total vitamin E analyses were done in triplicate and the mean concentration and standard deviation were calculated. Values were expressed as nmol/mg microsomal protein. All vitamin E concentration data are presented as total vitamin E except as noted.

The reduced form (only) of vitamin E was measured by the modification of Fukuzawa *et al.* (10), in which sample aliquots were placed directly into hexane, i.e., immediate hexane extraction. These samples were done in quadruplicate and the mean concentrations and standard deviations calculated. Values were expressed as nmol/mg microsomal protein. All vitamin E concentrations were determined by reverse phase high pressure liquid chromatography with fluorescence detection using a column (250 mm × 4.6 mm, internal diameter) packed with Econosphere C18, 5 micron (Alltech/Applied Science, Deerfield, IL/College Station, PA) as previously outlined (11).

When added directly to incubation aliquots immediately prior to vitamin E extraction, GSH did not increase vitamin E content above values obtained without GSH. Thus, GSH-dependent reduction of reversibly oxidized vitamin E to the reduced form did not occur during vitamin E assay procedures.

RESULTS

The microsomes used in this study to evaluate the requirement for vitamin E in the presence of GSH originated from rats fed a vitamin E deficient diet. For other comparisons, the microsomes used originated from rats fed a standard laboratory ration. There was concern that the two groups of microsomes would peroxidize at different rates (in the absence of GSH), precluding direct comparison. This concern was based on the different fatty acid compositions of the liver microsomal lipids from rats fed the two rations with differing dietary fat content. Microsomes from rats fed the vitamin E deficient diet contained a higher amount of arachidonic acid (data not shown) as a result of a greater amount of linoleic acid in the diet (12). Higher amounts of arachidonic acid have been associated with faster rates of MDA formation (13,14). When we tested the microsomes from the two sources we found that in spite of the difference in microsomal fatty acid composition, the rates of MDA production were similar (data not shown). Therefore, for the purposes of this study, microsomes from rats fed the vitamin E deficient or the standard laboratory diet were considered the same.

The validity of the methods for determining total and reduced vitamin E was evaluated by doing parallel assays under varying conditions. Immediate hexane extraction of samples was used to determine reduced vitamin E levels. Placement of samples in ascorbate containing ethanol/saturated NaCl (1:1, v/v) followed later by hexane extraction was used for total vitamin E levels. Evidence for the distinction between reduced and total vitamin E based on different assay procedures is as follows: When there was no lipid peroxidation (control data), results from the two assays were very similar. (There was no peroxidation in these controls because the lipid peroxidation systems were incomplete rather than inhibited by high levels of vitamin E. ADP-Fe^{+3} was present in some of the controls.) The similarity of results when iron was present also showed that iron dependent oxidation of vitamin E was not occurring during the immediate hexane extraction assay procedure. Ascorbate was necessary to prevent iron-dependent oxidation of vitamin E when the samples were first placed in 50% ethanol/saturated NaCl, i.e., when the procedure of Desai (9) was used.

Similar vitamin E values from both assay methods also were obtained when GSH was included in microsomal peroxidation incubation in which peroxidation was inhibited by vitamin E. This result was due to the ability of GSH to maintain vitamin E in the reduced form.

When using ascorbate (in ethanol/saturated NaCl, 1:1, v/v) followed later by hexane extraction, we found no decrease in vitamin E levels during inhibition of peroxidation by high vitamin E levels. Despite the results indicating an apparently constant level of vitamin E, it was probable that vitamin E was being oxidized during inhibition of peroxidation. This discrepancy between assay results, indicating no oxidation of vitamin E and an expected slow decline resulting from inhibition of peroxidation, can be explained if ascorbate was capable of reducing the oxidized vitamin E. Using the immediate hexane extraction method, a gradual decrease in vitamin E was measured. This result was more consistent with the expected role of vitamin E in inhibition of peroxidation. Others, e.g., Fukuzawa *et al.* (10) have obtained a similar slow decline in a liposome containing system.

The final piece of evidence supporting the difference between the assay methods and, hence, our use of reduced and total vitamin E, was obtained from peroxidation incubations in which vitamin E was insufficient to inhibit peroxidation. Under these circumstances, immediate hexane extraction assays showed a very rapid loss of vitamin

E prior to commencement of rapid peroxidation (MDA production). When the ascorbate method was used, vitamin E levels dropped much more slowly despite rapid MDA production and did not become unmeasurable for at least the first 10 min of the peroxidation incubation (data not shown).

In summary, the vitamin E assay in which there was immediate hexane extraction yielded only the reduced form of vitamin E, while the assay which used ascorbate prior to extraction yielded total vitamin E, i.e., both reversibly oxidized and reduced vitamin E presumably by allowing reduction of reversibly oxidized vitamin E to the reduced form.

Using the described rat liver microsomal NADPH and ADP-Fe^{+3} dependent lipid peroxidation system, we first tested the effects of adding GSH on the rate of MDA production. In the absence of GSH, and with (total) vitamin E concentrations of 0.004 or 1.17 nmol/mg protein, peroxidation occurred at a rapid rate (Fig. 1). Inhibition of peroxidation was observed at 2.85 nmol/mg protein concentration of vitamin E. This inhibitory amount of vitamin E is roughly 25-fold higher than the amount present in the microsomes from rats fed the standard laboratory ration. In the presence of GSH, peroxidation was inhibited at a lower concentration of vitamin E (1.17 nmol/mg protein) in addition to the higher concentration which was inhibitory in the absence of GSH. When microsomes from rats fed a vitamin E deficient diet (which contained 0.004 nmol vitamin E/mg protein) were used, the presence of GSH had little effect on peroxidation (Fig. 1). This indicated that the observed inhibition could not be attributed solely to GSH. We concluded from these studies that inhibition of lipid peroxidation required vitamin E, and the concentration of vitamin E necessary for inhibition was lowered by GSH addition.

As reported by Reddy *et al.* (3), we also found that the GSH had no effect when aged microsomes were used. Use of aged microsomes (1-month-old) resulted in rates of MDA production that were similar regardless of the presence or absence of GSH (Fig. 2). At the vitamin E concentrations used in these incubations, inhibition occurred when we used recently isolated microsomes. This phenomenon is probably due to the fact that aged microsomes have lost their GSH-dependent vitamin E reductase activity (3).

To determine if GSH was maintaining reduced vitamin E concentrations as has been proposed (3), we measured reduced vitamin E concentrations in reaction mixtures containing vitamin E sufficient to inhibit peroxidation in the presence of GSH. In incubations in which lipid peroxidation was completely inhibited by inclusion of GSH, vitamin E (reduced form) concentrations were stable at about 0.28 nmol/mg protein over the incubation (Fig. 3). During this time, the MDA concentration rose only slightly, indicating a vitamin E sparing effect of GSH. In incubations without GSH, MDA production occurred at a rapid rate and the vitamin E (reduced form) fell to below the limit of detection ($\leq$ 0.01) nmol/mg protein) by the first time point (5 min, Fig. 3). In incubations in which inhibition was incomplete with peroxidation beginning after a lag period, vitamin E (reduced form) concentrations declined with the increase in MDA production (data not shown) supporting the hypothesis that inhibition of peroxidation depends on whether propagation or termination reactions predominates, not *per se* on the presence of vitamin E.

To examine the effect of vitamin E concentration on lipid peroxidation, we determined the (total) vitamin E concentrations and the rates of MDA production in incubations with and without GSH. Microsomes containing

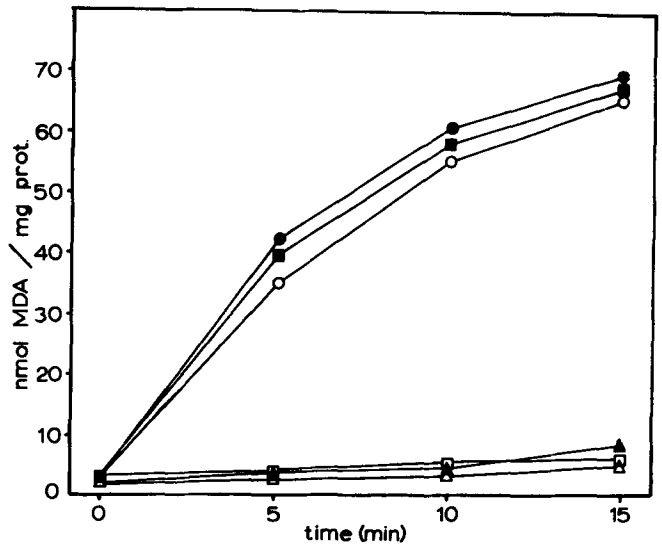

FIG. 1. The effect of adding GSH to an NADPH and ADP-Fe^{+3} dependent lipid peroxidation system utilizing rat liver microsomes containing varying amounts of vitamin E. Reaction mixutres contained microsomes (0.6 mg protein/ml), ADP-Fe^{+3} (500 μM ADP, 100 μM $FeCl_3$), and NADPH (0.4 mM) in 50 mM NaCl, pH 7.0. GSH (5 mM) was added at the time of initiation. Microsomal vitamin E concentrations were 0.004 (● ○), 1.17 (■ □) and 2.85 (▲ △) nmol/mg microsomal protein. Incubations with (○ □ △), and without (● ■ ▲) GSH.

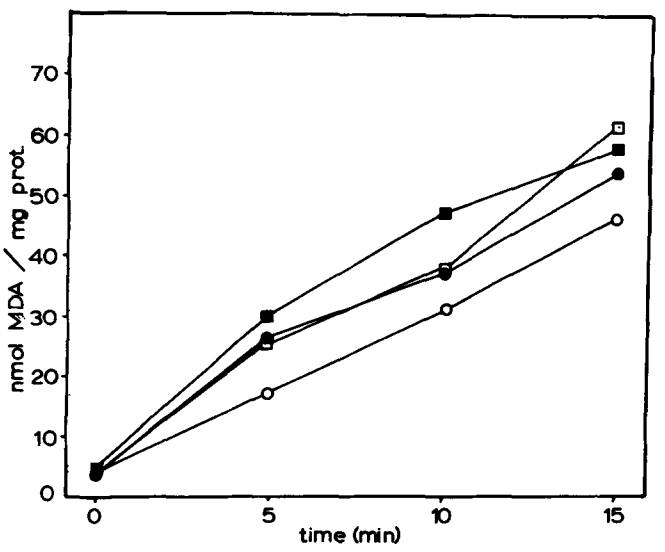

FIG. 2. The effect of adding GSH to an NADPH and ADP-Fe^{+3} dependent lipid peroxidation system utilizing aged (one-month-old) rat liver microsomes containing varying amounts of vitamin E. Reaction mixtures contained microsomes (0.6 mg protein/ml), ADP-Fe^{+3} (500 μM ADP, 100 μM $FeCl_3$), and NADPH (0.4 mM) in 50 mM NaCl, pH 7.0. GSH (5 mM) was added at the time of initiation. Microsomal vitamin E concentrations were 0.36 (■ □), and 0.56 (● ○) nmol/mg microsomal protein. Incubations with (□ ○), and without (■ ●) GSH.

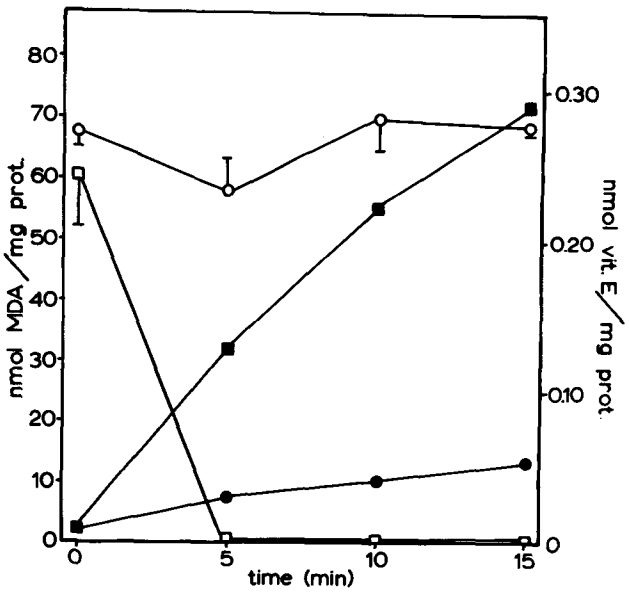

FIG. 3. The effect on vitamin E concentrations (reduced form) and malondialdehyde production over time of adding GSH to an NADPH and ADP-Fe^{+3} dependent lipid peroxidation system utilizing rat liver microsomes. Reaction mixtures contained microsomes (0.6 mg protein/ml), ADP-Fe^{+3} (500 μM ADP, 100 μM $FeCl_3$), and NADPH (0.4 mM) in 50 mM NaCl, pH 7.0. GSH (5 mM) was added at the time of initiation. Vitamin E concentrations (○□) and MDA (● ■) concentrations. Incubations with (○ ●), and without (□ ■) GSH.

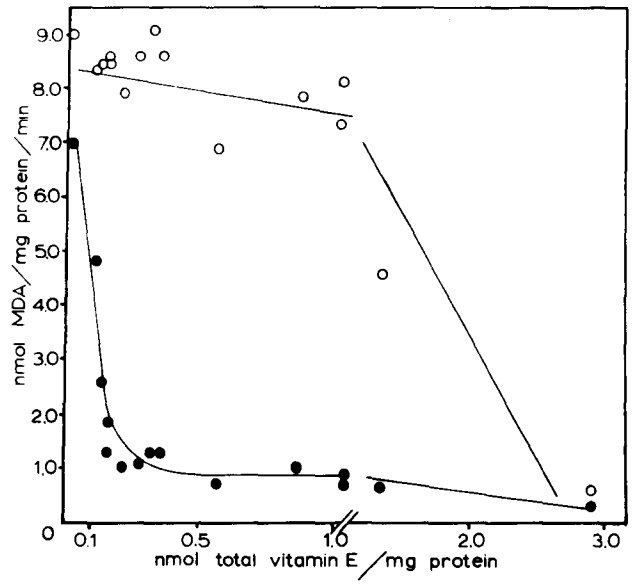

FIG. 4. The effect on the rate of lipid peroxidation of adding GSH to an NADPH and ADP-Fe^{+3} dependent lipid peroxidation system utilizing rat liver microsomes containing varying amounts of vitamin E. Reaction mixtures contained microsomes (0.6 mg protein/ml), ADP-Fe^{+3} (500 μM ADP, 100 μM $FeCl_3$), and NADPH (0.4 mM) in 50 mM NaCl, pH 7.0. GSH (5 mM) was added at the time of initiation. Incubations with (●), and without (○) GSH.

vitamin E varying from 0.004 to 2.85 nmol/mg protein were used. In the presence of GSH, rates of MDA production decreased rapidly with vitamin E concentrations from 0.004 to 0.3 nmol/mg protein (Fig. 4). In contrast, in the absence of GSH, no change in MDA production was observed at vitamin E concentrations up to about 1.0 nmol/mg protein. At the 0.004 nmol vitamin E/mg protein level, rates of peroxidation were 7.2 and 9.0 nmol MDA/mg protein/min with and without GSH, respectively (Fig. 4). With GSH and 0.11 nmol vitamin E/mg protein (the amount present in microsomes at isolation from rats fed the standard laboratory ration), the rate of MDA production sharply declined to 4.9 nmol/mg protein/min (Fig. 4). With vitamin E added to 0.22 nmol/mg protein, the rate of peroxidation was 1.06 vs 7.8 nmol MDA/mg protein/min with and without GSH, respectively, which represented an 86% inhibition of lipid peroxidation. Subsequent increases in the microsomal vitamin E concentration (up to 1.0 nmol/mg protein) did not increase the degree of inhibition, which remained between 85 and 90%. In the absence of GSH, rates of peroxidation were similar (7.8–9.1 nmol/mg protein/min) for all vitamin E concentrations of less than 1.0 nmol (total) vitamin E/mg protein. At vitamin E concentrations greater than 1.0 nmol/mg protein, peroxidation was increasingly inhibited until essentially complete. Complete inhibition of peroxidation occurred at levels of microsomal vitamin E around 3 nmol/mg protein whether in the presence or absence of GSH (Fig. 4).

DISCUSSION

In the absence of GSH, no inhibition of microsomal NADPH and ADP-Fe^{+3} dependent lipid peroxidation was observed until very high levels of vitamin E were attained (Fig. 1). In the presence of GSH, however, inhibition occurred at the intermediate and high levels of vitamin E, but not at the low level. These data support previous observations that inhibition of microsomal lipid peroxidation occurs at lower vitamin E concentrations in the presence of GSH (3–5). The occurrence of lipid peroxidation at the very low vitamin E concentration (0.004 nmol/mg microsomal protein) in the presence of GSH was in agreement with the observations of other investigators (3–5), and indicated that GSH did not have an inhibitory effect on peroxidation that was independent of vitamin E. This is in contrast to results of Franco and Jenkins (15) who reported a direct inhibitory effect of GSH on peroxidation of lung microsomes from rats fed a vitamin E deficient diet. It is unlikely, however, that the inhibition observed in their system was due to the participation of vitamin E and the membrane protein, GSH-dependent vitamin E reductase, because they found that the effect was not specific for GSH and was not abolished by heating. In addition, it occurred at very low vitamin E concentrations. Heating destroys the activity of the purported membrane associated GSH-dependent vitamin E reductase (3,4). In the present study, we found similar rates of peroxidation at 0.004 nmol vitamin E/mg microsomal protein regardless of the presence of GSH. This not only ruled out a direct inhibitory effect of GSH that was independent of vitamin E, but also indicated that there was no pro-oxidant effect of GSH, as has been reported (13,16). Thus, in our system GSH had little direct effect on lipid peroxidation and our data support the hypothesis that inhibition requires GSH and vitamin E.

Maintenance of vitamin E concentrations (reduced form) over time during GSH and vitamin E-dependent

inhibition of lipid peroxidation (Fig. 3) suggested that reversibly oxidized vitamin E ($I^{\bullet}$) was more rapidly reduced by GSH via the purported reductase (reaction 3) than $I^{\bullet}$ was produced by lipid peroxidation (reaction 2). This was deduced by considering the likely change in vitamin E concentrations during the partial inhibition of peroxidation that occurred between approximately 0.25 and 1.0 nmol vitamin E/mg microsomal protein. Without reduction of the reversibly oxidized vitamin E by GSH, vitamin E concentrations should have decreased slowly rather than remaining constant (Figs. 3 and 4). This contention is supported by the data of Fukuzawa *et al.* (10), who reported such a decrease in vitamin E concentrations (reduced form) in a liposomal lipid peroxidation system containing high concentrations of vitamin E that were sufficient to prevent peroxidation.

The difference in MDA production between freshly isolated and aged microsomes in the presence of GSH and vitamin E (Figs. 1 and 2) demonstrated that a storage labile membrane associated factor was necessary to cause inhibition of peroxidation at lowered vitamin E concentrations, as reported previously (3). Despite the presence of vitamin E and GSH, rapid MDA production in aged microsomes occurred (Fig. 2). This indicated that direct reduction of reversibly oxidized vitamin E by GSH was not an important mechanism in inhibition of microsomal lipid peroxidation. In other words, GSH required the participation of the putative membrane associated GSH-dependent vitamin E reductase. The requirement for a membrane protein for reduction of vitamin E during the peroxidation incubations differs from the results of Niki *et al.* (17), who found that direct reduction of reversibly oxidized vitamin E (the chromanoxyl radical) by GSH occurred with vitamin E dissolved in benzene. The most likely reason for the difference in results is because we used a membrane system in an aqueous suspension rather than vitamin E dissolved in benzene.

In previously published reports, inhibition of lipid peroxidation occurred with addition of GSH to microsomes isolated from rats fed vitamin E sufficient rations (3–5). In the present study, two-fold more vitamin E than the amount present at microsomal isolation from rats fed the standard laboratory ration was needed in order to achieve 85–90% inhibition (Fig. 4). This apparent difference in the inhibitory vitamin E concentration may have been due to the amount of vitamin E present in the isolated microsomes. Of the authors who reported this phenomenon, Reddy *et al.* (3) were the only ones to report vitamin E concentrations. They found three to five-fold more vitamin E present than reported here. When we used the higher concentrations, we also found inhibition.

In the absence of GSH, lipid peroxidation was inhibited at high vitamin E concentrations, while at the lower concentrations peroxidation was unaffected (Fig. 4). These results are similar to those obtained in liposomal lipid peroxidation systems in which peroxidation was inhibited when the vitamin E concentration exceeds a threshold value (10,11,16). Similarities between vitamin E dependent inhibition in liposomes in which vitamin E incorporation into the membrane is known to occur (18,19) and microsomes also indicated that the method used to add vitamin E to microsomes resulted in its incorporation into the membrane.

With the addition of GSH, 85–90% inhibition of lipid peroxidation was achieved with vitamin E between 0.2 and 1.0 nmol/mg microsomal protein; while in the absence of GSH, the rate of lipid peroxidation did not begin to decrease until vitamin E values were greater than 1.0 nmol/mg protein (Fig. 4). It is important to point out, however, that at 85–90% inhibition there was a gradual increase in MDA with time (Fig. 3). At a very high level of vitamin E, peroxidation was inhibited completely, regardless of the presence of GSH (Fig. 4). This result, in conjunction with the finding that the concentration of vitamin E (reduced form) was maintained in incubations where there was partial inhibition of MDA production (Fig. 3), suggested that at vitamin E concentrations sufficient to inhibit peroxidation (in the presence of GSH) there was still a limited amount of propagation occurring. Perhaps this was due to insufficient vitamin E present to react with all the lipid radicals generated. The limited mobility of vitamin E in the membrane may be responsible for this phenomenon (2).

In summary, we found that addition of GSH to a microsomal lipid peroxidation system lowered the concentration of vitamin E required to inhibit peroxidation in a concentration dependent manner. The amount of vitamin E necessary to achieve greater than 85% inhibition, however, was still two to five-fold higher than the amount present in the microsomes at isolation.

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Effect of Dietary α-Linolenic Acid on n-3 Fatty Acids of Rainbow Trout Lipids

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The effects of α-linolenic acid enriched diets on the lipid composition of rainbow trout *(Oncorhynchus mykiss)* muscle and liver were determined. Diets containing 0, 4, 10 or 20% (wt/wt) linseed oil were fed to 280 trout for up to 64 days. Linolenic acid levels in total lipid, neutral lipid and phospholipid of liver and muscle increased with duration of intake. The linolenic acid content of total lipid in muscle increased from 10 mg to 355 mg/100 g flesh. There was no significant increase in the elongated desaturated products, i.e., eicosapentaenoic or docosahexaenoic acids, in either tissue during the feeding period.
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Much research has been conducted concerning the necessity of dietary intake of n-3 fatty acids as well as the presence of an active Δ-6 desaturase in rainbow trout *(Oncorhynchus mykiss)* (1–5). Supplementation of a diet devoid of n-3 fatty acids with linolenic 18:3n-3 (LNA), eicosapentaenoic 20:5n-3 (EPA) or docosahexaenoic 22:6n-3 (DHA) acids prevented essential fatty acid deficiency signs in rainbow trout (1,6). Fish consuming diets containing only 1% LNA, as a n-3 polyunsaturated fatty acid (PUFA(source, showed no deficiency signs, suggesting that an active Δ-6 desaturase is present in rainbow trout (6). Takeuchi and Watanabe (7) reported an enhanced potency of the long chain n-3 PUFA, EPA and DHA, in preventing deficiency signs as compared to the precursor LNA in rainbow trout. This suggested that the Δ-6 desaturase may have limited or highly regulated activity.

Isolated hepatocytes from cold water acclimiated rainbow trout have greater Δ-6 desaturase activity than hepatocytes from warm water acclimiated fish (8,9). Hagve *et al.* (10) reported Δ-6 activity in rainbow trout liver similar to that of rat liver, and that LNA was a better substrate than linoleic acid 18:2n-6 (LA).

The percentage of n-3 PUFA in farm raised Atlantic Salmon *(Salmo salar)* is lower than that of their wild counterparts, presumably because of the lack of algae, zooplankton and other natural organisms containing n-3 PUFA in commercial diets (11). Enrichment of fish tissue with n-3 PUFA may be possible by dietary manipulation over relatively short periods of time (12). Few studies, however, have investigated the alteration of fish fatty acid composition for the improvement of human nutrition. When farm raised catfish were fed a diet containing 6% menhaden oil, the n-3 PUFA content was increased from 3 to 10% in 12 weeks (13). Satoh *et al.* (14) increased the n-3 PUFA content of channel catfish polar

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Abbreviations: EDTA, ethylenediaminetetraacetic acid; EPA, eicosapentaenoic acid (20:5n-3); DHA, docosahexaenoic acid (22:6n-3); LNA, linolenic acid (18:3n-3); NL, neutral lipids; PL, phospholipids; PUFA, polyunsaturated fatty acids; OL, oleic acid (18:1n-9); TBA, thiobarbituric acid; TRIS, tris(hydroxymethyl)aminomethane.

TABLE 1

Composition of Basal Diet Fed to Rainbow Trout

Ingredient	g/100 g
Herring meal	30.0
Full fat soybean meal	10.0
Corn gluten meal	15.0
Liver meal	5.0
Brewer's dried yeast	5.0
What middlings	8.0
Dried whey	2.0
Vitamin mix[a]	3.0
Mineral mix[b]	2.0
Added fat (see Table 2)	20.0

[a]Vitamin mix provided: 7,400 IU retinyl palmitate, 2000 IU cholecalciferol, 210 IU tocopherol per kg of diet and the following viatmins (mg/kg): ascorbic acid, 250; D-biotin, 0.4; folic acid, 5.0; choline chloride (70%), 1000; niacinamide, 0.150; D-calcium pantothenate, 85.0; pyridoxine-HCL, 25.0, riboflavin, 25.0; thiamin-HCL, 45.0; menadione, 5.0; ethoxygquin, 150; and carrier.

[b]Mineral mix provided per kg: $CaHPO_4.2H_2O$, 5.2 g; $NaH_2PO_4.H_2O$, 21.0 g; NaCl, 3.0 g; $MnSO_4.7H_2O$, 121 mg; KI, 6.0 mg; $ZnSO_4.7H_2O$, 126 mg; and carrier.

TABLE 2

Added Fat in Experimental Diets Fed to Rainbow Trout

Diet	Linseed oil g/100 g diet	Olive oil g/100 g diet
A	0	20
B	4	16
C	10	10
D	20	0

TABLE 3

Weight Percent Fatty Acid in Experimental Diets Containing 0 (A), 4 (B), 10 (C) and 20 (D) wt % Linseed Oil (mean ± SEM n = 8)

Fatty acid	Diet A	B	C	D
14:0	0.46 ± .11	0.67 ± .10	0.67 ± .10	0.65 ± .14
16:0	15.24 ± .30	14.89 ± .61	12.62 ± .30	10.73 ± .45
16:1	1.65 ± .05	1.49 ± .61	1.18 ± .03	0.86 ± .09
18:0	3.36 ± .02	3.13 ± .48	3.56 ± .03	3.73 ± .05
18:1	57.58 ± .25	47.43 ± 2.16	40.62 ± .85	24.12 ± 4.08
18:2	16.03 ± .04	18.05 ± .71	18.73 ± .15	21.33 ± .62
18:3n-3	1.70 ± .11	9.94 ± 1.25	18.51 ± .83	34.65 ± 3.97
20:1	1.27 ± .04	1.33 ± .05	1.34 ± .04	1.20 ± .15
20:5n-3	0.60 ± .03	09.61 ± .03	0.55 ± .07	0.65 ± .03
22:1n-9	1.41 ± .11	1.48 ± .10	1.51 ± .11	1.45 ± .05
22:6n-3	0.70 ± .09	0.99 ± .26	0.70 ± .11	0.63 ± .04

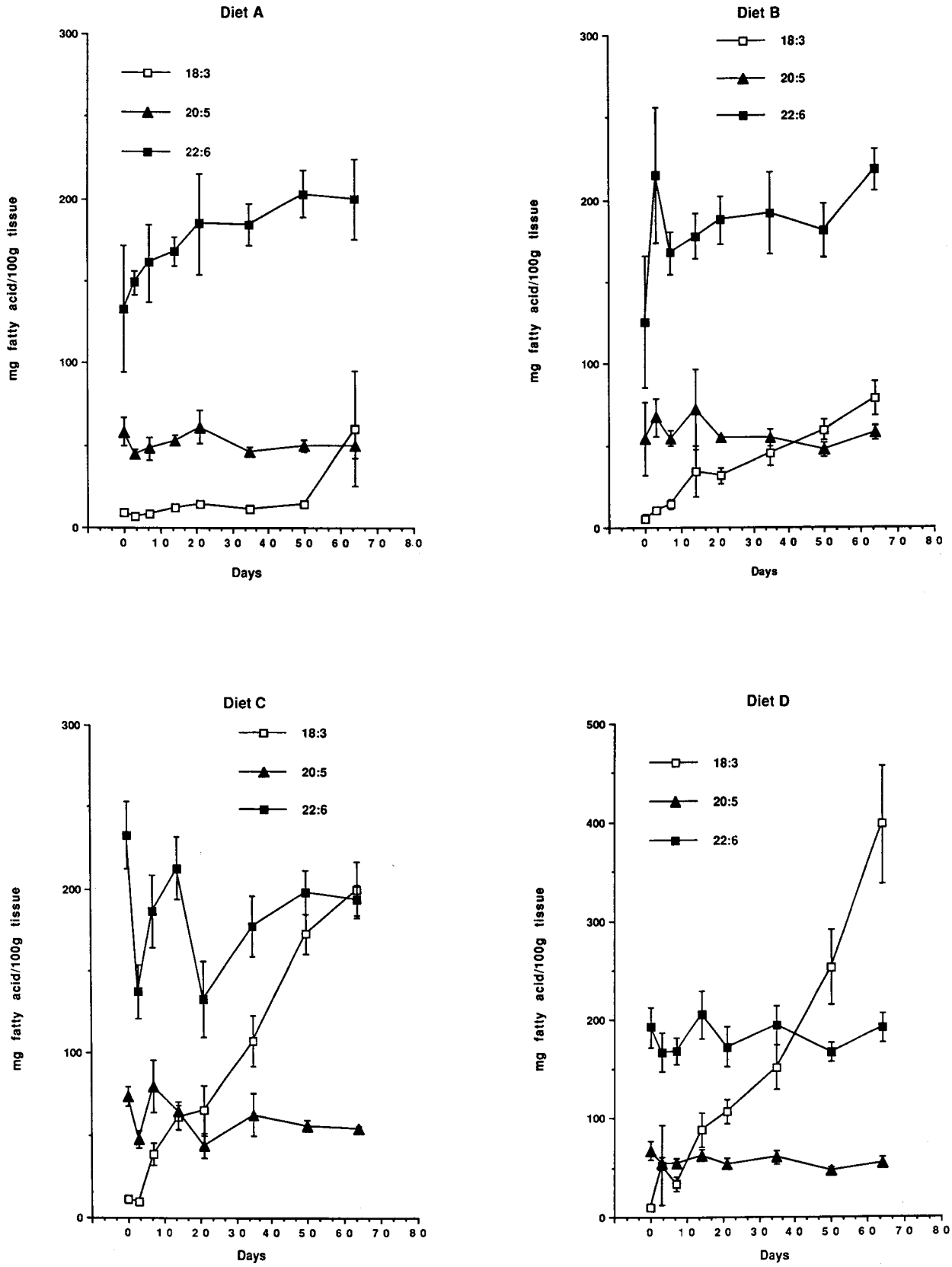

FIG. 1. Changes in fatty acid content of flesh (mg fatty acid/100 g fillet) for fish fed diets containing 0 (A), 4 (B), 10 (C) and 20 (D) wt % linseed oil during the experimental period (mean ± SEM, n = 9).

TABLE 4

The Proximate Composition of Rainbow Trout Maintained on Four Experimental Diets for 64 Days (mean ± SEM n = 3)

Diet	Market ready fish			Viscera		
	% Protein	% Lipid	% H_2O	% Protein	% Lipid	% H_2O
A	18.3 ± 1.0	8.4 ± 1.3	70.6 ± .3	11.5 ± .4	27.8 ± 1.7	55.4 ± 1.0
B	18.1 ± .5	7.9 ± 1.3	71.4 ± .4	10.8 ± .2	25.2 ± 1.8	58.2 ± .5
C	17.9 ± .5	7.2 ± 1.6	72.4 ± .5	10.8 ± 1.5	23.2 ± 3.3	59.8 ± 1.6
D	18.0 ± .2	7.8 ± .6	71.6 ± .2	10.8 ± 1.6	23.7 ± 2.9	59.8 ± 1.3

No significant differences between treatments were observed at $p \leq 0.05$.

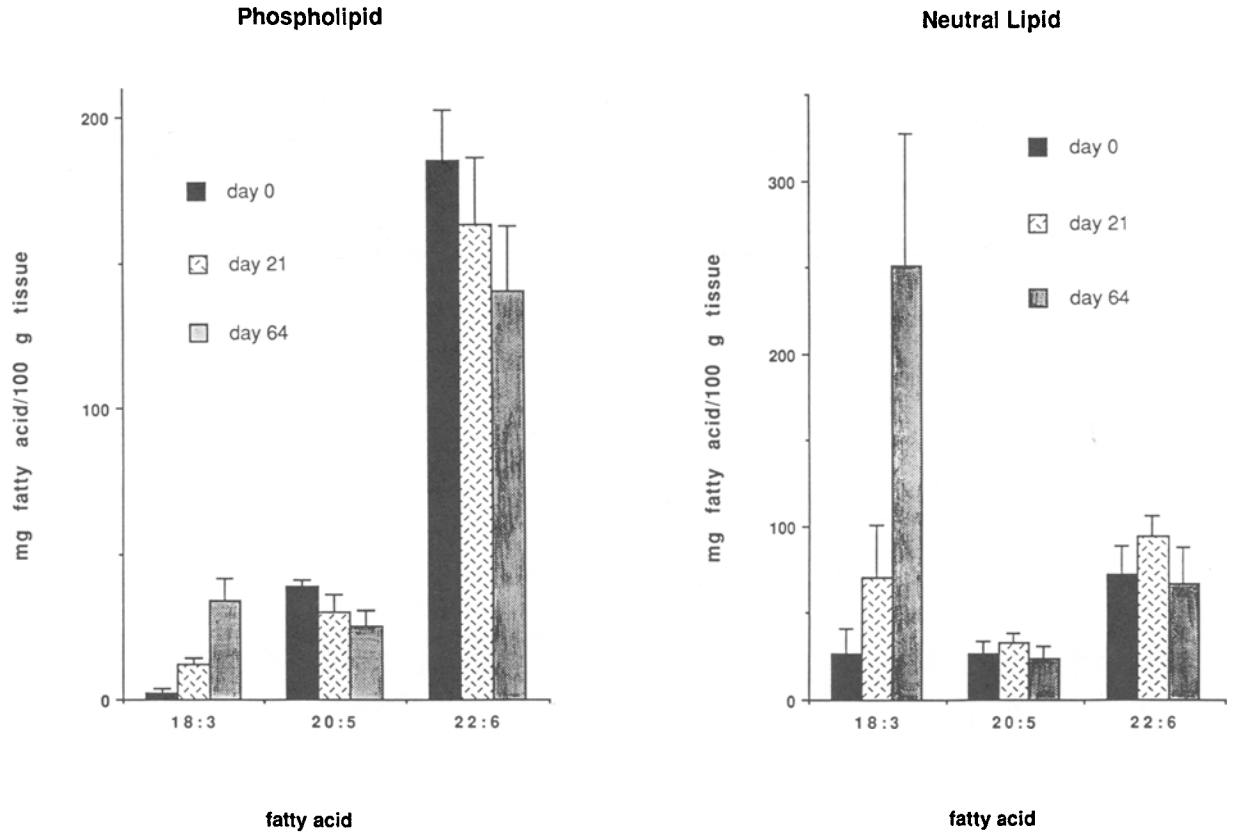

FIG. 2. Changes in fatty acid composition (mg fatty acid/100 g muscle tissue) of phospholipid and neutral lipid from rainbow trout fed a 20% linseed oil diet (mean ± SEM, n = 3).

liver lipid by feeding a diet high in LNA, as well as with diets high in EPA and DHA.

A finishing diet high in n-3 fatty acids might be employed to increase the n-3 fatty acids in rainbow trout produced by aquaculture. Feeding a diet high in LNA may elevate the elongation and desaturation products, EPA and DHA, in rainbow trout tissue. An enrichment of n-3 fatty acids in foods may be desirable in light of epidemiological and clinical research, suggesting that a diet high in n-3 PUFA is beneficial in ameliorating the risk of several chronic diseases, including cardiovascular disease (15).

The objectives of this research were to investigate the effect of dose and time on the uptake and desaturation/elongation of LNA (from linseed oil) to EPA and DHA in rainbow trout. Fish were fed four diets with 0, 4, 10 and 20 wt % linseed oil which resulted in diets containing 2, 10, 18 and 35 wt % LNA for 64 days, and the changes in fatty acid composition of flesh and liver were determined.

MATERIALS AND METHODS

Fish. Rainbow trout under one year of age (288 fish, mean weight = 92 g, SEM = 4.35) were randomly assigned to 12 experimental tanks (20 L capacity) supplied with 4.7 L/min of aerated well water at 9–10°C at the Tunison Laboratory of Fish Nutrition, Cortland, New York (24 fish per tank, three tanks per dietary treatment). The fish were allowed to acclimate to the tanks for one month prior to

initiating the feeding study. Daily feed levels were calculated from the weight of fish in each tank according to the method of Buterbaugh and Willoughby (16), using a hatchery constant of 8.5. The daily ration of feed was equal to about 1% of the body weight of fish. Fish were weighed weekly and feeding rates adjusted accordingly. Fish were fed five times daily, seven days a week between 0700 hr and 1600 hr.

Diets. Four isonitrogenous and isocaloric diets with 0, 4, 10, or 20 wt % linseed oil (Hain Pure Foods, Los Angeles, CA) were prepared bimonthly (Tables 1 and 2). Cold, pressed, olive oil was added to obtain diets containing 20 wt/wt % added fat. The total fat content of the diets was 26%. The experimental diets were mixed and pelleted without steam in a laboratory scale pellet mill and stored in double-ply plastic storage bags, flushed with nitrogen, and maintained at 4°C. Upon completion of the feeding phase of the study and prior to analyses, the diet samples were stored at −70°C. The fatty acid composition of the diets was determined as described below. A modified TBA test (17) was performed on the diets to determine the extent of lipid oxidation.

Reagents. All chemicals were of reagent grade and were purchased from commercial suppliers.

Sample collection and analyses. Fish were fed the experimental diets for up to 64 days. On days 0, 3, 7, 14, 21, 35, 50 and 64 of the feeding period, three fish were randomly selected from each of the twelve tanks and were sacrificed by decapitation. A section of the right fillet, distal to the dorsal fin (equivalent to one tenth of the total fish length), was excised for analysis. The whole liver (minus the gall bladder) and the fillet section were removed, washed of residual blood and frozen immediately in liquid nitrogen. The samples were held at −70°C until analyzed.

After partial thawing, the samples of fillet were skinned, cleaned of visible adipose tissue, weighed and homogenized (Brinkman Instruments, Westbury, NY) in a 250 mM sucrose, 10 mM tris(hydroxymethyl)aminomethane (TRIS) and 1 mM EDTA buffer (pH = 7.4) to give a 100 mg tissues/mL buffer homogenate. The lipids in tissue and diets were then extracted by the method of Bligh and Dyer (18). The fatty acid composition of the total lipid of the flesh and diets and the phospholipid of the liver were analyzed. The lipids extracted from the liver samples were separated into lipid classes on high performance thin-layer chromatography (HPTLC) plates (Merck, Darmstadt, West Germany) using chloroform/methanol (80:10, v/v) (19). The phospholipid bands were identified by comparing Rf values with known standards. Appropriate bands were scraped into tubes and 0.25 mL of toluene and 0.5 mL of 0.5 M potassium hydroxide in methanol were added, along with a known amount of pentadecanoic acid which served as an internal standard. The tubes were flushed with nitrogen and placed in an 86°C water bath for 8 min. After cooling, 1 mL 0.7 M hydrochloric acid in methanol was added. The free fatty acids were extracted twice with 1.5 mL hexane. The hexane was evaporated under a stream of nitrogen and the fatty acids methylated with diazomethane as described (19). The total lipids of flesh and diets were transesterified as described above.

The fatty acid methyl esters were quantified on a HP 5880a (Hewlett Packard, Avondale, PA) gas chromatograph with a Restek (Restek Corp., Bellefonte, PA) 0.53 mm × 30 m Stabilwax ethylene glycol column using hydrogen as the carrier gas. Fatty acids were identified according to their relative retention times as compared to a certified standard (NuChek Prep, Elysian, MN). The data were expressed as mg fatty acid per 100 g tissue.

On day 64, 15 fish from each dietary treatment were sacrificed and eviscerated for proximate analysis. Proximate analyses was run on both the viscera and the market ready fish to determine if fat, protein and moisture content of the edible portion differed between dietary treatments (20).

All fatty acid composition data were analyzed for difference with Duncan's mean separation test on SAS (21).

RESULTS AND DISCUSSION

The major difference in fatty acid composition between the four diets was the content of oleic acid, 18:1n-9 (OL) and LNA (Table 3). Diets A–D contained 57, 47, 40 and 24 wt % OL and 2, 10, 18 and 35 wt % LNA.

The concentration of malonaldehyde was approximately 45 μmoles/kg diet for each diet and di not change over the study period.

The mean weights of the fish on the four diets were similar, and all fish showed comparable weight gains (76 g) during the study period. Feed conversion (weight food fed/weight gain) for fish on the four diets was not

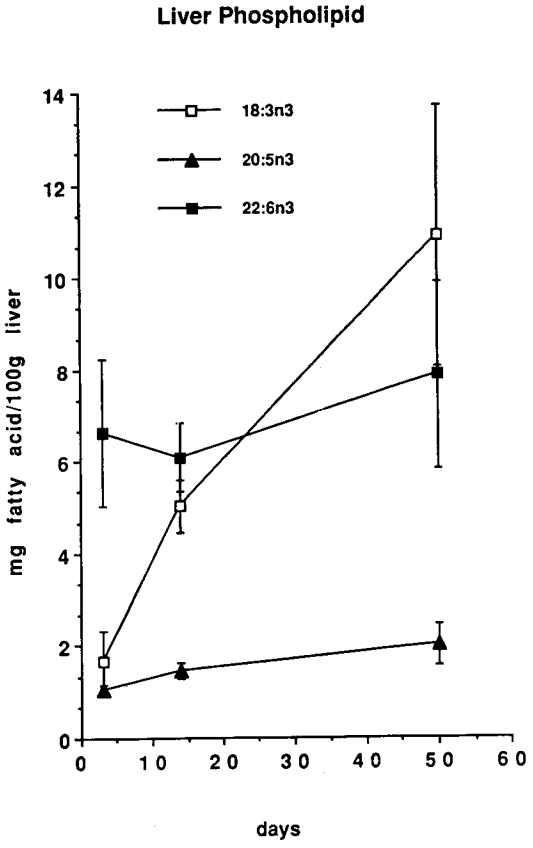

FIG. 3. Changes in fatty acid content (mg fatty acid/100 mg tissue) of liver phospholipid from trout fed 20% linseed oil (Diet D) for 3, 14 and 50 days (mean ± SEM, n = 9).

TABLE 5

Changes in Fatty Acid Composition in Trout Fillet Fed Diets Containing 0% (A), 4% (B), 10% (C) or 20% (D) Linseed Oil for Progressive Periods Up to 64 Days

Fatty acid	Day 0	Day 3	Day 7	Day 14	Day 21	Day 35	Day 50	Day 64
				mg fatty acid/100 g fillet (mean ± SEM)				
Period Fed Diet A								
14:0	118.0 ± 3.0^{a}	64.0 ± 6.7^{b,c}	90.6 ± 10.3^{a,b}	66.2 ± 5.2^{b,c}	82.7 ± 17.1^{b,c}	60.3 ± 7.3^{b,c}	50.2 ± 7.8^{d}	53.8 ± 8.5^{c}
16:0	271.4 ± 16.9^{a,b}	200.4 ± 18.1^{b}	243.9 ± 23.1^{a,b}	229.5 ± 15.6^{b}	276.5 ± 46.0^{a,b}	280.9 ± 32.8^{a,b}	255.6 ± 24.1^{a,b}	327.9 ± 32.8^{a}
16:1	137.5 ± 9.7^{a}	77.4 ± 8.6^{b}	98.9 ± 9.5^{a,b}	99.1 ± 11.7^{a,b}	112.6 ± 22.7^{a,b}	94.6 ± 10.3^{b}	85.6 ± 11.4^{b}	93.0 ± 15.8^{b}
18:0	35.1 ± 2.5^{a,c}	31.7 ± 2.6^{b}	40.3 ± 4.4^{b,c}	36.5 ± 2.4^{a,c}	46.9 ± 7.9^{b,c}	53.0 ± 6.4^{b}	50.0 ± 4.3^{b,c}	69.1 ± 4.7^{d}
18:1n-9	262.8 ± 18.8^{a}	182.9 ± 18.2^{a}	293.5 ± 30.2^{a}	390.1 ± 38.9^{a,b}	551.0 ± 98.3^{b,c}	692.2 ± 95.9^{c}	714.2 ± 87.3^{c}	941.3 ± 120.0^{d}
18:2n-6	31.9 ± 3.0^{a,b}	24.1 ± 2.1^{a}	43.5 ± 4.8^{a,b}	67.4 ± 8.3^{c}	97.0 ± 17.2^{c}	130.1 ± 17.7^{e}	142.1 ± 17.8^{e}	209.7 ± 20.9^{f}
18:3n-3	8.7 ± 2.0^{a}	6.8 ± 0.5^{a}	8.9 ± 0.7^{a}	12.8 ± 2.7^{a}	12.0 ± 2.4^{a}	11.1 ± 1.8^{a}	14.3 ± 1.5^{a}	58.9 ± 33.5^{b}
20:1n-9	140.4 ± 18.3^{a}	112.9 ± 11.1^{a}	140.0 ± 12.9^{a}	137.3 ± 10.8^{a}	149.0 ± 28.3^{a}	136.5 ± 14.0^{a}	133.1 ± 18.2^{a}	140.7 ± 22.1^{a}
20:4n-6	6.3 ± 1.5^{a}	5.7 ± 0.3^{a}	7.3 ± 0.5^{a}	8.6 ± 0.7^{a}	9.4 ± 1.5^{a}	13.2 ± 1.2^{a}	15.1 ± 1.3^{b}	20.0 ± 2.2^{c}
20:5n-3	58.8 ± 8.4^{a}	45.4 ± 2.6^{a}	54.2 ± 3.5^{a}	55.4 ± 3.6^{a}	52.6 ± 9.6^{a}	46.9 ± 3.2^{a}	120.4 ± 70.8^{a}	50.4 ± 8.4^{a}
22:1n-9	141.9 ± 25.4^{a}	109.7 ± 15.4^{a}	142.6 ± 15.2^{a}	127.2 ± 14.3^{a}	151.4 ± 28.7^{a}	125.5 ± 12.1^{a}	132.3 ± 19.5^{a}	130.6 ± 20.5^{a}
22:5n-3	25.6 ± 8.2^{a}	10.6 ± 0.9^{a}	13.1 ± 1.4^{b}	11.1 ± 1.7^{a}	11.9 ± 3.0^{b}	13.6 ± 2.7^{b}	15.6 ± 1.4^{b}	15.2 ± 2.1^{b}
22:6n-3	134.1 ± 38.5^{a}	152.0 ± 6.4^{a,b}	182.1 ± 13.2^{a,b}	174.9 ± 9.9^{a,b}	156.0 ± 25.5^{a,b}	184.6 ± 12.7^{a,b}	208.7 ± 14.1^{b}	201.8 ± 25.4^{b}
Period Fed Diet B								
14:0	104.6 ± 19.3^{a}	77.9 ± 11.7^{a,b}	85.5 ± 9.7^{a,b}	102.5 ± 41.2^{a}	67.0 ± 7.6^{a,b}	66.6 ± 6.6^{a,b}	45.3 ± 10.6^{b}	64.7 ± 8.6^{a,b}
16:0	190.5 ± 32.4^{a}	266.3 ± 47.1^{a}	219.2 ± 17.5^{a}	300.6 ± 91.0^{a}	233.7 ± 22.9^{a}	269.9 ± 19.2^{a}	271.0 ± 26.8^{a}	322.9 ± 26.7^{a}
16:1	228.5 ± 92.5^{a}	111.8 ± 18.6^{b}	111.3 ± 14.8^{b}	149.5 ± 63.5^{a,b}	93.6 ± 7.9^{b}	100.2 ± 10.1^{b}	75.3 ± 12.7^{b}	106.3 ± 11.9^{b}
18:0	23.8 ± 4.7^{a}	38.9 ± 6.6^{a,b}	31.0 ± 2.1^{a,b}	51.2 ± 14.3^{b,c,d}	45.1 ± 4.8^{b,c,d}	50.6 ± 3.0^{b,c,d}	55.3 ± 5.0^{c}	64.8 ± 5.9^{d}
18:1	240.3 ± 49.4^{a}	243.4 ± 38.7^{a}	287.7 ± 37.9^{a,b}	532.5 ± 264.4^{a,b,c}	461.3 ± 54.4^{a,b,c}	612.7 ± 83.0^{b,c}	682.0 ± 71.1^{c,d}	949.8 ± 109.3^{d}
18:2n-6	31.0 ± 7.1^{a}	33.0 ± 5.4^{a}	46.3 ± 7.1^{a}	172.7 ± 72.8^{b,c}	98.3 ± 14.1^{a,b}	136.7 ± 20.0^{b}	166.1 ± 16.7^{b,c}	230.3 ± 26.8^{c}
18:3n-3	5.6 ± 2.9^{a}	10.3 ± 2.0^{a,b}	14.0 ± 2.9^{b,c}	35.6 ± 16.3^{c,d,e}	32.1 ± 4.7^{b,c}	45.6 ± 7.4^{d,e}	59.0 ± 6.3^{e,f}	79.4 ± 10.2^{f}
20:1n-9	121.6 ± 31.1^{a}	129.4 ± 27.0^{a}	131.8 ± 10.0^{a}	187.5 ± 69.3^{a}	144.3 ± 9.1^{a}	144.5 ± 17.1^{a}	105.7 ± 16.6^{a}	147.3 ± 18.0^{a}
20:4n-6	5.3 ± 1.8^{a}	7.6 ± 1.3^{a,b}	7.2 ± 0.6^{a,b}	11.0 ± 3.6^{b,c}	8.2 ± 1.0^{a,b,c}	10.8 ± 1.2^{b,c}	12.6 ± 1.2^{c,d}	16.1 ± 0.9^{d}
20:5n-3	57.5 ± 16.4^{a}	68.7 ± 11.0^{a}	54.3 ± 5.4^{a}	75.1 ± 25.6^{a}	55.1 ± 1.3^{a}	55.3 ± 5.3^{a}	47.4 ± 4.7^{a}	58.2 ± 4.3^{a}
22:1n-9	91.6 ± 28.5^{a}	143.5 ± 22.3^{a}	122.8 ± 10.4^{a}	181.3 ± 71.0^{a}	144.2 ± 12.8^{a}	150.6 ± 20.1^{a}	179.8 ± 76.8^{a}	145.0 ± 20.2^{a}
22:5n-3	9.8 ± 5.1^{a}	16.4 ± 2.6^{a}	11.1 ± 1.6^{a}	19.7 ± 7.9^{a}	14.3 ± 2.5^{a}	15.4 ± 2.5^{a}	18.3 ± 6.3^{a}	21.6 ± 5.5^{a}
22:6n-3	128.8 ± 40.9^{a}	219.4 ± 38.2^{a}	166.2 ± 14.4^{a}	233.1 ± 71.7^{a}	188.5 ± 15.0^{a}	193.0 ± 24.7^{a}	180.2 ± 16.5^{a}	219.1 ± 12.2^{a}

(continued)

TABLE 5 *(Continued)*

Fatty acid	Day 0	Day 3	Day 7	Day 14	Day 21	Day 35	Day 50	Day 64
	mg fatty acid/100 g fillet (mean ± SEM)							
Period Fed Diet C								
14:0	82.0 ± 5.2^{a,b}	84.2 ± 10.7^{a,b}	111.8 ± 30.5^{a}	91.8 ± 10.9^{a,b}	81.4 ± 25.4^{a,b}	76.3 ± 24.5^{a,b}	51.6 ± 3.8^{b}	47.6 ± 4.5^{b}
16:0	429.6 ± 204.3^{a}	233.7 ± 22.7^{a}	253.4 ± 26.0^{a}	292.8 ± 25.8^{a}	207.1 ± 36.2^{a}	303.6 ± 49.8^{a}	269.6 ± 17.5^{a}	291.9 ± 22.8^{a}
16:1	127.7 ± 7.9^{a,b}	104.2 ± 14.0^{a,b}	159.4 ± 45.2^{a}	133.1 ± 16.7^{a,b}	74.7 ± 13.8^{b}	112.5 ± 31.26^{a,b}	79.1 ± 6.3^{b}	78.9 ± 6.8^{b}
18:0	34.1 ± 3.4^{a}	34.9 ± 3.7^{a}	38.7 ± 3.9^{a}	50.0 ± 4.8^{a,b}	40.3 ± 7.0^{a}	61.8 ± 6.7^{b,c}	60.9 ± 3.6^{b,c}	73.2 ± 6.6^{c}
18:1	301.0 ± 20.3^{a,b}	217.8 ± 23.8^{a}	404.21± 98.0^{a,b,c}	447.0 ± 48.7^{b,c,d}	339.5 ± 67.5^{a,b}	566.5 ± 92.1^{c,d}	627.1 ± 46.5^{d,e}	780.9 ± 65.8^{e}
18:2n-6	37.1 ± 2.4^{a}	29.5 ± 3.0^{a}	72.8 ± 16.1^{a,b}	100.1 ± 10.7^{b}	92.8 ± 20.1^{b}	155.9 ± 22.7^{c}	208.8 ± 15.5^{d}	257.3 ± 21.5^{e}
18:3n-3	11.8 ± 0.5^{a}	10.1 ± 0.9^{a}	39.1 ± 7.3^{a,b}	60.6 ± 7.7^{b}	62.7 ± 14.1^{b}	111.0 ± 15.6^{c}	171.2 ± 11.8^{d}	202.3 ± 17.9^{d}
20:1n-9	182.4 ± 14.8^{a,b}	133.3 ± 15.5^{a}	198.8 ± 54.3^{a,b}	163.8 ± 17.4^{a,b}	112.0 ± 19.7^{b}	162.5 ± 36.5^{a,b}	108.0 ± 7.4^{b}	123.4 ± 9.1^{a,b}
20:4n-6	9.8 ± 0.6^{a,d}	5.9 ± 0.5^{b}	9.5 ± 2.1^{a,c,d}	8.8 ± 0.7^{a,b,c}	6.2 ± 1.0^{a,b}	11.1 ± 1.8^{c,d}	12.1 ± 0.6^{c,d}	12.9 ± 0.8^{d}
20:5n-3	76.5 ± 5.3^{a,b}	50.7 ± 4.2^{b,c}	80.5 ± 17.1^{a}	64.3 ± 6.0^{a,b,c}	42.7 ± 6.9^{c}	64.3 ± 13.3^{a,b,c}	55.7 ± 3.3^{a,b,c}	54.4 ± 1.9^{c}
22:1n-9	183.3 ± 20.0^{a,b}	132.5 ± 18.1^{a,b}	206.5 ± 54.8^{a}	165.8 ± 21.6^{a,b}	111.3 ± 22.9^{b}	165.0 ± 34.6^{a,b}	109.6 ± 5.7^{b}	122.7 ± 9.9^{a,b}
22:5n-3	19.4 ± 1.9^{a,b}	10.6 ± 2.1^{b,c}	22.0 ± 6.6^{a}	15.7 ± 1.5^{a,b,c}	9.3 ± 2.2^{c}	20.4 ± 3.7^{a}	15.2 ± 1.6^{a,b,c}	14.9 ± 0.8^{a,b,c}
22:6n-3	242.3 ± 19.3^{a}	146.6 ± 14.6^{b}	237.4 ± 56.3^{a}	210.7 ± 19.5^{a,b}	128.9 ± 20.5^{b}	215.2 ± 41.3^{a,b}	197.6 ± 13.0^{a,b}	195.3 ± 9.3^{a,b}
Period Fed Diet D								
14:0	106.4 ± 21.3^{a}	72.0 ± 10.5^{a}	73.8 ± 6.2^{a}	73.6 ± 13.3^{a}	122.6 ± 62.9^{a}	61.2 ± 10.8^{a}	45.9 ± 8.6^{a}	46.0 ± 5.9^{a}
16:0	297.8 ± 49.9^{a}	231.6 ± 21.6^{a,b}	202.4 ± 9.9^{b}	215.4 ± 30.6^{a,b}	224.7 ± 24.6^{a,b}	220.5 ± 15.3^{a,b}	217.4 ± 27.3^{a,b}	232.2 ± 21.0^{a,b}
16:1	130.4 ± 28.1^{a}	92.8 ± 13.4^{a,b}	92.1 ± 8.3^{a,b}	104.3 ± 15.5^{a,b}	82.1 ± 9.2^{b}	83.4 ± 11.5^{b}	61.2 ± 9.0^{b}	69.8 ± 8.5^{b}
18:0	46.8 ± 6.8^{a,b,c}	38.8 ± 4.4^{b,c}	31.5 ± 1.5^{c}	35.4 ± 4.9^{b,c}	69.7 ± 22.5^{a}	47.6 ± 3.3^{a,b,c}	56.4 ± 6.8^{a,b}	63.1 ± 6.9^{a,b}
18:1	288.4 ± 55.5^{a}	232.5 ± 35.3^{a}	217.1 ± 16.2^{a}	281.4 ± 36.9^{a}	294.1 ± 40.9^{a}	305.7 ± 19.7^{a}	320.0 ± 40.8^{a}	473.2 ± 54.7^{b}
18:2n-6	35.9 ± 6.5^{a}	54.5 ± 26.0^{a}	43.4 ± 4.5^{a}	83.8 ± 13.7^{a,b}	98.5 ± 10.5^{a,b}	123.6 ± 14.3^{b}	188.2 ± 27.6^{c}	263.6 ± 41.1^{d}
18:3n-3	10.3 ± 2.0^{a}	52.1 ± 40.1^{a,b}	33.7 ± 7.1^{a}	88.6 ± 17.7^{a,b}	107.1 ± 12.6^{a,b}	149.5 ± 22.6^{b}	261.2 ± 38.6^{c}	355.4 ± 68.1^{c}
20:1n-9	183.9 ± 33.7^{a}	119.2 ± 15.8^{b}	129.1 ± 8.4^{b}	131.7 ± 18.1^{b}	123.7 ± 11.9^{b}	127.4 ± 13.4^{b}	92.8 ± 9.3^{b}	115.2 ± 14.5^{b}
20:4n-6	8.0 ± 0.9^{a}	7.0 ± 0.9^{a}	27.4 ± 20.7^{a}	8.3 ± 0.6^{a}	7.5 ± 0.8^{a}	9.3 ± 0.9^{a}	9.5 ± 1.1^{a}	14.3 ± 2.4^{a}
20:5n-3	67.2 ± 9.2^{a}	54.9 ± 6.6^{a}	54.8 ± 4.76^{a}	63.0 ± 5.6^{a}	53.4 ± 5.0^{a}	59.0 ± 6.3^{a}	48.1 ± 3.1^{a}	56.9 ± 5.4^{a}
22:1n-9	196.5 ± 35.3^{a}	115.5 ± 17.5^{b}	123.9 ± 8.3^{b}	123.6 ± 17.1^{b}	124.3 ± 13.4^{b}	134.6 ± 14.7^{b}	94.4 ± 8.9^{b}	118.6 ± 16.1^{b}
22:5n-3	13.9 ± 2.4^{a}	15.0 ± 2.1^{a}	12.1 ± 1.4^{a}	17.1 ± 2.9^{a}	12.3 ± 2.0^{a}	17.4 ± 2.2^{a}	12.1 ± 0.9^{a}	16.1 ± 1.5^{a}
22:6n-3	192.7 ± 18.9^{a}	170.1 ± 20.7^{a}	171.1 ± 13.9^{a}	207.0 ± 23.86^{a}	172.3 ± 19.0^{a}	190.4 ± 18.6^{a}	171.7 ± 9.9^{a}	198.8 ± 14.9^{a}

Means with different superscripts in the same row are significantly different ($p < 0.05$).

content of the flesh increased from 4.3 ± 0.14% to 6.95 ± 0.21% over the 64 day feeding period. The lipid content was within the range reported in this and other laboratories (22,23).

Significant changes in fatty acid composition, notably in OL and eicosaenoic acid (20:1n-9), occurred between fish fed different diets. Increases in olive oil led to increases in OL and the elongation product, eicosaenoic acid in the tissue. Because the objective of this research was to investigate the possibility of increasing EPA and DHA via elongation and desaturation of dietary LNA, the discussion focusses mostly on the changes in the n-3 PUFA of fish flesh.

The rainbow trout accumulated significant amounts of LNA in muscle tissue within three days following introduction of diets containing linseed oil (Fig. 1 and Table 5). Increasing levels of LNA in the diet caused commensurate increases in tissue LNA. However, at most time points and diets negligible changes occurred in either EPA or DHA levels in the total lipid of the muscle. However, fish consuming diet C had significantly lower levels of EPA and DHA at two time points, suggesting that high dietary LNA may decrease the elongation and desaturation of n-3 fatty acids. As the fish continued to consume diets high in LNA, the DHA level stabilized at approximately 200 mg/100 g tissue regardless of the level of linseed oil fed.

Further investigation of the phospholipids (PL) and the neutral lipids (NL) of the muscle tissue from trout fed Diet D (20% linseed oil) showed that LNA levels in neutral lipid are greater than that in PL while EPA and DHA levels are higher in PL (Fig. 2). With increasing dietary LNA, LNA increases in both the PL and NL. EPA and DHA do not change in NL and decrease in PL with increasing dietary LNA.

Other researchers have reported elongation and desaturation of LNA into EPA and DHA in trout previously maintained on lipid-free diets (24) or fed diets containing lower levels of LNA that were administered in this study (25-27). Other researchers focusing on LNA desaturation/elongation could not resolve chromatographic peaks containing LNA and eicosaenoic acid, a fatty acid prevalent in marine oil fed in the diet (27).

Inhibition of Δ-6 desaturase by high levels of dietary DHA and EPA has been proposed (26,28,29). In this study, there was negligible conversion of dietary LNA to EPA or DHA suggesting inhibition of the Δ-6 desaturase in these fish irrespective of dietary LNA levels. However, it should be noted that tissue levels of DPA and DHA were well maintained through the feeding period.

Regulation of Δ-6 activity to maintain a certain degree of membrane saturation has been proposed by several investigators (30). In this study the ratio of PUFA to saturated fatty acids in the total lipids increased with time in fish on all the diets. The PUFA/SAT ratio on day 0 was 0.63, and 1.23, 1.38, 1.78 and 2.65 on day 64 for diets A–D, respectively.

Livers from fish on diet D (20% linseed oil) were analyzed to determine if changes in the muscle tissue were paralleled by changes in the liver (Fig. 3). Similar relative changes in LNA, EPA and DHA occurred in both the PL and glycerolipid fractions of the liver as occurred in the flesh total lipid. There was an uptake of LNA but little concomitant increase in EPA or DHA in neutral glycerides or PL. The concentration of n-3 PUFA EPA and DHA were significantly higher in the PL than in the glyceride, perhaps because n-3 fatty acids are highly conserved in trout phospholipid (31).

This research shows that LNA can be enriched in rainbow trout flesh, but that the relative concentration of the long chain n-3 fatty acids, EPA and DHA, are affected to only a small degree by manipulation of dietary linolenic acid. This suggests that the Δ-6 desaturase in rainbow trout may have been inhibited by high concentrations of dietary LNA, and especially when EPA and DHA is concurrently supplied in the diet. Feeding an LNA enriched finishing diet to rainbow trout does not seem to be an effective method of increasing long chain n-3 PUFA. Further studies are planned to assess the effects of LNA in diets containing lower fat levels which also lack EPA or DHA and also to determine how effectively dietary EPA is incorporated nto muscle of trout.

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Incorporation of Hormone-Sensitive Lipase into Phosphatidylcholine Vesicles

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Enzymatically active, detergent-solubilized purified hormone-sensitive lipase (HSL) was incorporated into phosphatidylcholine (PC) vesicles, using a detergent-dialysis procedure with small PC vesicles, obtained by sonication, as phospholipid source and CHAPS, a zwitterionic bile-salt derivative, as detergent. Association of enzyme protein with the PC vesicles was verified by floatation in a discontinuous dextran gradient and by gel chromatography. An average of 35% of added HSL was incorporated into the vesicles. The vesicles were shown, by quasi-elastic light scattering and electron microscopy, to have a diameter of approximately 160 nm. The vesicle-associated HSL could be phosphorylated by cyclic AMP-dependent protein kinase. The vesicles were stable, both with regard to enzyme activity and size, for at least 4 days when stored at 4°C. The preparation of detergent-free, vesicle-associated and stable HSL provides new possibilities to study some of its properties, and supports and extends the previous report (Holm, C., Fredrikson, G., and Belfrage, P., *J. Biol. Chem. 261,* 15659-15661, 1986) which demonstrated the amphiphilic character of HSL.
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Hormone-sensitive lipase (HSL) regulates the mobilization of fatty acids from adipose tissue by catalyzing the rate-limiting step in the hydrolysis of stored triacylglycerols (1). HSL has been extensively purified from rat adipose tissue after solubilization with non-ionic detergents (2,3) and from bovine adipose tissue, using a hydrophobic interaction chromatography step (4). Recently the rat HSL cDNA was cloned and sequenced to give the entire cDNA sequence and deduced amino acid sequence (5,6).

The purified enzyme (2) has been found to have properties in common with integral membrane proteins. It requires detergent to be solubilized and the presence of detergent is necessary during purification (2-4). Charge-shift electrophoresis and phase separation in Triton X-114 have shown that the enzyme binds detergent (7) similarly to typical integral membrane proteins (8). Quantitative measurements of the detergent binding have not yet been possible due to limited availability of purified enzyme protein. The primary structure of HSL contained no apparent α-helical membrane spanning region, although several short hydrophobic stretches could be predicted based on Kyte and Doolittle (9) analysis (5).

Non-ionic detergents are known to inhibit the activity of HSL in analogy to what has been found for pancreatic lipase (10). This inhibition is presumably due to interference by the detergent with the substrate-enzyme interaction (11) as supported by the fact that the detergent inhibition varies with the type of substrate used (2). The fact that the purified HSL is associated with detergent, which is inhibitory, greatly complicates examination of its enzymological properties. This is especially true for studies of the mechanism behind its activation upon phosphorylation (12), since it is speculated that phosphorylation of HSL increases its binding to the triacylglycerol substrate (1). It has been shown that the detergent bound to HSL can be completely exchanged by adsorbing the enzyme to a hydroxyapatite column equilibrated in buffer containing the new detergent, washing it extensively, and then eluting it in the same buffer now containing phosphate at a high concentration (13). However, initial attempts to use hydroxyapatite chromatography to obtain HSL free from detergent by eluting with detergent-free buffers were unsuccessful, since this resulted in both low recovery and lability of the eluted enzyme (C. Holm, unpublished observations).

The aims of the present investigation were (i) establish if HSL could be incorporated into PC vesicles, as has been previously described for a number of integral membrane proteins (for review see 14-17), which would be further proof of its amphiphilic character, and (ii) to develop a simple protocol for preparing detergent-free, stable enzyme, to be used in studies of the enzyme which are hampered by the presence of detergent.

EXPERIMENTAL PROCEDURES

Materials. Hydroxyapatite and CHAPS were from Calbiochem. ^{3}H-labeled CHAPS was synthesized by Dr. Lennart Krabisch at this department; dialysis membrane (Spectrapor 2) was from Spectrapor (cutoff 12-14000); Sepharose CL-2B and dextran T40 from Pharmacia; $C_{13}E_{12}$ (Berol 058) from Berol Kemi AB (Stenungsund, Sweden); $C_{12}E_8$ from Nikko Chemicals (Tokyo, Japan) ($C_{13}E_{12}$ and $C_{12}E_8$ are alkyl polyoxyethylene ether detergents; general formula $C_nH_{2n+1}(OCH_2CH_2)_xCH$, abbreviated to C_nE_x); leupeptin and antipain from Peptide Institute Inc. (Osaka, Japan); and [^{3}H]glycerol from Amersham. The 1(3)-mono[^{3}H]oleoyl-2-*O*-oleyl-glycerol was synthesized and purified as described in (18). Bovine serum albumin (crystalline, delipidized) was from Sigma.

Enzyme. HSL was purified from rat adipose tissue up to and including the first hydroxyapatite chromatography step (2) and obtained in 0.5 M potassium phosphate, pH 7.0 (4°C), 50% (w/v) glycerol, 1 mM dithioerythritol and 0.1% (w/v) $C_{12}E_8$. In some experiments, the second gradient sievorptive chromatography and hydroxyapatite steps were exchanged for a HPLC purification step on Mono Q (Pharmacia, Sweden) (3). In these cases, the enzyme was obtained in 50 mM Tris-acetic acid, 30 mM potassium phosphate, pH 7.0, 0.15 M sodium acetate, 50% glycerol, 1 mM dithioerythritol and 0.2% (w/v) $C_{13}E_{12}$. After desalting the enzyme by dialyzing overnight (Spectrapor 2) at 4°C against 50% (w/v) glycerol, 2 mM potassium phosphate (pH 7.0 at 4°C), and

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Abbreviations: HSL, hormone-sensitive lipase; cDNA, complementary deoxyribonucleic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; HPLC, high performance liquid chromatography; Mono Q, monobeads with quaternary amino groups; CMC, critical micelle concentration; PC, phosphatidylcholine; SDS-PAGE, polyacrylamide gel electrophoresis in sodium dodecyl sulfate.

1 mM dithioerythritol, or by passing it through a Sephadex G-25 column (19), the enzyme was applied to a hydroxyapatite column (0.2 g hydroxyapatite/mL enzyme solution) equilibrated in 10 mM potassium phosphate (pH 7.0 at 4°C), 5% (w/v) glycerol, 1 mM dithioerythritol and 9 mM CHAPS (= equilibration buffer) in order to concentrate it and exchange the $C_{12}E_8$ or $C_{13}E_{12}$ for CHAPS. The column was washed extensively with at least 5 vol of equilibration buffer, and the enzyme was eluted with 0.5 M potassium phosphate in equilibrating buffer (pH 7.0 at 4°C).

Enzyme activity. The diacylglycerol lipase activity of HSL was determined using 1(3)-mono-[^{3}H]oleoyl-2-*O*-oleylglycerol (a monoether analogue of dioleoylglycerol) as a substrate (2). 1 U is equal to the relase of 1 μmol fatty acids/min at 37°C.

Phosphorylation of HSL. Phosphorylation of HSL was as in (20). Briefly, the enzyme was incubated at 37°C for 30 min with 0.2 mM [γ-^{32}P]ATP (10×10^6 cpm/nmol), 5 mM $MgCl_2$, 1 mM dithioerythritol and the catalytic subunit of cAMP-dependent protein kinase (3 μg/mL) purified to near homogeneity from rat adipose tissue (21). Phosphorylated samples were analyzed by SDS-PAGE as described by Laemmli (22) with modifications (23), followed by autoradiography.

Lipids. Soybean PC (Epicuron 200, >99% pure) was from L. Meyer (Hamburg, West Germany). 1-Palmitoyl-2-oleoyl-9,10[^{3}H]-PC (98.7% pure) was synthesized by Dr. Lennart Krabisch at this department. Lipid concentrations were determined by the ^{3}H-radioactivity.

Protocol for incorporation of HSL into PC vesicles. ^{3}H-labeled and unlabeled PC were mixed and the solvent was evaporated under nitrogen. Evaporation was repeated twice after adding 0.1 mL freshly distilled, dried diethyl ether. The resulting lipid film was placed under reduced pressure for at least 12 hr, and then allowed to swell for 30 min at room temperature in 20 mM Tris-HCl, pH 7.0 (4°C), 0.1 M NaCl, 1 mM EDTA and 1 mM dithioerythritol at a final concentration of 25 mg PC/mL. After swelling, the solution was sonicated with 0.5 s pulses (with 0.5 s intervals) for 45 min at 4°C under nitrogen with a microtip sonicator (Branson, model B-15P) at the setting of 50% of maximal intensity. The clear solution obtained was centrifuged at 100,000 × *g* for 60 min to remove any undispersed phospholipid, e.g. multilamellar vesicles, and titanium fragments from the microtip. The supernatant (1 vol) was mixed with CHAPS-solubilized enzyme (4 vol) from a hydroxyapatite column (see above) to yield the desired PC concentration and detergent:PC ratio. The mixture was incubated for 1–4 hr on ice and dialyzed for 36 hr against at least 1000-fold excess of 20 mM Tris-HCl, pH 7.0 (4°C), 0.1 M NaCl, 5% (w/v) glycerol, 1 mM EDTA, 1 mM dithioerythritol with at least one change of dialysis buffer.

In some experiments the enzyme pooled from the hydroxyapatite column was added directly to a PC film to yield the desired PC concentration and detergent:PC ratio, allowed to swell for 30 min on ice, sonicated for 20 min in a sonicator bath at 4°C and further incubated 1–4 hr on ice before the dialysis was started.

Floatation in a discontinuous dextran gradient. Floatation in a discontinuous 0%/10%/20% dextran gradient is given (24). Briefly, the sample was mixed with 20% (w/v) dextran in buffer (0.1 M NaCl, 20 mM Tris-HCl, pH 7.0 (4°C), 1 mM EDTA, 1 mM diethioerythritol), placed at the bottom of a 4.4 ml centrifuge tube and overlaid by 10% (w/v) dextran (in buffer) and further overlaid by buffer only. Samples were centrifuged in a SW 60 rotor at 100,000 × *g*, 4°C, for 45 min in a Beckman L2-65B ultracentrifuge. Vesicles were obtained at the 0%/10% dextran interphase, clearly visible as a distinct opalescent band. Fractions were collected from the bottom and analyzed for enzyme activity and PC concentration.

Gel chromatography. HSL-PC vesicles were applied to a Sepharose CL-2B column (0.3 × 50 cm), preequilibrated with several volumes of buffer (20 mM Tris-HCl, pH 7.0 (4°C), 0.1 M NaCl, 5% (w/v) glycerol, 1 mM dithioerythritol, 1 mM EDTA) with 0.2 mg/ml of sonicated PC vesicles (25). The column was equilibrated with at least 2 vol of buffer without PC vesicles before the sample was applied. Fractions were collected and analyzed for enzyme activity and PC concentration. The total volume of the column was determined using [^{3}H]glycerol.

Quasi-elastic light scattering. The light scattering equipment was a Malvern system 4600 equipped with a Malvern 7027 correlator and a Spectra Physics model 124B He/Ne laser. Measurements were made at a scattering angle of 90 degrees. Samples were prepared using buffers, which had been filtered through 0.22 μm membrane, throughout the whole reconstitution procedure. The z-average translational diffusion coefficient (D_t) was determined by means of a cumulant analysis of the intensity autocorrelation function (26,27). From the value of D_t the hydrodynamic radius r_h was calculated using Stoke-Einstein's equation:

$$r_h = kT/6\pi\eta D_t$$

where η is the viscosity of the solvent, k is Bolzmann's constant and T is the absolute temperature. A polydispersity program (Malvern PC S14) was used to estimate the size distribution of the vesicles from the autocorrelation function.

The measurements were performed at a PC concentration of approximately 40 μg/ml. Dilution up to four-fold reduced the value of the estimated vesicle diameter <10% for all types of vesicles investigated, indicating that there was no pronounced concentration dependence.

Electron microscopy. For negative staining, a drop of the sample solution was put onto a Formvar-coated copper grid and air-dried. The negative stain (2% phosphotungstic acid, adjusted with NaOH to pH 7.2) was then added. After 5–15 s the excess stain was removed and the grids air-dried. Negative stain preparations were examined with a Zeiss 10°C electron microscope. The electron microscopy studies were performed by Dr. Gunnel Karlsson at the University of Agriculutre in Svalöv, Sweden.

RESULTS

Incorporation of HSL into PC vesicles. HSL was incorporated into PC vesicles using a CHAPS-dialysis method. CHAPS is a zwitterionic, bile salt derivative detergent, with a high critical micelle concentration (1–10 mM) (28,29), allowing easy removal by dialysis. Preliminary results from CMC-determinations with an equilibration dialysis method (30, with some modifications; G.

Fredrikson and M. Lindström, unpublished results) demonstrated a CMC of approximately 4 mM in the described system. The CMC was dependent on the concentration of PC and glycerol, so that CMC decreased with increasing concentration of PC and increased with increasing glycerol concentration. In the presence of both PC and glycerol, the decrease in CMC from PC to a large extent suppressed the increase in CMC from glycerol. Using ^{3}H-labeled CHAPS, 99.9% of initial detergent was found to be removed in 40 hr in the presence of 5 mg/mL (6.7 mM) of PC and 5% (w/v) glycerol (not illustrated). The remaining detergent corresponded to less than 1 detergent molecule for every 200 PC molecules.

The protocol used for incorporation of HSL into PC vesicles was to add a concentrated solution of small, sonicated PC vesicles to the CHAPS-solubilized, concentrated HSL at the desired lipid concentration and detergent:lipid ratio. In order to standardize the conditions these vesicles were always used on the day of preparation. At that time they constituted a population of vesicles with a diameter of 53 nm as determined by light scattering (Table 1). Analysis of the size distribution of these vesicles demonstrated that they consisted of one major population with a peak diameter of approximately 40 nm (Fig. 1). A more commonly employed method used for incorporating proteins into phospholipid vesicles is to present the lipids as a film of dried phospholipids rather than as a solution of sonicated vesicles. The intention in both cases is to obtain the largest possible contact area between phospholipid and enzyme. An extensive comparison between these methods was not carried out, but results from some initial experiments indicated that presenting lipids as small, sonicated vesicles resulted in a somewhat higher (≈35%) extent of association of enzyme (not illustrated). This technique was therefore used in all experiments described below.

TABLE 1

Laser Light Scattering Results

Sample	$D_t \times 10^8$ (cm^2/s)	z-average diameter (nm)
Dialyzed vesicles with HSL	3.0	162
Dialyzed vesicles without HSL	2.3	212
Sonicated PC vesicles	9.1	53

[a]Dialyzed vesicles with and without HSL were the same preparations as described and shown in Figure 4A and B. Sonicated PC vesicles were the same preparation as described and shown in Figure 1.

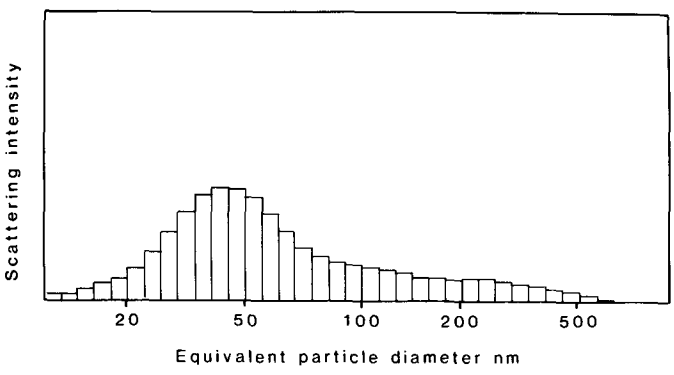

FIG. 1. Quasi-elastic light scattering analysis of the size distribution of sonicated PC vesicles. The bar histogram illustrates scattering intensity as a function of vesicle diameter (nm). Sonicated PC vesicles were prepared as described under Experimental Procedures and diluted to a PC concentration of 1.25 mg/mL.

A convenient method for separating vesicle-associated HSL from free HSL was floatation in a discontinuous 0%/10%/20% dextran gradient (24). Vesicle-associated HSL was obtained at the 0%/10% dextran interphase, clearly visible as a distinct opalescent band (Fig. 2A), whereas phospholipid-free HSL remained at the bottom of the centrifuge tube. The distribution of the sonicated PC vesicles used as the lipid starting material (Fig. 2B) and PC- and detergent-free HSL (= control, Fig. 2C), upon floatation in the same discontinuous dextran gradient is also illustrated. Both the enzyme and lipid recoveries during the dextran floatations were approximately 90%.

The average extent of HSL incorporation, calculated from data like those presented in Fig. 2A, was 35 ± 10% (x ± S.D., n = 7) of the total amount HSL added. Attempts to correlate the variability with any parameter and to increase the extent of incorporation have so far been unsuccessful. To further verify the association of HSL to the PC vesicles, gel chromatography analyses were performed. The problem of lipids adsorbing to the gel matrix was reduced by equilibrating the columns with several volumes of sonicated PC vesicles in buffer before applying the samples (25), resulting in a PC recovery of ≥80%. When analyzed on Sepharose CL-2B, vesicle-associated HSL was obtained in the void volume (Fig. 3A —top), whereas free HSL that had not associated with the vesicles (Fig. 3A) and also HSL from a control preparation, i.e. with no PC (Fig. 3B—bottom), were recovered in the included gel volume. The enzyme recovery in these analyses was around 50%.

Analyses of PC vesicle-associated HSL. The association of HSL to PC vesicles and floatation in the dextran gradient resulted in a 9-fold purification of HSL as judged by HSL specific activities before (6.0 U/mg protein) and after (54 U/mg protein) binding to the vesicles, and by SDS-PAGE analysis (not illustrated).

The vesicle-associated HSL could be phosphorylated by the catalytic subunit of cyclic AMP-dependent protein kinase in the presence of ATP and Mg^{2+} (12), to approximately the same extent as a detergent-solubilized reference HSL, when using comparable ratios between the kinase and the lipase (not illustrated).

The enzyme activity of the detergent-free, vesicle-associated HSL was stable for at least 4 days at 4°C (84 ± 7%, n = 7, of initial activity). During this time period there was no change in the vesicle size distribution, as judged by light scattering analyses (not illustrated).

Vesicle size and size distribution. The sizes and size distribution of the PC vesicles were examined using quasi-elastic light scattering and electron microscopy. The values for the translational diffusion coefficient and the corresponding vesicle diameter for the three types of vesicles analyzed by light scattering—dialyzed vesicles with associated HSL, dialyzed vesicles without HSL and sonicated PC vesicles—are summarized in Table 1. An analysis of the size distribution of the dialyzed vesicles

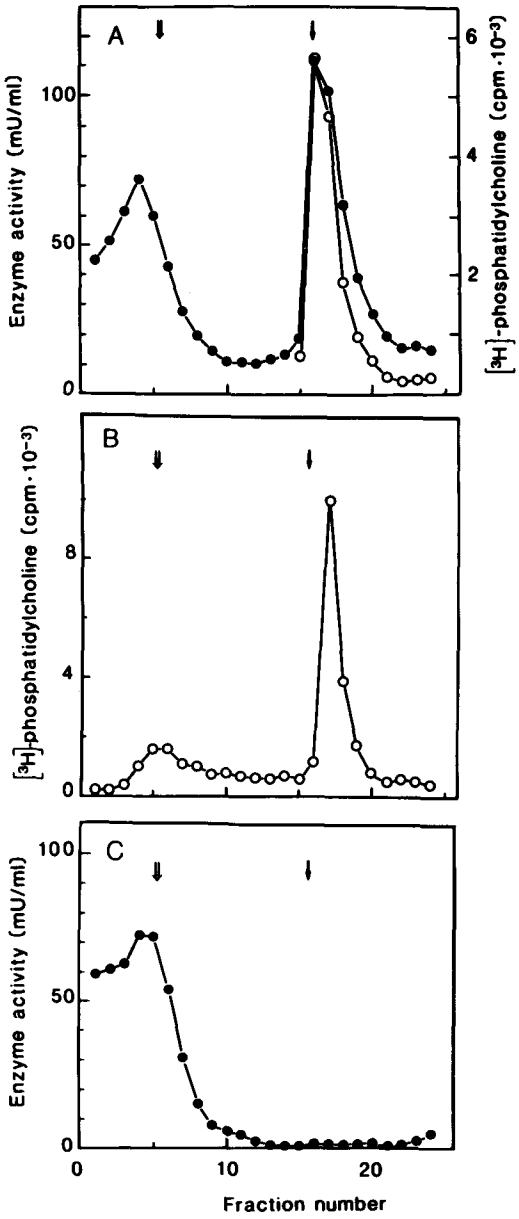

FIG. 2. Floatatoin of vesicle-associated HSL in a discontinuous dextran gradient. HSL was incorporated into PC vesicles as described under Experimental Procedures, using sonicated PC vesicles as lipid starting material. The concentrations of PC and CHAPS were 2.7 mM and 8.1 mM, respectively, at the start of the dialysis. After the 36 hr dialysis, the material was floatated in a discontinuous 0%/10%/20% dextran gradient. Fractions were collected from the bottom and analyzed for HSL activity and PC concentration. A, vesicle-associated enzyme. Unfilled (left) arrow indicates the position of the 10%/20% dextran interphase and filled (right) arrow indicates the position of the 0%/10% dextran interphase; B, distribution of sonicated PC vesicles used as lipid source; C, enzyme control treated as above but in the absence of phospholipids.

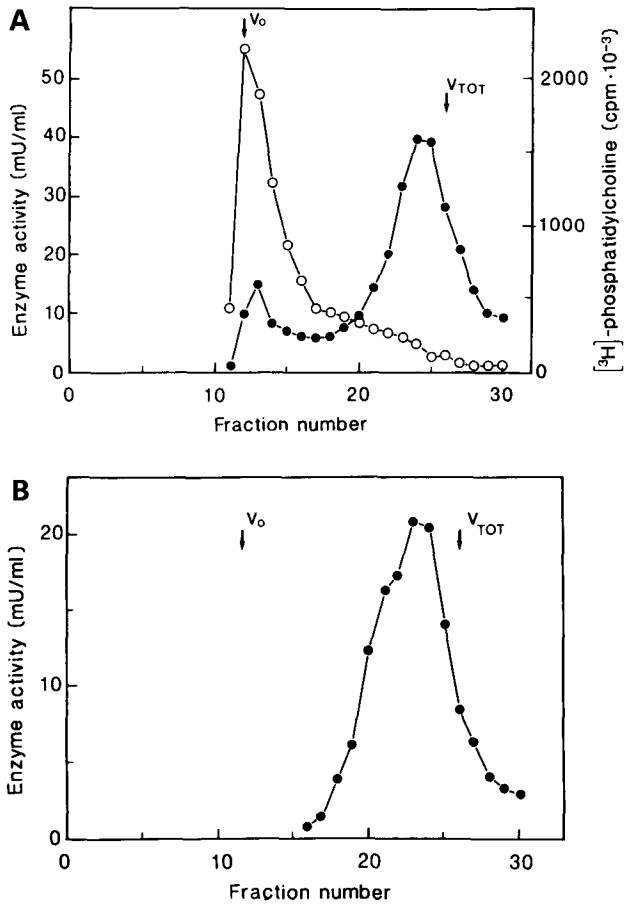

FIG. 3. Gel chromatography analysis of vesicle-associated HSL. Vesicle-associated HSL (A) and control (B) (the same preparation as in Fig. 2) were applied to a Sepharose CL-2B column, preequilibrated with buffer containing PC vesicles as described under Experimental Procedures. The void and total volumes of the column are indicated in the figure.

showed that two populations of vesicles were present, with the approximate peak diameters of 30 and 200 nm, respectively (Fig. 4). This size distribution is reasonable with regard to the z-average diameter of these vesicles (Table 1). Furthermore, electron microscopy analyses also demonstrated the presence of two populations of vesicles (Fig. 5), although the approximate peak diameters appeared to be somewhat smaller than those obtained by light scattering analyses. This apparent discrepancy is most likely due to some shrinkage of the vesicles during the negative stain preparation, which is a common problem encountered in this type of studies.

DISCUSSION

The results described in this paper demonstrate that HSL can be separated from detergent and at the same time associated with PC vesicles, as has previously been shown for a number of integral membrane proteins (14–17). The association was verified by floatation in a discontinuous 0%/10%/20% dextran gradient. The vesicle-associated HSL was obtained at the 0%/10% dextran interphase whereas free HSL remained at the bottom (Fig. 2). Gel chromatographic analyses showed that vesicle-associated HSL was eluted in the void volume and free HSL in the included gel volume (Fig. 3). The apparent discrepancy between the extent of association when analyzed by gel chromatography as compared to when analyzed by dextran floatation could be attributed to an exchange of HSL molecules between the PC vesicles loaded onto the column and the PC vesicles that were bound to the column

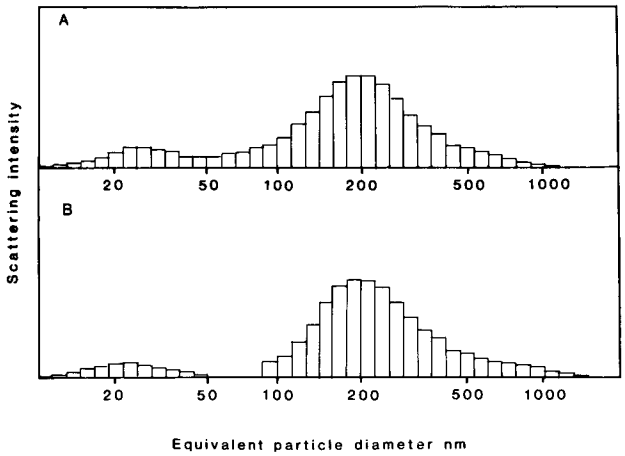

FIG. 4. Apparent size distribution of PC vesicles as determined by quasi-elastic light scattering. HSL was incorporated into PC vesicles (Experimental Procedures) using sonicated PC vesicles as lipid source (A). At the start of dialysis the concentrations of PC and CHAPS were 2.7 mM and 8.9 mM, respectively. Dialyzed vesicles without protein (B) were prepared according to the same procedure, except that proteins were excluded. The vesicles were floatated in a discontinuous 0%/10%20% dextran gradient. Vesicles obtained at the 0%/10% dextran interphase were diluted 50-fold and analyzed by a polydispersity program. Scattering intensity as a function of vesicle diameter (nm).

during the preequilibration (see above). Alternatively, the 50% loss in enzyme recovery during gel chromatographic analyses (see above) is due to a selective loss in activity of vesicle-associated HSL.

The finding that HSL is incorporated into PC vesicles is in agreement with a number of previous findings indicating the amphiphilic character of HSL i.e. HSL binds detergent similarly to typical membrane proteins (7). Furthermore, it requires detergent for solubilization, purification and stability (2). However, HSL is found in the infranatant fraction rather than the membrane fraction upon a high-speed centrifugation of an adipose tissue homogenate (31) and appears to lack a membrane-spanning region as shown by the Kyte and Doolittle analysis (9) of the predicted primary structure deduced from the cDNA sequence (5). In addition, the M_r of the primary translation product deduced from cDNA sequencing of 82,820 (5) is in very good agreement with the M_r in SDS-polyacrylamide gels of 84,000 (2). This indicates that HSL is not glycosylated, which is usually the case for all membrane proteins, including intracellular. Therefore, we postulate that HSL is a cytosolic protein, or possibly a peripheral membrane protein, that possesses hydrophobic surface domains that account for the detergent-binding and allow it to bind to phospholipids and its lipid substrate.

The mode of association of HSL to the vesicles is yet not known, but elucidating this could provide valuable information regarding the native form and intracellular localization of HSL. It is known that HSL appears in lipid-protein aggregates in the infranatant after a 110,000 $\times$ *g* centrifugation of an adipose tissue homogenate. These aggregates have a diameter of 20–60 nm and a density of 1.08–1.09 g/mL and contain phospholipid as the main lipid (31). However, it is doubtful whether these

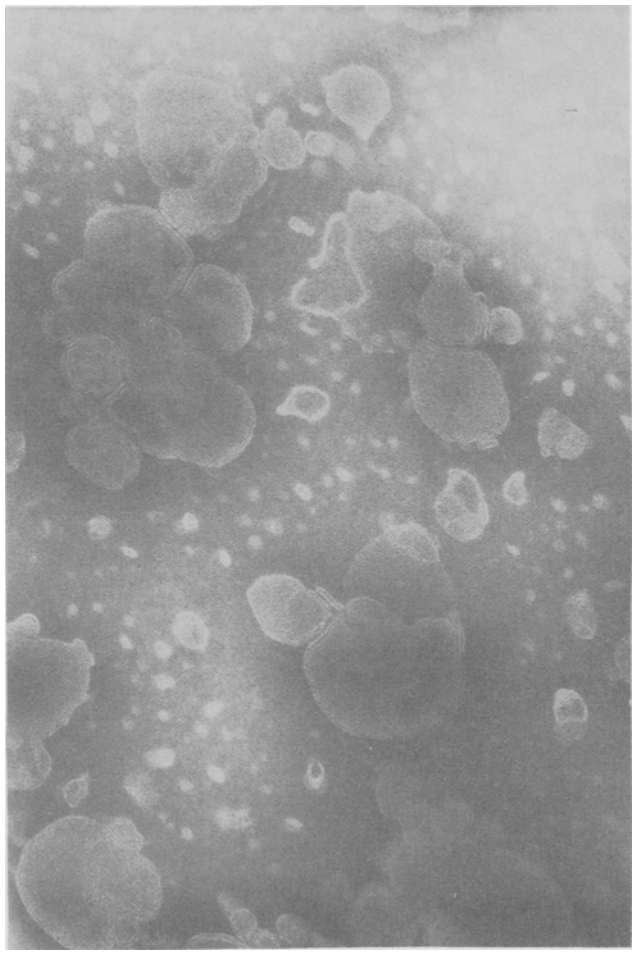

FIG. 5. Electron micrograph of PC vesicle-associated HSL. Vesicles from the same preparation, as shown in Figure 4A, were negatively stained with 2% phosphotungstic acid at pH 7.2. The bar is 0.2 μm.

particles, represent the true native form of the enzyme as has been suggested (31). It is equally possible that they represent artefacts, which are formed during the homogenization of the tissue. Upon centrifugation in a discontinuous 0%/10%/20% dextran gradient, these particles were found to remain at the bottom (not illustrated), indicating that they are denser and more protein-rich than the HSL-containing PC vesicles described in this paper.

Quasi-elastic light scattering and electron microscopy analyses were performed to obtain information on the diameter of the vesicles (Figs. 4, 5, Table 1). Using laser light scattering for this purpose involves several assumptions, e.g. that the vesicles are isotropic and spherical. The latter assumption is a prerequisite for the calculated hydrodynamic radius to be correct, since it is derived from the diffusion coefficient using Stoke-Einstein's equation. The electron microscopy gave some support that the vesicles were spherical (Fig. 5). Two distinct populations of vesicles with regard to size were found (Fig. 4), but it is not known if HSL is associated with both, or only one of them. Based on a number of assumptions—the vesicles were spherical bilayers with a diameter of 160 nm (Table 1, Fig. 4), the packing area of the phospholipid head group was 50Å^2 in both the outer and inner layer (32),

the specific activity of HSL was 200 U/mg protein (2) and the HSL molecules were evenly distributed between the vesicles and all had access to the substrate—it could be calculated that each vesicle contained between 1 and 2 HSL subunits (calculations made for the pooled fractions 15-20 in Fig. 2A).

CHAPS, described in detail (28,29), has previously been used for reconstituting the receptor of IgE (33,34), guanine nucleotide regulatory proteins (35) and (Na^+ + K^+)-ATPase (36). We chose to use CHAPS at a concentration of 9 mM, which is approximately twice the CMC in our system (see above). An extensive investigation into the appropriate conditions for achieving maximal solubilization and subsequent incorporation into phospholipid vesicles has been published (33). In this study a parameter ϱ is defined:

$$\varrho = \frac{[\text{detergent}]\text{-}CMC_{eff}}{[\text{phospholipid}]}$$

where CMC_{eff} is the CMC in the system under study. At ϱ = 1.5-2, maximal solubilization is achieved with minimal detergent/phospholipid ratio—as examined with CHAPS, octylglucoside and deoxycholate as detergents, two different sources of phospholipid and the receptor for IgE and a mixture of surface-labeled proteins (33). In our system the ϱ-value in most experiments was 1.9, and attempts to increase this, by increasing the detergent concentration, did not result in any higher extent of incorporation.

In conclusion, in this report it is shown that HSL associates with PC vesicles similarly to what has been described for many integral membrane proteins. The mode of association has not yet been established, but could give valuable clues to the native localization of HSL. The presence of detergent in purified preparations of the enzyme could possibly have some influence on the properties of the enzyme, e.g. substrate affinity. Phospholipid vesicles could be considered to provide an environment that resembles the conditions in the cell and thus give an enzyme preparation that more resembles the enzyme in its natural state. This could be valuable for studies of e.g. the mechanism of activation by phosphorylation. The protocol used for incorporating HSL into PC vesicles could thus serve as a protocol for preparing detergent-free, phospholipid-associated HSL.

ACKNOWLEDGMENTS

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Preparation and Physical Properties of Some C_{18} Unsaturated Fatty Esters Containing *L*-Amino Acid Residues and Methyl Esters of *N*-Stearoylamino Acids

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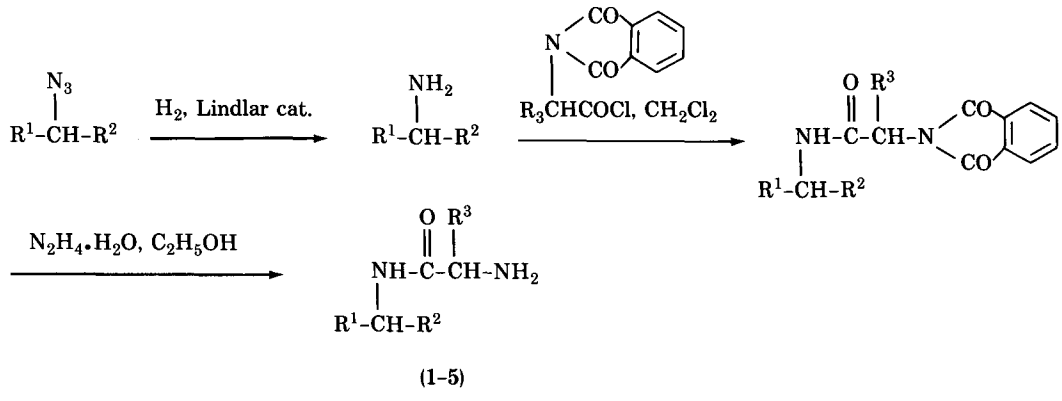
A series of five C_{18} unsaturated fatty esters (1-5) containing an *L*-amino acid residue (glycine, alanine, valine, leucine, phenylalanine) was prepared from methyl 12-amino-9-*cis*-octadecenoate and five methyl *N*-stearoylamino acid ester derivatives (6-10) from stearoyl chloride and the same *L*-amino acids. The infrared analysis of compounds 1-5 showed characteristic absorption bands at 3300 and 1665 cm^{-1} for the amino and amido functions, while the amido function in compounds 6-10 gave absorption bands at 3300 and 1680 cm^{-1}. The position of the amido group (peptide linkage) in compounds 1-5 was readily determined by mass spectral analysis. ^1H nuclear magnetic resonance (NMR) analysis showed a doublet at 7.15 ppm for the amide proton (NHCO) in compounds 1-5, while in compounds 6-10 the amide proton signal appeared at 6.0 ppm. In ^{13}C NMR, the amido carbonyl resonance appeared at 171-175 ppm. In compounds 1-5 the effect of the amide function on the ethylene carbon (C-9) gave a signal at 124.1-125.3 ppm, while the remaining ethylenic carbon appeared at 131.9-132.5 ppm. The methine carbon (C-12) of the alkenyl chain was shifted to 48.4-49.8 ppm, and the assignment of the various carbon nuclei in the amino acid residue was readily achieved. In the methyl *N*-stearoylamino acid ester derivatives (6-10), the methine carbon adjacent to the amido system and alpha to the carbomethoxy group appeared between 41.2-57.0 ppm depending on the type of alkyl group present in the amino acid moiety. The phenyl system in compounds 5 and 10 was confirmed by the ^{13}C signals in the 127-136 ppm range and by the proton signals in the 7.0-7.27 ppm region.
***Lipids* 25, 260-266 (1990).**

Numerous long-chain *N*-acylamino acids have been prepared for study and use as detergents or surfactants (1,2). Sodium *N*-lauroylsarcosine has been found to be an effective anti-caries agent for dental care (3). Sodium salts of *N*-acylamino acids (containing glycine, alanine or sarcosine) were recently found to be useful flotation agents in the purification of fluorite (4). Monolayers studies with *N*-stearoylamino acids (valine, leucine, isoleucine) have shown the temperature dependence of surface pressure to area, and that upon methylation of the amino acid residues the monolayers become unstable (5). *N*-Lauroylaspartic acid, *N*-stearoylglycine and *N*-stearoylglutamic acid were also shown to improve the quality of bread by enhancing proteolysis of wheat proteins (6,7).

While most past efforts have been directed towards the study of the properties and uses of *N*-acylamino acids, we have focused our attention on derivatives of unsaturated C_{18} fatty esters containing an amino acid residue attached to a specific carbon of the alkyl chain of the fatty ester. We also report on the nuclear magnetic resonance (NMR) spectroscopic properties of these *L*-amino acid-containing unsaturated fatty esters, as no such data are available in the literature. The derivatives are required for studies to investigate the effects of different types of amino acid residues on the rate of fatty acids metabolism in hepatocytes.

The present paper describes the preparation of five unsaturated C_{18} fatty esters (1-5) containing an amino acid residue (glycine, alanine, leucine, valine, phenylalanine) linked to C-12 of the alkenyl chain and of the respective methyl esters of *N*-stearoylamino acids (6-10). The spectroscopic properties of the compounds and the synthetic routes are illustrated in Schemes 1 and 2.



(1-5)

c

$R^1 = CH_3(CH_2)_5$; $R^2 = CH_2CH{=}CH(CH_2)_7COOCH_3$;

R^3 = H (1); CH_3 (2); $(CH_3)_2CH$ (3); $(CH_3)_2CHCH_2$ (4); $C_6H_5CH_2$ (5).

SCHEME 1. Preparation of methyl C_{18} unsaturated fatty ester derivatives containing *L*-amino acid residues (1-5).

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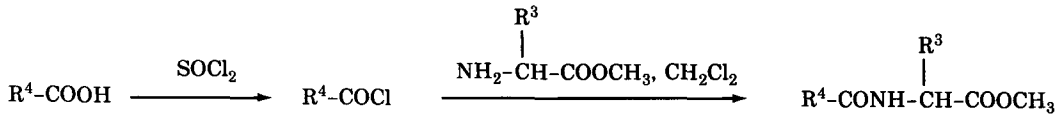


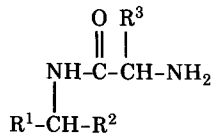
(6–10)

$R^4 = CH_3(CH_2)_{16}$; R^3 = H (6); CH_3 (7); $(CH_3)_2CH$ (8); $(CH_3)_2CHCH_2$ (9); $C_6H_5CH_2$ (10).

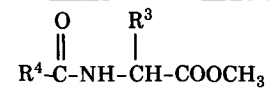
SCHEME 2. Preparation of methyl *N*-stearoylamino acid esters (6–10).

TABLE 1

Characterization of C_{18} Unsaturated Esters Containing *L*-Amino Acid Residues (1–5) and Methyl Esters of *N*-Stearoylamino Acids (6–10)

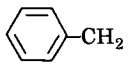
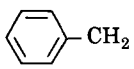


(1–5)



(6–10)

$R^1 = CH_3(CH_2)_5$; $R^2 = CH_2CH{=}^{c}CH(CH_2)_7COOCH_3$; $R^4 = CH_3(CH_2)_{16}$

Compound	R^3	TLC R_f (PEA)[a]	Formula		Microanalysis C	H	N (%)	Infrared (cm^{-1})
1	H	0.4	$C_{21}H_{40}O_3N_2$	found	68.43	10.94	7.60	3300 (m), 1740 (s), 1665 (s),
				calc.	68.11	11.12	7.30	1660 (w), 1505 (s)
2	CH_3	0.4	$C_{22}H_{42}O_3N_2$		68.78	11.22	7.06	3300 (m), 1740 (s), 1665 (s)
					69.06	11.07	7.33	1660 (w), 1500 (s)
3	$(CH_3)_2CH$	0.5	$C_{24}H_{46}O_3N_2$		70.15	11.56	6.49	3400 (m), 1740 (s), 1665 (s),
					70.19	11.29	6.83	1660 (w), 1505 (s)
4	$(CH_3)_2CHCH_2$	0.5	$C_{25}H_{48}O_3N_2$		71.38	11.56	6.51	3300 (m), 1740 (s), 1655 (s),
					71.22	11.46	6.37	1660 (w), 1505 (s)
5		0.5	$C_{28}H_{46}O_3N_2$		73.56	10.32	6.00	3300 (m), 3060 (w), 1740 (s),
					73.36	10.04	6.11	1665 (s), 1600 (w), 1505 (s)
6	H	0.5	$C_{22}H_{41}O_3N$		71.10	11.52	3.80	3400 (m), 1740 (s), 1680 (s),
					70.94	11.62	3.94	1630 (s), 1530 (m)
7	CH_3	0.5	$C_{22}H_{43}O_3N$		71.40	11.89	3.90	3390 (m), 1740 (s), 1690 (m),
					71.50	11.73	3.79	1630 (s), 1530 (m)
8	$(CH_3)_2CH$	0.6	$C_{24}H_{47}O_3N$		72.40	11.80	3.60	3300 (m), 1740 (s), 1680 (m),
					72.49	11.91	3.52	1630 (s), 1530 (m)
9	$(CH_3)_2CHCH_2$	0.6	$C_{25}H_{49}O_3N$		72.80	12.01	3.43	3300 (m), 1740 (s), 1680 (w),
					72.94	11.99	3.40	1630 (m), 1520 (w)
10		0.6	$C_{28}H_{47}O_3N$		75.50	10.58	3.20	3300 (m), 3050 (w), 1730 (s)
					75.46	10.63	3.14	1650 (m), 1630 (m), 1530 (m)

[a]PEA = petroleum ether/diethyl ether/acetone (1:4:1, v/v/v).

MATERIALS AND METHODS

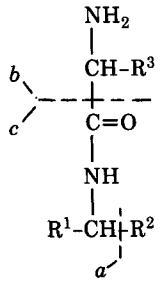
Materials. 12-Hydroxy-9-*cis*-octadecenoic acid (ricinoleic acid) was isolated from castor oil according to the partition procedure described by Gunstone (8). Stearic acid was purchased from Aldrich Chemical Co. (Gillingham, England, UK). *L*-Amino acids and their methyl ester hydrochloride salts were purchased from Sigma Chemical Co. (St. Louis, MO). Methyl 12-azido-9-*cis*-octadecenoate was prepared as described elsewhere (9). Methyl esters of *N*-stearoylamino acids (glycine, alanine, leucine, valine, phenylalanine) were prepared by the Schotten-Baumann reaction from stearoyl chloride, and the methyl esters of the corresponding *L*-amino acids as described by Iyer *et al.* (2).

*Methyl 12-amino-9-*cis*-octadecenoate.* A mixture of methyl 12-azido-9-*cis*-octadecenoate (5 g), methanol (50 ml) and Lindlar catalyst (0.1 g) was shaken in an atmosphere of hydrogen at 800 mm Hg pressure for 12 hr. The mixture was filtered and the solvent evaporated under reduced pressure. Column chromatographic purification on silica (50 g) using a mixture of petroleum ether/diethyl ether/methanol (1:3:1, v/v/v) furnished methyl 12-amino-9-*cis*-octadecenoate, 4.2 g, 91%; ECL = 19.90 (SE-30); IR (cm^{-1}): 3300 (w), 1740 (s), 1600 (w), 1250 (m); ^1H NMR ($CDCl_3$) δ 0.88 ppm (*t*, 3H, $C\underline{H}_3$), 1.2-1.4 (*m*, 18H, $C\underline{H}_2$), 1.72 (*s*, 2H, $N\underline{H}_2$), 2.3 (*t*, 2H, $C\underline{H}_2COOCH_3$), 2.7 (*m*, 1H, $C\underline{H}NH_2$), 3.66 (*s*, 3H, $COOC\underline{H}_3$), 5.2-5.4 (*m*, 2H, $C\underline{H}=C\underline{H}$).

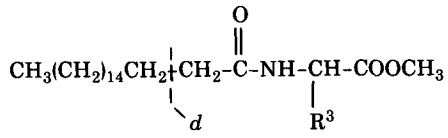
*General method for the preparation of methyl C_{18} unsaturated fatty esters containing an amino acid residue at C-12 as exemplified by the preparation of methyl 12-glycylamino-9-*cis*-octadecenoate* (1). A mixture of glycine (1.5 g) and phthalic anhydride (3 g) was heated with

TABLE 2

Mass Spectral Analysis of C_{18} Unsaturated Esters Containing *L*-Amino Acid Residues (1-5) and Methyl Esters of *N*-Stearoylamino Acids (6-10)

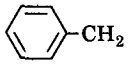
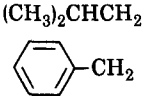


(1-5)



(6-10)

R^1= $CH_3(CH_2)_5$; R^2 = $CH_2CH{=}CH(CH_2)_7COOCH_3$

Compound	R^3	M^+	M-31	*a*	*b*	*c*	$R_1CH{=}\overset{+}{N}H_2$	*d* + 1	Others
1	H	368 (0.3)[a]	337 (0.1)	171 (34.6)	30 (99)	339 (0)	114 (100)	—	
2	CH_3	382 (0.6)	351 (2.1)	185 (28.6)	44 (98)	339 (3.3)	114 (100)	—	
3	$(CH_3)_2CH$	410 (3.2)	379 (10.1)	213 (24.7)	72 (100)	339 (16.4)	114 (58)	—	
4	$(CH_3)_2CHCH_2$	424 (4.1)	393 (8.5)	227 (26.0)	86 (98)	339 (18.0)	114 (100)	—	
5		458 (2.3)	427 (4.7)	261 (29.4)	120 (100)	339 (26.5)	114 (30)	—	
6	H	355 (0.8)	324 (0.2)	—	—	—	—	130 (100)	144 (15.5), 90 (27.8)
7	CH_3	369 (0.9)	338 (0.5)	—	—	—	—	145 (100)	104 (15.3), 86 (8.5)
8	$(CH_3)_2CH$	397 (0.4)	366 (0.2)	—	—	—	—	173 (10.8)	143 (13.7), 87 (63.4), 74 (35.6), 72 (65.7)
9	$(CH_3)_2CHCH_2$	411 (0.2)	380 (0.1)	—	—	—	—	187 (13.8)	131 (15.1), 86 (100)
10		445 (0.5)	414 (0.2)	—	—	—	—	221 (2.3)	162 (100), 120 (22), 88 (14.5)

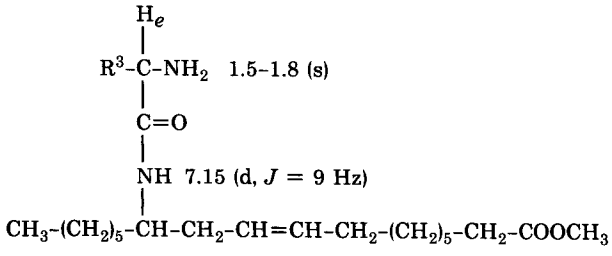
[a] *m/z* (intensity).

stirring at 160°C for 30 min. The cooled mixture was dissolved in hot methanol (20 ml) and diluted with water (20 ml) to precipitate the phthaloyl derivative of glycine (3.7 g, 90%). This derivative was heated with PCl_5 (4.2 g) in benzene (50 ml) at 60°C for 1 hr. The cooled reaction mixture was filtered, and evaporation of the solvent under reduced pressure furnished the acyl chloride of the phthaloyl derivative of glycine (3.9 g, 89%). The latter was mixed with methyl 12-amino-9-*cis*-octadecenoate (4.5 g) in dichloromethane (30 ml) and stirred overnight at room temperature. The reaction mixture was poured into dilute HCl (2 M, 15 ml) and extracted with dichloromethane (50 ml). The organic extract was successively washed with water (20 ml), dilute $NaHCO_3$ (10%, 10 ml), water (20 ml) and dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure and flash column silica chromatographic purification, using a mixture of petroleum ether/diethyl ether (1:4, v/v) as eluent, gave the phthaloyl protected methyl 12-glycylamino-9-*cis*-octadecenoate (3.9 g, 68%). The latter was refluxed with hydrazine hydrate (0.5 g) in ethanol (30 ml) for 1 hr. Water (30 ml) was added and the mixture extracted with diethyl ether (2 × 30 ml). The ethereal extract was washed with saturated aqueous NaCl (15 ml) and dried over anhydrous Na_2SO_4. Evaporation of the solvent furnished methyl 12-glycylamino-9-*cis*-octadecenoate (2.9 g, 90%).

General method for the preparation of methyl N-*stearoylamino acid derivatives as exemplified by the preparation of methyl* N-*stearoylglycinate* (6). A mixture of stearic acid (10.9 g) and thionyl chloride (5.3 g) was heated at 100°C for 1 hr. Excess thionyl chloride was evaporated under reduced pressure. Methyl glycinate hydrogen

TABLE 3

^{1}H NMR Chemical Shift Values of C_{18} Unsaturated Fatty Esters Containing *L*-Amino Acid Residues (1-5)[a]

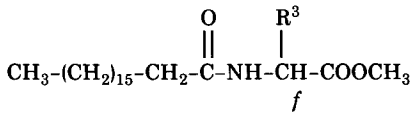


Compound	R^3	H_e
1	H	3.34 (s)
2	CH_3 1.3	3.47 (q, J = 7 Hz)
3	CH_3 \ CH CH_3 / 1.6 (m) 0.85 (d, J = 7 Hz)	3.20 (d, J = 7 Hz)
4	CH_3 \ 1.6-1.7 (m) CH-CH_2 CH_3 / 0.93 (d, J = 7 Hz)	3.41 (t, J = 7 Hz)
5	2.6-2.8 (m) CH_2 7.27 (s)	3.1-3.6 (m)

[a]Spectra were recorded on solutions in $CDCl_3$. Chemical shifts are reported in ppm downfield from tetramethylsilane used as internal standard.

TABLE 4

^{1}H NMR Chemical Shift Values of Methyl Esters of *N*-Stearoylamino Acids (6-10)[a]



Compound	R^3	H_f
6	H	4.04 (d, J = 5.3 Hz)
7	CH_3 1.40 (d, J = 7 Hz)	4.61 (quint., J = 7 Hz)
8	CH_3 \ CH CH_3 / 1.6-1.8 (m) 0.85 (d, J = 6.8 Hz)	4.5-4.65 (m)
9	CH_3 \ 1.6-1.8 (m) CH-CH_2 CH_3 / 0.9 (m)	4.5-4.8 (m)
10	CH_2 3.1 (d, J = 6 Hz) 7.0-7.2 (m)	4.8-5.0 (m)

[a]Spectra were recorded on solutions in $CDCl_3$. Chemical shifts are reported in ppm downfield from tetramethylsilane used as internal standard.

chloride (4.9 g) in dichloromethane (50 ml) was added with external cooling (0–5°C) followed by triethylamine (4.9 g). The reaction mixture was stirred for 16 hr at room temperature. Dilute HCl (2 M, 30 ml) was added and the organic layer isolated. The organic extract was washed with water (20 ml), dilute $NaHCO_3$ (10%, 20 ml), water (20 ml) and dried over anhydrous Na_2SO_4. The solvent was evaporated and the product isolated by silica column chromatography (190 g, silica) using increasing polar mixtures of petroleum ether/diethyl ether (200 ml portions of 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, v/v). Fractions (20 ml each) were collected and analyzed by thin-layer chromatography (TLC), and fractions 28–35 contained compound **6**, 6.4 g, 45%.

General methods. TLC was carried out on glass microscope slides coated with silica gel (about 0.1 mm thick, Kieselgel 60). The solvent system used was a mixture of petroleum ether/diethyl ether/acetone (1:4:1, v/v/v). Spots were visualized by exposing the plates to iodine vapor. Elemental analyses were carried out by Butterworth Laboratories (Teddington, Middlesex, England, UK). Infrared spectra were recorded on a Perkin Elmer model 577 spectrophotometer. ^1H and ^{13}C NMR spectra were recorded on a JEOL FX90 FT NMR spectrometer. Samples were dissolved in $CDCl_3$ (about 0.2 mmol concentration), and tetramethylsilane used as internal standard. Mass spectrometric analyses were carried out on a Hitachi RMS-4 spectrometer by direct insertion. Spectra were run at 70 eV with a source pressure of 10^{-6} torr and a temperature of 150–180°C.

RESULTS AND DISCUSSIONS

In the preparation of the series of C_{18} unsaturated fatty esters (**1–5**) with the amino acid residue linked to the amino group at C-12 of the alkenyl chain, the amino function of the *L*-amino acid (glycine, alanine, leucine, valine, phenylalanine) was protected with a phthaloyl group (10) prior to conversion of the carboxylic acid function to the acyl chloride. The reaction between methyl 12-amino-9-*cis*-octadecenoate and the phthaloyl protected amino acid chloride proceeded smoothly with an average yield of 78%. Deprotection of the phthaloyl group was achieved by refluxing the isolated product with hydrazine hydrate in ethanol. The methyl esters of *N*-stearoylamino acids (**6–10**) were prepared by reacting stearoyl chloride with the methyl ester of the respective *L*-amino acids (2).

The results of the TLC, elemental and infrared analyses of compounds **1–10** are presented in Table 1. The presence of a free (basic) amino group in compounds **1–5** increased the polarity of these derivatives considerably relative to methyl oleate on TLC when silica gel was used as the adsorbent. However, when alumina was employed as the absorbent for TLC analysis, these amine-containing derivatives are poorly separated and tend to streak. The presence of an amino and amido function was evident from the N–H stretching (3300 cm^{-1}) and bending (1660, 1505 cm^{-1}) vibrations and the strong C=O stretching (1665 cm^{-1}) vibration of the amide group in the infrared spectra of compounds **1–5**. The methyl esters of *N*-stearoylamino acids (**6–10**) were slightly less polar than compounds **1–5** on TLC and the amido group in these compounds was confirmed by the absorption bands at 3400, 1680 and 1630 cm^{-1} in the infrared spectrum.

In the mass spectra of compounds **1–5** (Table 2), two characteristic peaks are found. Ion fragments m/z = 171, 185, 213, 227 and 261 result from cleavage (*a*) between C-11 and C-12 of the alkenyl chain of compounds **1**, **2**, **3**, **4** and **5**, respectively. Ion fragment m/z = 114 is due to $CH_3(CH_2)_5CH{=}\overset{+}{N}H_2$. From these two peaks, the position of the amide (peptide) linkage in the alkenyl chain of the fatty ester can be determined.

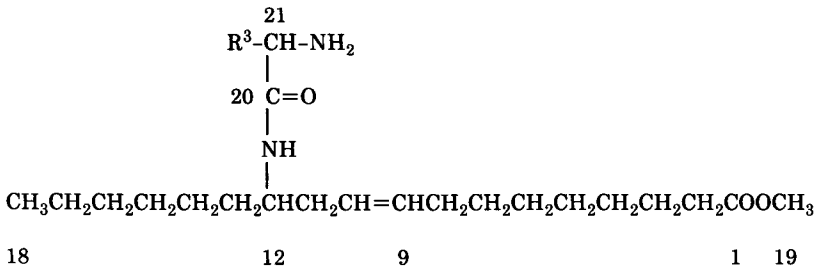
The structure of the amino acid moiety was also reflected by an intense peak resulting from ion fragments *b*. The mass spectral analysis of the methyl *N*-stearoylamino acid derivatives (**6–10**) showed β-cleavage at the amide function with bond cleavage between C-2 and C-3 of the C_{18} chain giving an intense peak, *d* + 1, for compounds **6** and **7**. In the case of compounds **8–10**, this ion fragment was less intense, as further fragmentation by rearrangement furnished other intense ions of lower mass unit (Table 2). It was not possible to ascertain from the mass spectra of compounds **6–10** the chain length of the alkyl group in these derivatives. In all these amino acid-containing fatty ester derivatives, the M^+ and M − 31 ions were very weak (<1%).

The ^1H and ^{13}C NMR chemical shifts for compounds **1–10** are summarized in Tables 3–6. Although both series contained an amido system, ^1H NMR could differentiate these two sets of derivatives by the appearance of a downfield signal at 7.15 ppm, resulting from the resonance of the amido (NHCO) group in compounds **1–5**, while the resonance of the proton of the amido group of compounds **6–10** was found at 6.0 ppm.

Although ^1H NMR spectral analysis provided adequate information regarding the chemical structure of these derivatives, ^{13}C NMR of compounds **1–10** was even more informative. With the exception of several signals associated with the methylene carbons (29.1–29.7 ppm) of the alkyl chain of compounds **6–10**, the signals for the remaining carbons in both sets of derivatives were readily assigned. In compounds **1–5** the α-effect of the amido system on the resonance of the C-12 carbon atom of the alkenyl chain was significant, causing this nucleus to resonate between 48.8–49.8 ppm relative to TMS. The effect of the same amido group on the ethylenic carbon caused an upfield shift for the C-9 carbon (124–125 ppm), while the remaining ethylenic carbon at C-10 (131–132 ppm) was barely affected by this amido function. Also, the γ-effect of the amido group influenced the resonance of the methylene carbon at C-14, which appeared at 25.8–26.0 ppm. The methyl carbon resonances of the isopropyl system in compounds **3** and **4**, and also in compounds **8** and **9** of the *N*-stearoylamino acid series, furnished two shift values due to the chemical nonequivalence of these methyl groups. The phenyl system in compounds **5** and **10** was characterized by signals appearing in the 126–138 ppm region for the aromatic carbons. In considering the chemical shift for the carbonyl carbon in the amido system in both series of derivatives, compounds **1–5** contained an amino group located β to the amido carbonyl carbon. This caused the shift for the latter to appear at 171.9 ppm in compound **1**, and between 173.4–175.1 ppm in compounds **2–5**. In the *N*-stearoylamino ester series, the chemical shift for the carbonyl carbon of the amido function appeared at about 172 ppm, except in the case of compound **6**, where the resonance occurred at 170.6 ppm. The effect of the phenyl system

TABLE 5

^{13}C NMR Chemical Shift Values of C_{18} Unsaturated Fatty Esters Containing *L*-Amino Acid Residues (1-5)[a]



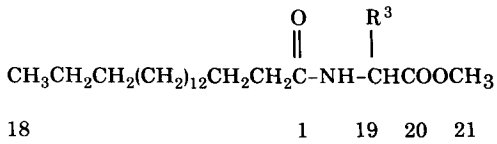
Compounds	R^3	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9	C-10	C-11	C-12	C-13
1	H	174.2	34.1	24.9	29.1	29.5	29.1	29.1	27.4	124.1	132.5	32.5	49.8	34.4
2	CH_3 22	174.1	34.0	24.9	29.1	29.6	29.1	29.1	27.4	125.3	132.3	32.6	48.7	34.5
3	$(CH_3)_2CH$ 24 23 22	174.6	34.7	24.7	28.8	28.9	28.8	28.8	27.0	125.1	131.9	32.3	48.4	34.1
4	$(CH_3)_2CHCH_2$ 25 24 23 22	174.1	33.9	24.9	29.0	29.5	29.0	29.0	27.3	125.2	132.2	32.5	48.6	34.4
5	26 CH(25 CH=CH 24)(23 C-CH2 22)(25 CH-CH 24)	174.2	34.1	24.9	29.1	29.4	29.1	29.1	27.4	125.1	132.4	32.5	48.8	34.4

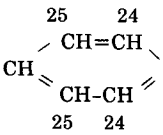
Compounds	R^3	C-14	C-15	C-16	C-17	C-18	C-19	C-20	C-21	C-22	C-23	C-24	C-25	C-26
1	H	26.0	29.5	31.5	22.6	14.0	51.4	171.7	44.8					
2	CH_3 22	26.0	29.8	31.8	22.6	14.0	51.3	175.1	50.9	21.9				
3	$(CH_3)_2CH$ 24 23 22	25.8	29.3	31.5	22.3	13.7	50.9	173.4	60.0	30.6	22.3	19.4		
4	$(CH_3)_2CHCH_2$ 25 24 23 22	25.9	29.4	31.7	22.5	14.0	51.2	174.7	53.7	44.3	23.3	21.4	22.5	
5	26 CH(25 CH=CH 24)(23 C-CH2 22)(25 CH-CH 24)	25.9	29.5	31.8	22.6	14.0	51.4	173.5	56.6	41.1	138.0	128.6	129.3	126.7

[a]Spectra were recorded on solutions in $CDCl_3$. Chemical shifts are reported in ppm downfield from tetramethylsilane used as internal standard.

TABLE 6

^{13}C NMR Chemical Shift Values of Methyl Esters of *N*-Stearoylamino Acids (6-10)[a]



Compound	R^3	C-1	C-2	C-3	C-4 to C-15	C-16	C-17	C-18	C-19	C-20	C-21	C-22	C-23	C-24	C-25	C-26
6	H	173.4	36.4	25.6	29.3-29.7	31.9	22.7	14.1	41.2	170.6	52.3					
7	CH_3 (22)	173.7	36.6	25.6	29.3-29.7	31.9	22.7	14.1	47.9	172.7	52.4	18.6				
8	$(CH_3)_2CH$ (24 23 22)	173.1	36.8	25.8	29.2-29.7	32.0	22.7	14.1	57.0	172.8	52.0	31.5	18.9	17.9		
9	$(CH_3)_2CHCH_2$ (25 24 23 22)	173.8	36.7	25.7	29.3-29.7	32.0	22.7	14.1	50.7	172.9	52.1	42.0	25.0	22.8	22.1	
10		172.7[b]	36.6	25.6	29.3-29.7	32.0	22.7	14.1	53.1	172.3[b]	52.2	38.1	136.1	128.6	129.3	127.1

[a] Spectra were recorded on solutions in $CDCl_3$. Chemical shifts are reported in ppm downfield from tetramethylsilane used as internal standard.
[b] Assignments are interchangeable.

on the shift of the carbonyl carbon of the amido and the carbomethoxy group made it difficult to assign with certainty the chemical shifts for these two nuclei in compound **10**. One very distinct feature in the ^{13}C NMR spectra of these derivatives (**1-10**) was the chemical shift of the methylene carbon of the glycine residue in compounds **1** and **6**, and the similarly positioned methine carbon in the other amino acid residues of the remaining derivatives, all of which appeared between 40-60 ppm. These chemical shift values were very much affected by the number and position of the methyl group(s) or the phenyl group present in the *L*-amino acid residue. However, these signals were readily differentiated from the signal arising from the carbon of the methyl group of the carbomethoxy (methyl ester) function by off-resonance decoupling.

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Effect of Dietary α-Linolenate/Linoleate Balance on Crescent Type-Anti-Glomerular Basement Membrane Nephritis in Rats

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Rats were fed diets with three different ratios of α-linolenate (18:3 n-3) and linoleate (18:2 n-6), and then crescentic-type anti-glomerular basement membrane (GBM) nephritis was induced. The urinary protein levels and the plasma urea nitrogen levels were significantly higher, and histological abnormalities of glomeruli were seen more frequently in the high-α-linolenate group than in the high-linoleate group. The differences in dietary α-linolenate/linoleate balances were reflected in the proportions of arachidonate and eicosapentaenoate in glomerular phospholipids. Our results indicate that dietary enrichment with α-linolenate causes unfavorable effects in this anti-GBM nephritis model.
Lipids 25, 267-272 (1990).

Cyclooxygenase products have been implicated in the development and progression of renal diseases; these compounds are vasoactive and have multiple action on renal hemodynamics (1-3). Lianos *et al.* (4) were the first to suggest that thromboxane A_2 (TXA_2), a vasoconstrictive eicosanoid produced by the glomerulus, was involved in the pathogenesis of anti-glomerular basement membrane (anti-GBM) nephritis. TXA_2 synthetase inhibitors were found to prevent the abnormal renal hemodynamics in this nephritis model. Subsequent reports have documented the role for TXA_2 in the pathogenesis of other types of experimental and clinical renal diseases (5-12). However, other investigators have failed to provide evidence for a role of TXA_2 (13-16), and instead have reported evidence implicating prostaglandin E_2 (PGE_2), a renal vasodilator, as the agent which maintains renal hemodynamics (13,14). In related work, the effects of dietary polyunsaturated fatty acids, precursors of eicosanoids, on various types of experimental renal diseases have been studied. In general, eicosapentaenoic acid (EPA, 20:5 n-3) has been found to be beneficial in preventing the progression of several types of immune-mediated renal diseases (17-23). However, beneficial effects of dietary linoleic acid (18:2 n-6) have also been reported (24-26). Thus the interpretation of the effects of dietary n-3 and n-6 polyunsaturated fatty acids on renal diseases appears to be complicated; in part, this may be due to the fact that no direct comparisons of n-3 and n-6 polyunsaturated fatty acids have previously been made.

In the present study, we examined the effect of dietary α-linolenate (18:3 n-3)/linoleate (18:2 n-6) balances on the development of an immune-mediated renal disease, crescentic type anti-GBM nephritis in rats. Rats were fed perilla seed oil (rich in 18:3 n-3) or safflower seed oil (rich in 18:2 n-6) or mixtures of the two oils. These two vegetable oils contain very similar proportions of saturated and monoenoic fatty acids and differ mainly in the proportions of 18:3 n-3 and 18:2 n-6. Thus feeding experiments with these two oils allow us to evaluate the comparative effectiveness of n-3 and n-6 polyunsaturated fatty acids in the diets.

MATERIALS AND METHODS

Animals and diets. Male Sprague-Dawley rats (Shizuoka Laboratory Animal Co., Ltd., Shizuoka, Japan) at 3 weeks of age (40-60 g) were divided into three groups, 15 to 16 rats each. Rats in each group were fed diets supplemented with (i) α-linolenate-rich perilla seed oil, (ii) linoleate-rich safflower seed oil, or (iii) a mixture (65:35, w/w) of perilla oil and safflower oil (Table 1). The basal diet was prepared by extracting conventional laboratory chow (CE-2, Nippon Clea Co., Tokyo, Japan) with n-hexane by a method commonly used to prepare vegetable oils, and then supplementing the defatted chow with a vitamin mixture (2%, w/w) (Nippon Clea Co., Tokyo) and the respective oils (10%, w/w) described above.

Induction of crescentic-type anti-GBM nephritis. Rabbit anti-rat GBM antiserum was prepared as described previously (28). After 21 days of feeding (i.e. at 6 wk of age), each of 10 rats in each group was injected intravenously with 0.75 mL of anti-GBM antiserum (day 1), followed, on the next day, by subcutaneous immunization with 6.5 mg of rabbit γ-globulin (Sigma, St. Louis, MO) as an emulsion with Freund's complete adjuvant (Difco Laboratory, Detroit, MI) (10,11). On day 1, there was no difference in the mean body weights of animals in the three dietary groups (169 ± 8.6 g in the high-α-linolenate group, 170 ± 9.2 g in the high-linoleate group, and 168 ± 9.5 g in the mixture group). The treated rats were fed the respective diets throughout the experimental period. The remaining 5 to 6 rats in each group were also kept as untreated controls.

Biochemical and histological assessments of the severity of glomerulonephritis. In order to collect 24-hr urines, rats were orally administered with 8 mL of water per animal by gastric intubation and then placed in metabolic cages on days 1, 4, 11, 20, 30 and 39 after the injection of anti-GBM antiserum. Protein contents in 24-hr urines were determined by the sulfosalicylic acid method (29). On days 8, 16, 28 and 36, blood samples (0.3 to 0.5 mL) were taken from the tail vein, and plasma was prepared. Urea nitrogen and total cholesterol in plasma were determined by the urease-indophenol method (30) and by using a Determina TC-5 kit (Kyowa Medix Co., Ltd., Tokyo, Japan), respectively. On day 43, rats were anesthesized with sodium pentobarbital (30 mg/kg), and each kidney was perfused with 20 mL of ice-cold phosphate-buffered saline.

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Abbreviations: GBM, glomerular basement membrane; PG, prostaglandin; TX, thromboxane.

TABLE 1

Fatty Acid Compositions of Experimental Diets (% of total fatty acids)[a]

Fatty acids	High-α-linolenate (Perilla seed oil)	Mixture	High-linoleate (Safflower seed oil)
14:0	0.1	0.1	0.1
16:0	7.9	8.1	8.1
18:0	1.8	1.8	2.3
18:1 n-9	17.3	15.6	11.8
18:2 n-6	17.1	37.0	74.2
18:3 n-3	51.7	33.9	1.0
20:4 n-6	0.1	0.1	—
20:5 n-3	0.7	—	0.7
22:6 n-3	0.3	0.3	0.3
18:3 n-3/ 18:2 n-6 ratio	3.0	0.9	<0.1

[a]The fatty acid composition of each diet was analyzed by gas-liquid chromatography using an EGSS-X (Shimazu, Tokyo, Japan) as stationary phase (27). The diets contained 10% perilla oil (high-α-linolenate), 10% safflower oil (high-linoleate), or a 65:35 (w/w) mixture of perilla oil and safflower oil (mixture). Fatty acids are designated by the carbon chain:the number of double bonds. The position of the first double bond numbered from the methyl terminus is designated as n-9, n-6 or n-3.

Immediately after the perfusion, the kidneys were harvested and one-half of one kidney from each rat was fixed and dehydrated in solutions containing increasing concentrations of ethanol (30 to 100%) in phosphate-buffered saline at 4°C. The renal tissue was embedded into paraffin and then cut into 3 μm-thick sections. The sections were stained with Masson's trichrome. Fifty glomeruli in a section from each rat were examined under a light microscope. The occurrences of (i) crescent formation and fibrinoid necrosis in glomeruli, and (ii) adhesion of the capillary wall to Bowman's capsule were quantified.

Isolation of glomeruli and analysis of fatty acid composition of glomerular phospholipids. The residual portions of kidney from one or two rats were placed in ice-cold phosphate-buffered saline. Cortical and medullallary tissue were separated by dissection, and the cortical tissue was minced. The minced cortices were first passed through a 106 μm sieve, and then trapped onto a 75 μm sieve. The glomerular-enriched fraction trapped onto the sieve was washed with ice-cold phosphate-buffered saline, collected and suspended in the same solution. The purity of glomeruli was determined microscopically to be 84 ± 4.9% (mean ± SD). The impurities were mostly tubular materials. The total lipids of the isolated glomeruli were extracted with chloroform/methanol according to the method of Bligh and Dyer (31). The phospholipids were separated by one-dimensional thin-layer chromatography on Silica Gel (Merck 60) using petroleum ether/diethyl ether/acetic acid (80:30:1, v/v/v) as a solvent. Fractions were located by spraying with ethanolic Rhodamin 6G. Phospholipids were recovered from the adsorbent by extracting with chloroform/methanol/6%-ammonium hydroxide (6:5:1, v/v/v), and the fatty acids in the phospholipids were transmethylated with 5% HCl/MeOH. Fatty acid methyl esters were then quantitated by gas-liquid chromatography using heptadecanoic acid as an internal standard (32).

Statistical analysis. All data are expressed as mean ± SD. Statistical analyses of the data were performed with the analysis of variance (ANOVA) using Turkey's or Scheffe's multiple comparison and the Student's t-test.

RESULTS

Biochemical parameters in urine and plasma. After i.v. injection of anti-GBM serum, all rats in the three dietary groups developed glomerulonephritis as judged by proteinuria apparent on day 1 (150–240 mg/day) (Fig. 1). The untreated control rats (fed the mixture diet) excreted about 10 mg of protein/day into the urine. The protein contents in 24-hr urines dropped to 80–120 mg/day on day 4, but thereafter the proteinuria in the high-α-linolenate and the mixture groups developed progressively; an approximately two-fold difference over 4-day values was observed on days 20, 30 and 39. The progression of proteinuria in the high-linoleate group was significantly reduced compared to the other two groups.

Urea nitrogen levels in plasma from rats in all the anti-GBM serum-treated groups tended to be higher ($p<0.05$, Student's t-test) than those in the untreated group (fed the mixture diet) on day 9, 16 and 36 (Fig. 2a). The urea nitrogen levels were slightly higher in the high-α-linolenate and the mixture groups than in the high-linoleate group only on day 36 ($p<0.05$ and $p<0.01$, respectively). The total plasma cholesterol levels in all the treated groups were markedly elevated as compared with those of the untreated group (Fig. 2b). However, the differences among the three dietary groups were relatively small, and the pattern of elevations of total plasma cholesterol among the three dietary groups did not parallel the severity of glomerulonephritis as estimated by proteinuria (Fig. 1). Both body weights and 24-hr urine volume were quite similar among the three dietary groups after the induction of nephritis (data not shown). Therefore, the elevation of plasma urea content is not due to the difference in urine volumes, but represents the decreased amount of urea excreted in the high-α-linolenate and the mixture groups.

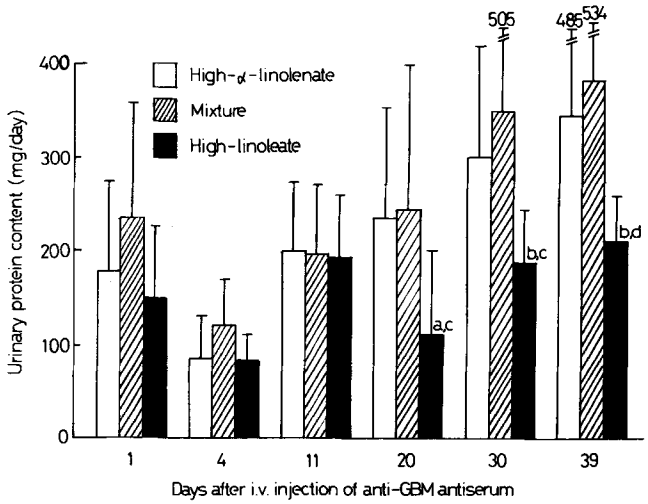

FIG. 1. Effect of dietary fatty acids on the progression of proteinuria in rats with crescentic-type anti-GBM nephritis. Male Sprague-Dawley rats were fed the test diets for 21 days prior to i.v. injection of anti-GBM antiserum. Urinary protein contents in 24-hr urines were determind by the sulfosalicylic acid method (28). Figures are averages (± SD) of 9 to 10 rats in each group. The untreated rats excreted approximately 10 mg of protein per day into the urine throughout the experimental period. Values denoted with symbols represent statistically significant differences in the Student's t-test as follows: a) $p<0.05$ and b) $p<0.01$ for the high-linoleate vs the mixture groups, c) $p<0.02$ and d) $p<0.01$ for the high-linoleate vs the high-α-linolenate groups. In ANOVA test, these differences were not significant.

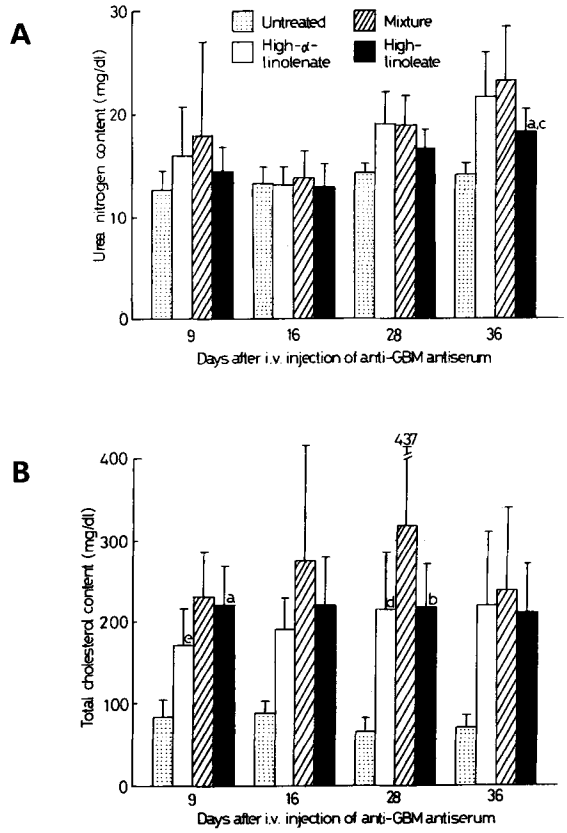

FIG. 2(a,b). Effects of dietary fatty acids on urea nitrogen content and total cholesterol content in plasma from rats with crescent-type anti-GBM nephritis. Blood samples were obtained from rats with (n=9 or 10) or without (untreated, n=6) crescentic-type anti-GBM nephritis on days 9, 16, 28 and 36 after an i.v. injection of anti-GBM antiserum. Urea nitrogen content (a) and total cholesterol content (b) in plasma were determined enzymatically (see text for details). Values denoted with symbols represent statistically significant differences in the Student's t-test as follows: a) $p<0.05$ for the high-linoleate vs the high-α-linolenate groups, b) $p<0.05$ and c) $p<0.01$ for the high-linolenate vs the mixture groups, d) $p<0.05$ and e) $p<0.02$ for the high-α-linolenate vs the mixture groups. In ANOVA test, these differences were not significant.

Histological evaluation of the severity of glomerulonephritis. Characteristic histological abnormalities of rat kidneys with anti-GBM nephritis are crescent formation and fibrinoid necrosis in glomeruli, and adhesion of the glomerular tuft to Bowman's capsule (33). The frequencies of these abnormalities were determined microscopically (Fig. 3). Crescent formation in glomeruli and adhesion were both significantly higher in the high-α-linolenate and the mixture groups than in the high-linoleate group. There were no statistically significant differences in the occurrence of fibrinoid necrosis among the three dietary groups.

Diet- and anti-GBM antiserum-induced changes in the fatty acid compositions of glomerular phospholipids. Fatty acid compositions of glomerular phospholipids from rats both in the treated groups and the untreated groups at the end of the experiments (on day 43) are compared in Table 2. The differences in the α-linolenate/linoleate ratios of the diets were reflected in the proportions of n-6 and n-3 polyunsaturated fatty acids in glomerular phospholipids. The major changes were noted in the proportions of arachidonate (20:4 n-6), eicosapentaenoate (20:5 n-3), 22:4 n-6 and 22:5 n-3. In contrast, the proportions of 22:5 n-6 and 22:6 n-3 remained relatively constant. These diet-induced changes were qualitatively similar in the treated and the untreated groups. However, the proportions of some 20 and 22 carbon polyunsaturated fatty acids (20:4 n-6, 20:5 n-3, 22:5 n-6) in glomerular phospholipids were slightly but significantly lower in the treated groups than in the untreated groups.

The time courses of diet-induced changes in the proportions of arachidonate (20:4 n-6) and eicosapentaenoate (20:5 n-3) are shown in Figure 4. The proportions of these fatty acids changed markedly during the first 20 days after the shift in diets, and then remained at the new plateau levels throughout the remainder of the experiments. These results establish that the fatty acids of kidney phospholipids had undergone diet-induced changes before administration of anti-GBM antiserum.

DISCUSSION

The crescentic-type anti-GBM nephritis model used in this study is characterized by a heavy and progressive proteinuria which occurs in a biphasic pattern (28). The earlier phase is termed the heterologous phase, and it occurs within a day after anti-GBM antiserum injection. The later phase is termed the autologous phase

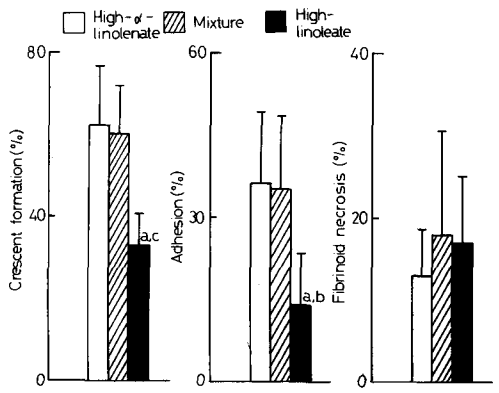

FIG. 3. Effect of dietary fatty acids on histological abnormalities of glomeruli. On day 43 after an i.v. injection of anti-GBM antiserum, kidney were harvested, dehydrated and embedded into paraffin. Sections (3 μm) were prepared and stained with Masson's trichrome. Fifty glomeruli in a section from each rat were examined under a light microscope and the percentages of occurrences of histological abnormalities were calculated. Values denoted with symbols represent statistically significant differences in the Student's t-test as follows: a) $p<0.001$ for the high-linoleate vs the high-α-linolenate groups, b) $p<0.01$ and c) $p<0.001$ for the high-linoleate vs the mixture groups. Crescent formations in the high-α-linolenate and the mixture groups were significantly ($p<0.05$) higher than in the high-linoleate group, but the difference in adhesion (%) was not significant among the dietary groups, when estimated by ANOVA.

and it occurs 7 to 10 days thereafter. The heterologous phase is thought to be elicited by a reaction between anti-GBM antibody and host GBM, thereby activating complement systems and causing emigration of polymorphonuclear leukocytes (34). The development of the autologous phase is dependent on the production of antibody against rabbit γ-globulin which reacts with the GBM-bound heterologous antibody; these reactions cause monocytes/macrophages to participate (35,36). Eicosanoids derived from n-3 and n-6 polyunsaturated fatty acids appear to be involved in all these events but the mechanisms of their involvement have not been defined.

Feeding α-linolenate-rich diets decreases arachidonate (20:4 n-6) and increases eicosapentaenoate (20:5 n-3) levels in cellular phospholipids (33,37), thereby decreasing the synthesis of eicosanoid from arachidonate. We have shown previously that this dietary manipulation suppresses an immunoinflammatory response (33) and thrombotic tendency (37) in rats. Unexpectedly, however, feeding α-linolenate-rich diets exacerbated anti-GBM nephritis. This finding was surprising considering the reported suppressive effects of fish oil on the progression of nephritis in other animal models (17,19-23) and in humans (18). Feeding fish oil containing 22:6 n-3 is known to result in the accumulation of 22:6 n-3 in tissues and in the induction of peroxisomes (38), but feeding high-α-linolenate oil did not accumu-

TABLE 2

Effects of Dietary Fatty Acids on the Fatty Acid Composition of Glomerular Phospholipids (% of total fatty acids)[a]

Fatty acids	Treated groups High-α-linolenate (n=4)	Treated groups mixture (n=4)	Treated groups High-linoleate (n=4)	Untreated groups High-α-linolenate (n=3)	Untreated groups mixture (n=3)	Untreated groups High-linoleate (n=3)
14:0	0.1 ± 0.1	0.1 ± 0	0.1 ± 0	0.3 ± 0.1	0.2 ± 0	0.1 ± 0.1
14:1	0.1 ± 0.1	0.1 ± 0	0.1 ± 0.1	0.1 ± 0	0.1 ± 0.1	0.1 ± 0.1
16DMA	2.3 ± 0.4*	2.4 ± 0.2*	2.2 ± 0.1*	1.4 ± 0.2	1.6 ± 0.2	1.6 ± 0.2
16:0	18.5 ± 0.5*	18.0 ± 0.3*	18.2 ± 0.7	17.2 ± 0.5	17.1 ± 0.3	17.8 ± 0.2
16:1	0.4 ± 0.4	0.5 ± 0.1	0.3 ± 0.2	0.8 ± 0.1	0.4 ± 0.3	0.5 ± 0.1
18DMA	1.6 ± 0.3	1.8 ± 0.2*	1.7 ± 0.1	1.0 ± 0.4	1.2 ± 0.1	1.5 ± 0.2
18:0	21.4 ± 0.3	21.4 ± 0.4	21.4 ± 0.6	21.1 ± 0.6	21.2 ± 0.5	20.9 ± 0.2
18:1	7.8 ± 0.5[b,d]	6.3 ± 0.2[c]	4.6 ± 0.2	7.0 ± 0.3[b,d]	5.9 ± 0.1[c]	5.1 ± 0.3
18:2 n-6	12.2 ± 0.8	12.8 ± 0.4	11.7 ± 1.0	13.2 ± 0.5[b]	14.1 ± 0.7[c]	11.7 ± 0.5
18:3 n-6	tr	tr	tr	ND	tr	tr
18:3 n-3	2.1 ± 0.1[b,d]	1.3 ± 0.1[c]	0.5 ± 0.1	2.5 ± 0.1[b,d]	1.4 ± 0.7[c]	0.4 ± 0.1
20:1 n-9	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0
20:3 n-6	1.0 ± 0.1	1.1 ± 0.1	0.8 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	0.7 ± 0.1
20:4 n-6	12.8 ± 0.5[b,d,*]	19.0 ± 0.7[c,*]	23.4 ± 1.1*	14.0 ± 0.8[b,d]	21.4 ± 0.6[c]	27.6 ± 1.3
20:5 n-3	5.8 ± 0.5[b,d*]	1.7 ± 0.2[c]	tr	7.3 ± 0.8[b,d]	1.7 ± 0.1[c]	tr
22:4 n-6	0.7 ± 0.1[b,d,*]	1.6 ± 0.1[c,*]	4.3 ± 0.3*	0.4 ± 0[b,d]	0.9 ± 0.1[c]	2.7 ± 0.7
22:5 n-6	3.6 ± 0.6[b,*]	3.6 ± 0.3[c,*]	4.8 ± 0.6	4.9 ± 0.3	5.0 ± 0.2	5.3 ± 0.7
22:5 n-3	5.0 ± 0.7[b,d,*]	3.4 ± 0.2[c,*]	0.4 ± 0.1	3.9 ± 0.1[b,d]	2.4 ± 0.1[c]	0.3 ± 0
22:6 n-3	2.3 ± 0.1	2.5 ± 0.2	1.8 ± 0.4	2.2 ± 0.2	2.3 ± 0.1	1.9 ± 0.3
n-3	15.1 ± 0.3[b,d]	8.8 ± 0.3[c]	2.7 ± 0.4	15.9 ± 1.1[b,d]	7.8 ± 0.1[c]	2.7 ± 0.3
n-6	30.3 ± 1.8[b,d]	38.0 ± 0.9[c]	44.9 ± 1.3	33.5 ± 1.2[b,d]	42.4 ± 0.3[c]	47.9 ± 0.9

[a]For each assay, glomeruli from 1 or 2 rats were used.
[b]Statistically significant differences ($p<0.05$) among the dietary groups were observed when analyzed by ANOVA using Turkey's multiple comparison for the high-linoleate vs the high-α-linolenate groups.
[c]The high-linoleate vs the mixture groups.
[d]The high-α-linolenate vs the mixture groups.
*Significant differences ($p<0.05$) between the treated and the untreated groups were evaluated by the Student's t-test.

Abbreviations: DMA, dimethylacetal; tr, trace; ND, not detected.

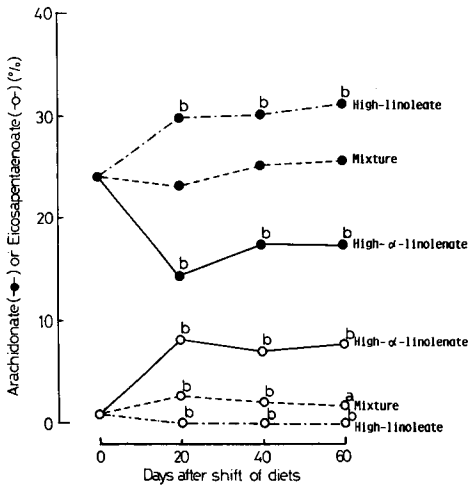

FIG. 4. Changes in the levels of arachidonic acid and eicosapentaenoic acid in kidney phospholipids. On days 0, 20, 40 and 60 after feeding the test diets (high-α-linolenate —— , mixture ——, high-linoleate——·——), kidneys were harvested and the fatty acid compositions of kidney phospholipids were determined by gas-liquid chromatography. Each point represents the mean of three determinations. Standard deviations were all below 7% of the mean values. Statistically significant differences between the mean values at each time point after the feeding (on days 20, 40, 60) and day 0 are denoted as follows: a) $p<0.01$, b) $p<0.001$ (Student's t-test). Both arachidonate and eicosapentaenoate levels in kidney phospholipids were significantly different ($p<0.05$) among the three dietary groups, when estimated by ANOVA.

late 22:6 n-3 in kidney (Table 2). However, it is unclear whether or not this difference accounts for the differential effects of these oils on nephritis.

Our previous observations (10,11) that TXA_2 synthetase inhibitors ameliorate anti-GBM nephritis are apparently inconsistent with the lack of suppressive effects of the high-α-linolenate diet (Fig. 1). Inhibition of TXA_2 synthesis may change the metabolic flow of liberated arachidonate resulting in increased PGE_2 synthesis, thereby increasing renal blood flow. The effectiveness of vasodilatory and vasoconstrictive eicosanoids may be different depending on the stages and type of nephritis. In fact, the unfavorable effects of high-α-linolenate diets observed in the current study of anti-GBM nephritis were not seen in murine autoimmune lupus glomerulo-nephritis in female MRL/1 pr mice (S. Watanabe *et al.*, unpublished observations).

The autologous phase of anti-GBM nephritis is initiated by the appearance of antibody against rabbit γ-globulin in the host (35). The dietary α-linolenate/linoleate balance might affect the immune system, but the antibody titers against rabbit γ-globulin in plasma estimated by a passive hemaglutinin test were similar among the three dietary groups (data not shown). Further studies are necessary to determine if there are diet-induced modifications in the production of anti-rabbit γ-globulins.

Recent studies in our laboratory revealed beneficial effects of feeding high-α-linolenate diet in several kinds of chronic diseases in animals (32,33,37,39-41). The present study is the first demonstration of an unfavorable effect of a high-α-linolenate diet. Undoubtedly, the present conclusion does not apply to all types of nephritis, and the applicability of the anti-GBM nephritis models in rats to human nephritis is not certain. However, we believe that it is quite important to note that there are pathogenic conditions in which high-α-linolenate, low-linoleate diets exert detrimental rather than protective effects.

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Metabolism of Phosphatidylinositol in Plasma Membranes and Synaptosomes of Rat Cerebral Cortex: A Comparison Between Endogenous Vs Exogenous Substrate Pools

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The metabolism of phosphatidylinositols (PI) labeled with [^{14}C]arachidonic acid within plasma membranes or synaptosomes was compared to the metabolism of PI prelabeled with [^{14}C]arachidonic acid and added exogenously to the same membranes. Incubation of membranes containing the endogenously-labeled PI pool in the presence of Ca^{2+} resulted in the release of labeled arachidonic acid, as well as a small amount of labeled diacylglycerol. Labeled arachidonic acid was effectively reutilized and returned to the membrane phospholipids in the presence of adenosine triphosphate (ATP), CoA, and lysoPI. Although Ca^{2+} promoted the release of labeled diacylglycerol from prelabeled plasma membranes, this amount was only 17% of the maximal release, i.e., release in the presence of deoxycholate and Ca^{2+}. This latter condition is known to fully activate the PI-phospholipase C, and incubation of prelabeled plasma membranes resulted in a six-fold increase in labeled diacylglycerols. On the other hand, when exogenously labeled PI were incubated with plasma membranes in the presence of Ca^{2+}, the labeled diacylglycerols released were 59% of that compared to the fully activated condition. The phospholipase C action was calcium-dependent, regardless of whether exogenous or endogenous substrates were used in the incubation. In contrast to plasma membranes, intact synaptosomes had limited ability to metabolize exogenous PI even in the presence of Ca^{2+}, although the activity of phospholipase C was similar to that in the plasma membranes when assayed in the presence of deoxycholate and Ca^{2+}. These results suggest that discrete pools of PI are present in plasma membranes, and that the pool associated with the acyltransferase is apparently not readily accessible to hydrolysis by phospholipase C.
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Although phosphatidylinositol (PI) constitutes less than 4% of total phospholipids in brain membranes, this phospholipid is metabolically more active than other phospholipids. PI is known to participate in several enzymic reactions. One important class of reactions is associated with a deacylation-reacylation process, mediated by phospholipase A_2 and lysophospholipid:acyl-CoA acyltransferase (1). This process is important for enriching PI with arachidonoyl groups, since the preferred substrate for this acyltransferase is arachidonoyl-CoA (1). Besides these reactions, PI is converted to phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP_2) by the action of PI-and PIP-kinases, which have distinct properties and subcellular distributions (2). A third pathway for metabolism of PI and its phosphorylated derivatives is the hydrolysis by phospholipase C (3). In brain, several phospholipase C species are present (4–7) and exist in both membrane-bound and soluble forms (8–10).

Since the various enzymes promoting metabolism of PI may be present together within the same type of membrane, the metabolic fate of this phospholipid may be controlled by the topological distribution of the enzymes or substrates. To address this issue, we have taken advantage of the abundance of LPI acyl transferase activity (11) to generate a pool of 1-acyl-2-[^{14}C]arachidonoyl-*sn*-glycero-3-phosphoinositol ([^{14}C]arachidonoyl-PI) within the membrane. The metabolism of this radiolabeled, endogenously generated PI pool by enzymes such as phospholipase A_2 and phospholipase C was studied and compared with that of [^{14}C]arachidonoyl-PI exogenously added to the membrane suspension.

MATERIALS AND METHODS

[1-^{14}C]Arachidonic acid (specific radioactivity 54.9 mCi/mmol) was purchased from New England Nuclear Corp. (Boston, MA). Lysophosphatidylinositols (LPI), sodium deoxycholate (DOC), dithiothreitol, bovine serum albumin (BSA), adenosine triphosphate (ATP) and CoA were obtained from Sigma Chemical Co. (St. Louis, MO).

Preparation of synaptosomes and a non-synaptic plasma membrane fraction from rat cerebral cortex. Sprague-Dawley rats were decapitated and cerebral cortices were quickly removed and suspended in 20 vol of ice-cold 0.32 M sucrose with 50 mM Tris-HCl (pH 7.4) and 1 mM EDTA. Brain tissue was homogenized using a motor-driven glass homogenizer fitted with a Teflon pestle. The brain homogenate was centrifuged at 278 × *g* for 10 min. After discarding the pellet, the supernatant fraction was centrifuged at 18,800 × *g* for 20 min to obtain the P_2 pellet. Synaptosomes were prepared from the P_2 pellet by layering the suspension on 0.8 M sucrose and subsequently centrifuging at 43,500 × *g* for 20 min. Under this condition, myelin membranes appeared at the interface between 0.32 M and 0.8 M sucrose, and synaptosomes were pelleted together with a small amount of free mitochondria (less than 10%). Synaptosomes were suspended in a Krebs-Ringer bicarbonate buffer containing: 122 mM

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ATP, adenosine triphosphate; BSA, bovine serum albumin; DG, diacylglycerol; DOC, deoxycholate; FFA, free fatty acids; GLC, gas liquid chromatography; HPTLC, high performance thin-layer chromatography; LPI, lysophosphatidylinositol; PA, phosphatidic acid; PC, phosphatidylcholine; PI, phosphatidylinositol; PL, phospholipid; PS, phosphatidylserine; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; TG, triacylglycerols.

NaCl, 3 mM KCl, 1.2 mM $MgSO_4$, 0.4 mM KH_2PO_4, 25 mM $NaHCO_3$ and 10 mM D-glucose. Final protein concentration was 10–15 mg protein/ml. Calcium in specified amounts was added prior to incubation.

A non-synaptic plasma membrane fraction was prepared from the post-mitochondrial supernatant according to the procedure described by Sun *et al.* (12), except that BSA (0.5 mg/ml) was added to the supernatant prior to the centrifugation. This step was added to remove excess fatty acids from the membranes. The BSA wash also yielded membranes that were more active in acyltransferase activity. The membrane pellet obtained was rinsed several times with the sucrose-Tris buffer and resuspended in the same buffer at a final protein concentration of 3–4 mg/ml.

Prelabeling membranes with [^{14}C]arachidonic acid. Aliquots of synaptosomes or plasma membranes were prelabeled by incubating the membranes (4–5 mg protein) with [1-^{14}C]arachidonic acid (5 μCi per tube), 131 nmol of LPI, 10 mM $MgCl_2$, 2.5 mM ATP, 0.1 mM CoA and 0.3 mM dithiothreitol in a total volume of 5 ml with 0.32M sucrose-Tris buffer (13,14). Incubation was carried out at 37°C for 30 min. The reaction was terminated by adding 15 ml ice-cold sucrose-Tris buffer containing BSA (0.5 mg/ml) and centrifuged at 19,250 × *g* for 15 min. This procedure was repeated in order to remove excess labeled arachidonic acid. The membrane pellet was resuspended in 1 ml sucrose-Tris buffer. In a typical preparation, the plasma membranes yielded approximately 100,000 cpm/mg protein with over 90% of the radioactivity in the PI fraction.

Preparation of PI containing [^{14}C]arachidonic acid. In order to isolate PI containing [^{14}C]arachidonic acid, plasma membranes were incubated with [1-^{14}C]-arachidonic acid, LPI and the same cofactors, except that the label used was 10 times more than that used in normal incubations. The reaction was terminated by adding 4 ml of chloroform/methanol (2:1, v/v). The aqueous phase was again extracted with 2 ml of chloroform/methanol (4:1, v/v). The two lower phases were combined, dried under nitrogen, and applied to a high performance thin-layer chromatography (HPTLC) plate. Lipids were separated by the two-dimensional procedure described by Horrocks and Sun (15). After visualization with 2′,7′-dichlorofluorescein, the PI spot was scraped into a test tube and extracted with chloroform/methanol (2:1, v/v).

Assay for Phospholipase A_2 and Phospholipase C activity in prelabeled membranes or in membranes with labeled PI. Aliquots of prelabeled membranes containing approximately 20,000 cpm were transferred to separate test tubes and incubated in a shaking incubator in the presence of cofactors such as Ca^{2+} (1.5 mM), sodium deoxycholate (1 mg/ml) and/or EGTA (1 mM) in a total volume of 0.5 ml, either with sucrose-Tris buffer (for plasma membranes) or with Krebs-Ringer bicarbonate buffer (for synaptosomes). Incubation was carried out at 37°C for 30 min. Background activity was assessed by using similar membrane suspensions without incubation. Incubation was terminated by adding 4 vol of chloroform/methanol (2:1, v/v), followed by extraction and centrifugation for phase separation. The lower phase was then filtered through anhydrous Na_2SO_4, and the solvent was taken to dryness. The samples were applied to HPTLC plates for lipid separation.

For experiments involving exogenous labeled PI, the substrate was first added to test tubes, organic solvent evaporated and then mixed with buffer prior to adding the membranes.

Lipid separation. For separation of DG and fatty acids, aliquots of the lipid extract were applied to silica gel 60 HPTLC plates (E. Merck, Darmstadt, Federal Republic of Germany) and developed in a system containing hexane/diethyl ether/glacial acetic acid (80:20:3, by vol). The plates were developed twice in the same system. After development, the plates were dried and exposed to iodine vapor for visualization of lipid bands. The solvent system is known to give good separation of DG (together with cholesterol), fatty acids and triacylglycerols (TG), with phospholipids remaining at the origin. The lipid fractions were scraped into scintillation vials, and radioactivity was counted with a Beckman LS5800 scintillation spectrometer (Beckman, Palo Alto, CA).

A two-dimensional HPTLC system similar to that described by Horrocks and Sun (15) and Sun (16), was used to analyze phospholipids after prelabeling with [^{14}C]arachidonic acid. This procedure was used because PI can be clearly separated from phosphatidylserine (PS) and PA which may contain a small amount of the labeled arachidonate. Samples were spotted on 10 × 10 cm silica gel 60 HPTLC plates and developed in the first solvent system containing chloroform/methanol/15M NH_4OH (135:60:10, by vol). After development, the plates were dried briefly and then exposed to HCl fumes for 3 min to cleave the alkenyl ether bond of plasmalogen. After removing the HCl fumes, the plates were developed in the second solvent system containing chloroform/methanol/acetone/glacial acetic acid/0.1 M ammonium acetate (70:30:27:2.5:5, by vol). The lipid spots were visualized by exposing the plates to iodine. Lipid fractions were scraped into scintillation vials and counted.

The same two-dimensional HPTLC system was used for the separation of phospholipids which were used for quantitative analysis of the acyl groups of PI and phosphatidylcholine (PC) by gas liquid chromatograph (GLC). After development, the HPTLC plates were sprayed with 2′,7′-dichlorofluorescein and the lipid spots were visualized under UV light. The acyl groups of PI and PC were converted to their fatty acid methyl esters by base-catalyzed methanolysis (0.5 M NaOH in methanol) (16). The methyl esters were quantitatively analyzed by GLC together with an internal standard (17:0).

RESULTS

Incubation of brain membranes with [1-^{14}C]arachidonic acid, LPI and cofactors results in rapid incorporation of radiolabel into phospholipids as reported previously (13,14). Following removal of residual [1-^{14}C]arachidonic acid by washing plasma membranes with BSA, 90% of the radioactivity incorporated into phospholipids was present in PI. Similar labeling of synaptosomes resulted in 55% of the radiolabel in PI and 40% in PC. Complete removal of all radiolabeled fatty acid was not

achieved with the washing procedure employed, which could account for 6-10% of total radioactivity in the prelabeled membranes.

When plasma membranes or synaptosomes containing labeled PI were incubated in the presence of 1.5 mM Ca^{2+}, there was a decrease in the relative amount of radiolabel in phospholipids and a corresponding increase in labeled fatty acids and DG (Table 1). When the cofactors ATP and CoA were included in the incubation medium, a significant decrease was observed in the proportion of label in the fatty acid pool. This apparent enhancement of reacylation activity was further promoted by the addition of LPI, a preferred substrate for the acyltransferases in brain (1).

To examine additional factors affecting the hydrolysis of labeled PI in plasma membranes, incubations were carried out in the presence of Ca^{2+}, EGTA and/or deoxycholate. As shown in Figure 1, no appreciable amount of labeled DG was formed from the prelabeled membranes either during incubation alone or incubation in the presence of EGTA. However, a nearly three-fold increase in the relative amount of labeled DG was observed following incubation in the presence of Ca^{2+}. When deoxycholate and Ca^{2+} were both present a further six-fold increase in label (to 17.3% of the total label) was found in DG. These latter incubation conditions have been shown to result in maximal activation of PI-phospholipase C activity in brain (7). Figure 1 also illustrates the ability of plasma membranes to metabolize exogenously added [1-^{14}C]arachidonoyl-PI. Incubation of plasma membranes with labeled PI (dispersed in buffer by vortexing) resulted in a two-fold increase of labeled DG, as compared to controls without incubation. This increase was inhibited by EGTA, but strongly enhanced by the addition of Ca^{2+}, which resulted in a 10-fold increase in label compared to controls without incubation. As with the endogenously labeled membranes, the addition of deoxycholate and Ca^{2+} resulted in a further increase in labeled DG, and the maximal extent of DG release was nearly the same for both endogenously and exogenously labeled PI.

To quantify the amount of PI in plasma membranes which is susceptible to hydrolysis by phospholipase C, unlabeled plasma membranes were analyzed for PI and PC content by GLC both before and after incubation under various conditions described above. As shown in Table 2, there was no significant change in the amount of PC under any of the incubation conditions, whereas the PI content was decreased significantly (by 20%) after incubation in the presence of Ca^{2+} and deoxycholate.

We also tested the response of synaptosomes containing labeled PI under similar incubation conditions as described above for the plasma membranes. When prelabeled synaptosomes were incubated with different combinations of EGTA, Ca^{2+}, and deoxycholate, large increases in labeled DG were observed in those systems containing deoxycholate alone or deoxycholate together with Ca^{2+} (Fig. 2a). On the other hand, when labeled PI was added exogenously to synaptosomes, an increase in labeled DG was produced only by the presence of both deoxycholate and Ca^{2+} (Fig. 2b). Synaptosomes also were examined for their ability to release labeled arachidonic acid from labeled PI (either generated endogenously or added exogenously). As shown in Figure 2, the presence of Ca^{2+} alone was the only condition that gave an apparent increase in labeled arachidonic acid from either endogenously or exogenously labeled PI.

DISCUSSION

In agreement with previously reported results (11,14), we have demonstrated that brain plasma membranes can actively incorporate labeled arachidonic acid into PI through the acyltransferase pathway. The labeled arachidonic acid was shown to be subsequently re-

TABLE 1

Effects of ATP, CoASH and LPI on Phospholipid Pools in Synaptosomes and Plasma Membranes[a]

Conditions	PL	DG	FFA
Plasma membranes:			
No incubation	91.4 ± 0.88	2.0 ± 0.33	6.6 ± 1.02
I	88.1 ± 1.34*	2.6 ± 0.52	9.2 ± 0.80*
I + ATP	91.9 ± 1.56**	2.4 ± 0.30	5.7 ± 1.37**
I + ATP + CoA	91.5 ± 1.05**	2.5 ± 0.22	6.0 ± 0.89**
I + ATP + CoA + LPI	95.6 ± 0.66**	2.5 ± 0.45	1.9 ± 0.40**
Synaptosomes:			
No incubation	87.5 ± 0.88	2.1 ± 0.13	10.4 ± 0.83
I	81.8 ± 1.32*	3.5 ± 1.11*	14.8 ± 0.44*
I + ATP	86.9 ± 0.96**	2.7 ± 0.29	10.4 ± 0.73**
I + ATP + CoA	88.2 ± 1.29**	3.3 ± 0.43	8.6 ± 0.86**
I + ATP + CoA + LPI	93.1 ± 1.68**	2.8 ± 0.45	4.1 ± 1.20**

[a]Plasma membranes or synaptosomes were first prelabeled by incubating with [1-^{14}C]arachidonic acid in the presence of ATP, Mg^{2+}, CoASH and LPI as described in the text. The prelabeled membranes were washed with BSA, resuspended in Krebs-Ringer bicarbonate buffer with 10 mM glucose and 1.5 mM Ca^{2+}, and further incubated under different conditions. Incubations (I) were carried out at 37°C for 30 min. Data depict the percent distribution of radioactivity (mean ± S.D. from triplicate incubations) among phospholipids (PL), DG and FFA (free fatty acids). Values that are significantly different based on one-way ANOVA ($p<0.05$) are marked: *, comparing incubation (I) to no-incubation controls; **, comparing cofactors to incubation alone. Data are from one of three experiments which gave similar results.

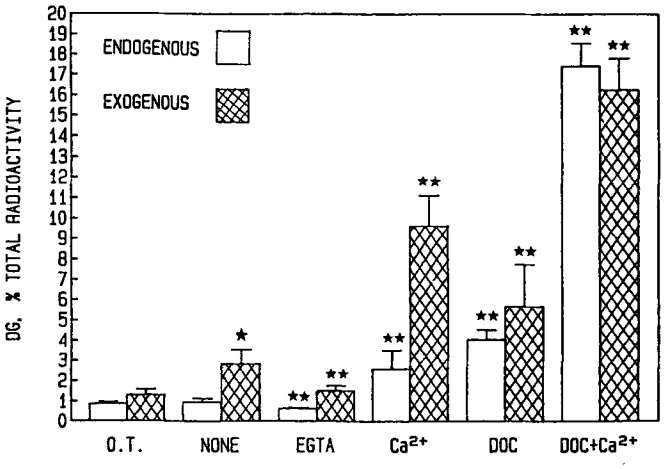

FIG. 1. Metabolism of endogenous and exogenous PI labeled with [1-^{14}C]arachidonic acid by brain plasma membranes. Labeled PI was either generated endogenously within the plasma membranes via the acyltransferase route or added exogenously to the incubation system. The incubation system contained freshly prepared plasma membranes and agents such as EGTA (1mM), Ca^{2+} (net 1.5 mM), deoxycholate (DOC) (1 mg/ml), or DOC plus Ca^{2+} (1.5 mM). Incubations were carried out for 30 min at 37°C. Data depict the radioactivity of DG as percent of total radioactivity. Values are mean ± S.D. from four samples for each condition. The experiment was repeated two times and similar results were obtained. Data that are significantly different based on one-way Analysis of Variance (ANOVA) ($p<0.05$) are marked: *, labeled DG formed due to incubation of samples (NONE) as compared to no incubation (O.T.); **, DG formed due to incubation of samples with EGTA, Ca^{2+} and/or DOC as compared to incubation (NONE) without these agents.

TABLE 2

Effects of Incubation in the Presence of Calcium and/or Deoxycholate on Levels of PI and PC in Brain Plasma Membranes[a]

Conditions	PI	PC
	nmol/mg protein	
1. No incubation	7.3 ± 0.5	83.6 ± 7.7
2. Incubation (I)	8.2	86.1 ± 12.2
3. I + Ca^{2+}	7.9 ± 1.0	81.2 ± 6.0
4. I + DOC	9.2 ± 1.7	78.5 ± 8.6
5. I + DOC + Ca^{2+}	5.9 ± 0.7*	91.0 ± 5.7

[a]Plasma membranes freshly isolated from brain cortex were incubated in the presence of Ca^{2+} and/or DOC at 37°C for 30 min. After incubation, lipids were extracted and separated by the two-dimensional HPTLC procedure as described in text. The amount of phospholipid in the plate was quantitatively determined by GLC analysis of the fatty acids after converting to their methyl esters. Values are nmol of phospholipids/mg protein (mean ± S.D.) from four samples except the PI in two which was obtained from average of two samples. Values that are significantly different ($p<0.05$) from no incubation control (condition in 1.) based on one way ANOVA are depicted by the symbol*.

we have demonstrated that brain plasma membranes can actively incorporate labeled arachidonic acid into PI through the acyltransferase pathway. The labeled arachidonic acid was shown to be subsequently released from either prelabeled plasma membranes or synaptosomes upon incubation in the presence of Ca^{2+}. The release of labeled fatty acids was coincident with a decrease in labeled phospholipids, suggesting that the release is mediated by the action of phospholipase A_2 under these conditions. The fatty acids released upon incubation could readily be reincorporated into membrane phospholipids in the presence of ATP and appropriate cofactors. The relative activities of acyltransferases and phospholipase A_2 thus may regulate the changes in the concentrations and the metabolism of arachidonic acid. Nevertheless, the data do not completely exclude the possibility that some arachidonic acid may be derived through hydrolysis from DG by the action of DG lipase or monoglyceride lipase, since these enzymes are also present in the brain membranes (17,18). However, the low level of labeled DG and the lack of an obvious precursor-product relationship between released DG and fatty acids are an argument against this idea (14).

The highly active deacylation-reacylation reactions with PI contrast with the low accessibility of this phospholipid to the actions of phospholipase C. Although PI can be effectively labeled in plasma membranes through the acyltransferase route, this labeled pool of PI is apparently not readily available to the hydrolytic action of phospholipase C. This lack of activity with phospholipase C is not due to an absence of the enzyme since a tightly bound form of phospholipase C has been described (8), and since high activity is expressed in the presence of Ca^{2+} and deoxycholate, a detergent known specifically to activate PI-phospholipase C (6–8). Furthermore, it is also unlikely that the results are due to the activity of a cytosolic phospholipase C, which is retained in the membrane vesicles, since enzymic activity could not be depleted even after repeated centrifugation (data not shown). One explanation for these results, therefore, is a topological distribution of phospholipase C which limits access to substrate (PI labeled by the acyltransferase) or to required activators. Support for this explanation comes from studies which show that phospholipase C is a peripheral protein with active sites exposed to the membrane surface (probably the cytoplasmic side). Such a location of the enzyme is in agreement with the observation that labeled PI, when added exogenously, is converted to DG in the presence of Ca^{2+}. Furthermore, when membranes were incubated in the presence of Ca^{2+} and deoxycholate together, approximately the same amount of labeled PI was degraded by phospholipase C regardless of whether the substrate was generated endogenously or added exogenously. Therefore, deoxycholate appears to make available all of the PI to the phospholipase C.

Some differences in the metabolism of PI were observed between synaptosomes and plasma membranes. Most significantly, intact synaptosomes were not able to hydrolyze effectively exogenous PI, even in the presence of Ca^{2+}. This result also suggests a distinct localization for the membrane-bound phospholipase C and possible confinement, largely within the synaptosomes.

Due to the presence of multiple pathways for metabolism of PI and related phospholipids, it is not surprising that these compounds would be present in

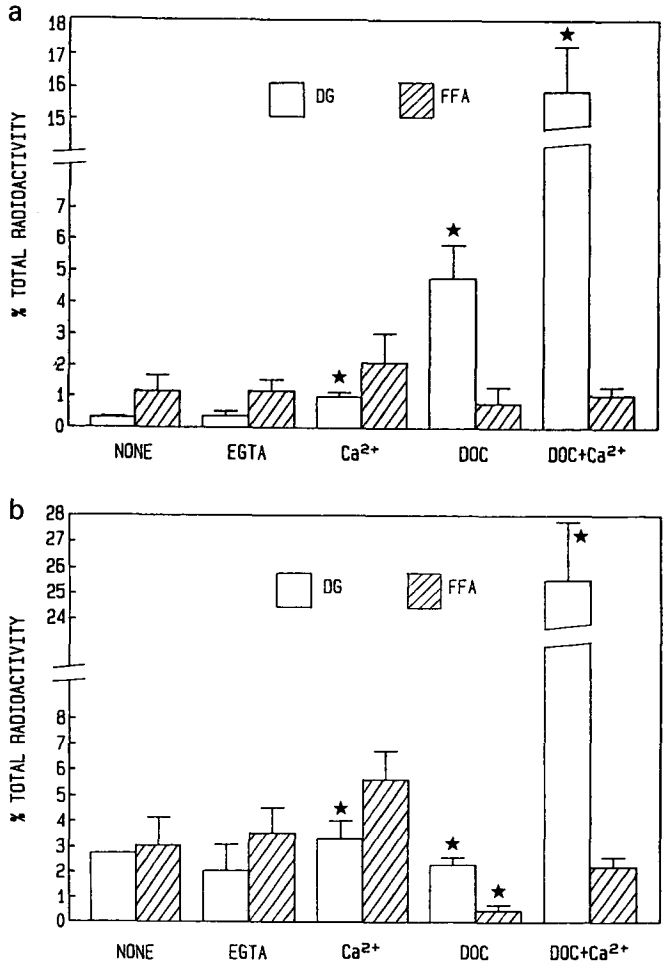

FIG. 2. Metabolism of PI labeled with [1-^{14}C]arachidonic acid in synaptosomes (a) generated endogenously via the acyltransferase or (b) added exogenously to the incubation mixture. Prelabeled synaptosomes were suspended in the Krebs-Ringer bicarbonate buffer and incubated for 30 min at 37°C as described in text. Results depict the percent of total radioactivty of DG (open bar) and free fatty acids (shaded bar) formed due to incubation, i.e., values were subtracted from the no incubation control. Values are mean ± S.D. from four samples for each condition. Values obtained from incubation in the presence of EGTA (1 mM), Ca^{2+} (1.5 mM) and/or deoxycholate (DOC, 1 mg/ml) that are significantly different from controls (NONE) based on one-way ANOVA are marked: *p<0.05.

discrete pools within the membrane. In this study, we showed that labeled PI, synthesized via the acyltransferase, appears to be sequestered into functionally distinct pools within the membrane. Segregation of this type may explain the difficulty in using the endogenously labeled pool as a substrate for other enzymes. The phenomenon may be especially pertinent to studies involving phospholipase C since there is considerable interest in elucidating the mechanism by which this enzyme interacts with its substrate (phosphoinositides) and functions as part of the receptor-mediated signal transduction mechanism (19-21).

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Effect of Dietary Fat on Phospholipid Class Distribution and Fatty Acid Composition in Rat Fat Cell Plasma Membrane

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The effect of dietary fats on phospholipid class distribution and fatty acid composition was studied in rat fat cell plasma membrane. Three groups of male Wistar weanling rats were fed for 8 wk three diets differing in the amount and nature of the fats: 1.5% sunflower oil (low fat control; LFC), 10% sunflower oil (high fat, unsaturated; HFU), 1.5% sunflower oil + 8.5% cocoa butter (high fat, saturated; HFS). Plasma membranes were prepared from epididymal adipocytes. The amount and type of dietary fat significantly altered membrane phospholipid distribution. Phospholipid content was lowered with HFU as compared to LFC or HFS diets, but no changes were observed for cholesterol. Phosphatidylinositol (PI) and phosphatidylserine (PS) were less affected by dietary changes than were other phospholipid classes. Major changes were detected for phosphatidylcholine (PC), phosphatidylethanolamine (PE) and sphingomyelin (SM) contents. No large changes in PC and PE fatty acid compositions were observed between the LFC and HFS groups, but the HFU diet induced several changes. Correlations with plasma membrane 5'-nucleotidase activities are discussed.
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The influence of dietary fat on membrane lipid composition has been widely investigated in recent years. It has been demonstrated that (i) membrane lipid composition can rapidly and profoundly be modified by the diet and that (ii) many membrane-associated functions can be altered by diet-induced changes in lipid composition (1). Most studies have been devoted to the dietary modification of the fatty acyl moieties of phospholipids (PL). Less attention has been paid to the influence of diet on PL class distribution (2-4). This is surprising because polar head group shape, size and charge are known to determine many structural and, consequently, functional characteristics of phospholipids (5,6), and because changes in phospholipid class distribution have been associated with various pathological states (7) as well as the physiological process of aging (8). Until recently, it was quite accepted that PL fatty acid composition could rapidly be modified by dietary fat whereas PL distribution would not be modified as readily. Indeed, some studies done on total PL extracts have shown no effect of dietary fat on PL class distribution (9-11). Diet-induced changes in membrane lipid composition were often found to be tissue specific or even membrane specific. For example, Tahin *et al.* have shown that rat liver, heart and brain subcellular fractions respond to different degrees, to dietary fatty acids (12). More recently, Brasitus *et al.* have demonstrated that different regions of enterocyte plasma membrane (i.e. microvillus and basolateral) had different patterns in response to changes in diet lipid composition (3). In the same way, studies on particular membrane fractions (as opposed to total PL) have shown that the PL profile could be influenced by dietary fat (2-4, 13-16).

Stored adipose tissue and structural lipids are known to be highly responsive to dietary fat intake (16). Recently, it was also shown that insulin binding to fat cells is modified in parallel with diet-induced changes in the fatty acyl moieties of plasma membrane PL (17). The present study was designed to investigate the effect of both the level and the type of dietary fat on PL distribution in the fat cell plasma membrane.

MATERIAL AND METHODS

Seventy-two male Wistar rats (4-wk old) weighing 55-60 g (IFFA-CREDO, l'Arbresle, France) were divided into three groups and fed a diet containing either 1.5% (w/w) sunflower oil (low fat, LFC diet), 10% sunflower oil (HFU diet) or 1.5% sunflower oil/8.5% cocoa butter (HFS diet). The compositions of the diets are given in Table 1, and the fatty acid profiles in Table 2. Animals were housed individually in wire-bottomed cages in a temperature-controlled room on a 12-hr light/dark cycle. Rats were fed *ad libitum* during a period of 8 wk, at the end of which the animals were killed by decapitation.

Epididymal fat pads were collected, and adipocytes isolated by incubation with crude collagenase (1 mg/g of tissue, Worthington, Cooper Biomedical, Marne-la-Vallée, France) as described by Rodbell (18) using Krebs Ringer biocarbonate buffer containing 4% (w/v) bovine serum albumin and 25 mM HEPES. Cell diameter was measured according to Lavau *et al.* (19). The diameter of about 200 cells was measured for each preparation.

The plasma membrane-enriched fraction was prepared by the Percoll gradient method of Belsham *et al.* (20). Briefly, adipocytes were homogenized in two volumes of buffer (0.25 M sucrose, 10 mM Tris, 2 mM EGTA, pH 7.4) in a Potter-Elvejehm homogenizer (3 strokes, 1200 rpm). The crude extract was defatted by centrifugation (1000 × *g* for 30 sec), and infranatant (referred to as "defatted homogenate") was removed after the fat plug had congealed. The particulate material was collected by centrifugation at 30,000 × *g* for 30 min and was applied to self-forming Percoll gradient (16,000 × *g* for 15 min). The plasma membrane fraction was recovered below the surface of the gradient (d 1.04) and was washed by centrifugation (30,000

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Abbreviations: LFC, low fat control; HFU, high fat, unsaturated; HFS, high fat, saturated; PL, phospholipid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; LPC, lysophosphatidylcholine; PEMT, phosphatidylethanolamine methyltransferase.

TABLE 1

Diet Composition

Ingredients g/kg	Diets[a] Low fat control (LFC)	High fat unsaturated (HFU)	High fat saturated (HFS)
Sucrose	250	220	220
Cellulose	40	40	40
Wheat starch	445	390	390
Mineral mix[b]	35	35	35
Vitamin mix[c]	10	10	10
DL-methionine	3	3	3
Choline chloride	2	2	2
Casein	200	200	200
Sunflower oil	15	100	15
Cocoa butter	—	—	85

[a]All diets contained less than 0.01% (w/w) cholesterol.
[b]Salt mixture (g/100 g): cupric sulfate, 0.143; manganese sulfate, 0.440; magnesium sulfate, 6.571; ferric citrate, 1.686; potassium iodide, 0.009; calcium carbonate, 20.714; potassium chloride, 20.857; sodium phosphate, 17.143; calcium phosphate, 32.285; zinc carbonate, 0.152.
[c]Vitamin mixture (mg/100 g): retinyl acetate (500,000 UI/g), 4.0; cholecalciferol (100,000 UI/g), 4.0; alpha-tocopherol (250 UI/g), 160; thiamin hydrochloride, 2.0; riboflavin, 3.0; pyridoxine hydrochloride, 10.0; niacin, 10.0; folic acid, 0.1; *p*-aminobenzoic acid, 10.0; vitamin B_{12}, 0.5; inositol, 20.0; biotin, 2.0; calcium pantothenate, 10.0; menaquinone, 0.2; nicotinamide, 0.2.

× *g* for 10 min) in a large volume of the NaCl-based medium described by Belsham *et al.* (20) to remove the Percoll present. The pellet is referred to as "plasma membrane" fraction. For each membrane preparation, the pooled adipocytes of six animals were used.

Protein was determined by the method of Lowry *et al.* (21) using bovine serum albumin as a standard. Enrichment in plasma membrane was judged by determining 5′-nucleotidase specific activities in the defatted homogenate and in plasma membrane fractions. The 5′-nucleotidase was assayed as the rate of [^{3}H]adenosine release from [^{3}H]AMP (19.3 Ci/mM Amersham, Les Ulis, France) (22).

Although at the end of the feeding period, body and adipose tissue weights were higher in the 10% fat groups (HFU and HFS diets) than in the LFC group (data not shown), the recovery of plasma membrane proteins per g of adipose tissue was not different in the three groups (Table 3). The 5′-nucleotidase specific activity was significantly higher when animals were fed 10% fat, but relative enrichment was not different in the three groups (Table 3). Total lipids were extracted from membrane vesicles by the method of Folch *et al.* (23). Antioxidant BHT (0.02%) was added to all solvents used for lipid analysis. Cholesterol was measured by a colorimetric procedure (Boehringer, CHOD-PAP). Neutral lipids and phospholipids were separated on silica Supelco cartridges (Serlabo, Paris, France) by successive elution with chloroform, then methanol. The eluates were concentrated and kept in a known amount of chloroform until analysis. The HPLC equipment was a Beckman Model 332 gradient liquid chromatography system consisting of two Model 110A pumps, a Model 420 system controller, and a Model 210 sample injection valve. Phospholipids were detected using a LKB detector, Model 2158 Uvicord SD, set at 206 nm. The fraction collector was a FRAC 100, Pharmacia Model. Organic solvents were of HPLC grade (Prolabo, Paris, France) and water was purchased from J.T. Backer (Deventer, the Netherlands). Solvent mixtures were deaerated under vacuum before use.

The separation of phospholipid classes was achieved using a Beckman 250 × 10 mm column, packed with 5 μm Ultrasphere Si. All separations were done at room temperature. The chromatographic system was programmed for gradient elution using mobile phases according to the method of Geurts van Kessel *et al.*

TABLE 2

Diet Fatty Acid Composition (wt %)

Fatty acid	LFC and HFU	HFS
16:0	5.7	21.8
16:1	0.2	0.1
18:0	4.4	30.0
18:1	19.3	32.1
18:2	68.9	14.5
18:3	—	0.1
Others	1.5	1.4
P/S ratio[a]	6.0	0.3

[a]Polyunsaturated fatty acid/saturated fatty acid ratio in the diet.

TABLE 3

Protein Recovery and 5′-Nucleotidase Activity in the Plasma Membrane-Enriched Fraction[a]

Diets	LFC	HFU	HFS
Protein recovery (mg/g of tissue)	0.13 ± 0.01	0.15 ± 0.02	0.14 ± 0.01
5′-Nucleotidase			
Specific activity[b]	1310 ± 77*	1813 ± 52**	1759 ± 68**
RSA[c]	32 ± 2	37 ± 3	32 ± 2

[a]Results are means ± SEM for four different membrane preparations. Each membrane preparation represents a pool of six rats.
[b]Specific activity is expressed as nmol/mg protein/hr.
[c]RSA refers to the specific activity of the enzyme in plasma membrane divided by the specific activity in defatted homogenate.
*Values within a row with different superscript are statistically different ($p<0.05$). Values in a row with no superscripts or common symbols are not different.

(24)—Solvent A, n-hexane/2-propanol/water (41:54:5, v/v/v); Solvent B, n-hexane/2-propanol/water/(39:52:9, v/v/v). The gradient elution was modified in accordance with the column characteristics. A linear gradient from 0 to 100% of solvent B was formed between 10 and 20 min; the flow-rate was 2.5 ml/min. The phospholipid extracts (1–2 mg) were injected into the column using 500 μl of n-hexane/2-propanol (3:4, v/v). Before injection, the column was washed with Solvent A until a stable baseline was obtained. An aliquot of each fraction collected was subjected to thin-layer chromatography (25) in order to determine its purity as compared to phospholipid standards (Sigma Chemical Co., St. Louis, MO). Because phospholipid quantification is not possible by the UV detection mode, phospholipids were collected and quantified by phosphorus analysis according to the method of Bartlett (26). Recoveries for the various membrane PL were tested using Sigma standards and were all > 96%.

Fatty acids of phospholipid extracts were transmethylated according to Berry *et al.* (27), and fatty acid methyl esters were analyzed on a Carlo-Erba 4180 gas-liquid chromatograph equipped with an on-column injector and a flame ionization detector and coupled with a Nelson integrator. The capillary column (mean diameter 0.30 mm, length 50 m) was coated with FFAP (0.45%), and the column temperature was programmed from 54°C to 200°C at 3°C/min.

Data are presented as means ± SEM. Comparisons between groups were made by the Student's *t*-test.

RESULTS AND DISCUSSION

Cholesterol and PL contents and PL class distributions are given in Table 4. All diets contained less than 0.01% (w/w) of cholesterol, and the data show that cholesterol content in the membranes was not significantly modified by the diet.

Addition of fat to the diet resulted in a modification of PL content in the membranes. The PL content (μg/mg of membrane protein) was not different between the low fat control (LFC) diet and the high fat saturated (HFS) diet, but it was significantly lowered when animals were fed the high fat unsaturated (HFU) diet.

The LFC and HFU diets only differed by the amount of fat (1.5 vs 10% of sunflower oil, Table 1), and not in their fatty acid compositions. The lowered PL content in membranes of rats fed the HFU diet (when compared to LFC animals) indicated that the amount of dietary fat is a determinant in modulating the membrane PL content. The HFU and HFS diets only differed in their fatty acid compositions, in particular in their P/S ratios (6.0 and 0.27 respectively, Table 2) and not in their fat contents (10% in both diets). The lowered PL content of HFU animals (when compared to HFS animals) indicated that, besides the amount of dietary fat, the nature of fatty acids and presumably the amount of essential polyunsaturated fatty acids (EFA) maybe equally important in modulating membrane PL content.

Cell size was larger with the high fat diets (HFU and HFS) than with the low fat diet (LFC) (data not shown). No differences in cell size were observed between the two high fat diets. Thus, the lowered PL content in the HFU group cannot be due to decreased fat cell size. High fat diets are known to increase cell size by comparison with low fat diets (28). In agreement with this, a lowered PL content was observed in enterocyte (3) and liver (13) plasma membranes of rats when animals were fed at least 20% of their calories

TABLE 4

Cholesterol Content and Phospholipid Class Distribution of Fat Cell Plasma Membrane[a]

Diet	LFC	HFU	HFS
Total cholesterol (μg/mg protein)	52 ± 18	43 ± 7	69 ± 10
Total phospholipid[b] (μg/mg protein)	280 ± 21*	180 ± 30**	310 ± 40*
PC	144 ± 3* (51)	90 ± 2** (50)	140 ± 5* (46)
PE	65 ± 1* (23)	45 ± 3** (25)	91 ± 5*** (29)
PI	9 ± 1 (3)	10 ± 1 (6)	15 ± 5 (5)
PS	25 ± 2 (9)	16 ± 3 (9)	22 ± 1 (7)
SM	21 ± 5* (8)	5 ± 2** (3)	26 ± 4* (8)
LPC	8 ± 6*,** (3)	4 ± 1* (2)	0.3 ± 0.5** (0.1)
X[c]	7 ± 2* (3)	8 ± 1 (4)	12 ± 1** (4)
PC/PE	2.2 ± 0.1*	2.1 ± 0.2*	1.6 ± 0.1**
SM/PC	0.15 ± 0.03*,**	0.06 ± 0.03*	0.19 ± 0.03**

[a]Values are means ± SEM of four different membrane preparations. Values within a row with different superscript letters are statistically different ($p<0.05$).

[b]Values are expressed in μg/mg protein. Values in parentheses represent the percentage of total PL.

[c]X, unidentified phospholipid.

as polyunsaturated fat. Equally consistent is the fact that no change of PL content was observed in enterocyte brush-border membranes of pigs fed EFA-deficient diets containing 15% of their caloric intake as fat (29). It was thus tempting to speculate that there is a threshold level of dietary EFA beyond which there is a decrease in PL *de novo* biosynthesis. It has been demonstrated that the activity of a key enzyme in PL *de novo* biosynthesis, CTP:phosphocholine cytidyltransferase, is regulated by a translocation process (30). The enzyme is activated when it attaches to endoplasmic membranes, and is inactivated by translocation to the cytosolic fraction. Translocation has been shown to be energy-dependent through substrate availability and cAMP-dependent phosphorylation (30,31). To our knowledge, the effect of large amounts of dietary polyunsaturated fatty acids on the regulation of PL biosynthesis has not been studied. In light of our observations and of those of others (3,13,29), this topic would deserve further investigation.

The PL profiles of fat cell plasma membranes were significantly modified by both the amount of fat and the fatty acid compositions of the diets (Table 4). Since total PL content was modified by dietary fat, somewhat different conclusions on modulation of PL profile can be drawn depending on whether the results are expressed per mg of membrane protein or as percentage of total PL. Both ways of expressing these data are meaningful. The PL classes which on a protein basis were not altered by dietary fat include the anionic phosphatidylserine (PS) and phosphatidylinositol (PI). However, the percentage contribution of PI to total membrane PL was increased with both high fat diets (HFS and HFU) when compared to controls. The most commonly studied modulation of PL head group distribution concerns the more abundant PL classes phosphatidylcholine (PC) and phosphatidylethanolamine (PE). The present study clearly demonstrates that both the amount and the fatty acyl compositions of dietary fat are modulators of PC and PE levels. In agreement with the observations of others (3,13), PC levels in plasma membranes were decreased when animals were fed the HFU diet and were not modified by the HFS diet. PE levels were also decreased with the HFU diet and increased with the HFS diet. On a percentage basis, PC levels were unchanged with HFU and decreased with the HFS diet, and PE levels were unchanged with the HFU diet and increased with the HFS diet. In whatever terms the results are expressed, the PC/PE ratio was significantly decreased when animals were fed the saturated diet (HFS) and unchanged with the HFU diet when compared to the LFC group.

Sphingomyelin (SM) levels were considerably lowered with HFU and remained unchanged with HFS. As a consequence the SM/PC ratio was decreased and the total choline PL content (SM + PC + LPC) was lowered with HFU.

Lysophosphatidylcholine (LPC) levels were significantly lowered with the HFS diet.

Because the major changes observed concerned the PC and PE contents, the fatty acid compositions of these classes were also investigated. Results are presented in Table 5.

The fatty acid composition of the cell plasma mem-

TABLE 5

Fatty Acid Composition of Phosphatidylcholine (PC) and Phosphatidylethanolamine (PE) of Fat Cell Plasma Membrane (wt %)[a]

	PC			PE		
Fatty acids[b]	LFC	HFU	HFS	LFC	HFU	HFS
14:0	<1.0	1.1	1.3	<1.0	<1.0	<1.0
16:0	22.2	18.1	19.4	15.0	10.0	9.7
16:1 n-7	2.3	1.0	<1.0	2.0	1.1	<1.0
18:0	19.9	24.4	23.1	18.7	23.9	23.0
18:1 n-9	14.9	7.9	15.4	15.0	11.6	18.2
18:1 n-7	3.0	1.5	1.6	2.6	1.6	1.5
18:2 n-6	12.1	21.7	13.9	11.4	15.3	9.3
20:2 n-6	1.1	<1.0	<1.0	3.5	1.9	<1.0
20:4 n-6	7.5	10.0	9.4	18.5	23.1	25.6
22:0	2.2	3.2	2.9	<1.0	<1.0	<1.0
22:3 n-6	1.6	1.2	<1.0	1.5	<1.0	<1.0
22:4 n-6	<1.0	<1.0	<1.0	1.1	2.5	1.8
22:5 n-6	<1.0	<1.0	<1.0	<1.0	1.7	<1.0
22:6 n-3	1.5	<1.0	1.2	<1.0	<1.0	<1.0
24:0	3.6	3.2	3.8	1.7	<1.0	1.3
Total saturated	48.8	50.9	51.1	36.6	35.5	35.0
Total MUFA	20.8	11.7	19.3	20.9	16.1	21.7
Total n-6 PUFA	24.1	35.6	25.9	38.0	45.6	39.0
Total n-3 PUFA	2.7	1.6	2.5	3.7	1.9	2.0
PUFA/saturated	0.5	0.7	0.5	1.1	1.3	1.2

[a]Individual values are only reported for fatty acids present at a concentration higher than 1.0%. However all values are taken into account for presentation of total fatty acids. Values are means of 2 experiments with different membrane preparation. Variation was less than 10%.

[b]MUFA: Monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.

brane was different for PC and PE dependent upon the diet, as earlier described for rat liver (32). PC was characterized by a larger amount of saturated fatty acids than in PE (50% vs 35%), and especially in palmitic acid (16:0). PE was characterized by a high content of n-6 fatty acids. In particular, the arachidonic acid (20:4 n-6) content was more than two-fold higher in PE than in PC. As a consequence, unsaturation as judged by P/S ratio was much higher in PE than in PC.

When compared with the LFC diet, addition of saturated fat (HFS) did not change the overall contents of saturated, monounsaturated and polyunsaturated fatty acids in both PC and PE, but increased stearic acid (18:0) and arachidonic acid (20:4 n-6) levels, thus favoring the esterification of elongated fatty acids at the expense of their precursors (16:0 and 18:2 n-6, respectively). Addition of polyunsaturated fat to the diet (HFU) did not modify the overall content of saturated fatty acids, but resulted in an increase in the overall n-6 fatty acid content in both PC and PE, although more in PC (32). Both the n-6 precursor (18:2 n-6) and elongated (20:4 n-6) forms were increased with HFU at the expense of monounsaturated fatty acids (mainly oleic acid).

In short, addition of saturated fat to the diet (HFS) was found to result in minor changes in the fatty acid profiles of PC and PE, but in important changes in the relative proportion (PC/PE) of the two PL classes in the membrane. Conversely, addition of polyunsaturated fat was found to alter the fatty acid profile, but did not modify the PC/PE ratio in the membrane. These results suggest that dietary fat presumably interferes with different regulatory steps in the biosynthesis of membrane PL.

Most information on PL synthetic pathways has been derived from studies on rat liver and brain. The primary site for *de novo* biosynthesis of the more abundant PL (i.e. PC and PE) by CDP-base pathways has been shown to be the endoplasmic reticulum (30), although recent evidence suggests that certain PL destined for lipoprotein secretion may also be synthesized in Golgi (33). PC can also be synthesized via the PE methylation pathway. The physiological importance of this pathway in extrahepatic tissues has been questioned and the existence of one or two phosphatidylethanolamine methyltransferase (PEMT) is still controversial (34). PEMT activity was mainly located in endoplasmic membranes of the liver and the pituitary (34,35), but could also be demonstrated in plasma membranes of nonhepatic tissues (35-37). Synthesis of PI and PS occurs in the microsomal fraction (30), and SM synthesis takes place in both microsomal and plasma membrane fractions (38,39). Intracellular transport of PL molecules from the synthesis site to the plasma membrane is thus an essential step in membrane biogenesis, although it is not well understood (40). Alterations of membrane PL polar head groups could result either from altered *de novo* biosynthesis and subsequent transport or, by analogy with the *in situ* retailoring of PL fatty acyl moieties, from the fine tuning of the polar head groups in the membrane. The present study did not distinguish between these different processes, although we assume that the highly decreased PL content observed with the HFU diet is presumably due to a decreased *de novo* PC biosynthesis.

Lipoprotein profiles and lipoprotein composition are known to be modified by dietary fat (37,41). We cannot exclude that alterations of PL content of the fat cell plasma membrane may at least in part result from altered lipoprotein metabolism.

Information on the specific lipid dependence of membrane enzymes has been obtained mainly from delipidation-reconstitution studies (42). It has been demonstrated, in particular, that 5′-nucleotidase was active only in the presence of sphingomyelin. In our study, 5′-nucleotidase specific activity was shown to be dependent on the level of dietary fat (higher with HFU and HFS than with LFC diet, Table 3) and independent of the level of sphingomyelin in the membranes (decreased with HFU, Table 4). This suggests that the SM level is not the sole factor regulating *in vivo* 5′-nucleotidase activity and that *in vivo* specific lipid requirements are difficult to be inferred from *in vitro* studies.

In conclusion, our results show that both the amount and the nature of dietary fat induce changes in the distribution and the fatty acid profile of PL classes in the fat cell plasma membrane. Interestingly, the acidic PL (PI and PS) seemed to be less affected by dietary fat changes. The levels of all other classes of phospholipids were modified to different extents by dietary fat. Increasing evidence that PL, apart from their structural role, are active participants in cell/environment interactions and that PL metabolic disorders are implicated in pathological states (7,43,44) emphasizes the necessity of a better understanding of the *in vivo* regulation of PL metabolism. The fact that in adipocytes (the present study) and in other cell types (3,4,11,13) dietary fat was found to be involved in the regulation would certainly deserve further investigation.

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METHODS

Analysis of North Atlantic and Baltic Fish Oil Triacylglycerols by High-Performance Liquid Chromatography with a Silver Ion Column

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Triacylglycerols from Atlantic herring (*Clupea harengus*), sandeel (*Ammodytes sp.*) and Baltic herring (*Clupea harengus membras*) have been fractionated by silver ion high-performance liquid chromatography. An ion exchange column loaded with silver ions was the stationary phase, and a gradient in the mobile phase from 1,2-dichloroethane/dichloromethane (1:1, v/v) to acetone and then to acetone/acetonitrile (2:1, v/v) was used to effect the separation with light-scattering (i.e., mass) detection. Fractions were collected via a stream-splitter, and fatty acid methyl esters were prepared by transesterification in the presence of an internal standard for identification and quantification by gas liquid chromatography. Triacylglycerols were separated according to the number of double bonds in the fatty acyl residues. Resolution was excellent at first, when the least unsaturated molecules eluted (trisaturated to dimonoene-monodiene fractions). Base-line resolution could no longer be achieved when molecules containing trienoic or more highly-unsaturated fatty acids began to elute because of overlapping components. Nonetheless, some valuable separations of species containing two saturated and/or monoenoic fatty acids and one polyenoic fatty acid were achieved. Double bond indices (average number of double bonds in each triacylglycerol molecule) were calculated to estimate the separations possible. Fractions containing at least 11–14 double bonds per molecule were obtained.
***Lipids* 25, 284–291 (1990).**

Reversed-phase high-performance liquid chromatography (RP-HPLC) has been widely used to analyze natural triacylglycerols (1). The separation is based both on the combined chain-lengths of the fatty acyl residues and on the total number of double bonds, each of the latter reducing the retention time of the molecules by an equivalent of about two methylene groups. Often, natural fat samples are so complex that adequate separation of triacylglycerols cannot be achieved by RP-HPLC alone because of overlapping components, and other techniques must be used in a complementary manner.

Silver ion chromatography is a powerful technique for separating molecules according to degree of unsaturation, resolution being due to weak interactions of Ag^+ and the π-electrons in double and triple bonds. This technique has been used most extensively with thin-layer chromatography (TLC) with silver ions incorporated into the silica gel layers (2). More recently, a method for using HPLC with silver-loaded ion exchange column has been developed and excellent separations have been obtained from various triacylglycerol samples (3,4). This method is rapid, reproducible and permits the collection of clean fractions uncontaminated by silver ions.

Fish oils contain a large variety of different fatty acids including long-chain polyunsaturated components such as 20:5(n-3) and 22:6(n-3). The chain-lengths of the fatty acids vary between 14 and 24 carbon atoms, and the number of double bonds from zero to six (5). This wide range results in a complex mixture of molecular species of triacylglycerols. It is obvious that the use of only one chromatographic method will not permit separation of all the different molecules. The complexity of fish triacylglycerols may be one reason why their structures have not been studied more extensively. Bottino (6) used silver ion TLC and Dolev and Olcott (7) used a column of silica gel impregnated with silver ions to partially separate marine oil triacylglycerols according to the total number of double bonds in the fatty acyl residues. A combination of RP-HPLC and gas liquid chromatography (GLC) techniques was used by Wada *et al.* (8) to separate fish oil triacylglycerols first by partition number, and then by carbon number. In addition, they analyzed a fish oil sample containing a very low amount of polyunsaturated fatty acids (<3 mole%), first fractionating the sample by silver ion TLC followed by separation according to the combined chain-lengths of the fatty acid constituents by RP-HPLC (9). Takeuchi and Ackman (10) utilized reversed-phase HPLC for the analysis of the triacylglycerols of the rock crab. As well as analytical scale studies, a semi-preparative HPLC fractionation of fish oil components rich in n-3 fatty acids, especially 20:5(n-3) and 22:6(n-3), has been published (11).

Silver ion HPLC has been used in this research to separate fish oil triacylglycerols according to differences in the level of unsaturation and to evaluate the utility of this technique in separations of highly polyunsaturated components.

EXPERIMENTAL

Samples and reagents. Atlantic herring (*Clupea harengus*) and sandeel (*Ammodytes spp.* or "sand launce") oils from Norway were recovered by an industrial press-

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Abbreviations: GLC, gas liquid chromatography; RP-HPLC, reversed-phase high performance liquid chromatography; TLC, thin-layer chromatography.

ing process after which the crude oil was washed with water to remove proteinaceous material and alkali refined to eliminate free fatty acids. Baltic herring (*Clupea harengus membras*), which is a brackish water fish, was obtained from Finland, and the lipids were extracted from the flesh with chloroform/methanol (2:1, v/v). Triacylglycerol fractions were obtained, prior to silver ion HPLC analysis, by HPLC on a column of silica gel (Spherisorb^{TM}—5 micron; 250 × 5 mm i.d. column) with hexane/tetrahydrofuran (99:1, v/v) as the mobile phase at a flow-rate of 1 ml/min. All solvents and reagents were Analar (BDH Ltd., Poole, U.K.) or HPLC grades and were supplied by FSA Scientific (Loughborough, U.K.).

Silver ion high-performance liquid chromatography. HPLC analyses were performed with a Spectra-Physics Model 8700 solvent delivery system (Spectra-Physics, St. Albans, U.K.) together with an ACS Model 750/14 mass detector [Applied Chromatography Systems (ACS), Macclesfield, U.K.] and a Spectra-Physics SP 4290 integrator. The silver ion column was prepared by loading the sulfonic acid moieties of an ion exchange column, Nucleosil^{TM} 5SA (250 × 4.6 mm i.d.), with silver ions as described previously (3). A stream-splitter (approx. 10:1) was installed between the column and the detector to be used when required.

During silver ion HPLC analysis, a complex gradient requiring three different solvent mixtures was used: (A) 1,2-dichloroethane/dichloromethane (1:1, v/v); (B) acetone; and (C) acetone/acetonitrile (2:1, v/v). With the fish oil samples, the solvent gradient started at 100% A and changed to 50% A/50% B over 10 min, then to 70% B/30% C over a further 30 min, to 50% B/50% C after another 20 min, and finally to 100% C after an additional 20 min and was maintained for 5 min in this final state. The flow-rate of the mobile phase was 1.0 ml/min and the column was kept at ambient temperature.

Samples for analysis were dissolved in 1,2-dichloroethane and volumes of 10–25 μl (1–2 mg) were injected on to the column. Fractions were collected manually via the stream-splitter. A standard solution of methyl nonadecanoate in methanol (70 μl, 0.18 mg/ml) was added to each fraction as an internal standard.

Gas liquid chromatography. Fatty acid methyl esters were prepared by sodium methoxide-catalyzed transesterification (12). GLC analyses of fatty acid methyl esters were performed on a Carlo Erba Model 4130 capillary gas chromatograph equipped with a split/splitless injector and a fused silica capillary column (25 m × 0.22 mm i.d.; film thickness, 0.2 μm) coated with Carbowax 20M (Chrompack, London, U.K.); hydrogen was the carrier gas. The column temperature was programmed from 165°C (at which it was held for 3 min) to 195°C at a rate of 4°C/min and was kept at the final temperature for 22 more minutes. Components were identified by reference to a standard fish oil (cod liver oil) and were quantified by electronic integration.

RESULTS AND DISCUSSION

In this study, triacylglycerols from Atlantic herring, sandeel and Baltic herring were analyzed as representative marine species. Using HPLC with the silver ion column and the ternary solvent gradient system, the triacylglycerols from each species were resolved into molecular fractions as represented in Figures 1A, 1B and 1C, respectively. In order to determine the fatty acid compositions of different unsaturated molecules, the triacylglycerols were divided manually into 20–26 fractions via the stream-splitter installed in front of the detector. Each of the fractions was transesterified in the presence of an internal standard for identification and quantification by GLC. (The response of the light-scattering detector cannot be used for quantification without careful calibration). The fatty acid compositions of the samples, expressed as mole %, as well as the proportions of the different molecular types for each fish species are listed in Tables 1, 2 and 3, respectively. To check the recoveries, the fatty acid composition of the whole was reconstituted from the relative proportions in each of the fractions. For all samples, the fatty acid recoveries were good. Some 14:0 can be lost if too vigorous a stream of nitrogen is used to evaporate the solvent while preparing the fatty acid methyl esters. As single molecular entities are not readily obtained from polyunsaturated fractions with this silver ion system, double bond indices (the average number of double bonds in one triacylglycerol molecule) were calculated from the fatty acid compositions and are also listed in the Tables. Numbered fractions in the Tables refer to the corresponding ones in the Figure 1.

Typically, the fish oils contained approximately 30 mole % saturated, 40 mole % monoenoic and 30 mole % polyenoic fatty acids, with the exception of Baltic herring which contained equal amounts of saturated, monoenoic and polyenoic fatty acids. The most abundant fatty acids in Atlantic herring and sandeel were 14:0, 16:0 and 22:1, while in Baltic herring 16:0 and 18:1 dominated. Baltic herring differs markedly from the other samples studied in that it contains less than 1 mole % of 20:1 and 22:1 fatty acids while the other two contained approximately 22 mole %. The difference may be due to the diet or environment of Baltic herring, which is a brackish-water fish. For example, it is known that fresh-water fish contain lower levels of 20 and 22 carbon fatty acids and higher levels of 18 carbon unsaturated fatty acids than do marine fish (13). The amount of eicosapentaenoic acid [20:5(n-3)] varied between 6.4 and 9.3 mole %, and the amount of docosahexaenoic acid [22:6(n-3)] between 7.4 and 10.4 mole % in different samples. Virtually all fatty acids greater than 0.05% of the total were identified, but many of the minor components are listed simply as "others"; these include odd-chain, branched-chain and polyenoic fatty acids. Occasionally, some of these assume greater proportions in specific HPLC fractions; for example, 20:2 and 16:3 fatty acids are usually much more abundant in fractions containing 18:2 and 18:3, respectively. The monoenoic components consist of several isomers, but for convenience of presentation these are not differentiated in the Tables.

Atlantic herring, sandeel and Baltic herring triacylglycerols showed similar elution behavior on silver ion chromatography, although those from Baltic herring differed slightly in that they had components eluting at about 30 min, for example, that were not

found in appreciable amounts in the other samples. Also, from the chromatograms, it can be seen that triacylglycerols from Atlantic herring contained less of the most unsaturated species than did those from other samples. For example, triacylglycerols from Atlantic herring all eluted before 60 min, while Baltic herring has many components which eluted after this point. The resolution in the chromatograms is excellent at first (up to peak 8), i.e., the area where the more saturated molecules elute. As noted previously (3), the elution order for the molecular species is SSS, SSM, SMM, SSD, MMM, SMD and MMD, where S, M and D denote saturated, mono- and dienoic fatty acid residues, respectively. Later in the text, T, Te, P and H are used to denote tri-, tetra-, penta- and hexaenoic fatty acids, respectively. Specific positional distribu-

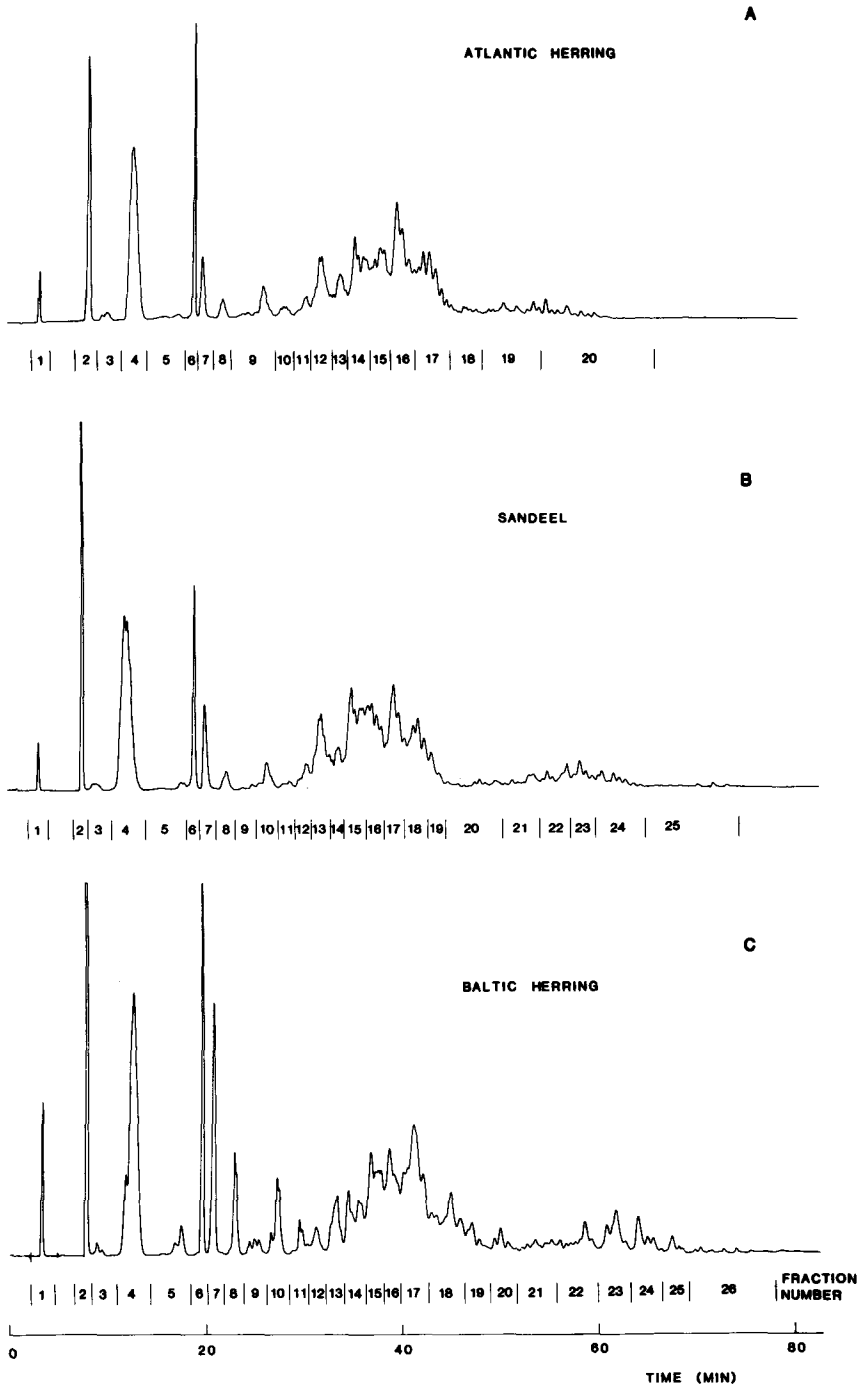

FIG. 1. Separation of the triacylglycerols from Atlantic herring (A), sandeel (B) and Baltic herring (C), by HPLC with a silver ion column and mass detection. The chromatographic conditions are described in the Experimental Section. Fraction numbers are the same as in Tables 1, 2 and 3, respectively.

TABLE 1

Fatty Acid Compositions (Mole% of the Total), Proportions and Double Bond Indices of Triacylglycerol Fractions, Obtained by Silver Ion HPLC from Atlantic Herring

Fatty acid	Total	Fraction (1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
14:0	11.1	36.0	27.4	15.5	12.0	7.9	1.2	9.8	1.4	13.2	4.3
15:0	0.8	2.8	2.0	1.4	0.9	0.7		0.9		1.2	0.4
16:0	17.8	52.7	36.0	28.0	20.7	16.1	2.3	19.6	3.7	24.8	10.3
16:1	6.6		5.2	14.1	11.2	13.0	26.0	5.5	13.3	3.8	10.9
16:2	0.4					1.2		5.7	7.5	0.7	
16:4	0.3										1.1
17:0	0.3	0.8	0.6								
18:0	1.2	5.1	2.4	1.4	0.7	1.1	0.2	0.9		1.3	1.2
18:1	11.1		9.2	12.9	19.2	12.9	33.8	7.2	20.8	5.5	16.3
18:2	1.9					7.9	0.6	26.2	22.0	2.5	0.4
18:3(n-3)	2.1									27.6	23.3
18:4(n-3)	4.7										1.3
20:1	8.2		5.1	6.0	12.4	12.4	16.7	7.1	11.3	5.6	9.0
20:4(n-6)	0.3										1.4
20:4(n-3)	0.7										
20:5(n-3)	6.4										
22:1	14.5		9.9	9.2	21.0	22.2	16.5	12.9	16.3	8.0	12.8
22:5(n-3)	0.5										
22:6(n-3)	8.7										
Other	2.5	2.6	2.3	11.5	1.8	4.6	2.8	4.1	3.6	5.8	7.3
Amount (mol %)		1.5	7.9	1.3	14.5	2.0	4.1	2.3	1.4	3.6	2.0
Double bond index		0.0	0.9	1.3	1.9	2.4	2.9	3.0	3.7	3.7	4.4

		(11)	(12)	(13)	(14)	(15)	(16)	(17)	(18)	(19)	(20)	Recombinant composition
14:0	11.1	18.3	10.7	7.6	9.3	10.4	9.6	4.7	2.8	6.8	3.3	10.2
15:0	0.8	1.7	0.8	0.7	0.8	0.9	0.9	0.4	0.3	0.6	0.3	0.8
16:0	17.8	30.5	17.0	19.5	23.0	18.7	21.6	6.3	7.7	15.8	9.0	18.6
16:1	6.6	1.7	4.7	6.4	3.3	6.1	3.1	7.5	8.9	1.9	2.9	6.9
16:2	0.4							0.4	2.0	0.4		0.4
16:4	0.3	3.9	1.1							1.0	0.6	0.3
17:0	0.3			0.4	0.4	0.3	0.5			0.2		0.2
18:0	1.2	2.0	0.6	2.1	1.7	2.0	2.4	0.2	0.6	2.9	0.9	1.4
18:1	11.1	2.1	5.8	11.1	6.9	10.3	6.7	15.6	12.8	3.9	5.6	11.3
18:2	1.9	1.4	0.8	0.4	0.9	1.2	0.7	1.6	9.8	1.2	0.6	2.0
18:3(n-3)	2.1	1.5	0.9		0.4	0.7	0.3	1.0	8.2	9.1	0.8	2.5
18:4(n-3)	4.7	19.3	27.4	17.0	2.1	1.5	0.2	0.9	6.3	16.1	12.9	5.0
20:1	8.2	2.4	7.4	6.2	5.3	7.2	6.0	11.4	3.4	2.5	3.2	7.6
20:4(n-6)	0.3	2.8	0.6						0.4	0.9	0.4	0.2
20:4(n-3)	0.7	2.3	4.1	1.9	0.4				0.8	3.2	1.9	0.7
20:5(n-3)	6.4			13.1	28.1	17.0	2.3	1.2	3.1	11.4	23.1	6.3
22:1	14.5	7.2	14.0	11.0	12.3	9.6	15.6	16.5	5.6	5.9	4.6	13.0
22:5(n-3)	0.5		2.7		2.1	2.0	0.4			1.3	1.8	0.6
22:6(n-3)	8.7					10.4	28.1	29.9	22.4	8.4	26.4	9.1
Other	2.5	2.8	1.3	2.7	3.1	1.7	1.7	2.3	5.1	6.5	1.8	2.9
Amount (mol%)		2.3	6.1	3.8	9.6	2.3	9.6	10.4	2.9	4.8	7.4	
Double bond index		4.0	5.5	5.3	5.8	6.1	6.5	7.5	7.9	7.4	11.0	

tions within the molecules are not implied. All samples analyzed had a small fraction, 1.0–1.5 mole %, of trisaturated triacylglycerols (fraction 1). The fatty acid distribution in fraction 2 was 67.4–68.3 mole % saturated and 29.4–30.8 mole % monoenoic fatty acids, so these molecules contain two saturated and one monoenoic fatty acids (SSM). Other fractions were identified in a similar way. Between SSM and SMM in all samples analyzed, a small fraction (0.8–1.3 mole %, number 3) eluted with a ratio of saturated to monoenoic fatty acids of 1:1, indicating that these molecules may be glycerol ether diesters, the alkyl moiety of which would not be seen under the GLC conditions here. Lipids of this kind have been found in fish samples by other workers (14,15). Presumably, molecular species of glycerol ether diesters are also present at low levels

TABLE 2

Fatty Acid Compositions (Mole % of the Total), Proportions and Double Bond Indices of Triacylglycerol Fractions, Obtained by Silver Ion HPLC from Sandeel

Fatty acid	Total	Fraction (1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)
14:0	10.0	34.3	24.2	11.7	12.0	9.5	1.2	7.7	2.3	11.5	9.2	4.4	12.8
15:0	0.5	2.0	1.2	0.8	0.6	0.5		0.5		0.9	0.6	0.4	0.8
16:0	17.7	55.6	39.7	33.6	22.5	20.3	3.2	21.5	5.8	32.0	19.9	12.3	30.1
16:1	8.3		6.3	11.2	13.3	12.6	31.3	6.6	16.6	2.6	6.7	12.1	3.1
16:2	0.7					1.8	0.0	6.0	9.2	1.6	1.0		0.4
16:4	1.3											4.5	10.7
17:0	0.2	0.8	0.4							0.4			0.4
18:0	1.3	4.7	2.8	2.0	0.6	1.3	0.2	0.9	0.6	2.8	1.0	1.8	2.4
18:1	8.4		8.2	7.9	16.9	11.6	22.7	8.3	11.5	3.9	6.3	11.9	3.3
18:2	2.2					8.2	0.9	23.9	18.3	7.7	2.7	0.5	2.0
18:3(n-3)	1.9									13.1	22.9	20.2	2.8
18:4(n-3)	6.2											0.6	15.4
20:1	8.9		6.6	9.0	14.2	12.2	16.6	8.5	13.6	4.8	9.7	11.1	4.5
20:4(n-6)	trace											0.7	1.7
20:4(n-3)	0.4												0.8
20:5(n-3)	9.3												
22:1	12.6		8.7	14.9	19.0	17.0	21.5	12.3	18.1	8.7	12.9	13.3	6.1
22:5(n-3)	0.5												
22:6(n-3)	7.4												
Other	2.1	2.5	1.9	9.0	0.9	5.0	2.5	3.7	3.9	9.8	7.1	6.1	2.9
Amount (mole %)		1.0	6.3	1.0	14.3	1.7	3.5	3.0	1.5	1.1	2.5	1.2	2.3
Double bond index		0.0	0.9	1.3	1.9	2.2	2.8	2.9	3.5	2.7	3.8	4.2	4.3

		(13)	(14)	(15)	(16)	(17)	(18)	(19)	(20)	(21)	(22)	(23)	(24)	(25)	Recombinant composition
14:0	10.0	7.9	5.8	8.1	7.3	5.0	3.4	0.5	3.6	4.2	3.4	1.3	2.7	0.9	8.1
15:0	0.5	0.5	0.4	0.5	0.4	0.4	0.3		0.3	0.4	0.3		0.2		0.5
16:0	17.7	16.4	21.3	23.2	14.5	20.0	9.2	2.4	9.2	12.5	13.6	2.4	9.0	2.5	18.1
16:1	8.3	5.6	6.9	4.6	7.0	5.9	4.9	9.5	6.7	3.0	4.3	3.3	3.3	1.1	7.8
16:2	0.7	0.2		0.2	0.4	0.4	0.5	0.7	2.3		0.3	0.3	0.4	0.3	0.7
16:4	1.3	4.2	1.1	0.3	0.3			0.4	2.4	4.3	4.5	2.0	0.4	2.9	1.2
17:0	0.2	0.2	0.3	0.3	0.2	0.4			0.1						0.1
18:0	1.3	1.0	2.4	2.5	1.4	3.4	0.5	0.3	0.8	0.9	1.8	1.0	0.5		1.4
18:1	8.4	6.4	8.9	5.0	8.6	5.4	9.3	17.6	5.3	3.1	3.7	5.6	4.1	1.4	8.8
18:2	2.2	0.6	0.3	0.9	1.2	0.9	2.4	3.4	7.7	0.7	0.8	1.0	1.1	1.3	2.3
18:3(n-3)	1.9	0.7	0.3		0.4	0.4	0.4	3.4	7.7	7.1	0.5		1.2	2.8	1.9
18:4(n-3)	6.2	26.5	17.4	4.9	1.9	0.9	0.4	3.5	11.4	24.7	0.5	16.5	3.7	18.7	5.5
20:1	8.9	9.2	8.4	7.1	11.7	9.1	14.4	14.6	4.8	4.5	5.0	7.2	4.5	1.4	9.5
20:4(n-6)	trace								0.4	0.6	0.6				0.1
20:4(n-3)	0.4	2.0	1.0						0.8	1.9	1.8	1.3	0.2	1.2	0.4
20:5(n-3)	9.3	0.3	13.3	27.1	22.8	6.1	2.8	4.0	8.8	16.2	39.3	28.5	30.1	32.8	9.8
22:1	12.6	15.8	10.1	12.2	13.6	14.9	18.6	10.0	6.8	6.7	5.0	7.8	4.1	1.1	12.9
22:5(n-3)	0.5			1.2	1.4	1.3			0.4	0.7	2.2	1.3	1.0	1.0	0.5
22:6(n-3)	7.4				4.8	23.4	31.2	26.9	14.6	6.1	9.2	18.2	30.6	28.0	7.8
Other	2.1	2.3	2.0	1.7	2.0	2.0	1.6	2.6	5.8	2.5	3.2	2.3	2.9	2.7	2.6
Amount (mole %)		6.6	2.5	9.6	7.8	6.7	7.0	2.3	3.8	3.1	3.0	2.9	3.3	1.8	
Double bond index		5.2	5.4	5.8	6.1	6.6	7.7	8.0	8.0	8.7	9.4	10.9	11.4	13.4	

in many of the later fractions. It was not possible to remove these by adsorption chromatography (HPLC), because the wide range of fatty acid components caused the triacylglycerols to elute as broad bands.

After SMM, another small fraction eluted that contains the SSD type of molecule, although it was only abundant in Baltic herring (fraction 5), where it is predominantly 14:0/16:0-14:0/16:0-18:2 (again, specific positional distributions within the molecules are not implied). This same fraction also contains molecules with monoenoic fatty acids and may be a glycerol ether diester.

When molecules containing 18:3 and other polyunsaturated fatty acids started to elute, base-line resolution could no longer be obtained, and the identification of molecular types became more difficult, or even impossible, because of overlapping components. Nonetheless, it was evident that fractions 9–17 from Baltic

TABLE 3

Fatty Acid Compositions (Mole % of the Total), Proportions and Double Bond Indices of Triacylglycerol Fractions, Obtained by Silver Ion HPLC from Baltic Herring

Fatty acid	Total	Fraction (1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)
14:0	7.9	24.5	17.3	10.7	9.4	15.5	1.4	10.3	1.0	10.9	6.4	3.4	10.3	5.3
15:0	0.5	1.6	1.0	0.8	0.5	0.9		0.4		0.8	0.4	0.3	0.8	0.4
16:0	22.6	66.6	46.9	32.5	24.5	38.3	7.7	21.3	2.7	39.0	20.8	14.8	34.9	25.6
16:1	9.7		8.0	16.6	19.8	6.1	26.8	10.8	17.8	1.0	7.6	13.2	3.1	7.4
16:2	0.1					0.6		1.2	1.3	0.5				
16:4	trace												0.5	
17:0	0.2	0.9	0.4							0.4				0.3
18:0	1.1	4.2	1.8	1.1	0.6	1.2	0.3	0.6		1.7	0.9	1.1	1.6	2.0
18:1	23.2		22.5	22.3	41.2	9.5	52.7	21.8	43.2	1.8	23.8	27.6	5.7	22.3
18:2	5.6					23.6	5.6	31.2	29.7	18.6	8.8	2.7	9.3	3.9
18:3(n-3)	3.3									21.9	26.5	25.4	10.4	4.2
18:4(n-3)	3.0											4.5	13.8	19.4
20:1	0.5		0.4	0.5	0.6		0.9		0.7		0.4	0.8		0.5
20:4(n-6)	0.3											1.6	5.0	0.9
20:4(n-3)	0.4												2.1	2.8
20:5(n-3)	8.9													2.8
22:1	0.2				0.3		0.4							
22:5(n-3)	trace													
22:6(n-3)	10.4													
Other	2.0	2.2	1.7	15.4	3.1	4.2	4.2	2.3	3.7	3.4	4.3	4.6	2.5	2.4
Amount (mole %)		1.4	8.0	0.8	12.0	2.1	6.2	3.8	2.6	1.5	3.4	1.8	1.6	3.8
Double bond index		0.0	0.9	1.2	1.9	2.0	2.8	3.0	3.8	3.2	4.0	4.5	4.3	4.7

		(14)	(15)	(16)	(17)	(18)	(19)	(20)	(21)	(22)	(23)	(24)	(25)	(26)	Recombinant composition
14:0	7.9	9.2	5.7	10.5	5.2	2.7	3.6	2.8	4.6	3.3	2.2	0.8	2.0	1.2	7.0
15:0	0.5	0.8	0.5	0.8	0.5	0.2	0.4		0.4	0.3	0.3				0.5
16:0	22.6	32.3	26.4	35.8	20.6	6.8	16.0	10.2	17.0	14.9	13.7	6.9	3.0	4.0	22.3
16:1	9.7	5.2	7.4	2.8	8.9	13.7	9.2	5.0	3.6	4.4	2.9	4.4	4.5	1.2	10.2
16:2	0.1				0.1	0.1	0.8	0.6							0.1
16:4															0.0
17:0	0.2		0.2	0.2	0.2					0.2					0.1
18:0	1.1	1.4	2.2	1.3	1.4	0.4	0.6	0.5	1.0	0.9	0.8	0.7			1.0
18:1	23.2	13.6	19.3	8.4	22.2	33.2	11.2	15.0	10.6	10.0	8.4	16.1	14.4	2.6	23.6
18:2	5.6	0.7	2.2	1.1	3.2	5.0	19.3	21.0	3.3	2.1	1.1	1.8	6.5	2.5	5.8
18:3(n-3)	3.3		0.9	0.4	0.7	1.4	5.3	10.4	15.7	5.8	0.9	1.5	3.3	4.6	3.4
18:4(n-3)	3.0	7.3	1.5	0.8	0.6	1.3	3.8	3.2	10.4	12.3	5.8	2.0	4.1	9.9	3.0
20:1	0.5	0.4	0.3	0.4	0.4	0.8			0.3	0.2	0.3		0.5		0.4
20:4(n-6)	0.3	0.2							1.5	1.6				0.7	0.3
20:4(n-3)	0.4	1.0	0.3		0.1		0.6	0.7	1.5	1.6	0.7			1.2	0.4
20:5(n-3)	8.9	22.6	27.2	7.2	9.0	3.5	2.0	8.7	16.2	25.5	34.5	26.2	11.8	31.7	7.6
22:1	0.2	0.5		0.3	0.3	0.4									0.2
22:5(n-3)		0.7	1.1	0.7	0.5				0.5	0.8	0.8			0.8	0.2
22:6(n-3)	10.4		2.1	26.7	22.3	26.2	24.0	14.6	10.3	13.7	25.2	37.8	48.1	37.2	10.6
Other	2.0	4.0	2.5	2.6	3.6	4.3	3.1	7.4	3.2	2.3	2.5	1.9	1.9	2.4	3.3
Amount (mole %)		5.0	1.8	4.5	12.7	7.8	1.9	1.9	2.9	3.7	3.8	2.6	1.2	1.2	
Double bond index		5.2	5.9	6.6	6.8	7.4	7.5	7.3	8.0	9.4	11.1	11.8	12.2	13.7	

herring and sandeel and fractions 9–16 from Atlantic herring had two molecules of saturated plus monoenoic fatty acids per molecule of triacylglycerol. The component that varied in these fractions was the polyunsaturated fatty acid, which was first 18:3, then changed through 18:4 to 20:5, and at last to 22:6. The highest calculated double bond indices for these fractions were 6.5–6.8 which, together with the fatty acid distributions, suggested that the last fraction, for example, consisted mainly of SMH type molecules. Because of the complexity of the fish oil triacylglycerols, it was impossible to separate the samples into individual peaks and the fractions were inevitably more or less mixed. For example, fraction 9 from Baltic herring had 52.8 mole % saturated, 2.8 mole % monoenoic, 19.1 mole % dienoic and 21.9 mole % trienoic fatty acids. This fraction was probably a mixture of SST composed of 14:0/16:0–14:0/16:0–18:3, SDD comprising 14:0/16:0–18:2–

18:2 mainly, and perhaps a small amount of MMD. By calculation from the fatty acid analysis, SST comprised 65.7 mole %, SDD 27.0 mole % and MMD 3.3 mole % of the total triacylglycerols in this fraction. As another example, fraction 12 may have been a mixture of SSTe plus SDT having fatty acid compositions as 14:0/16:0-14:0/16:0-18:4 and 14:0/16:0-18:2-18:3. Similarly, fraction 13 from Atlantic herring could be a mixture of SMTe and SMP molecular species and fraction 15 a mixture of SMP and SMH. In spite of the complexity, recognizable SMT, SMTe, and SMP and SMH fractions could be separated from the three species studied. Sandeel fractions 10 (SMT), 13 (SMTe), 15 (SMP) and 17 (SMH) especially contained only small amounts of other fatty acids, and this was also true of the corresponding fractions from the other species. In these fractions, the ratio of the different types of saturated unsaturated fatty acids in the molecules was very close to 1:1:1.

In the SMT, SMTe, SMP and SMH fractions from sandeel and Atlantic herring, the proportion of the 22:1 fatty acid was approximately 40% of the total monoenes, e.g., sandeel fraction 14, which was probably a mixture of SMTe and SMP molecules, had a lower 22:1 content and higher 16:1 and 18:1 contents than fraction 13, which was formed from SMTe species. The difference in retention might be due to how strongly the molecules complex with the silver ions. Shorter-chain monoenoic fatty acids appear to form a stronger complexes than do longer-chain ones. However, the double bonds are generally closer to the carboxyl group in the former, and this may be the reason for the effect rather than chain-length per se. Fraction 18 from sandeel and Baltic herring and fraction 17 from Atlantic herring all contained nearly 50 mole % monoenoic fatty acids. These fractions were formed predominantly from SMH and MMH molecules.

After these fractions, molecules started to elute with other unsaturated fatty acids with two or more double bonds in addition to the pentaenoic acids and 22:6. In the last fraction from Baltic herring and sandeel, the proportion of saturated plus monoenoic fatty acids had decreased to less than 10 mole % of the total. Triacylglycerol molecules with 20:5 and 22:6 together with, for example, a tetraenoic fatty acid, would be expected to elute here.

The double bond indices increased steadily as elution proceeded (Tables 1-3). In a few places, the indices of adjacent fractions were almost the same, or in some cases a peak had a slightly smaller value than the previous one. Such apparent inconsistencies were due to the fact that the affinity of silver ions for double bonds is greater if the bonds reside within one fatty acid chain rather than being distributed over several acyl structures. In this HPLC system, one dienoic fatty acid is retained more strongly than two monoenes and a triene has the same mobility as two dienes per molecule (3). Similar effects are known with silver ion thin-layer chromatography (2). With the latter technique, the position to which fatty acid is bound on the glycerol backbone is important; if the unsaturated fatty acid is in position *sn-2*, it forms weaker complexes with silver ion than if it is in the outer positions (2,6). Such an effect is probably not of great importance with the HPLC system used here (3). The double bond indices confirmed that molecules with 11-14 double bonds were separated by this technique, and the number of double bonds in those components with the longest elution times must have been even higher.

It appears then that the single main molecular species in Atlantic herring and sandeel was SMM (fraction 4), forming 14.3 and 14.5 mole % of total triacylglycerols, respectively. Other large components from sandeel were fraction 15 (9.6 mole %) containing SMP, and fraction 16 (7.8 mole %) containing probably MMP and SSH, and from Atlantic herring fraction 17 (10.4 mole %) containing SMH and MMH and fractions 14 and 16 (both 9.6 mole %) containing SMP and SMH. The most abundant fractions from Baltic herring were SMH 12.7 mole % (fraction 17) and SMM 12.0 mole % (fraction 4). Errors in the amounts of different molecular types might arise during the separation because of difficulties in recognizing where fractions begin and end in the region where most of the polyunsaturated components eluted.

With such complicated samples as the triacylglycerols of fish oils, silver ion HPLC can be used to efficiently separate many molecular fractions by degree of unsaturation. Even though the chromatograms appear very complex, they are highly reproducible, even in the polyunsaturated regions. However, when molecules containing polyunsaturated fatty acids start to elute, the number of different kinds of fatty acid combination increases, molecular fractions start to overlap, and base-line separation can no longer be achieved. Bearing in mind the wide fatty acid composition of fish oils and the complexity of the factors controlling the order of elution from silver ion HPLC, it is not possible to identify single components using this technique alone. It would be interesting to combine silver ion fractionation with reversed-phase HPLC because molecules would be separated first according to degree of unsaturation and then subsequently by the combined chain-lengths of the fatty acyl residues. This approach is now being followed in our laboratory.

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Lipoprotein Lipase as a New Tool in Steryl Ester Analysis

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A new method for the quantitative hydrolysis of steryl esters is described. Using lipoprotein lipase (triacylglycero-protein acylhydrolase, EC 3.1.1.34), steryl esters are cleaved to yield free sterols and fatty acids. In comparison to alkaline hydrolysis, the method is more gentle, more accurate and less laborious. Quantification of the hydrolysis products was done with capillary gas chromatography. The practicability of the method is demonstrated for authentic steryl esters and steryl esters as they occur in needles of Scots pine (*Pinus sylvestris* L.).

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Steryl esters occur in all higher organisms, but still very little is known about their function. It has been suggested that steryl esters play a role in sterol transport processes and in the regulation of sterol biosynthesis (1,2).

In contrast to the analysis of free sterols the analysis of steryl esters is more difficult to perform, which has been the reason for the keen interest in the development of new methods (3). The identification of intact steryl esters by capillary gas chromatography, for example, by using columns which are stable even at very high temperatures, has led to unsatisfactory results. Thus the separation of steryl esters differing in the degree of unsaturation in the fatty acid moiety is usually incomplete or impossible (4,5). Other methods, such as capillary gas chromatography-electron impact and chemical ionization-mass spectrometry (6) or gas chromatography-mass spectrometry (7), are expensive. Despite its long tradition, alkaline hydrolysis of steryl esters and the identification of the products, free sterols and fatty acids (8,9), have the major disadvantage of creating artifacts in the fatty acid fraction (10).

To learn more about the biological role of steryl esters, it is necessary to develop a method which allows the hydrolysis of steryl esters without alterations of both the sterol and fatty acid moiety. We introduce here an enzymatic method to cleave steryl esters. The enzyme used is lipoprotein lipase (triacylglycero-protein acylhydrolase, EC 3.1.1.34). The method is gentle, accurate and easy.

MATERIALS AND METHODS

Isolation and purification of steryl esters from Scots pine. Needles from Scots pine trees (stand near Munich) and increment borings from both the trunk and one of the main roots were frozen with dry ice immediately after collection. After lyophilization, the material was homogenized with liquid nitrogen using a micro dismembrator.

Twenty mg of the needle and the wood powder (the outermost annual ring tissues without the bark) was extracted with 1 mL of chloroform/methanol (2:1, v/v) for 30 min and washed twice with 0.5 mL of the chloroform/methanol mixture. The combined extracts were dried under a stream of nitrogen. The residues were redissolved with 50 μL chloroform/methanol and recovered by TLC (0.2 mm Silica gel 60 on aluminum). Cholesteryl palmitate served as standard.

Plates were developed three times in petroleum ether (b. r. 40-60°C)/diethyl ether/acetic acid (90:10:1, v/v/v) (11). Spots carrying the authentic cholesteryl palmitate were visualized by spraying with sulfuric acid/acetic acid (1:1, v/v) and heating (3 min at 360°C) (12). Unsprayed plate areas corresponding to cholesteryl palmitate were scraped off and eluted with chloroform/methanol as described above.

Standard incubation mixture. Authentic cholesteryl esters (100 nanomoles) and cholesteryl esters extracted and purified from Scots pine were incubated with 200 μl of potassium phosphate buffer (0.05 M. pH 8.0) containing 7.8 mM sodium taurocholate (5β-cholan-24-oic acid N-[2-sulfoethyl]amid-3α, 7α, 12α-triol, sodium salt) and 0.1 % Triton X-100. Dihydrocholesterol (3β-hydroxycholestane, 100 nanomoles) served as an internal standard. After vigorous mixing for 30 sec (Vortex mixer), lipoprotein lipase (E. Merck, Darmstadt, FRG) was added. Final volume was 225 μL. Final enzyme concentration was 7.5 U/mL.

To obtain information about the requirements of the enzyme, the concentrations of the above reagents were varied and so were incubation time, incubation temperature and pH (buffers: 0.05M citric acid/sodium citrate [pH 3-5], 0.05M monobasic potassium phosphate/dibasic potassium phosphate [pH 6-8], 0.05M *tris*/HCl [pH 8.2-9] and sodium carbonate/sodium bicarbonate [pH 9.2-10.5]).

Derivatization. After termination of the incubation, the aforementioned mixtures were dried in a stream of nitrogen. To produce derivatives of fatty acids (fatty acid methyl esters, FAME) and sterols (trimethylsilyl ethers), respectively, residues were treated with 0.1 mL of boron trifluoride (14 % in methanol) for 30 min at 80°C. After adding 1 mL of water, the mixtures were extracted three times with diethyl ether. The combined ether extracts were washed two times with 1 mL of water and then taken to dryness. Residues (after extraction with diethyl ether) were treated with 0.1 mL of a mixture containing anhydrous pyridine and **bis**(trimethylsilyl)trifluoroacetamide (BSTFA: 5:1, v/v) and heated for 15 min at 80°C. Before injection into the gas chromatograph, specimens were diluted with chloroform to a final concentration of approximately 50 ng/μL of FAME and sterol derivatives, respectively.

Gas chromatography. A DANI 8500 Capillary Gas Chromatograph with a Programmed Temperature Vaporizer (PTV) injection system and flame ionization detector was used.

Sterol determination. The trimethylsilyl ethers of sterols were determined on a fused silica 25 m SE-30-CB (polydimethylsiloxane) column (Macherey Nagel, Düren

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Abbreviations: BSTFA, *bis* (trimethylsilyl) trifluoroacetamide; FAME, fatty acid methyl esters; HCl, hydrochloric acid; KOH, potassium hydroxide; LPL, lipoprotein lipase; PTV, Programmed Temperature Vaporizer; TLC, thin-layer chromatography.

FRG) with an internal diameter of 0.25 mm and a film thickness of 0.25 μm. The column temperature program was a two-minute hold at 200°C followed by a 15°C/min ramp to 280°C. This final temperature was held for ten min.

The carrier gas was helium at an inlet pressure of 1.6 bar yielding a flow rate of 2 mL/min. Detector gases were hydrogen at 56 mL/min and air at 180 mL/min, the make-up gas was nitrogen at 40 ml/min. We used the splitless injection mode with solvent split. The injection volume was 1 μL. The split flow was 40 mL/min (split ratio 1:20). The temperature of the PTV was 60°C for 6 sec, followed by a very rapid heating ca. 900°C/min) to 300°C for 15 min. The detector temperature was 300°C. A Shimadzu C-R3A integrator was used to collect data.

FAME determination. A fused silica 30 m DB-225 (50% cyanopropylphenyl - 50% dimethylpolysiloxane) column (J&W Scientific) with an internal diameter of 0.25 mm and film thickness of 0.25 μm was used. The column temperature program was two min at 150°C, followed by a 10°C/min ramp to 210°C. This final temperature was held for 15 min.

Helium served as carrier gas with an inlet pressure of 2.0 bar (flow rate: 3 ml/min). All other conditions were as described above, with exception of the final PTV-temperature and the detector-temperature of 230°C.

Alkaline hydrolysis. Steryl esters isolated and purified from Scots pine were heated with 2.5% ethanolic KOH (w/v) at 80°C for 3 hr. After neutralization with HCl, the saponification products were extracted three times with 1 mL of diethyl ether. Derivatization was done as described above.

Evaluation. To ascertain "% cleavage" we determined the amounts of cholesterol recovered from authentic cholesteryl esters by enzymatic hydrolysis in percentage. Cholesterol was quantified by capillary gas chromatography relative to dihydrocholesterol as an internal standard. Statistical significance was assessed at the 95% level using two-tailed t-tests.

Accuracy of the method was proven by determining the amounts of cholesterol recovered from authentic cholesteryl stearate incubated under optimal conditions. Molar amounts of cholesteryl stearate and the recovered cholesterol were not significantly different. Experimental error, expressed as the 99% confidence interval, was about 4% of the mean. Similar results were obtained when authentic cholesteryl stearate was added to the pine needle extraction solvent and recovered by TLC in the steryl ester fraction.

RESULTS AND DISCUSSION

Lipase(glycerol ester hydrolase, EC 3.1.1.3), cholesterol esterase (steryl ester hydrolase, EC 3.1.1.13) and esterase (carboxylic ester hydrolase, EC 3.1.1.1) from various microbial sources and from porcine and bovine pancreas, respectively, have no or only little hydrolytic activity when incubated with steryl esters. Lipoprotein lipase (LPL), however, shows *in vitro* a high activity on steryl ester hydrolysis. Under *in vivo* conditions, LPL cleaves triglycerides in chylomicrons, which are the major transport form of nutritional fats in mammalian blood vessels (13). LPL is activated by apolipoprotein C-II and inhibited by apolipoprotein C-I and C-III (II). Apolipoprotein C-II is not commercially available, but can be isolated by means of DEAE-cellulose chromatography (14).

Our incubation mixtures were without this activator. The absence of apolipoprotein C-II, however, can be overcome by extension of the incubation time. Figure 1 shows the degree of cleavage of various cholesteryl esters differing in the fatty acid moiety when incubated with LPL for 2 hr at 37°C. It becomes clear that individual cholesteryl esters are hydrolyzed to varying extents. This behavior is known to be typical for lipases when different triglycerids and wax esters are used as substrates (15, 16). Some of the differences between various cholesteryl esters were significant (Fig. 1), but there was no attempt made to correlate cleavage with chain length or degree of unsaturation. To optimize our method, we chose cholesteryl stearate for subsequent experiments, although it was not the best substrate for lipoprotein lipase.

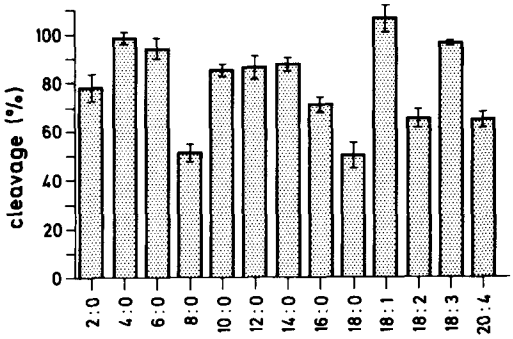

FIG. 1. Affinity of lipoprotein lipase to various cholesteryl esters differing in the chain length and degree of unsaturation of their fatty acid moiety. Substrates are as defined in Standard Incubation Mixture. Incubation time was 2 hr at 37°C. The data are expressed as means ± SD (vertical bars, n=6).

Figure 2 depicts the influence of incubation time and temperature on the hydrolysis of cholesteryl stearate by lipoprotein lipase. Seventeen hr of incubation at 37°C proved to be sufficient for complete cleavage of the substrate. All steryl esters tested were completely hydrolyzed when incubated for 17 hr.

Figure 3 shows the pH-dependency of lipoprotein lipase activity. The enzyme exhibits a broad pH spectrum, with an optimum at about pH 8.2.

Bile acids have been reported as activators as well as inhibitors of lipases (17). At inframicellar concentrations, bile acids stabilize lipases against unfolding at the oil-water interface. By increasing concentrations, lipase activity is progressively inhibited due to blockage of the interface or by removing (displacing) the enzyme from the interface.

Quantitative hydrolysis of steryl esters by lipoprotein lipase depends on the presence of bile acids. The nature of the bile acid (we used the sodium salts of cholic acid -(3α, 7α, 12α-trihydroxy-5β-cholan-24-oic acid)—, deoxychol-

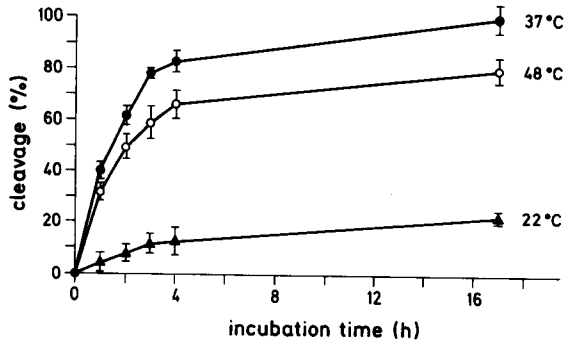

FIG. 2. Cleavage of cholesteryl stearate by lipoprotein lipase as function of incubation time and temperature. Substrates are as defined in Standard Incubation Mixture. Means ± SD (vertical bars, n=6) are given.

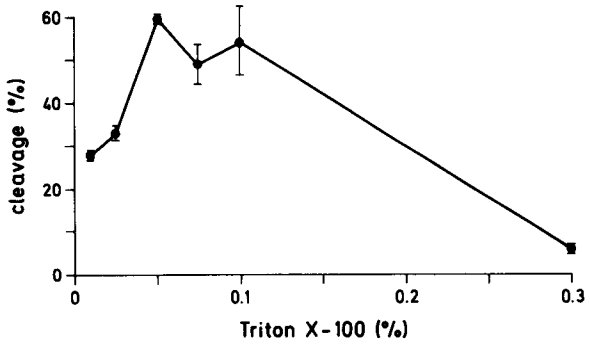

FIG. 5. Influence of Triton X-100 on lipoprotein lipase activity with cholesteryl stearate as substrate. Substrates are as defined in Standard Incubation Mixture. Incubation time was 2 hr at 37°C. Data are expressed as means ± SD (vertical bars, n=6).

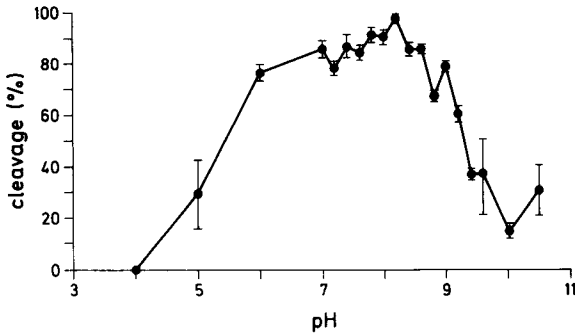

FIG. 3. The pH dependence of lipoprotein lipase activity with cholesteryl stearate as substrate. Substrates are as defined in Standard Incubation Mixture. Incubation time was 17 hr at 37°C. Data are expressed as means ± SD (vertical bars, n=6).

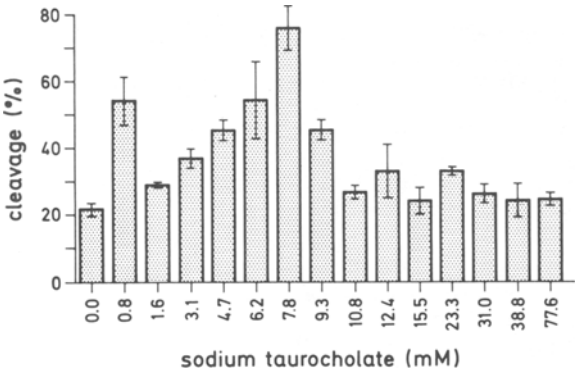

FIG. 4. Influence of sodium taurocholate on lipoprotein lipase activity with cholesteryl stearate as substrate. Substrates are as defined in Standard Incubation Mixture. Incubation time was 2 hr at 37°C. Data are expressed as means ± SD (vertical bars, n=6).

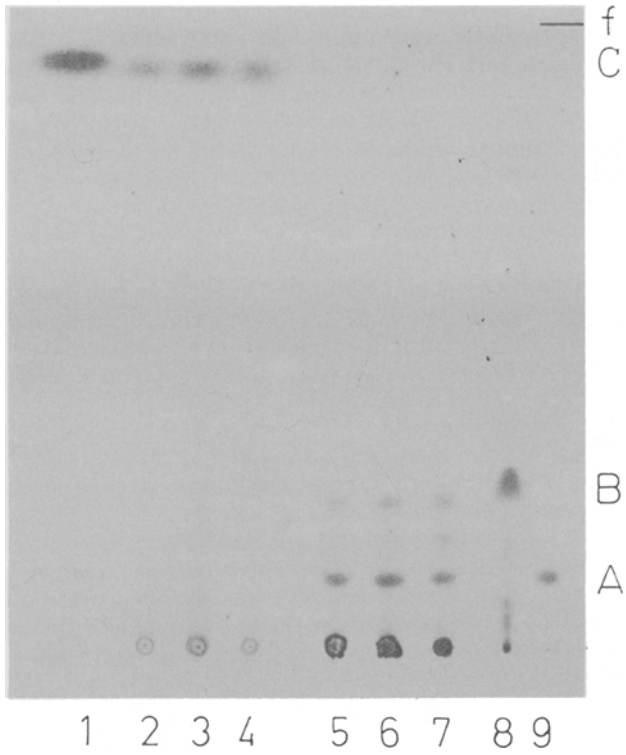

FIG. 6. Thin-layer Chromatogram of authentic cholesteryl palmitate (1) and steryl esters from Scots pine isolated from the outer part of one of the main roots (2) and the trunk (3) and from current year needles (4). The right side of the plate shows hydrolysis products of steryl esters from Scots pine rootwood (5), trunkwood (6) and needles (7) after incubation with lipoprotein lipase. Linoleic acid (8) and cholesterol (9) served as standards. A, sterols; B. fatty acids; C, steryl esters; F, front.

ic acid—(5β-cholan-24-oic acid-3α, 12α-diol)—, and taurocholic acid—(5β-cholan-24-oic acid N-[2-sulfoethyl] amide-3α, 7α, 12α-triol) is of secondary importance. Figure 4 shows the influence of sodiumtaurocholate on the hydrolytic activity of lipoprotein lipase. A concentration of 7.8 mM taurocholate proved to be optimal.

Figure 5 shows the influence of Triton X-100 on the activity of lipoprotein lipase. A final concentration of 0.1% of Triton X-100 in the test tube is optimal for enzyme activity. Solubilization of the substrate is not the only function of Triton X-100. When this detergent is replaced

TABLE 1

Experimental Errors by Enzymatic and Alkaline Cleavage of Steryl Esters Isolated from Pine Needles

	Enzymatic hydrolysis		Alkaline saponification	
	M[a]	cv[b]	M[a]	cv[b]
Sterols				
Cholesterol	0.105	4	0.075	7
Campesterol	0.235	6	0.224	6
β-Sitosterol	5.109	5	5.100	5
Fatty Acids				
Palmitic acid	0.928	13	1.256	34
Stearic acid	0.638	14	0.525	49
Oleic acid	1.332	29	1.093	60
Linoleic acid	1.816	10	3.155	44
Linolenic acid	0.791	10	1.135	37

Sample size n = 10.
[a]M means (μmol/g dry weight).
[b]Coefficient of variation cv is (%).

by other surfactants, *e.g.* phosphatidylcholine in similar concentrations, the activity of the lipoprotein lipase decreases rapidly. Higher concentrations of Triton X-100 show an inhibitory effect on the hydrolytic activity.

We incubated steryl esters isolated from needles and wood (both trunk and root) of Scots pine with lipoprotein lipase and recovered the hydrolysis products by TLC. The results of this experiment are depicted in Figure 6. On the left side of the plate, steryl esters from Scots pine and authentic cholesteryl palmitate appeared as red spots. The right side of the chromatogram shows products of steryl esters after treatment with lipoprotein lipase. It is obvious that steryl esters from Scots pine are split into at least two substances which show the same chromatographic behavior as authentic cholesterol and authentic linoleic acid. Figure 6 shows that the hydrolysis of steryl esters from Scots pine by lipoprotein lipase is complete.

The accuracy of the method was proven by the fact of quantitative recovery of cholesterol from authentic cholesteryl stearate, even when mixed with pine-needle powder.

We compared the reproducibility of steryl ester cleavage using both the alkaline hydrolysis and the enzymatic method. Needles from Scots pine were used as substrate. As depicted in Table 1, no significant differences between the amounts of sterols recovered were found with both methods. The experimental error is about 6% of the average value (given as coefficients of variation). The same is true for the recovery of the fatty acid moiety of steryl esters. The experimental error in the case of fatty acids, however, is much higher using alkaline hydrolysis as compared with the enzymatic method. This holds true even when the oxidation of double bonds was counteracted by adding antioxidants. This latter result clearly demonstrates the advantage of the enzymatic method compared with the traditional hydrolysis using alkaline reagents.

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COMMUNICATIONS

Existence of Cholinephosphotransferase in Mitochondria and Microsomes of Liver and Lung of Guinea Pig and Rat[1]

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We reported earlier on the occurrence of cholinephosphotransferase in the mitochondria of guinea pig lung. In order to determine whether organ and/or species specificities exist in regard to the cholinephosphotransferase activity in mitochondria, we have compared the subcellular distribution of the enzyme in the liver and lungs of rats and guinea pigs. Even though the activity of the enzyme was higher in microsomes than it was in mitochondria, the mitochondrial activity was authentic in both tissues of both species. The authenticity of mitochondrial activity was established by marker enzyme studies and ultrastructural examination of mitochondrial preparations.
***Lipids* 25, 296-300 (1990).**

Cholinephosphotransferase, the terminal enzyme of the CDPcholine pathway, was generally presumed to be located exclusively in the endoplasmic reticulum (1). However, our laboratory had reported the presence of the enzyme in the mitochondria of guinea pig lung earlier (2,3). We further established that the enzyme is located in the outer mitochondrial membrane (3). Although the presence of cholinephosphotransferase has been observed by others in mitochondria of yeast (4), rat liver (5-7) and intestine (8), there are several reports on rat liver (9-12), rabbit lung (13) and fetal rat lung (14,15) which contradict these findings.

It is presently not known whether there is a species and/or tissue specificity as far as the presence of cholinephosphotransferase in mitochondria is concerned. We have therefore compared the distribution of cholinephosphotransferase in mitochondria and microsomes of the liver and lung of both rats and guinea pigs.

MATERIALS AND METHODS

Materials. All biochemicals were purchased from Sigma Chemical Company (St. Louis, MO). Radioactive cytidine diphosphocholine was purchased from New England Nuclear (Boston, MA). n-Butanol was purchased from Malinckrodt, Inc. (Paris, KY). Universol cocktail was purchased from ICN Radiochemicals (Irvine, CA).

Subcellular fractionation. Hartley strain male guinea pigs (350-400 g) and Wistar male rats (180-200 g) obtained from Camm Laboratory Animals (Wayne, NJ) were killed by decapitation. Liver and lung were quickly excised, washed in ice cold saline (0.9% NaCl), blotted dry and weighed. The tissues were then minced with scissors and homogenized in 4-6 volumes of 0.25 M sucrose/1 mM EDTA (pH 7.4), using a Potter Elvehjem homogenizer.

The nuclear fraction was prepared by centrifugation of whole homogenate at 600 × *g* for 10 min in a refrigerated Sorvall RC-5 centrifuge using a SS-34 rotor. The nuclear pellet was washed once with 0.25 M sucrose/1 mM EDTA (pH 7.4). The supernatants were combined and centrifuged at 10,000 × *g* for 10 min to obtain the mitochondrial fraction. In most cases, the supernatant was centrifuged in a Beckman L8-M ultracentrifuge at 105,000 × *g* for 60 min using a 70.1 Ti rotor to obtain the microsomal and cytosolic fractions. However, for guinea pig lung, the post-mitochondrial supernatant was first centrifuged at 20,000 × *g* for 10 min to collect an intermediary fraction. The post 20,000 × *g* supernatant was used to collect microsomes and cytosol. The crude mitochondrial fraction was purified by linear sucrose density gradient as described earlier (3). Protein content was determined by the method of Lowry *et al.* (16).

Biochemical assays. Cholinephosphotransferase activity was assayed by measuring the incorporation of radioactivity from cytidine diphospho-[*methyl*-^{14}C]-choline into phosphatidylcholine using dioleoylglycerol as a substrate, as described earlier (2). We have previously reported that dioleoylglycerol is the most preferred substrate for both guinea pig lung mitochondrial and microsomal cholinephosphotransferase (17).

Succinic INT-reductase, a mitochondrial marker, was assayed as described by Possmayer *et al.* (18). Rotenone-sensitive NADPH cytochrome c reductase (19), as well as glucose-6-phosphatase (20), were used as microsomal markers.

For the electron microscopic examination, mitochondrial pellets were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3), followed by osmium tetroxide in the same buffer (21). The specimens were dehydrated through an upgraded ethanol series at room temperature. Preparations were embedded in Araldite-502. Post-staining was done in a saturated solution of uranyl acetate in 50% ethanol followed by Reynold's lead citrate. Sections were examined with a Phillips 300 transmission electron microscope.

RESULTS AND DISCUSSION

The subcellular distribution of cholinephosphotransferase and marker enzyme activity in the liver and lung of rats is shown in Table 1. The specific activity of cholinephosphotransferase in either mitochondria or microsomes is not significantly different between liver and lung. The microsomal activity is higher than mitochondrial activity in both tissues. As shown in Figure 1, the relative specific activity of microsomal

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TABLE 1

Specific Activity of Cholinephosphotransferase and Marker Enzymes in Subcellular Fractions of Rat Liver and Lung[a]

Fractions	Liver				Lung		
	A	B	C	D	A	B	C
Whole homogenate	0.5 (0.1)	44.6 (2.4)	14.5 (0.8)	25.9 (2.2)	0.3 (0.0)	3.2 (0.6)	2.4 (0.4)
Nucleus	0.5 (0.1)	40.0 (3.5)	12.2 (1.0)	23.4 (1.0)	0.1 (0.0)	2.6 (0.2)	1.7 (0.3)
Mitochondria	0.7 (0.1)	54.6 (1.5)	10.2 (1.0)	2.9 (0.7)	0.6 (0.2)	6.1 (0.4)	4.7 (0.9)
Microsomes	0.9 (0.1)	2.8 (0.5)	46.4 (2.2)	58.2 (1.3)	1.2 (0.2)	1.0 (0.4)	19.2 (3.2)
Cytosol	0.1 (0.0)	2.0 (0.4)	3.9 (0.3)	6.0 (0.2)	0.1 (0.0)	2.0 (0.6)	1.6 (0.3)

[a]The specific activities of enzymes are expressed in nmol/min per mg protein. A represents cholinephosphotransferase, B represents succinic INT reductase, C represents NADPH cytochrome c reductase, and D represents glucose 6-phosphatase. Values are mean ± S.E. of seven experiments.

cholinephosphotransferase was 1.25-fold and 2.17-fold higher than that of mitochondrial fraction in the liver and lung, respectively. Succinic INT-reductase activity was distributed in mitochondria almost 19-fold more than in microsomal fraction in liver and 6-fold more in the lung. NADPH cytochrome c reductase activity of microsomes was significantly higher than that of mitochondria in both liver (4.65-fold) and lung (3.98-fold), respectively.

The apparent occurrence of NADPH cytochrome c reductase in mitochondria may be explained by the presence of pyridine nucleotide transdehydrogenase in the mitochondria which, in conjunction with the mitochondrial NADH cytochrome c reductase and NAD^+, would catalyze the oxidation of NADPH by cytochrome c (19). Therefore, the presence of NADPH cytochrome c reductase activity in the mitochondrial fraction does not necessarily indicate the presence of microsomal vesicle in this fraction. To exclude the possibility of microsomal contamination of mitochondria, we have also measured another microsomal marker—glucose-6-phosphatase—in the liver. The relative specific activity was about 20-fold higher in microsomes than in mitochondria. Moreover, the ratio of relative specific activity of cholinephosphotransferase to that of succinic INT-reductase is higher than the ratio of relative specific activity of cholinephosphotransferase to that of NADPH cytochrome c reductase in mitochondria of both the lung and liver, suggesting a true cholinephosphotransferase activity in mitochondria in both tissues.

Table 2 presents the data on cholinephosphotransferase and marker enzymes in the guinea pig lung and liver. The distribution patterns of these enzymes are shown in Figure 2. In contrast to the rat, the specific activity of cholinephosphotransferase in guinea pig is higher in the lung than in the liver in all fractions studied, except the nucleus. The relative specific activity of the enzyme in microsomes was found to be 1.65-fold and 1.8-fold higher, as compared to that of mitochondria in liver and lung, respectively. Succinic INT-reductase activity is mainly enriched in mitochondria, approximately 8-fold and 7-fold more in mitochondria than in the microsomes of the liver and lung, respec-

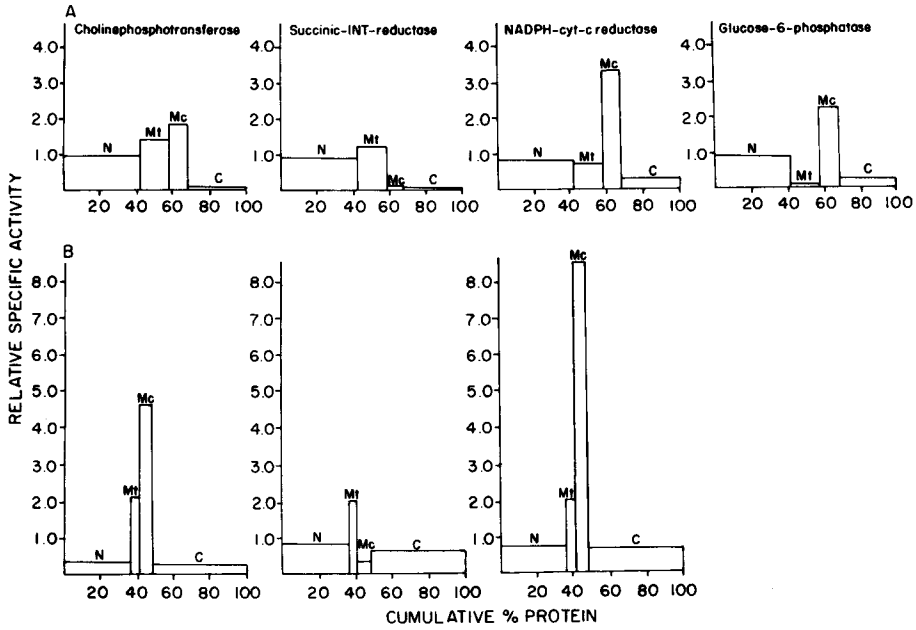

FIG. 1. Subcellular distribution of cholinephosphotransferase and marker enzyme activities of rat liver (A) and lung (B). Relative specific activity (the ratio of the specific activity of the respective fraction over that of the whole homogenate) is plotted as a function of cumulative percent protein. Relative specific activities were determined from the data in Table 1. N, nucleus; Mt, mitochondria; Mc, microsomes; and C, cytosol.

TABLE 2

Specific Activity of Cholinephosphotransferase and Marker Enzymes in Subcellular Fractions of Guinea Pig Liver and Lung[a]

Fractions	Liver			Lung		
	A	B	C	A	B	C
Whole homogenate	0.3 (0.1)	24.8 (2.2)	19.1 (2.6)	0.3 (0.1)	12.8 (0.3)	41.2 (1.2)
Nucleus	0.3 (0.1)	28.4 (6.7)	20.8 (3.4)	0.3 (0.1)	6.2 (0.2)	32.5 (1.2)
Mitochondria	0.5 (0.1)	99.9 (8.9)	26.6 (2.4)	1.1 (0.2)	89.2 (1.2)	42.1 (2.0)
20,000 × *g*				2.3 (0.1)	45.2 (2.0)	59.2 (2.0)
Microsomes	0.9 (0.2)	12.5 (3.2)	69.1 (5.9)	2.0 (0.4)	13.1 (0.7)	99.9 (4.5)
Cytosol	0.1 (0.0)	15.5 (4.8)	8.2 (1.2)	0.2 (0.1)	4.3 (0.2)	21.2 (2.3)

[a]The specific activities of enzymes are expressed in nmol/min/mg protein. A represents cholinephosphotransferase, B represents succinic INT reductase, and C represents NADPH cytochrome c reductase. Values are mean ± S.E. of eight experiments. For lung, the postmitochondrial fraction was centrifuged at 20,000 × *g* to collect an intermediary fraction.

tively. The relative specific activity of NADPH cytochrome c reductase was 2 to 2.5-fold higher in microsomes than mitochondria in both tissues. It should further be noted that the electron microscopic analysis of mitochondrial fraction from liver and lung of both species showed no contamination by endoplasmic reticulum. A typical electron micrograph of guinea pig liver mitochondria is shown in Figure 3.

There are some conflicting reports on the presence of cholinephosphotransferase in Golgi bodies. Although van Golde *et al.* (10) found no cholinephosphotransferase activity in Golgi bodies, others (5,22) reported considerable activity in the same organelle. Cholinephosphotransferase activity also has been detected in the plasma membrane of rat brain (23). Since the electron micrograph of the mitochondrial preparation of liver and lung of either guinea pig or rat did not show any contamination with Golgi bodies (Fig. 3), we did not study any marker for them. Furthermore, the mitochondrial preparation had negligible 5′-nucleotidase activity (marker for plasma membrane) (24). Therefore, the cholinephosphotransferase activity in mitochondria cannot be attributed to plasma membrane contamination.

In the present study, the activity of cholinephosphotransferase in the mitochondrial fraction is a true representation of the mitochondrial enzyme. This further confirms the previous observation on dual localization of the enzyme in the lung of guinea pigs (3) and rats (25), as well as in alveolar type II cells of fetal rat lung (26). The dual localization of the cholinephosphotransferase in both mitochondria and microsomes reported here is not unique. Glycerophosphate phosphatidyltransferase, a key enzyme in the synthe-

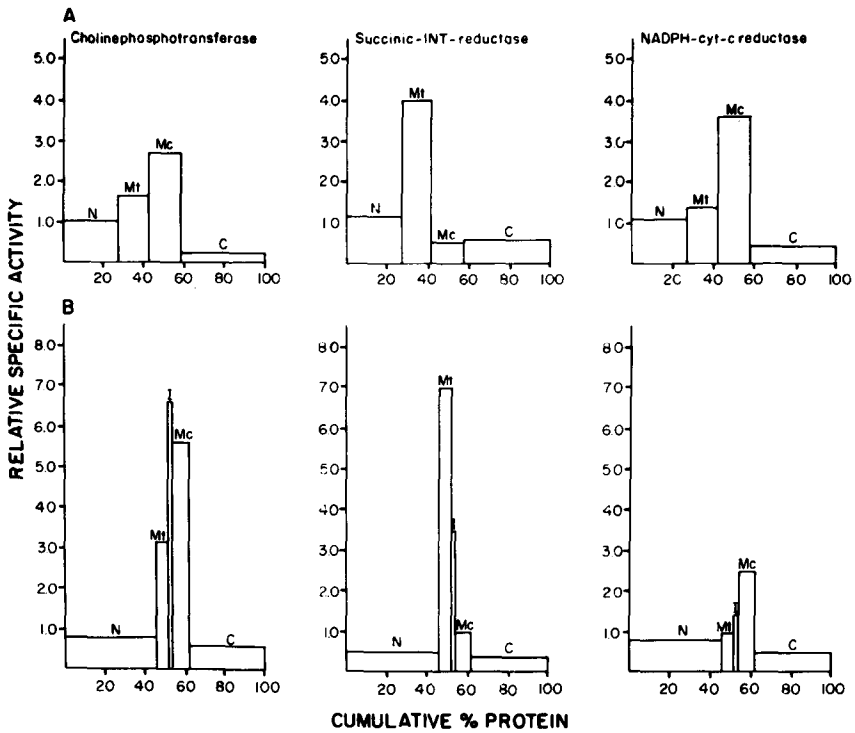

FIG. 2. Subcellular distribution of cholinephosphotransferase and marker enzyme activities of guinea pig liver (A) and lung (B). Relative specific activity (the ratio of the specific activity of the respective fraction over that of the whole homogenate) is plotted as a function of cumulative percent protein. Relative specific activities were determined from the data in Table 2. N, nucleus; Mt, mitochondria; I, 20,000 × *g* pellet; Mc, microsomes; and C, cytosol.

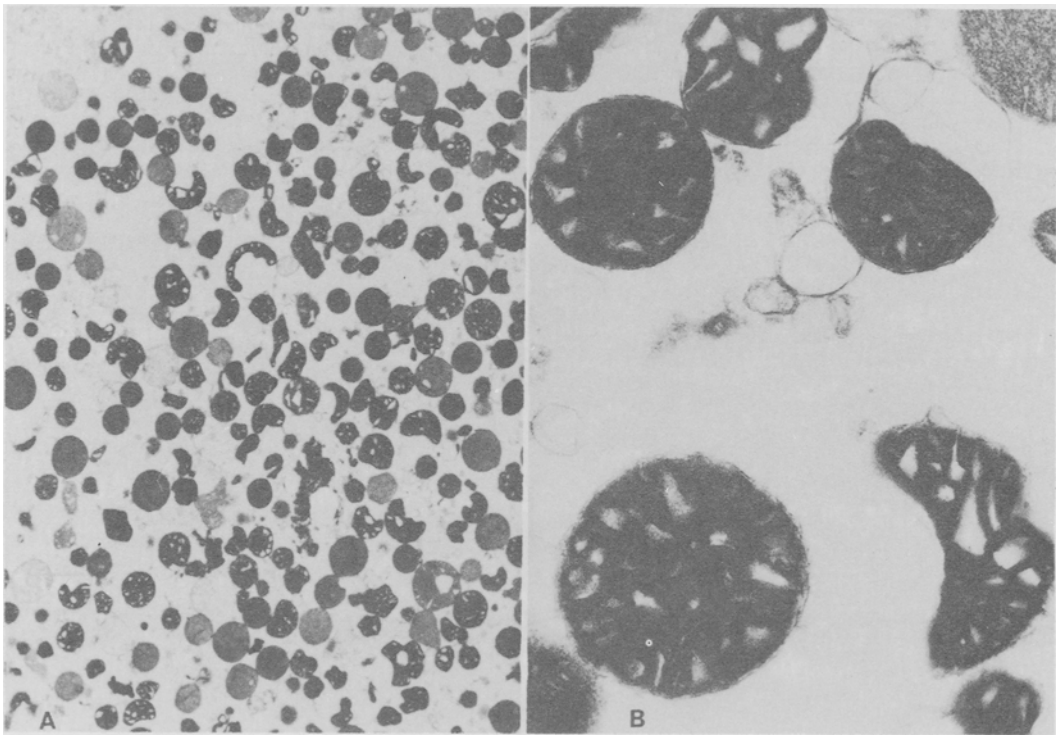

FIG. 3. A typical electron micrograph of guinea pig liver mitochondria (A at magnification × 5,440 and B at magnification × 34,850).

sis of phosphatidylglycerol, is present in both microsomes and mitochondria (10,26,27). CTP: cholinephosphate cytidyltransferase, which is thought to be located in microsomes and cytosol (28), has recently been reported to be present in human lung mitochondria (29). Hunt and Postle (29) reported that as much as 59% of human lung cytidyltransferase activity was associated with mitochondria, while the microsomal fraction accounted for only 4% of the activity. Stith and Das (2) reported a high cholinephosphotransferase activity in the mitochondrial fraction of the guinea pig lung between 45 and 60 days of gestation. The activity in fetal mitochondria was more than twice of that in fetal microsomes. Therefore, it is possible that mitochondrial cholinephosphotransferase may also be involved in phosphatidylcholine biosynthesis.

The results from the present study strongly suggest that the cholinephosphotransferase is localized in mitochondria, as well as in the endoplasmic reticulum in the liver and lungs of both species. However, there is some species and tissue specificity as far as the specific activity of cholinephosphotransferase is concerned. We have initiated studies to investigate the role of mitochondrial cholinephosphotransferase in phosphatidylcholine biosynthesis.

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Phospholipase A Activity of Cultured Rat Ventricular Myocyte Is Affected by the Nature of Cellular Polyunsaturated Fatty Acids

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Fatty acid composition of membrane phospholipids of cultured cardiomyocytes can be modified by the type of polyunsaturated fatty acids (n-3 or n-6 PUFA) constituting the culture medium. In this study, we investigated the effect of fatty acid modification on the activities of the key enzymes involved in the deacylation-reacylation cycle of membrane phospholipids.

Results showed that cardiomyocytes grown in the presence of n-6 PUFA exhibited a higher specific alkaline phospholipase A (mainly A_2) activity (+34%) and a moderately lower lysophospholipase activity (−17%) than when incubated with n-3 PUFA. AcylCoA:lysophosphatidylcholine acyltransferase, acid lysosomal phospholipase A_1 and acylCoA synthetase activities were not significantly altered by changes in cellular PUFA composition. It was demonstrated that the differences between phospholipase A activities of the two types of cultured cells were linked neither to a differential leakage of enzyme nor to oxidative injury to the enzyme through blockage of essential sulfhydryl groups. One likely explanation is that the PUFA-induced changes in membrane composition alter membrane physical properties which, in turn, affect membrane-bound phospholipase A activity. Possible beneficial effects of the n-3 PUFA-induced changes on membrane stability are discussed. ***Lipids 25,*** **301-306 (1990).**

A wide variety of membrane-associated enzymes have been shown to be affected by the physicochemical properties of the surrounding host membrane phospholipids. This becomes evident when manipulating the polyunsaturated (PUFA) to saturated fatty acid ratio or the n-6/n-3 PUFA ratio of the dietary fat, which, in turn, modifies the physicochemical properties of the membrane. Under such conditions, alteration of membrane-bound enzyme activities has been reported in various tissues (1,2). In murine hearts, this was demonstrated for $Ca^{2+}Mg^{2+}$-ATPase (3) and Ca^{2+} transport activity (4) of sarcoplasmic reticulum, for rat sarcolemmal 5′-nucleotidase (5) and for rat mitochondrial ATPase (6). It is, however, not possible to draw general conclusions concerning the modulation of enzyme activity by such diet-induced modification of the degree of unsaturation in membranes. This means that a diet-induced increase in membrane fluidity may either decrease or increase membrane-bound enzyme activity depending on the type of enzyme involved.

In this context, the behavior of membrane-associated phospholipase A is of great interest since this activity is involved in membrane phospholipid turnover, a process that plays a key role during cardiac ischemia and reperfusion (7,8). Interestingly, the particularity of phospholipase A is that the membrane is host as well as substrate. Thus, any change in membrane lipid composition may affect phospholipase activity in two ways: one is the modification of the physical state of the micro-environment surrounding the enzyme; the other is the modification of membrane susceptibility to hydrolysis. These two parameters have been studied for rat liver microsomal phospholipase A (9,10). Increasing the rigidity of the microsomal membrane resulted in an increase in the specific activity of phospholipase A_2 when an exogenous substrate was used (9). On the opposite, when endogenous membrane phospholipids were used as substrate, a lesser extent of hydrolysis was observed than with more fluid membranes (10). To date, studies on the modulation of phospholipase A activity by dietary fat have received little attention. Lee *et al.* (11) recently reported that liver microsomal phospholipase A_2 activity was significantly lower in rats fed a diet with a low n-6/n-3 ratio than in animals fed a diet with a high n-6/n-3 ratio. Similar observations were made using the method of endogenous hydrolysis of liver microsomal phospholipids by microsomal phospholipase A_2 (12). A higher rate of hydrolysis was obtained in rats fed a diet rich in n-6 PUFA compared with a saturated fat diet poor in n-6 PUFA. However, rat gastric phospholipase A_2 was shown to be enhanced by 30% in animals fed a fish oil diet rich in n-3 PUFA, compared with a corn oil diet rich in n-6 (13).

Studies devoted to dietary-induced changes in heart phospholipase A activity are scarce. In rats fed 17% (w/w) corn oil, we observed that the endogenous hydrolysis of whole membrane cardiac phosphatidylcholine (PC) and phosphatidylethanolamine (PE) was about 40% higher than in rats fed either a low-fat or saturated-fat diet (14). However, using exogenous radioactive dioleoyl PC as the substrate and the same whole membrane fraction as the enzyme source, we could not confirm these differences. Thus, we concluded that the higher endogenous hydrolysis observed with a corn oil diet was the result of a higher susceptibility of membrane phospholipids to hydrolysis.

In a recent study, it was shown that the use of fatty acid-controlled serum substitutes in primary culture allowed the production of purified ventricular myocytes with a characteristic n-6 or n-3 profile (15). The object of the present study was to observe the influence of these changes on phospholipase A activity as well as on other key enzymes involved in the deacylation-reacylation pathway of membrane phospholipids.

MATERIALS AND METHODS

Cell culture preparation and fatty acid-controlled defined media. Monolayer cultures of rat ventricular myocytes were prepared as previously described (16). Cultures were

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Abbreviations: PUFA, polyunsaturated fatty acids; PC, phosphatidylcholines; PE, phosphatidylethanolamines; SM3, n-3 PUFA-enriched synthetic media; SM6, n-6 PUFA-enriched synthetic media; ACLAT, acylCoA:lysophosphatidylcholine acyltransferase; BME, betamercaptoethanol; PI, phosphatidylinositol; DPG, diphosphatidylglycerol; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; SPH, sphingomyelin.

incubated in a humidifed atmosphere containing 5% CO_2, 19% O_2 and 76% N_2. The culture medium was changed 24 hr after seeding and thereafter every 48 hr. Two synthetic media were prepared as previously described (15), SM3 and SM6 containing only n-3 and n-6, respectively, as PUFA. Briefly, fatty acids (sodium salt, NuCheck Prep, Elysian, MN) were bound to fatty acid-free bovine serum albumin (fraction V, Sigma) in a 6:1 molar ratio. This fatty acid/albumin mixture was added to a chemically defined serum substitute (Ultroser-G IBF), sonicated for 30 sec and stirred for 12 hr at 37°C. The solutions obtained were incorporated into Ham's F10 medium. The compositions of the two fatty acid-enriched synthetic media (SM3 and SM6) are reported in Table 1. Cells were incubated for 5 days using conventional serum-containing medium and then for 24 hr either SM3 or SM6. The calcium concentration in SM3 and SM6 was standardized to 1.2 mM by sterile addition of 0.2 M $CaCl_2$ (16). Under these conditions, only the fatty acid composition of the media was variable. The cells were then washed three times in Ham's F10 medium (Seromed), harvested and homogenized at 4°C (in a Tris-HCl 0.01M buffer, pH 7.0) with a glass Potter-Elvejem, and treated either for lipid analysis or stored at −80°C for subsequent measurements of enzyme activities.

Protein and enzyme assays. Proteins were assayed according to Lowry *et al.* (17) using serum albumin as standard. Phospholipase A was assayed as previously described (18) using di[1-^{14}C]oleoylPC (NEN-Paris, France) with a specific radioactivity of 50 Bq/nmol. Alkaline phospholipase A (EC 3.1.1.4 + EC 3.1.1.32) was assayed at pH 8.4 at a final calcium concentration of 5 mM and acid phospholipase A_1 (EC 3.1.1.32) at pH 4.9, both for 20 min. Lysophospholipase (EC 3.1.1.5) was assayed at pH 8.0 for 20 min (18) using 1-[1′-^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine (NEN) with a specific radioactivity of 20 Bq/nmol. Acyl-CoA:lysoPC acyltransferase (ACLAT) (EC 2.3.1.23) activity was determined for 15 min at pH 7.4 according to the method published (19). The substrate was identical to that used for the lysophospholipase assay and oleoyl-CoA (Sigma, Paris) served as the donor. Acyl-CoA synthetase (EC 6.2.1.3) was assayed at pH 7.5 as recently described (20) using [1-^{14}C]oleic acid (NEN) complexed to bovine serum albumin. The reaction was initiated by addition of this complex and continued for 15 min at 37°C.

All the enzyme assays were done with 40 μg of protein which was in the linear range of the protein concentration dependence. All assays were done in duplicate.

Lipid analysis. Cell lipids were extracted according to Folch *et al.* (21). The total PL fraction was separated from the non-phosphorus lipids (22), and the fatty acids were analyzed by gas chromatography on a glass capillary column after transmethylation with BF_3/methanol.

Phospholipid composition was determined by high performance liquid chromatography using a Waters Assoc. system (Waters, Milford, MA) equipped with two pumps (M6000A and M45), an automatic injector (Wisp 710 A), a peak integrator (M730) and a gradient controller (M720). Phospholipids were separated on a 30 cm × 4.6 mm (i.d.) column packed with LiChrospher T^M 5m silica (Merck, Darmstadt, FRG). Elution started with 100% solvent A (chloroform/methanol/water, 72:26:2, by vol) for 5 min, followed by a continuous gradient from 100% A to 100% solvent B (chloroform/methanol/water, 43.2:50.0:6.8, by vol) for 10 min. The flow rate was 1.2 ml/min. All the phospholipids were eluted in 40 min with the order of elution being: DPG, PE, PI, LPE, PC, SPH, LPC. Detection was achieved with a DDL 10 light-scattering detector (Cunow, Clichy, France), and 82.5% of the eluted compounds were derived for the collection of individual phospholipids and subsequent quantification by phosphorus determination (23).

Association of di-[1-^{14}C]oleoyl PC with membranes of cardiomyocytes. This experiment was conducted to estimate the extent of the association between di-[1-^{14}C]-oleoyl PC of the exogenous substrate and phospholipids of the endogenous membranes under phospholipase A assay condition. Liposomes of di[1-^{14}C]oleoyl PC were first centrifuged at 100,000 × *g* for 1 hr at 5°C to sediment undispersed multilamellar particles. The limpid supernatant containing unilamellar liposomes of di-[1-^{14}C]oleoyl PC served as the substrate. For each type of cells (SM3 or SM6), 500 μg of membrane protein were incubated with 527 nmol of radioactive dioleoyl PC using conditions strictly identical to those used for the phospholipase A assay including 5 mM calcium as final concentration. The reaction proceeded for 20 min and was stopped at 4°C. The mixture was then immediately centrifuged at 100,000 × *g* for 1 hr at 5°C. The supernatant was removed and the pellet rinsed with 0.05 M Tris HCl buffer at pH 8.4, then resuspended in this buffer and centrifuged again at 100,000 × *g* for 1 hr. This operation was repeated three times. The membranes were resuspended, and the radioactivity counted. Repartition of the radioactivity was determined after lipid extraction of the pellet (21) and thin-layer chromatography separation of the lipids (F1500, Schleicher and Schüll, Dassel, FRG).

Statistical analysis. The experiment was repeated three times (A, B, C) at 1-month intervals. In each experiment, 6 dishes each of SM3 and SM6 were analyzed. A two-way analysis of variance was applied in which the block factor (experiment) was hierarchized to the PUFA (SM3 or SM6) factor (24). Studies involving enzyme release into the bathing fluid and β-mercaptoethanol (BME) effect were performed in two experiments (n = 2 × 6 for each SM3 and SM6), and a similar statistical treatment was

TABLE 1

Fatty Acid Composition (Weight %) of the Chemically Defined Media[a]

Fatty acid	SM 3	SM 6
16:0	5.3	4.6
18:0	2.5	2.3
16:1	2.3	1.9
18:1	27.0	26.4
18:2n-6	4.2	30.1
20:4n-6	0.2	33.1
18:3n-3	28.3	0.3
20:5n-3	29.9	0.8
n-6/n-3	0.08	57.5
Fatty acids (μg/ml)	47	51

[a]Methyl esters of fatty acids were separated by gas-liquid chromatography. Values are the means of three determinations.

applied except for the BME study in which a three-way analysis of variance (24) was performed (PUFA factor, BME factor and experiment factor).

RESULTS

Phospholipid composition of membranes of cardiomyocytes. Table 2 shows the membrane phospholipid composition and the fatty acid composition of total membrane phospholipids of cardiomyocytes grown for 5 days in conventional medium, and then incubated for 24 hr in SM3 or SM6. Phospholipid composition did not show any marked changes, and the membrane phospholipid fatty acid composition globally corresponded to that of the culture medium—that is to say that cells grown in SM3 medium enriched their membranes in 18:3 n-3 and 20:5 n-3, whereas those grown in SM6 medium were enriched in 18:2 n-6, 20:4 n-6 and 22:4 n-6. Consequently, the n-6/n-3 ratio was about 14 times higher in SM6 cells than in SM3 cells which is consistent with previous studies. The polyunsaturated/saturated ratio was about 58% higher in SM6 cells as compared to SM3 cells.

Enzyme activities. Cellular protein concentration was not affected by the type of PUFA added to the media.

TABLE 2

Composition (Weight %) of Whole Membrane Phospholipids of Ventricular Myocytes Grown for 24 hr on SM3 or SM6[a]

Lipid	SM 3	SM 6
Phospholipid		
DPG	2.45	2.02
SPH	6.41	5.76
PC	45.21	45.54
PE	27.88	27.09
Other	8.29	5.75
Fatty acid		
16:0	16.3	11.7
18:0	14.4	13.6
Other	3.3	2.2
Σ saturated	34.0	27.5
16:1	2.8	2.1
18:1	20.7	19.2
Other	1.4	1.2
Σ monounsaturated	24.9	22.4
18:2	6.2	18.3
20:4	8.0	18.5
22:4	1.5	3.3
Σ polyunsaturated n-6	17.1	41.8
18:3	4.7	0.6
20:5	8.6	0.3
22:5	3.1	1.0
22:6	1.2	1.0
Other	0.8	0.3
Σ polyunsaturated n-3	18.4	3.2
Σ DMA	5.8	5.3
n-6/n-3	0.9	13.1
P/S	1.04	1.64

[a]Values are the means of two determinations for phospholipids and three determinations for fatty acids. SEM were omitted but were in the range of 10% of the mean. DMA, dimethylacetal derivatives; P/S, polyunsaturated-to-saturated ratio. Methyl esters of fatty acids were separated by gas-liquid chromatography, and phospholipids by HPLC. DPG: diphosphatidylglycerol; SPH: sphingomyelin.

For the three experiments, it was 1.13 ± 0.066 and 1.09 ± 0.056 mg/ml in SM3 and SM6 cells, respectively (mean ± SEM, n = 18). In a previous paper, we reported that about 95% of the alkaline phospholipase A activity of the cardiomyocyte was directed towards the *sn*-2 position (25). In the present experiment, alkaline phospholipase A_2 activity remained higher than A_1 activity although the latter activity was slightly higher than previously reported (25). In SM3 cells, the percentages of A_2 and A_1 were 84.2 ± 5.9 and 15.8 ± 5.9, respectively, whereas in SM6 cells they were 91.3 ± 8.7 and 8.7 ± 8.7, respectively (n = 3). This slight discrepancy between the present and previous (25) results may reflect a different culture-induced expression of alkaline A_1 and A_2 activities. Thus, total alkaline phospholipase A_1 + A_2 activity was measured with di-[1-^{14}C]oleoyl PC. Consequently, we controlled in our phospholipase A assay the lysophospholipase contribution to the release of [1-^{14}C]oleic acid. This contribution, calculated as previously described (18), accounted for only 5 to 7% of the radioactive oleic acid liberated from both types of cells and was not considered in the calculation of phospholipase A activity. We also determined the percentage of phospholipase A bound to the membrane. Cell homogenate was submitted to ultracentrifugation (90 min; 100,000 × *g*). The pellet and the corresponding supernatant were assayed for alkaline phospholipase A. About 85% (mean of two experiments) of the total recovered activity was found associated with the membrane peallet.

Alkaline phospholipase A activity was significantly higher (+34%) in SM6 cells than in SM3 cells ($p < 0.01$) (Table 3). Statistical analysis revealed a significant variability between the culture preparation (experiments A, B, C), but no cross-interaction. This indicates that the differences between the groups of cells (SM3 and SM6) do not depend on the culture preparation. This also allows us to conclude that the higher phospholipase A activity in SM6 cells is specifically related to the nature of the membrane fatty acids in the cells. Lysosomal acid phospholipase A_1 activity was not significantly altered by the PUFA, though a weak effect of the culture was observed. A moderate but significantly ($p < 0.05$) higher lysophospholipase activity (+20%) was measured in SM3 cells although there existed a strong effect of the culture preparation on the lysophospholipase activity, but no cross interaction. For both ACLAT and acyl-CoA synthetase activities, statistical analysis again revealed a significant variability among the experiments but no significant effect of the type of PUFA. In one experiment (B), enzyme activities were measured in cells before they were separated for SM3 and SM6 experiments. Values are included in Table 3, but cannot be compared with those obtained under SM3 and SM6 conditions because factors other than fatty acid profile, such as medium composition, may interfere with cell metabolism. These values show that substitution of a conventional medium by a synthetic medium did not induce a drastic change in the activities of the enzymes of phospholipid metabolism.

The enzyme activities were measured in the bathing fluids, and the results are presented in Table 4. Alkaline phospholipase A exhibited a higher activity (+44%) in the bathing fluid of SM6 cells. Lysophospholipase did not show any significant variation whereas other enzyme activities were essentially undetectable.

TABLE 3

Enzyme Activities in Ventricular Myocytes Grown on SM3 or SM6[a]

	Alkaline phospholipase		Acid phospholipase		Lysophospholipase		ACLAT		AcylCoA synthetase	
Experiment	SM3	SM6	SM3	SM6	SM3	SM6	SM3	SM6	SM3	SM6
A	3.78 ± 0.37	5.16 ± 0.37	3.68 ± 0.07	4.20 ± 0.05	24.4 ± 1.54	20.2 ± 1.33	79.0 ± 2.1	75.6 ± 3.7	n.a.	n.a.
B	7.13 ± 0.29	9.42 ± 0.41	3.11 ± 0.46	3.13 ± 0.24	17.5 ± 0.64	14.5 ± 0.43	114.2 ± 6.1	132.2 ± 1.3	207.3 ± 26.7	247.6 ± 23.3
C	9.9 ± 0.70	12.8 ± 0.46	6.23 ± 0.42	7.33 ± 0.66	12.3 ± 0.4	12.2 ± 0.80	196.5 ± 8.4	194.0 ± 7.4	454.6 ± 23.6	440.8 ± 43.3
	$p < 0.01$		NS		$p < 0.05$		NS		NS	
(B)	(5.00 ± 1.17)		(3.98 ± 0.72)		(17.9 ± 2.5)		(152.0 ± 48)		n.a.	

[a]Enzyme activities are expressed in nmol/hr/mg protein and are, for each experiment, the mean ± SEM of 6 determinations (dishes), each assay being performed in duplicate. Alkaline phospholipase A was assayed at pH 8.4, and acid phospholipase A_1 at pH 4.9. ACLAT; Acyl-CoA:lysophosphatidylcholine acyltransferase. (n.a., not assayed; NS, not significant) The statistical analysis reported refers to the difference between SM3 and SM6 cells. For each enzyme the between-experiments' variability was significant, and the cross-interaction was not. Values in parentheses were obtained in experiment B and refer to the activities obtained in cells cultivated in conventional medium before they were separated for the SM3 and SM6 purposes (n = 6 for each enzyme).

TABLE 4

Enzyme Activities Released in the Bathing Fluid of Ventricular Cells Grown in SM3 or SM6[a]

	Alkaline phospholipase	Acid phospholipase	Lysophospholipase	ACLAT
SM3	1.13 ± 0.12	nd	0.95 ± 0.15	nd
SM6	1.62 ± 0.17	nd	0.80 ± 0.14	nd
	$p < 0.01$		NS	

[a]Enzyme activities are expressed in nmol/hr/mg protein and are the mean ± SEM of assays performed from experiments A and B (n = 2 × 6 for each SM3 or SM6) (nd; not detectable). Total protein content of SM6 and SM3 was not significantly different.

TABLE 5

Effect of Betamercaptoethanol on the Alkaline Phospholipase A Activity in SM3 or SM6 Cells[a]

		SM3	SM6
Control		5.87 ± 0.61	9.81 ± 0.61
+ BME		9.17 ± 1.00	13.86 ± 0.58
	PUFA factor		$p < 0.01$
Statistics	BME factor		$p < 0.01$
	Experiment factor		$p < 0.05$

[a]Phospholipase A activity is expressed in nmol/hr/mg protein, and values are means ± SEM of assays performed in duplicate (n = 2 × 6). The binary and ternary cross-interactions were not significant.

In a previous study (26), we showed that the activity of the alkaline phospholipase A_1, isolated from rat heart cytosol, was strongly dependent on the presence of thiol groups (in their reduced form), and that BME afforded a protection of the activity. The essentiality of the thiol group was also recently demonstrated for the detergent-solubilized form of phospholipase A_1 of rat cardiac sarcoplasmic reticulum (27). In order to determine if the PUFA composition of the cells might have differently affected the oxidation state of the sulfhydryl group of phospholipase A, assays were repeated in the presence of BME with a final concentration of 10mM. Results clearly showed (Table 5) that BME activated alkaline phospholipase A by about 50% in both types of cells. The differences between SM3 and SM6, however, persisted. The absence of any significant cross-interaction demonstrated that the effect of BME was the same in both groups of cells as well as among the experiments.

Association of di-[1-^{14}C]oleoyl PC with membranes of ventricular myocytes. During the conventional phospholipase A assay, it is highly probable that the exogenous, labelled substrate dispersed in the form of unilamellar liposomes interacts with the myocyte membranes. Thus, we conducted experiments to estimate if this process might be different depending on the nature of the fatty acid composition of the membrane. Under conditions strictly identical to those of the phospholipase A assay, we measured the amount of radioactive di-[1-^{14}C]oleoyl PC associated with membranes. Results, presented in Table 6, showed that this amount represented about 50 and 55% of total phospholipids in SM3 and SM6 cells, respectively. Separation of lipids by thin-layer chromatography revealed that 99% of the radioactivity associated with membranes was associated with PC.

DISCUSSION

Within the last two decades, several studies have been devoted to the beneficial or detrimental effect of various types of fat on health. However, by changing the dietary fat, one modifies the PUFA composition of the membrane. In the heart, such modifications have been demonstrated (14,28–31), and their influence on cardiovascular parameters has been investigated using either whole heart (32,33) or purified ventricular myocytes (15). Since heart membrane stabilization is crucial, especially during ischemia and reperfusion, any attempt to prevent membrane degradation may be of great physiological interest. Thus, dietary-mediated modulation of the enzyme involved in membrane phospholipid turnover appears to be a very attractive field of investigation. The results obtained in the present study using cultured rat ventricular myocytes clearly demonstrated that, among the enzymes studied—namely alkaline phospholipase A (A_1 + A_2), lysophospholipase, lysosomal phospholipase A_1, ACLAT, and acyl-CoA synthetase—only the first two enzymes

TABLE 6

Association of di[1-^{14}C]oleoyl PC with Membranes of Myocytes During Phospholipase A Assay[a]

	SM6	SM3
Endogenous PL	61.0	67.0
[1-^{14}C]DOPC incubated	527.0	527.0
[1-^{14}C]DOPC associated	73.0	85.0
Total PL recovered	145.0	153.0
[1-^{14}C]DOPC associated/total PL (%)	49.2	55.5

[a]Phospholipids (PL) are in nmol. Values are the mean of 2 determinations performed with the pool of 3 dishes for each type of cells. Recovery of radioactivity was about 90%. The value of total PL recovered was obtained after phosphorus assay of the pellet. This explains that this value does not exactly correspond to the sum of endogenous PL and DOPC associated.

responded to changes in PUFA composition of cellular lipids. Under standard assay conditions, the specific activity of alkaline phospholipase A was around 34% higher in SM6 cells than in SM3 cells. This difference appears restricted to the alkaline species since the acid lysosomal phospholipase A_1 did not show any significant changes. These changes probably affected mainly the A_2 species since it represented at least 85% of the alkaline activity. Several hypotheses could account for the difference we observed in this study: i) The release of phospholipase A into the bathing fluid may be more pronounced in SM3 cells, which, in turn, lowers the specific activity of the intracellular activity. ii) Rat heart phospholipase A is sensitive to the redox state through the essential sulfhydryl groups (26,27), and this redox balance may be altered differently by the nature of cellular PUFA. iii) The modification of the PUFA composition within the micro-environment surrounding the enzyme may influence its activity. iv) Phospholipase A activity may be genetically regulated by cellular PUFA. The first and second possibly can be ruled out for the following reasons. Firstly, our results clearly show that SM3 cells released lesser amounts of alkaline phospholipase A than did SM6 cells. Secondly, even in the presence of BME, a well-known protective agent of the SH groups, the difference between SM3 and SM6 persisted and became even more marked. The third possibility needs careful examination of the protocol used: when assaying phospholipase A with exogenous liposomal substrate, the amount of PC is about 8 times that brought by the membranes of the myocyte. Thus, during the time of incubation, an association of exogenous PC with endogenous membrane phospholipids certainly occurs, which allows the hydrolysis of substrate. This phenomenon, described during mitochondrial phospholipase A_2 assay using either PE or PC as exogenous phospholipids (34), was shown to be strongly enhanced by addition of calcium. Inasmuch as this association might be differently affected by the nature of PUFA esterifying the membrane phospholipids, artifactual changes in enzyme activity may occur. We confirmed that under our assay conditions exogenous liposomal dioleoyl PC associated with whole myocyte membranes during the 20 min phospholipase A assay. However, as this association is quite similar in the two types of preparations (SM3 or SM6), it probably did not induce different changes in phospholipase activity. Also, it is possible that the hydrolysis of endogenous phospholipids is higher in SM3 than in SM6 cells, which, in turn, might lead to the observed decrease in exogenous substrate hydrolysis by SM3 cells. This is unlikely since the amount of exogenous substrate greatly exceeded endogenous substrate. Thus, it can be reasonably suggested that PUFA-induced changes in the micro-environment of the phospholipase are responsible for the differences observed. The fourth suggestion assumes that the PUFA-mediated effect would be specific for the alkaline species since the acid lysomal enzyme was not affected. It is interesting to note that a linoleic-enriched diet often results in an increase in alkaline phospholipase A activity (11,12). Thus, a regulatory mechanism of enzyme synthesis by PUFA can presently not be excluded as well as a possible PUFA-induced change in the level of cytosolic inhibitors. In rats fed various high-fat diets (14), no difference in activity was observed when assaying membrane-bound phospholipase A activity of whole heart with exogenous dioleoyl PC as substrate. The fact that we now are able to observe differences may be due to the use of pure cardiomyocytes as the membrane-bound enzyme source. Except for lysophospholipase that showed a slightly higher activity (around 20%) in SM3 cells, other enzymes were not significantly affected by the nature of the PUFA. Combined with the lower phospholipase A activity, the higher lysophospholipase activity in SM3 cells may lead to a reduced level of intracellular lyso PC, a well-known cytolytic agent.

In conclusion, n-3 PUFA enrichment in cardiomyocyte lipids leads to a reduced degradative activity towards membrane phospholipids which may benefit membrane stability. That these modifications may or may not favor membrane phospholipid homeostasis during lipid metabolism disorders, such as hypoxia and reoxygenation, is the subject of ongoing research.

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Elevated Levels of Nonesterified Fatty Acids in the Myocardium of Alloxan Diabetic Rats

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Myocardial nonesterified fatty acids (NEFA) increase markedly within the first two days after the induction of insulin-dependent diabetes mellitus in rats by intravenous injection of alloxan. After initial variability, NEFA levels in diabetic hearts remain constant at approximately 450 nmol/g tissue (16 nmol/μmol lipid P), which is about three times higher than that in control hearts. Nonesterified linoleic acid is significantly increased in diabetic heart whereas both arachidonic and docosahexaenoic acids are decreased compared to controls.
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Heart disease remains the most serious complication of insulin-dependent diabetes mellitus, even with relatively good control of blood sugar levels by insulin injections. Although diabetic patients have a markedly increased risk of atherosclerosis and myocardial infarction (1), a significant portion of diabetic heart disease involves instead cardiac pump failure from a cardiomyopathy which is not related to changes of the coronary vasculature (2,3). In the alloxan diabetic rat, this cardiomyopathy involves not only the cardiac muscle cells but also their supporting capillaries and extracellular matrix (4).

Nonesterified fatty acids (NEFA), especially arachidonic acid, are known to be significantly increased in the myocardium as the result of acute ischemic injury, and this phenomenon has been correlated with cardiocyte injury and the loss of contractile function (5-7). In contrast, very little information is available on NEFA levels in diabetic myocardium and their potential involvement in the development of diabetic cardiomyopathy.

Early work with perfused rat heart showed higher levels of NEFA in alloxan diabetic than in normal myocardium, depending on the composition of the perfusion media (8). In contrast, Kraupp *et al.* (9) reported no difference in NEFA levels between normal and alloxan diabetic rat hearts, but the levels in both appeared to be unrealistically high. In these and other studies, reported NEFA levels in normal mammalian myocardium vary widely from 45 nmol to 25 μmol/g wet wt and are subject to a number of experimental uncertainties as discussed by Hunneman and Schweickhardt (10). These include overestimation due to post mortem lipolysis, incomplete purification of NEFA followed by transesterification of esters, or contamination of glassware and solvents. Loss of NEFA can also occur through incomplete extraction or oxidation prior to analysis.

To address the effects of insulin-dependent diabetes mellitus on myocardial NEFA, we carried out quantitative compositional analyses of NEFA in rat myocardium over a period of 6 wk after alloxan-induced diabetes. We observed that NEFA began to increase within 1 day, more than doubled within 2 to 7 days, and remained stable at this level for the duration of the study.

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Abbreviations: NEFA, nonesterified fatty acids; EDTA, ethylenediaminetetraacetic acid.

MATERIALS AND METHODS

Induction of diabetes. Male Sprague-Dawley rats weighing 180-200 g were purchased from a commercial supplier (Biolab Corp., St. Paul, MN) and randomly assigned to two groups. Those in one group were injected intravenously with alloxan monohydrate (Sigma Chemical Company, St. Louis, MO) at a dose of 55 mg/kg body wt as described previously (4). Animals in the second group were injected with an equal volume of saline and served as the control group. Two days after alloxan injection and at weekly intervals, nonfasting blood glucose levels were estimated using an Accu-Chek II blood glucose meter (Boehringer Mannheim Diagnostics, Indianapolis, IN). Glycosylated hemoglobin was estimated according to the method of Abraham *et al.* (11) using Isolab affinity columns (Isolab Inc., Akron, OH). Urine glucose was monitored by Chemstrip (Boehringer Mannheim Diagnostics, Indianapolis, IN). Only those alloxan-injected rats with plasma glucose levels greater than 300 mg/dl and showing little or no weight gain were considered sufficiently diabetic and included in the experiments (4).

Preparation of heart tissue and analysis of free fatty acids. Alloxan-injected diabetic and saline-injected control rats were killed by cervical dislocation 1 to 45 days after alloxan or saline injection, and hearts were quickly removed and rinsed in three changes of ice-cold saline. Each heart was then immediately perfused for 15-30 sec with 90 mM NaCl, 25 mM KCl, and 25 mM KH_2PO_4, pH 7.3 to 7.4 to clear the myocardial vasculature of blood, and to arrest the heart in diastole, then rapidly frozen in liquid N_2. Samples of 60-90 mg of frozen heart were taken from the left ventricular free wall midway between base and apex and homogenized in ice-cold methanol containing BHT using a Dounce homogenizer. After addition of 10 μg heptadecanoic acid as an internal standard, the homogenate was extracted with twice the volume of chloroform (12). After addition of 0.25 vol of 0.9% aqueous NaCl containing EDTA and vortexing, the lower chloroform layer was removed and the upper layer was re-extracted with chloroform/methanol/water (86:14:1, v/v/v). The pooled extracts were evaporated under N_2 and redissolved in chloroform. An aliquot of this lipid extract was assayed for lipid phosphorus. Another aliquot was analyzed for NEFA after methylation with diazomethane (13). The lipid extract in $CHCl_3$ was dried under N_2, a freshly prepared diethyl ether/methanol (9:1, v/v) mixture was added, and the mixture was treated with diazomethane for 10-15 min to form methyl esters of the nonesterified fatty acids. This sample was applied to a small silicic acid column and washed with hexane. Fatty acid methyl esters were eluted from the column using hexane/diethyl ether (9:1, v/v) as eluant, and were then analyzed by gas liquid chromatography using a Packard Model 428 gas chromatograph equipped with dual 1/8" ID × 12 ft columns packed with SP2330 on 100/120 Chromosorb WAW (Supelco) and flame ionization detectors. Conditions for the separation were: initial temperature

TABLE 1

Parameters of Alloxan-Induced Diabetes[a]

Rats	Number	Diabetes (days)	Body weight (g)	Weight gain (g/wk)	Blood glucose (mg/dl)	Glycosylated hemoglobin (%)
Control	4	—	200 ± 2.1	—	84.7 ± 9.2	2.0 ± 0.2
Diabetic	4	1	191 ± 0.8	—	474.0 ± 85.1[b]	4.3 ± 0.2[b]
Control	4	—	209 ± 10.3	—	94.0 ± 15.2	3.5 ± 0.4
Diabetic	4	2	188 ± 4.4	—	527.0 ± 82.2[b]	5.3 ± 0.3[b]
Control	4	—	259 ± 1.5	64.5 ± 3.3	85.2 ± 4.5	3.5 ± 0.2
Diabetic	4	7	199 ± 3.5	11.3 ± 5.0[b]	547.0 ± 64.0[b]	9.8 ± 0.5[b]
Control	4	—	352 ± 18.6	35.5 ± 11.8	98.5 ± 8.8	4.2 ± 0.2
Diabetic	4	28	202 ± 5.9	1.9 ± 1.0[b]	507.0 ± 22.7[b]	15.4 ± 1.0[b]
Control	5	—	444 ± 13.6	38.6 ± 2.0	88.2 ± 15.1	4.2 ± 0.2
Diabetic	5	45	193 ± 26.4	−1.6 ± 3.6[b]	457.0 ± 45.9[b]	16.6 ± 2.2[b]

[a] $\bar{x}$ ± S.D. [b] $P < 0.01$ compared to age-matched control.

TABLE 2

Phospholipid Content and Levels of Nonesterified Fatty Acids (NEFA) in Normal and Diabetic Rat Heart[a]

Diabetes (days)	Number	Lipid P (μmol/g tissue)		NEFA (nmol/g tissue)	
		Control	Diabetic	Control	Diabetic
1	4	28.6 ± 6.6	28.0 ± 0.3	158.3 ± 60.0	228.5 ± 108.3
2	4	24.8 ± 3.1	25.0 ± 3.0	143.5 ± 36.2	361.4 ± 168.8[b]
7	4	28.5 ± 1.3	28.3 ± 0.6	222.0 ± 17.8	454.0 ± 49.2[c]
28	4	29.6 ± 1.9	29.6 ± 1.5	137.7 ± 23.4	420.0 ± 59.6[c]
45	4	27.5 ± 1.4	28.7 ± 1.4	133.6 ± 33.5	468.1 ± 23.7[c]

[a] $\bar{x}$ ± S.D. [b] $P \leq 0.05$ compared to age-matched controls. [c] $P \leq 0.01$ compared to age-matched controls.

180°C, rising to a temperature of 225°C at 2°C/min. The detector and injection port temperatures were 260°C and 250°C, respectively. The peaks were identified by comparison with commercial standards (Nu-Chek-Prep, Elysian, MN) and quantified with a Spectra Physics 4270 computing integrator. Results were confirmed by analysis of hydrogenated methyl esters.

Statistics. For rate of weight gain, blood glucose, glycosylated hemoglobin, myocardial phospholipid, and myocardial NEFA, each diabetic group was compared to the age-matched control group by one-tailed Student's t-test. For NEFA composition, fatty acid mol% values for each diabetic group were compared to 1-day and 28-day control groups by one-tailed Student's t-test.

RESULTS

Alloxan-induced diabetes. Alloxan is specifically toxic to the pancreatic β-cell (14,15) inducing insulin deficiency and type 1 diabetes. As demonstrated by the data in Table 1, alloxan-injected animals in this study rapidly developed and maintained severe hyperglycemia and exhibited little or no weight gain throughout the experiment. Glycosylated hemoglobin values also increased significantly. Urine glucose values for the diabetic animals were, without exception, above the Chemstrip maximum of 1 g/100 mL. Therefore, alloxan diabetes is an excellent model for this study, and we have previously used it for structural studies of the heart (4).

Nonesterified fatty acids. Levels of myocardial phospholipids, expressed as lipid phosphorus, and NEFA are listed in Table 2. Although NEFA varied considerably as in other studies (10,16–18), it is clear that diabetic hearts contained two to three times higher NEFA levels than control hearts. There was essentially no change in phospholipid content, and when NEFA were expressed on the basis of total lipid phosphorus, the data illustrated in Figure 1 were obtained. After some variability within the first 2 days after alloxan injection, NEFA levels in diabetic hearts remained constant at about 16 nmol/μmol lipid P, as compared to 5 nmol/μmol lipid P for control hearts.

NEFA composition. The composition (mol%) of rat heart NEFA after different periods of diabetes is listed in Table 3 and compared to representative control groups from the 1-day (A) and 28-day (B) experiments. In diabetic heart, linoleate (18:2n-6) was significantly increased and arachidonate (20:4n-6) decreased in NEFA as observed previously for total and phospholipid-linked fatty acids of the diabetic heart and other tissues (19–21). We also observed a marked decrease in nonesterified docosahexaenoic acid (22:6n-3).

DISCUSSION

Our present results demonstrate that myocardial NEFA increased markedly within the first few days after the induction of insulin-dependent diabetes mellitus and

TABLE 3

Composition (mol%) of Nonesterified Fatty Acids in Diabetic Rat Heart[a]

Fatty acid[b]	Control A[c]	Control B[c]	Duration of diabetes (days) 1	2	7	28	45
16:0	23.2 ± 2.9	19.7 ± 2.8	20.4 ± 2.7	28.1 ± 5.6[d]	20.1 ± 1.5	24.1 ± 1.8[d]	24.2 ± 2.0[d]
16:1	2.8 ± 1.2	2.7 ± 0.5	2.1 ± 0.4	2.6 ± 0.3	1.7 ± 0.3[d]	2.5 ± 0.1	2.0 ± 0.4
18:0	19.8 ± 2.9	18.5 ± 1.2	20.9 ± 2.6	21.1 ± 2.6	19.1 ± 2.6	18.3 ± 2.2	19.2 ± 1.3
18:1	18.5 ± 5.8	15.9 ± 4.0	13.1 ± 2.6	17.7 ± 2.1	21.6 ± 6.9	19.4 ± 2.2	21.4 ± 0.9[d]
18:2 n-6	13.7 ± 3.0	18.6 ± 1.4	16.4 ± 3.4	21.4 ± 4.2[e]	31.8 ± 5.2[f,g]	29.7 ± 2.4[f,g]	29.8 ± 2.6[f,g]
20:4 n-6	14.3 ± 4.5	15.1 ± 2.6	16.5 ± 4.0	6.3 ± 2.4[e,g]	3.8 ± 1.2[f,g]	2.5 ± 0.1[f,g]	1.5 ± 0.9[f,g]
22:6 n-3	4.3 ± 2.7	7.1 ± 3.9	3.8 ± 1.9	1.3 ± 0.4[d]	1.0 ± 0.5[e,d]	2.0 ± 0.3[d]	1.1 ± 0.3[e,d]

[a] $\bar{x}$ ± S.D. (n = 4).

[b] Also present in small amounts: 14:0, 22:0, 24:0, 22:4 n-6.

[c] Control group A = 1 day after saline injection; Control group B = 28 days after saline injection.

[d] $P \leq 0.05$ compared to control B.

[e] $P \leq 0.05$ compared to control A.

[f] $P \leq 0.01$ compared to control A.

[g] $P \leq 0.01$ compared to control B.

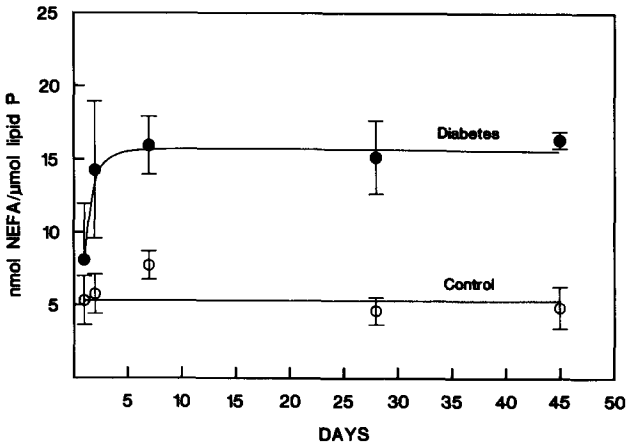

FIG. 1. Nonesterified fatty acid levels in rat myocardium 1, 2, 7, 28, and 45 days after the intravenous injection of alloxan (diabetic) or saline (control). Each value is the mean and standard deviation of measurements from four animals.

remained stable at this elevated level as the disease progressed. This occurred without changes in the phospholipid content of the tissue. Within 1 wk, the percentage of linoleate (18:2) doubled while the percentages of arachidonate (20:4) and docosahexanoate (22:6) decreased by at least 70% and remained low or even decreased further.

Analysis and quantification of NEFA is subject to a number of technical problems. Although we have rigorously standardized all procedures in attempts to keep artifactual liberation of fatty acids to a minimum, it is possible that some fatty acids, especially arachidonate, could have been released from phospholipids post mortem as observed with ischemic heart (5) and, more dramatically, with brain (22,23). However, the immediate rinsing in ice-cold saline and the short time from the removal of the heart to freezing (<1 min) make significant lipolysis highly unlikely. Methylation with diazomethane avoids potential transesterification from ester-linked fatty acids, and thus makes it unnecessary to purify NEFA prior to their conversion to methyl esters. Previous assays of NEFA in rat heart found levels which differed from one another by several orders of magnitude. Reported levels range from 45 nmol to 25 µmol/g wet wt as summarized by Hunneman and Schweickhardt (10). These authors used mass fragmentography of methyl esters prepared by the diazomethane technique to determine NEFA levels in dog heart at about 56 nmol/g wet wt (10). Our present data on NEFA levels in normal rat heart agree with the lowest values obtained from frozen hearts pulverized at dry ice temperature followed by extraction with chloroform-methanol (16) or trichlorofluoromethane (18).

Our finding that diabetic rat heart contains about three times more NEFA than nondiabetic heart is in general agreement with early observations by Garland and Randle, who showed that the concentration of NEFA in rat heart and diaphragm muscles perfused or incubated with medium containing glucose and insulin is increased by the induction of alloxan diabetes (8). Later work (9) which found no difference in NEFA levels between normal and diabetic rat hearts (~10 µmol/g wet tissue) can be disregarded due to the extremely high values reported, which were most likely caused by massive post mortem lipolysis. In a more recent study on the performance of normal and diabetic rabbit hearts in the presence of different substrates, myocardial NEFA levels were found to be slightly higher (178 ± 16 nmol/g wet tissue) than in control hearts (140 ± 15 nmol/g) (24). These authors also noted that performance of hearts from diabetic rabbits, 2 wk after alloxan injection, was more adversely affected by high exogenous NEFA levels than that of controls (24). The percentage increase in nonesterified linoleic acid and decrease in arachidonic acid which we observed agree with published data on total or phospholipid fatty acids in various diabetic tissues, including the heart (14-16), and may be due to inhibition of the desaturases responsible for polyunsaturated fatty acid synthesis (25). Reported percentage changes of docosahexaenoic acid in the lipids of diabetic tissues vary widely depending on

the type and duration of diabetes (19-21). Very little change of docosahexaenoate content was observed in the phospholipids of alloxan-diabetic rat heart (21).

It is well known that plasma NEFA levels are increased in both human and experimental diabetes (26-28) and elevated NEFA levels in diabetic heart may be due, at least in part, to higher concentrations provided by the circulation. This may also result in the observed increases of myocardial triacylglycerols (29,30). On the other hand, rates of lipolysis are known to be altered in diabetic heart as are activities of lipolytic enzymes (31,32). Myocytes prepared from rat hearts 3-4 days after alloxan injection showed NEFA levels approximately three times higher than control myocytes (33).

Contractile dysfunction has been reported to occur as early as 3 days (34) after induction of diabetes with alloxan, and altered NEFA levels in the heart are likely to contribute to this cardiomyopathy. Increased NEFA are known to depress myocardial contractility (35-37), but the mechanisms by which this occurs are not clear. NEFA may amplify ischemic myocardial stress by increasing the oxygen demand of the cells beyond the ability of the vasculature to support them (38,39). In fact, agents which block fatty acid oxidation were shown to have a protective effect (40,41). Increased levels of NEFA also lead to the accumulation of their metabolites (37,42), particularly acyl-CoA which can inhibit adenine nucleotide translocation in the heart (43), and acyl carnitine which can inhibit myocardial Na^+, K^+ ATPase activity (44,45) and interfere with calcium transport across the sarcoplasmic reticular membrane (46). In addition, fatty acids themselves may specifically damage the myocardium directly, as indicated by the demonstration that NEFA inhibited cytochrome-c oxidase from bovine heart *in vitro* but had no effect on the same enzyme from bovine liver (47).

If the increased NEFA levels arise, even in part, from membrane phospholipids, then membrane integrity could be affected which, in turn, could change the activities of a number of membrane-bound enzymes (48). A defective cycle of deacylation and reacylation of membrane phospholipids has been implicated in ischemia-reperfusion injury of the myocardium (5,49) and may be a factor in diabetic damage as well.

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Modification of Phospholipid Polar Head Group with Monomethylethanolamine and Dimethylethanolamine Decreases Cholesteryl Ester and Triacylglycerol Synthesis in Cultured Human Fibroblasts

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Modification of the phospholipid polar head group was achieved by supplementation of the growth medium of cultured human fibroblasts with the choline analogues monomethylethanolamine (ME) or dimethylethanolamine (DE) at a concentration of 80–200 μg/mL for 48 hr. The maximum concentration of phosphatidylmonomethylethanolamine (PME) or phosphatidyldimethylethanolamine (PDE) reached without affecting the phospholipid/protein ratio was about 45% of total phospholipids. Incorporation of oleic acid into cholesteryl esters and triacylglycerols was markedly inhibited after supplementation with ME or DE, and accounted for 60% and 40% of controls, respectively, at 200 μg/mL, whereas incorporation into phospholipids was not affected. AcylCoA:cholesterol acyltransferase (ACAT) and diacylglycerol acyltransferase (DGAT) activities measured on cell-free extracts appeared to be decreased also by phospholipid polar head group modification, whereas the overall phospholipid acyltransferase activity remained unchanged. The intracellular content of cholesteryl esters and triacylglycerols, determined by the isotopic equilibrium method with radioactive cholesterol and glycerol, was found to be diminished to 50–60% and 40–50% of controls, respectively, after supplementation with the choline analogues. The study showed that modification of the phospholipid polar head group affects the activity of membrane-bound enzymes involved in the metabolism of neutral lipids.
Lipids 25, 311–315 (1990).

Phospholipids are important structural components of cell membranes. Modification of phospholipid composition of cultured cells either in the fatty acid or the polar head group moiety can be achieved by supplementation of the growth medium with fatty acids or choline analogues (1,2). Modification of the phospholipid polar head group was shown to induce perturbations in the activity of membrane-bound enzymes. In mouse LM cells, the activity of adenylate cyclase was enhanced (3), whereas the activity of stearoyl-CoA desaturase was decreased (4). In C6 glial cells, the activity of the microsomal enzyme 3-hydroxy-3-methylglutaryl-CoA reductase was decreased by enrichment of cell membranes with PDE (5). Inclusion of choline analogues in the growth medium also modified cellular functions such as phagocytosis and pinocytosis (6), intracellular transport and secretion of immunoglobulins (7), and differentiation of myeloid leukemia cells into macrophages (8). We previously also demonstrated that receptor-mediated endocytosis of low density lipoproteins by cultured human fibroblasts was decreased by supplementation of the growth medium with the choline analogue ME (9).

In the present study, we investigated the effect of phospholipid polar head group modification on the incorporation of oleic acid into cholesteryl esters and triacylglycerols. We found that both cholesterol esterification and triacylglycerol synthesis were inhibited by supplementation with ME or DE due to a decrease in ACAT and DGAT activities. Phospholipid synthesis was shown to be unaffected by such modulation.

MATERIALS AND METHODS

Materials. ME, DE and oleoyl-CoA were from Sigma (St. Louis, MO); sodium [^{32}P]orthophosphate (625 mCi/mmol), [4-^{14}C]cholesterol (52 mCi/mmol), [1(3)-^{14}C]glycerol (54 mCi/mmol) and [*methyl*-^{3}H]thymidine (25 Ci/mmol) were from CEA (Saclay, France); [1-^{14}C]oleic acid (52 mCi/mmol) and [1-^{14}C]oleoyl coenzyme A (57 mCi/mmol) were from Amersham (Buckinghamshire, United Kingdom); Dulbecco's modified Eagle Medium and fetal calf serum were from Gibco (Grand Island, NY). MRC5 human fetal lung fibroblasts were purchased from Bio-Mérieux (Paris, France). The serum substitute Ultroser G was from Industries Biologiques Françaises (Villeneuve la Garenne, France). Silica gel plates F 1500 were from Schleicher und Schuell (Kassel, West Germany).

Cell culture. Cells were routinely cultured in 60 mm Nunc tissue culture Petri dishes containing 2 mL Dulbecco MEM medium supplemented with 20 mM Hepes buffer (pH 7.4), 100 units/mL penicillin, 100 μg/mL streptomycin and 10% (v/v) fetal calf serum, in a humidified atmosphere of 5% CO_2 at 37°C. For modification of the phospholipid polar head group, cells were grown for 48 hr in medium supplemented with the serum substitute Ultroser G 2% and the choline analogue at the concentration of 80–200 μg/mL. The pH of the medium was adjusted to 7.4. Experiments were performed with confluent cells at about 100 μg cellular protein/dish.

Determination of phospholipid composition and intracellular cholesteryl ester and triacylglycerol content. The isotopic equilibrium method was used. During the 48 hr supplementation with choline analogues, 50 μCi/mL sodium [^{32}P]orthophosphate, 0.2 μCi/mL [^{14}C]cholesterol in ethanol or 2 μCi/mL [^{14}C]glycerol was added to the culture medium. After harvesting, an aliquot of the cell suspension was directly applied onto the silica gel plate for chromatographic analysis (10). Phospholipids were separated by two-dimensional chromatography, using chloroform/methanol/H_2O (65:25:4, v/v/v) in the first direction and tetrahydrofurane/methanol/formaldehyde-

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Abbreviations: ME, monomethylethanolamine; DE, dimethylethanolamine; PME, phosphatidylmonomethylethanolamine; PDE, phosphatidyldimethylethanolamine; ACAT, acylCoA:cholesterol acyltransferase; DGAT, diacylglycerol acyltransferase.

dimethylacetal/2N NH_4OH (49:20:20:4, by vol) in the second direction. Free cholesterol, cholesteryl esters and triacylglycerols were separated by thin-layer chromatography using hexane/diethyl ether/acetic acid (70:30:2, v/v/v) as solvent. The lipid fractions were made visible by autoradiography, cut and measured by liquid scintillation counting with an Intertechnique instrument. Protein was measured by the method of Lowry *et al.* (11). Results are expressed in percentages for phospholipid compositions and in pmoles of precursor incorporated/mg protein for cholesteryl ester and triacylglycerol intracellular content.

Incorporation of thymidine. After modification of the phospholipid polar head group during 48 hr, incorporation of [^{3}H]thymidine was performed for 4 hr with 50 μCi/mL. Cells were then washed 3 times and measured by liquid scintillation counting. Results are expressed in DPM/μg cell protein.

Incorporation of oleic acid into lipids. After modification of the phospholipid head group, [^{14}C]oleic acid 1 μCi/mL resuspended in 0.2 mg/mL albumin was added. After a further 1 hr incubation, cells were washed 3 times and lipid analysis was performed by thin-layer chromatography with chloroform/methanol/acetic acid/water (50:30:8:4, by vol) for phospholipids and hexane/diethyl ether/acetic acid (70:30:2, v/v/v) for neutral lipids. Following autoradiography, the radioactive spots were cut and measured by liquid scintillation counting with an Intertechnique instrument. Results are expressed in pmol precursor incorporated/mg cell protein and calculated as percentages of controls.

For determination of oleic acid uptake, cells were incubated for short periods of 5, 10 and 20 min with the radioactive fatty acid, before washing, harvesting, and liquid scintillation measurement of cellular radioactivity. Results are expressed in DPM/μg cell protein.

Acyltransferase assays. After modification of the phospholipid polar head groups during 48 hr, cells were washed four times with a phosphate-buffered solution pH 7.4, and harvested. After centrifugation, the cell pellet was resuspended in 0.15M NaCl containing 5×10^{-3}M NaF. The acyltransferase activities were measured on sonicated cellular homogenates with a method previously described (12), using [^{14}C]oleoyl-CoA as substrate. The assay mixture contained about 100 μg of protein, 50000 dpm of [^{14}C]oleoyl-CoA diluted with unlabeled oleoyl-CoA (10^{-4}M final concentration), $MgCl_2$ 5×10^{-3}M, cholesterol 50 μg/mL in acetone solution (1% final concentration) and phosphate buffer 0.1M pH 7.4, in a total volume of 100 μL. Incubations were performed for 5 min at 37°C. The reaction was stopped on ice. Radioactive cholesteryl esters, triacylglycerols and phospholipids were separated by thin-layer chromatography as described above and measured by liquid scintillation counting.

RESULTS AND DISCUSSION

The phospholipid composition of cells grown for 48 hr in the presence of different amounts of the choline analogues was determined first. The results are shown in Table 1. Cells became progressively enriched with PME and PDE in the presence of increasing amounts of ME and DE, respectively. PME and PDE accounted for approximately 45% of the total phospholipids at 200 μg/mL ME or DE, thereby becoming the most abundant cell phospholipids. Under these conditions, the proportions of phosphatidylcholine and phosphatidylethanolamine became progressively decreased, whereas the proportions of sphingomyelin, phosphatidylinositol and other minor phospholipids (phosphatidic acid, diphosphatidylglycerol) remained unchanged. It is noteworthy that the phospholipid/protein ratio was not significantly affected by such modulation. The modification of the phospholipid polar head group was not accompanied by changes in cellular morphology or adherence. Furthermore, under our experimental conditions, DNA synthesis as assessed by radioactive thymidine incorporation was not significantly altered (Table 2). The data show that manipulation of the

TABLE 1

Phospholipid Composition of Human Cultured Fibroblasts Grown in Medium Containing Monomethylethanolamine or Dimethylethanolamine[a]

Addition	SM	PC	PI	PE	PME	PDE	Others	Phospholipid/ protein (CPM/μg)
None	7.2 ± 2.3	66.9 ± 4.5	8.3 ± 0.7	15.3 ± 0.9	—	—	2.3 ± 0.3	2,040 ± 178
Monomethylethanolamine (μg/mL								
80	8.7 ± 2.2	41.2 ± 4.2	9.9 ± 1.5	7.6 ± 1.2	29.5 ± 2.1	—	3.1 ± 0.3	2,248 ± 232
120	10.4 ± 1.5	36.7 ± 2.3	9.1 ± 0.4	4.6 ± 0.3	37.2 ± 1.1	—	2.0 ± 0.2	2,399 ± 212
160	9.1 ± 1.2	34.0 ± 3.1	9.9 ± 0.3	4.4 ± 0.2	39.4 ± 1.2	—	3.2 ± 0.2	2,188 ± 179
200	6.9 ± 1.7	31.2 ± 2.5	11.8 ± 0.5	3.1 ± 0.2	43.9 ± 1.5	—	3.1 ± 0.2	2,518 ± 221
Dimethylethanolamine (μg/mL)								
80	10.8 ± 1.7	39.8 ± 3.1	8.2 ± 0.5	11.8 ± 1.2	—	27.1 ± 2.4	2.3 ± 0.2	1,895 ± 205
120	7.2 ± 1.5	38.1 ± 2.7	10.6 ± 1.1	7.1 ± 1.3	—	34.2 ± 5.3	2.8 ± 0.3	1,929 ± 213
160	9.1 ± 2.5	35.4 ± 4.6	9.2 ± 0.9	6.1 ± 1.0	—	38.0 ± 3.1	2.2 ± 0.4	2,225 ± 230
200	11.3 ± 2.1	30.3 ± 3.1	8.5 ± 1.1	3.8 ± 0.5	—	44.1 ± 3.8	2.0 ± 0.3	1,896 ± 280

[a]Cells were preincubated 48 hr with the choline analogues at the indicated concentrations in the presence of 50 μCi/mL sodium [^{32}P]orthophosphate. Radioactive phospholipids were then separated by two-dimensional chromatography. Results are expressed as percentages of total radioactivity. Mean of 3 determinations ± s.d. SM: Sphingomyelin; PC: phosphatidylcholine; PI: phosphatidylinositol; PE: phosphatidylethanolamine; PME: phosphatidylmonomethylethanolamine; PDE: phosphatidyldimethylethanolamine.

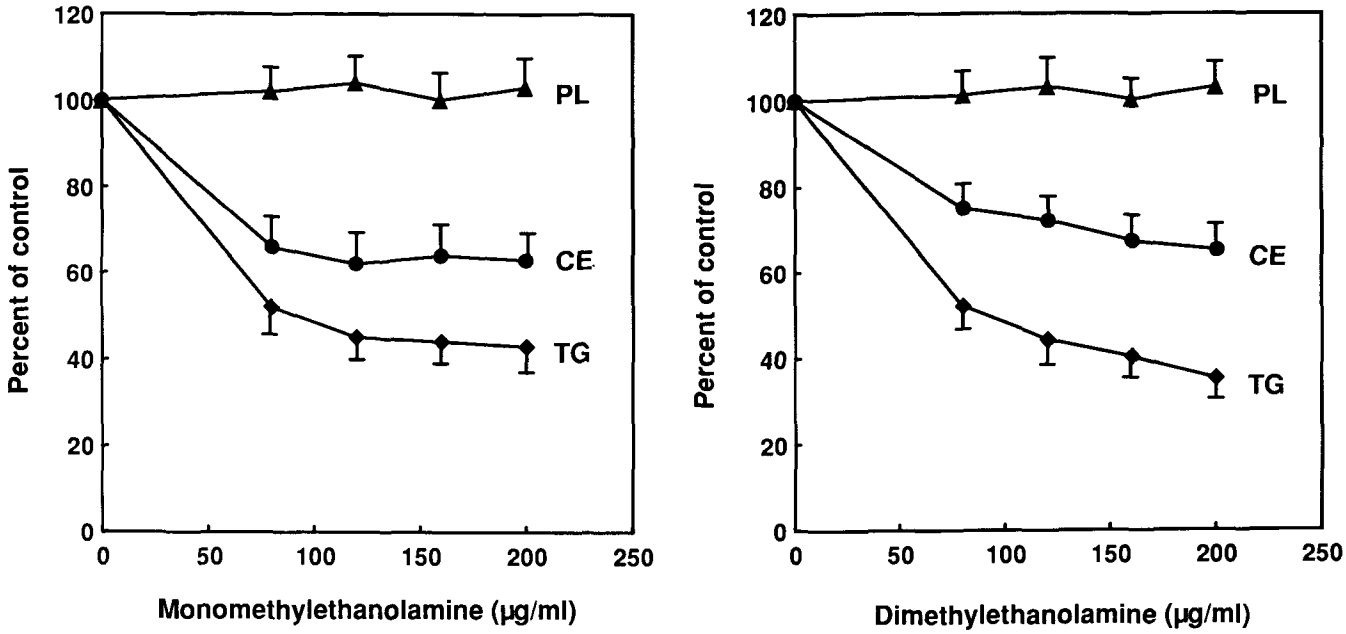

FIG. 1. Effect of monomethylethanolamine and dimethylethanolamine supplementation on oleic acid incorporation into phospholipids, cholesteryl esters and triacylglycerols. Cells were preincubated for 48 hr with ME (1a) or DE (1b) before addition of [^{14}C]oleic acid (1 μCi/mL) during 1 hr. Cellular lipids were then separated by thin-layer chromatography. ▲: phospholipids; ●: cholesteryl esters; ■: triacylglycerols. Results are expressed as percentages of controls. The absolute values are in pmoles incorporated/mg protein: phospholipids, 4,623; cholesteryl esters, 143; triacylglycerols, 56. Means of 6 determinations ± s.d.

TABLE 2

Effect of Phospholipid Polar Head Group Modification on [^{3}H]Thymidine Incorporation by Human Cultured Fibroblasts[a]

Addition	DPM/μg protein
None	262 ± 31
Monomethylethanolamine	
80 μg/mL	290 ± 43
120 μg/mL	242 ± 35
160 μg/mL	316 ± 41
200 μg/mL	322 ± 39
Dimethylethanolamine	
80	254 ± 32
120 μg/mL	219 ± 25
160 μg/mL	287 ± 22
200 μg/mL	282 ± 37

[a]Cells were preincubated for 48 hr with the choline analogues before incubation with [^{3}H]thymidine (50 μCi/mL) for 4 hr. Means of 4 experimental values ± s.d.

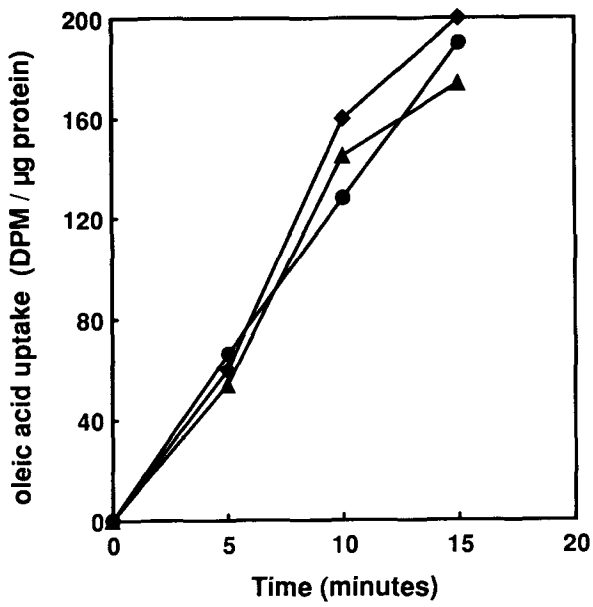

FIG. 2. Effect of monomethylethanolamine and dimethylethanolamine supplementation on oleic acid uptake. Cells were preincubated for 48 hr with ME or DE before addition of [^{14}C]oleic acid (1 μCi/mL). ▲: control; ●: ME; ■: DE. Means of 3 determinations.

phospholipid polar head group provides a valuable tool to study the interrelationship between the phospholipid structure and composition on one hand and the activities of membrane-bound enzymes on the other.

The effect of supplementation with the choline analogues on oleic acid incorporation into different lipid classes was also investigated. Figure 1 shows that supplementation with either ME (Fig. 1a) or DE (Fig. 1b) induced a decrease in fatty acid incorporation into cholesteryl esters and triacylglycerols. Maximum inhibition was essentially attained with 80 μg/mL ME or DE. Higher concentrations of ME in particular did not further increase inhibition. Overall incorporation into phospholipids was not affected. The relative incorporation of fatty acid into different phospholipid classes resulted in the phospholipid composition shown in Table 1. In view of the fact that incorporation into phospholipids was not affected, decreased incorporation into cholesteryl esters and

TABLE 3

Effect of Monomethylethanolamine or Dimethylethanolamine Supplementation on *in Vitro* Oleic Acid Transfer from Oleoyl Coenzyme A to Various Lipid Classes[a]

Addition	Cholesteryl esters (ACAT activity)	Triacylglycerols (DGAT activity)	Phospholipids (phospholipid acyltransferase activities)
None	209 ± 18	573 ± 62	625 ± 63
Monomethylethanolamine (μg/mL)			
80	127 ± 11	372 ± 32	623 ± 59
120	123 ± 59	327 ± 30	569 ± 58
160	109 ± 8	287 ± 22	538 ± 61
200	96 ± 9	235 ± 21	531 ± 57
Dimethylethanolamine (μg/mL)			
80	184 ± 17	275 ± 22	613 ± 56
120	134 ± 11	223 ± 21	575 ± 53
160	111 ± 10	212 ± 18	543 ± 46
200	113 ± 9	209 ± 16	538 ± 51

[a]Cells were preincubated 48 hr with the choline analogues before measurement of acyltransferase activities with [^{14}C]oleoyl CoenzymeA, as described in the text. Results are expressed in pmoles/5 min/mg protein. Means of 4 experimental values ± s.d.

TABLE 4

Effect of Monomethylethanolamine or Dimethylethanolamine Supplementation on Intracellular Cholesteryl Ester and Triacylglycerol Levels[a]

Precursor	[^{14}C]Cholesterol			[^{14}C]Glycerol
Addition	Free cholesterol	Cholesteryl esters	Total	Triacylglycerols
None	9,197 ± 102	5,249 ± 532	14,446 (100%)	1,140 ± 121
Monomethylethanolamine (μg/mL)				
80	10,050 ± 112	4,650 ± 424	14,700 (102%)	775 ± 72
120	9,933 ± 107	2,963 ± 256	12,896 (89%)	513 ± 49
160	10,370 ± 120	3,254 ± 319	13,624 (94%)	445 ± 36
200	8,824 ± 98	2,558 ± 246	11,382 (79%)	388 ± 33
Dimethylethanolamine (μg/mL)				
80	8,093 ± 95	3,764 ± 367	11,857 (82%)	695 ± 62
120	8,277 ± 78	3,512 ± 321	11,789 (82%)	467 ± 36
160	7,187 ± 87	3,448 ± 316	11,265 (78%)	410 ± 35
200	7,725 ± 84	3,155 ± 287	10,880 (75%)	353 ± 31

[a]Cells were preincubated 48 hr with the choline analogues in the presence of [^{14}C]cholesterol (0.2 μCi/mL) or [^{14}C]glycerol (2 μCi/mL) before analysis of cellular lipid by chromatography. Results are expressed in pmoles incorporated/mg protein. Means of 4 experimental values ± s.d.

triacylglycerols most likely was not due to impaired fatty acid uptake or activation. In subsequent experiments, oleic acid uptake by the cells was determined for short pulse periods of 5 to 20 min: again no change in incorporation rate was found in the presence of the choline analogues (Fig. 2).

To further determine whether the results obtained with oleic acid were due to alterations in acyltransferase activities, ACAT and DGAT activities were measured on cell homogenates with oleoyl-CoA as substrate. Table 3 shows that modification of the phospholipid polar head group with ME and DE induced a dose-dependent decrease in ACAT and DGAT activities. At 200 μg/mL of either choline analogue, ACAT and DGAT activities accounted for approximately 50% and 40% of controls, respectively. As expected, the activities of the phospholipid acyltransferases were not significantly affected, with only a slight decrease of 15% at 200 μg/mL ME or DE. Since it has been suggested that the activity of ACAT might be regulated by membrane microviscosity (13), one could postulate that the presence of PME or PDE may alter the physical environment of the cell membranes. However, a study of the physical properties of the membranes enriched in PME or PDE, as judged by fluorescence polarization (14) or electron spin resonance spectroscopy (15), showed that these parameters remained unaffected by such modulation. Another explanation could be that modification of the phospholipid polar head

group is accompanied by a modification of the fatty acid composition of the phospholipids, as it has been shown that ACAT activity can be modulated by changes in the fatty acid composition in microsomal membranes (16,17).

To determine whether the intracellular contents of cholesteryl esters and triacylglycerols were lowered as a consequence of decreased synthesis, isotopic equilibrium experiments with radioactive cholesterol and glycerol were performed. After long-term (48 hr) incubation with the radioactive precursor, the radioactivity found in different lipid species would be expected to be proportional to the lipid amounts present in the cell. Using this approach, we found that modification of the phospholipid polar head group induced a decrease in the amount of cholesteryl esters and triacylglycerols in the cultured cells (Table 4). The decrease was somewhat more pronounced for triacylglycerols, which amounted to 40-50% of controls at 200 μg/mL ME or DE. At the same ME or DE concentrations, cholesteryl esters amounted to approximately 50-60% of controls. The intracellular contents of free cholesterol or total cholesterol were not significantly affected. However, a slight decrease in the total cholesterol content was noted at the highest concentration of the choline analogues used. The observed decrease in ACAT and DGAT activities clearly resulted in changes of the intracellular levels of triacylglycerol and cholesteryl esters in human fibroblasts.

It should be emphasized here that modulation of the phospholipid polar head groups had no effect either on phospholipid/protein (Table 1) or on cholesterol/protein ratios (Table 4). The observed changes in ACAT and DGAT activities can most likely be explained by altered organization in cell membrane structures due to altered interactions between lipids and proteins, which in turn causes changes in the conformation of membrane-bound proteins and in the activities of membrane-bound enzymes. ACAT activity has been shown to be decreased in the presence of amphiphilic drugs, such as phenothiazines (18,19), which partition into the lipid bilayer. In this regard, it would be possible that the difference in sensitivity between ACAT or DGAT and the phospholipid acyltransferases could arise from their different location within the membrane.

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Effect of Dietary Fat on the Lipid Composition and Utilization of Short-Chain Fatty Acids by Rat Colonocytes

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The objective of the present studies was to examine the effect of dietary fat on the lipid composition of rat colonocytes and their utilization of short-chain fatty acids (SCFA). Rats were fed 14% beef fat, fish oil or safflower oil plus 2% corn oil in a semi-synthetic base diet for 4 wk. Colonocytes were isolated and their lipid composition was examined. Feeding beef fat and fish oil resulted in an increase in monounsaturated fatty acids and a reduction in ω-6 fatty acids. Feeding fish oil resulted in an enrichment with ω-3 fatty acids. There was no dietary influence on the amount of either cholesterol or phospholipids of colonocytes. Fish oil feeding resulted in significant increase in colonocyte free fatty acids (FFA) as compared to other diets. Dietary fat was found to have no effect on SCFA utilization by colonocytes. Colonocytes were found to utilize SCFA in the order of butyrate ≥ acetate ≥ propionate. The presence of acetate and propionate in the medium had no effect on the rate of butyrate utilization.
***Lipids* 25, 316-320 (1990).**

It has been shown that dietary fatty acid composition influences the lipid composition of plasma membranes (1,2), nuclear membranes (3), microsomal membranes (4), and mitochondrial membranes (5) of several types of cells. Some of these dietary alterations were found to influence several functional properties of these membranes. These include uptake of nutrients (6,7), membrane-bound enzymes (2,8,9) and the rate of mitochondrial respiration (5,10).

Epidemological and experimental studies suggest an effect of dietary fatty acid composition on the development of colon cancer. Epidemologic data points to an association between increased incidence of colon cancer in populations consuming diets with low polyunsaturated/saturated fat ratios (11,12). In support of these findings, saturated fat feeding has been shown to increase colon carcinogenesis in laboratory animals (13,14). Several mechanisms have been proposed for the role of dietary fat in the development of colon cancer. These include the effect of dietary fat on the production of bile acids (15,16) or prostanoids (17,18). In addition, the effect of dietary fat may be mediated through its influence on the structure and function of membranes (19,20). In line with the latter hypothesis, the present studies were designed to test the possibility that dietary fat may alter the structure of colonocytes in such a way that may interfere in their utilization of the main fuel. Several investigators have shown that short-chain fatty acids (SCFA) such as acetic acid, propionic acid and butyric acid are the main fuel for colonocytes (21-23). SCFA are produced in the colon by the gut microflora. In the present studies, we fed rats a high fat diet with different fatty acid compositions, saturated (beef fat), ω-3 rich polyunsaturated (fish oil) and ω-6 rich polyunsaturated fatty acids (safflower oil) to test this hypothesis.

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Abbreviations: SCFA, short-chain fatty acids; PBS, phosphate buffer saline; BSA, bovine serum albumin; FFA, free fatty acids.

MATERIALS AND METHODS

Animals and diets. A total of 32 Sprague-Dawley weanling male rats were used in four experiments. Animals were fed a semisynthetic high fat diet containing 14% by weight of beef fat, safflower oil or Menhaden fish oil. Beef fat was obtained locally, safflower oil was obtained from U.S. Biochemical (Cleveland, Ohio) and Menhaden oil was a gift from Zapata Haynie Corp. (Reedville, VA). Butylated hydroxytoluene was added at 0.02% by weight as an antioxidant to the oils and fats. In addition, all diets contained 2% corn oil (Mazola oil) to assure the adequacy of essential fatty acids. Diets were mixed in small batches and kept at −60°C under nitrogen until used. The composition of the semisynthetic diets and their fatty acid compositions are given in Tables 1 and 2, respectively. The vitamin and mineral mixes used in these diets are those recommended by the American Institute of Nutrition (24). Rats were kept in a temperature-controlled environment (22 ± 1°C) at a 12-hr light/12-hr dark cycle and had free access to tap water. Diets were fed to the experimental animals *ad libitum* for 4 wk. Records of body weights at the start and termination of the experiments, and food consumption per diet-group were kept.

Colonocyte isolation. The method of Roediger and Truelove (25) was used to isolate colonocytes after killing the animals by decapitation. The entire colon, from the caecum to rectal ampulla, was rapidly removed and flushed with O_2-saturated phosphate buffer saline (PBS), pH 7.2. This buffer contained 136 mM NaCl, 4.7 mM KCl, 0.1 mM $MgSO_4$, 1.2 mM KH_2PO_4, 4.0 mM Na_2HPO_4, 1.0 mM NaH_2PO_4 and 1.3 mM $CaCl_2$ and was maintained at 37°C. The proximal end of the colon was ligated and

TABLE 1

Composition of Semisynthetic Diets

Ingredients	Weight %
Cornstarch	30.0
Casein	26.0
Sucrose	16.5
Fat or oil[a]	14.0
Celufil (fiber source)	6.0
Mineral mix[b]	4.0
Corn oil	2.0
Vitamin mix[b]	1.0
DL-methionine	0.4
Choline chloride (70%)	0.1

[a] Beef fat, safflower oil or menhaden oil.
[b] AIN (18).

TABLE 2

Fatty Acid Composition of the Experimental Diets[a]

Fatty acid[b]	Diets		
	Beef fat (%)	Fish oil (%)	Safflower oil (%)
12:0	tr[c]	tr	tr
14:0	3.0	8.6	10.4
14:1	1.4	0.5	tr
16:0	20.7	15.6	—
16:1	5.2	12.3	—
17:0	—	1.8	—
18:0	11.8	4.4	3.2
18:1	44.8	13.9	22.5
18:2	11.4	9.0	62.9
18:3	0.5	1.3	—
20:3	—	1.4	—
20:4	—	1.0	—
20:5	—	13.6	—
22:5	—	2.2	—
22:6	—	8.3	—
Σ Saturated	35.5	30.4	13.6
Σ Monounsaturated	48.7	26.7	22.5
Σ ω-6	11.9	12.6	62.9
Σ ω-3	0	24.1	0
Unsaturation index[d]	0.75	1.98	1.50

[a]The difference between the sum of the percentages and 100 represents unidentified fatty acids.

[b]Number of carbon atoms:number of double bonds.

[c]≤0.5% (trace).

[d]Unsaturation index refers to average number of double bonds/fatty acid.

everted over a rod. The everted colon was rinsed again with PBS and was maximally distended using 10–15 mL PBS containing 0.3% (w/v) dialyzed bovine serum albumin (PBS-BSA). The method of Krebs *et al.* (26) was used to dialyze the bovine serum albumin. The distal end was then ligated. The colons of 2–3 animals were then placed in a flask containing 100 mL PBS-BSA buffer to which 5.0 mM EDTA was added and the $CaCl_2$ was omitted. The flask was continuously gassed with O_2 while incubating at 37°C in a shaking water bath at 60 oscillations/min.

The incubation buffer was replaced with 75 mL of PBS-BSA buffer and further incubated while gassing under O_2 for 40 min and shaking at 70–80 oscillations/min. The released cells in this medium were collected by centrifugation at 500 × *g* for 3 min. Cells were washed twice with PBS-BSA containing 5.0 mM dithiothreitol and then suspended in PBS containing 2.5% BSA and kept at 22°C. Cell suspensions were counted using a hemocytometer, and were adjusted to contain 1×10^7 live cells/mL. Trypan blue exclusion was used to test for cell viability. Aliquots of these cell suspensions were used for metabolic studies and lipid analysis as follows.

SCFA utilization by colonocytes. One mL of cell suspension containing 1×10^7 live cells was incubated at 37°C in 25-mL plastic flasks containing 4.0 mL of O_2-saturated PBS containing 2.5% BSA, 10 mM of a test SCFA and its radioactive form (2 μCi) as a tracer. The tracers [1-^{14}C]butyrate (55 mCi/mmole), [1-^{14}C]acetate (55 mCi/mmole) and [1-^{14}C]propionate (55 mCi/mmole) were obtained from American Radiolabeled Company (St. Louis, MO). Each incubation flask was equipped with a plastic center well that hung from a rubber stopper. Flasks were gassed with 100% O_2 for 20 sec and shaken at 55–65 oscillations/min for 1 hr in a 37°C bath.

Incubations were terminated by injecting 0.5 mL of 10% perchloric acid into the cell suspension. $^{14}CO_2$ was collected in the well by injecting 0.4 mL of 10 M NaOH. The flasks were allowed to shake for an additional 3 hr to trap released CO_2. Aliquots of the well content were taken for counting in a liquid scintillation solution (27). To correct for subsequent volatility of short-chain fatty acids, blanks were used in each experiment. The blanks were flasks containing identical components of each experimental flask, with the exception that blanks contained no cells. In the case of testing butyrate utilization by colonocytes in the presence of propionate and acetate, the concentration of each of these SCFA was 3.33 mM.

Lipid analysis. Lipids were extracted from cell suspension by the method of Folch *et al.* (28). Aliquots of lipid extract were used to measure phospholipids (29), cholesterol (29), triglycerides (30) and FFA (31). To examine the fatty acid composition of phospholipids, additional aliquots were used to isolate phospholipids on thin-layer plates (32). Identification of the fractions containing phospholipids was done by comparison with authentic standards obtained from Nu-Chek-Prep (Elysian, MN). The phospholipid fraction was scraped off, and the fatty acids were methylated according to the method of Lepage and Roy (33). Fatty acid analysis was carried out using a Shimadzu gas chromatograph (Columbia, MD) equipped with a flame ionization detector and a 6-ft glass column packed with 10% SP-2330 on 100/120 chromosorb WAW. Nitrogen was used as the carrier gas. The temperatures of the oven and the injection port were maintained at 190°C and 260°C, respectively. Authentic fatty acid methyl ester standards (Nu-Chek-Prep, Elysian, MN) were used to identify the peaks, and the areas under the peaks were measured using an integrator (Shimadzu, C-RIA).

Statistical analysis. Analysis of variance was used to analyze the data, and the differences between means were tested for significance by the method of Newman-Keuls (34). The least significant difference was used to test the significance between the percentages of fatty acid analysis (34).

RESULTS

Body weight and food consumption. Feeding the experimental diets for 4 wk resulted in average daily gains of 6.72 g for beef fat-fed animals, 7.20 g for fish oil-fed animals and 6.62 g for safflower oil-fed animals. Analysis of variance of the individual observations revealed that neither the diet nor the experiment had any effect on this parameter. The average food consumption, calculated from the total food consumed per diet-group, was 16 g/rat/day for animals fed the beef fat diet, which was not different from the other two diet groups.

Effect of dietary fat on colonocyte lipids. The data on phospholipid, cholesterol, and FFA contents of colonocytes of different food groups are given in Table 3. We

TABLE 3

Lipid Content of Colonocytes from Animals Fed the Experimental Diet

Lipid	Diets		
	Beef fat (3)[a]	Fish oil (4)	Safflower oil (3)
Phospholipids[b]	341 ± 12	406 ± 34	346 ± 71
Cholesterol[b]	86 ± 2.5	110 ± 8.5	96 ± 22
Phospholipid/ cholesterol	3.97 ± 0.03	3.69 ± 0.25	3.67 ± 0.17
Free fatty acids[c]	128 ± 10	270 ± 47[d]	193 ± 20
Triglycerides	N.D.[e]	N.D.	N.D.

[a] Number between parentheses refers to n of samples, each a pool of 2–3 colons.

[b] Values (μmol/l × 10^7 cells) are means ± S.E.M.

[c] Values (nmol/l × 10^7 cells) are means ± S.E.M.

[d] Significantly ($P \leq 0.001$) different from either that of beef fat or safflower oil.

[e] Not detected.

TABLE 4

Fatty Acid Composition of Colonocyte Total Lipids of Animals Fed the Experimental Diets[a]

Fatty acids	Diets		
	Beef fat (%)	Fish oil (%)	Safflower oil (%)
12:0[b]	tr[c]	tr	tr
14:0	0.8 ± 0.1	1.2 ± 0.2	0.8 ± 0.1
14:1	2.3 ± 0.2	3.0 ± 0.4	2.5 ± 0.1
16:0	18.2 ± 0.5	17.4 ± 0.5	17.2 ± 1.0
16:1	0.8 ± 0.1[d]	2.3 ± 0.2[e]	tr[c]
17:0	2.2 ± 0.1	3.0 ± 0.1	2.4 ± 0.4
18:0	11.9 ± 0.2[d]	12.6 ± 0.4[d,e]	13.5 ± 0.2[e]
18:1	27.3 ± 0.6[d]	19.6 ± 0.4[e]	14.8 ± 0.6[f]
18:2	10.4 ± 0.1[d]	9.7 ± 0.2[d]	20.0 ± 0.5[e]
18:3	1.3 ± 0.1[d]	tr[e]	0.6 ± 0.1[f]
20:4	14.4 ± 1.0[d]	6.9 ± 0.3[e]	15.5 ± 1.8[d]
20:5	tr[d]	9.4 ± 0.3[e]	tr[d]
22:4	2.0 ± 0.6[d]	tr[e]	2.2 ± 0.2[d]
22:5	tr[d]	1.9 ± 0.3[e]	tr[d]
22:6	tr[d]	4.5 ± 0.5[e]	tr[d]
Σ Saturated	33	34.2	33.9
Σ Monounsaturated	30.4	24.9	17.3
Σ ω-6	28.1	16.6	37.7
Σ ω-3	tr	15.8	tr
Unsaturation index[g]	1.21	1.55	1.29

[a] Values are (mean ± S.E.M.) of 3–4 samples, each is a pool of 2–3 colons. The difference between the sum of all percentages and 100 represents unidentified fatty acids plus traces of the identified fatty acids.

[b] Number of carbon atoms:number of double bonds.

[c] ≤0.5% of fatty acids.

[d–f] Values carrying the different superscripts in one row are significantly ($P \leq 0.05$) different.

[g] Unsaturation index refers to the average number of double bonds/fatty acid.

were not able to detect any measurable triglycerides in these cells by the enzymatic method used. Analysis of variance of the lipid data indicated that diet had no effect on either phospholipid or cholesterol content of colonocytes. However, colonocyte FFA were found to be significantly increased following fish oil feeding as compared to those of the animals fed either the safflower oil or beef fat diet. The colonocyte FFA content from the latter groups was not significantly different.

Effect of dietary fat composition on the fatty acid composition of colonocytes. The fatty acid composition of total lipids of colonocytes (Table 4) as well as their phospholipids (Table 5) was examined. The data show that dietary fat influences these lipids in a similar fashion. Feeding beef fat resulted in a decrease whereas fish oil resulted in an increase in the unsaturation index as compared to the safflower oil diet. Unsaturation index is defined as the average number of double bonds per fatty acid. Results indicate that both beef fat and fish oil feeding cause an increase in monounsaturates and a reduction of ω-6 fatty acids in total lipids and phospholipids of isolated colonocytes. In addition, fish oil resulted in an enrichment of ω-3 fatty acids in these lipids.

Effect of dietary alterations in colonocyte lipid composition on in vitro *utilization of SCFA.* Rates of $^{14}CO_2$ production by colonocytes upon incubation with radioactive SCFA are given in Table 6. Analysis of these data suggest that dietary fatty acid composition had no effect on the rate of SCFA utilization by colonocytes. Regardless of diet, butyric acid was utilized at a significantly faster rate as compared to acetate. Additionally, acetate was utilized significantly faster than propionate. Butyrate utilization was not influenced by the presence of either acetate or propionate.

DISCUSSION

The objective of the present studies was to examine the effect of dietary fatty acid composition on colonocyte lipid composition and its utilization of SCFA. SCFA are considered the main fuel for colonocytes (21–23) and are shown to be influenced by dietary carbohydrates (35) and protein (36). The effect of dietary fat on colon SCFA production in the colon is not known. Our results indicate that significant alterations in fatty acid composition and free fatty acids of colonocytes can be induced by dietary lipids. However, these alterations did not result in changes in the rate by which colonocytes utilize the SCFA *in vitro.* The utilization of SCFA was in the order of butyrate ⩾ acetate ⩾ propionate. There was no interference of the use of the preferred SCFA, butyrate, by the presence of the other two fatty acids.

Our present data suggest that both total lipid and phospholipid fatty acids are similarly altered by dietary means. This was not surprising since phospholipids make up the bulk of the cellular lipids of colonocytes. Feeding a saturated fatty acid-rich diet, such as beef fat, resulted in an increase in monounsaturated fatty acids and a reduction in 18:2 content of these cells and their phospholipids as compared to feeding an unsaturated safflower oil diet. This is in agreement with the findings in other tissues (37–39), however the magnitude of these changes is smaller. Feeding Menhaden oil-rich diet resulted in reduction in total ω-6 fatty acids (18:2 and 20:4)

TABLE 5

Fatty Acid Composition of Colonocyte Phospholipids of Animals Fed the Experimental Diets[a]

Fatty acids	Diets		
	Beef fat (%)	Fish oil (%)	Safflower oil (%)
12:0[b]	1.6 ± 0.8[c]	tr[c]	0.7 ± 0.4[d]
14:0	1.1 ± 0.1	1.6 ± 0.4	0.9 ± 0.1
14:1	3.6 ± 0.3	3.5 ± 0.9	3.1 ± 0.6
16:0	21.0 ± 0.8	22.2 ± 1.3	19.2 ± 1.5
16:1	1.0 ± 0.1	2.2 ± 0.3	tr
17:0	2.9 ± 0.3	3.3 ± 0.8	3.7 ± 0.4
18:0	16.4 ± 0.8	16.2 ± 0.7	13.8 ± 1.8
18:1	23.3 ± 1.7[c]	18.1 ± 0.9[d]	13.0 ± 0.1[e]
18:2	7.2 ± 0.2[c]	7.7 ± 0.3[c]	16.4 ± 1.2[d]
18:3	1.0 ± 0.1[c]	tr[d]	1.8 ± 0.7[e]
20:4	11.8 ± 0.5[c]	7.3 ± 0.3[d]	13.7 ± 1.3[c]
20:5	tr[c]	6.9 ± 1.0[d]	tr[c]
22:4	tr[c]	3.3 ± 0.8[d]	2.9 ± 0.8[d]
22:5	tr	0.7 ± 0.4	tr
22:6	tr[c]	3.4 ± 0.8[d]	tr[c]
Σ Saturated	28	43.3	38.3
Σ Monounsaturated	27.9	23.8	16.1
Σ ω-6	20	18.3	33.0
Σ ω-3	tr	11.0	tr
Unsaturation index[f]	0.92	1.40	1.22

[a] Values are (mean ± S.E.M.) of 3-4 samples, each is a pool of 2-3 colons. The difference between the sum of all percentages and 100 represents unidentified fatty acids plus traces of identified fatty acids. Trace = ≤0.05%.

[b] Number of carbon atoms:number of double bonds.

[c-e] Values carrying different superscripts in one row are significantly ($p \leq 0.05$) different.

[f] Refers to the average number of double bonds/fatty acid.

TABLE 6

Short-Chain Fatty Acid (SCFA) Utilization by Colonocytes of Animals Fed the Experimental Diets[a]

SCFA	$^{14}CO_2$ Production (pmol Co_2/hr 1 × 10^7 cells)		
	Beef fat	Fish oil	Safflower oil
Acetate (2:0)[b]	112 ± 14 (3)	96 ± 22 (4)	122 ± 6 (3)
Propionate (3:0)	74 ± 1 (3)	44 ± 7 (4)	82 ± 13 (4)
Butyrate (4:0)	158 ± 25 (4)	160 ± 4 (3)	131 ± 7 (3)
Butyrate in the presence of 2:0 and 3:0	154 ± 5 (3)	138 ± 37 (4)	164 ± 3 (3)

[a] Values are means ± S.E.M. of (n). Each sample is a pool of cells obtained from 2-3 animals.

[b] Number of carbon atoms:number of double bonds.

and in a moderate increase in monounsaturated fatty acids. In addition, feeding Menhaden oil resulted in 16-18% enrichment of ω-3 fatty acids in total and phospholipid fatty acids of colonocytes, respectively. Similar results in different tissues were obtained by other investigators (40-42). However, the enrichment level with ω-3 fatty acids in colonocytes was much smaller than that reported in rat liver by Garg *et al.* (43), where ω-3 fatty acid made up 30% of fatty acids.

Although dietary fatty acid composition did not influence the percentage of saturated fatty acids in phospholipids, the modification of ω-3 or ω-6 fatty acid contents of these lipids resulted in alteration in their unsaturation index. This may suggest an influence on membrane fluidity. Fish oil feeding resulted in a significant decrease in the percentage of arachidonic acid in the phospholipids. Yerram and Spector (44) have recently reported similar findings on smooth muscle cells in a tissue culture system supplied with 22:6 (ω-3) fatty acid. Swanson and Kinsella (45) demonstrated a decrease in 20:4 in cardiac lipids in rats fed 20% fish oil without a decrease in 18:2. The difference between the latter and the present studies may reflect differences in the response of tissues to dietary modification.

The present studies indicate that feeding fish oil resulted in a significant increase in colonocyte FFA as compared with either beef fat or safflower oil feeding. Recently, Grataroli *et al.* (46) demonstrated an increase in the activity of phospholipase A_2 in gastric mucosa of mice fed fish oil. Other possibilities for the source of FFA may also exist such as the inhibition of triglyceride synthesis as shown in other tissues isolated from animals fed an ω-3 fatty acid-enriched diet (47). The issue regarding the source of excess FFA in colonocytes of animals fed ω-3 fatty acids deserves more investigation.

The present studies showed that colonocytes have a preferential order for the use of SCFA, i.e. butyrate > acetate > propionate. This is in agreement with the findings of Roediger (21). Since under physiological conditions these SCFA exist in the lumen as a mixture, we examined the effect of the presence of acetate and propionate, which are less preferred, on the utilization of butyrate. The data suggest that butyrate is utilized without any interference from either acetate or propionate. Roediger (21) reported that the presence of acetate results in a decrease in butyrate utilization. This discrepancy may be due to differences in the methods used. Roediger (21) used 20 mM acetate and 10 mM propionate in the presence of 10 mM butyrate, whereas 3.33 mM of each was used in our incubation medium.

The lack of dietary influence on SCFA utilization by colonocytes may be due to the modest alterations observed in membrane lipids in response to dietary modifications, and/or to the possible well-controlled homeostatic mechanism that may exist to preserve this critical metabolic function.

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Arachidonic Acid Regulates the Binding of Human Interferon in Human Skin Fibroblasts

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The induction of the antiviral state in human fibroblasts by human interferon is inhibited by arachidonic acid, its analogues 5,8,11,14-eicosatetraynoic and 5,8,11-eicosatriynoic acids, as well as by sodium arachidonate. The fatty acids myristic or oleic acid and sodium palmitate do not inhibit the antiviral action of interferon. Experiments were conducted to investigate the mechanism by which arachidonic acid could inhibit the action of interferon. No correlation between cellular lipoxygenase activities and the inhibition of antiviral action of interferon was observed in the fatty acid treated cells. Likewise, the cyclooxygenase inhibitors indomethacin and oxyphenylbutazone do not inhibit the interferon-induced antiviral state. Taken together, the inhibition of interferon action by arachidonates is unlikely to be mediated by cyclooxygenase or lipoxygenase-generated intermediates, even though arachidonates are known to affect the activity of these enzymes *in vitro*. Measurement of interferon receptors in the fatty acid treated cells showed that arachidonic acid, sodium arachidonate and its analogues decreased the number of human type I interferon receptors available for binding, and inhibited the transcription of the interferon-induced 6-16 gene and the induction of cellular (2′-5′)-oligoadenylate synthetase, suggesting the mechanism of inhibition is mediated at the level of the interferon receptor. The significance of the finding that arachidonic acid, a common fatty acid of cells and serum, can affect the antiviral action of interferon is discussed.
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Signal transduction of several growth factors is reported to involve the breakdown of cellular lipids into second messenger molecules such as diacylglycerol and inositol phosphates (1). These compounds can activate protein kinase C directly or indirectly which, in turn, can initiate other biochemical reactions necessary for the intracellular actions of these ligands (2-4). Recently there have been reports of transient increases in the intracellular concentrations of diacylglycerol and Ca^{2+}, as well as the involvement of protein kinase C in cells incubated with cytokines such as interferons and interleukin 1 (5-12). These findings raise the question whether such transient rises in second messengers are involved in the signal transduction mechanisms of these ligands. Specific inhibitors and activators of cytokine action have been used to address these questions (10-19). Substances which are general inhibitors of gene transcription (20) or of protein synthesis inhibit the action of interferon by blocking the production of new proteins necessary for the expression of the antiviral state (21). Besides these, a variety of other unrelated substances, such as H7, modulate interferon action and have been used to elucidate the signal transduction pathway(s) for interferon (11-15,22-27). Changes in arachidonate or lipid metabolism in interferon treated cells or in livers of suckling mice have been observed by several groups (28-35).

We have investigated whether the metabolites of arachidonic acid are required for the antiviral action of interferon, but found no evidence to support their involvement. Instead, we observed that arachidonic acid and two analogues of arachidonic acid can suppress the ligand-induced antiviral activity of human type I interferon (α and β interferon) in human fibroblasts. This inhibition appears to be mediated at the level of the interferon receptor and appears specific for arachidonic acid.

EXPERIMENTAL

Interferons, cells and cell viability. Betaseron (36), a stable form of recombinant human β interferon with a substituted serine at amino acid residue 17, was a gift from Cetus Corporation (Emeryville, CA). Recombinant human α_{2b} interferon was purchased from Schering Corp. (Bloomfield, NJ). Pure recombinant human α_{2b} interferon (previously referred to as α interferon) was a gift from Dr. Paul Trotta of Schering Corp. Human foreskin fibroblasts were obtained from explants of human foreskins and maintained in culture with minimal Eagle's medium containing 1000 U/ml of penicillin, 100 μg/ml of streptomycin, 2 mM glutamine and 1.5 mg/ml of sodium bicarbonate (hereafter referred to as growth medium) containing 10% fetal calf serum purchased from Hyclone Laboratories (Logan, UT). The viability of the cells after treatment with each test compound was affirmed by staining them with 0.2% trypan blue in phosphate-buffered physiologic saline, as well as by measuring their ability to re-attach to and divide on tissue culture plastic dishes. In some experiments, mouse L and A_9 cells were used.

Treatment of cells with test compounds. Confluent cultures of human foreskin fibroblasts were grown in 96 well Micro-test II plates in growth medium containing 10% fetal calf serum. The cells were then incubated with growth medium containing 1% or 10% fetal calf serum in the presence of oleic acid, myristic acid, sodium palmitate, sodium arachidonate, arachidonic acid, indomethacin, oxyphenylbutazone (Sigma Chemical Co., St. Louis, MO), 5,8,11,14-eicosatetraynoic acid, 50,8,11-eicosatriynoic acid, 7,7-dimethyleicosadienoic acid and 5,6-dehydroarachidonic acid (Biomol, Plymouth Meeting, PA) under the conditions indicated in Tables 1, 2 and 3. The test compounds were prepared in dimethyl sulfoxide as 50 mM stock solutions. The cells were washed twice with 200 μl of growth medium containing 10% fetal calf serum to remove the test compounds, and then used to assay the antiviral activity of a given preparation of interferon (1000 I.U./ml) by incubating the cells with a serially

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Abbreviations: MOPS, 3-[N-Morpholino]propanesulfonic acid.

diluted preparation for 2 hr. The antiviral titer was compared with that obtained from controls which were treated under similar conditions with medium containing the same concentration of dimethyl sulfoxide.

Interferon-induced antiviral state. Human fibroblasts were derived from explants of human foreskin. The antiviral activity of interferon was assayed by the method of Armtrong (37), as adapted for human fibroblasts (38) The antiviral state was scored as % protection against the virus-induced cytopathic effect. In a typical experiment, cells were incubated with 200 μl of a serially (two-fold diluted) stock of interferon (1000 I.U./ml of interferon) for 1 or 2 hr. Interferon was removed from the cells and a challenge virus of vesicular stomatitis virus (m.o.i. of 1) added to the cells. Viral and cell controls were included for all experiments. The cells were examined 24 or 48 hr after viral challenge when the cells in the non-interferon treated controls were completely destroyed. The definition of 1 I.U./ml of interferon is that amount of interferon which confers protection upon 50% of cells treated with a viral challenge. All experiments were performed in duplicate and the results represent average readings of duplicate experiments. The interferons used were standardized to human interferon standards I-WHO-GO2-901-527 and GO2-902-527 for human α and β interferon, respectively.

Binding of ^{125}I-labelled interferon to cells. Pure human @ interferon was labelled with ^{125}I using Iodogen (39). After labelling, bovine serum albumin was added to the labelled interferon to a final concentration of 5 mg/ml. The labelled interferon was separated from free ^{125}I by gel filtration through a Sephadex G-75 column (0.7 × 25 cm) (40). The labelled interferon was stored at 4°C, and was assayed for biological activity and concurrently analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis before use to ensure that the labelled ligand was not degraded and was still biologically active. In all experiments the interferon was used within 10 days after labelling. Using this procedure, the specific activity of the labelled interferon ranged from 300-600 cpm/I.U. of human α interferon. The labelled interferon was used to study the binding of interferon to human foreskin fibroblasts (41) in the presence or absence of the test compounds. Different amounts of the labelled interferon (25, 50, 125, 500, 750 and 1000 I.U./ml) were incubated with human fibroblasts (1.56×10^5 cells) grown in 35 mm diameter petri dishes (Nunc, Kamstrup, Denmark) for 110 min in the presence or absence of a 100-fold excess of unlabelled human interferon. An incubation period of 110 min was adopted because equilibrium binding of interferon to cells occurred at 110 min at 4°C after the cells were exposed to labelled interferon either in the presence or absence of 100-fold excess of unlabelled interferon. The cells were then washed three times with 4 ml ice-cold phosphate buffered saline to remove the unreacted labelled and unlabelled interferon. They were then solubilized in 1 ml of 0.5% sodium dodecylsulfate, and the radioactivity in the solubilized mixture counted by a γ counter (LKB-Wallac 1282, CompuGamma, Uppsala, Sweden). The data were analyzed by the method of Scatchard (42).

Northern blot hybridization and cDNA probes. Total RNA was isolated from human foreskin fibroblasts by the method of Chirgwin *et al.* (43). Approximately 100 μg of RNA was isolated from batches of 15×10^6 human fibroblasts pretreated with interferon and/or the test compounds under the conditions indicated later in Figure 2. Northern blot hybridization was carried out by procedures described previously (44). Samples of RNA (20 μg) were denatured in 50% formamide, 6.25% formaldehyde in 3-(N-Morpholine)propanesulfonic acid (MOPS) buffer, pH 7.0 containing 5.3 μM sodium acetate and 1 μM EDTA. After heating at 65OC for 15 min, the samples were electrophoresed on a 1.2% agarose gel containing 0.66 M formaldehyde in MOPS buffer. Blotting by capillary action was done using Hybond-N membranes purchased from Amersham Corp. (Buckinghamshire, U.K.). Complementary 6-16 (45) and actin DNA were gifts from Dr. A. Porter (Oxford University, U.K.) and Dr. K.M. Hui (Institute of Molecular and Cell Biology, Singapore), and were labelled with ^{32}P using an oligonucleotide-labelling kit purchased from Pharmacia (Uppsala, Sweden). The 6-16 probe routinely cross-hybridized with 28S ribosomal RNA. The autoradiograms from these experiments were scanned with the LKB Ultroscan XL laser densitometer (Bromma, Sweden). The intensity of each band was expressed as arbitrary absorbance units and the values were normalized against values obtained for α-actin mRNA hybridized on the same blot.

Cyclooxygenase, lipoxygenases and [2=-5=]-oligoadenylate synthetase. The activity of endogenous (2'-5')-oligoadenylate synthetase was measured as previously described (46). For the cyclooxygenase and 5-, 12- and 15-lipoxygenases assays, the cells were labelled with [^{14}C]arachidonic acid from Amersham. The lipid-soluble metabolites were extracted (47) and analyzed by reverse-phase HPLC (48) using prostaglandin E_2, 5(S)-, 12(S)- and 15(S)-hydroxyeicosatetraenoic acids purchased from Biomol as standards.

Protein and RNA synthesis. Human foreskin fibroblasts were grown to confluency ($\sim 1.0 \times 10^5$ cells per dish) in 3.5 cm diameter tissue culture plastic dishes purchased from Nunc. The cultures were then incubated with growth medium containing 1% fetal calf serum in the presence or absence of the test compound. The cells were washed once with growth medium and incubated with fresh medium containing 1 mCi/ml ^{14}C-labelled amino acid mixture purchased from Amersham for 30 min at 37OC. The cultures were then washed three times with 3 ml of phosphate-buffered saline, pH 7.4, three times with 3 ml of cold 5% (w/v) trichloroacetic acid, and finally with 3 ml of cold methanol. The cells were solublized in 0.3 ml of 2% (w/v) sodium dodecylsulfate and the radioactivity of the extracts measured. Similarly, the effect of the test compounds on cellular RNA synthesis was studied using 1 μCi/ml of [^{3}H]uridine (Amersham) under similar conditions.

RESULTS

Inhibition of interferon-induced antiviral state. Arachidonic, 5,8,11,14-eicosatetraynoic, 5,8,11-eicosatriynoic acids and sodium arachidonate dose-dependently suppressed the antiviral action of human α and β interferons on human fibroblasts (Table 1), and that of mouse interferon on mouse L or A_9 cells (data not shown). Inhibitions of 83-95% were measured in human cells incubated with 32 μM to 200 μM of arachidonic and 5,8,11,14-eicosateraynoic acid in growth medium containing 1% or 10% fetal calf serum, respectively (Table 1). At these concentra-

TABLE 1

Inhibition of the Interferon-Induced Antiviral State in Human Fibroblasts by Arachidonate and its Analogues[a]

Compound (μM)	% Inhibition of antiviral state induced by	
	Human α interferon	*Human β interferon*
Arachidonic acid (1)	0	0
Arachidonic acid (10)	30	0
Arachidonic acid (32)	83	83
Arachidonic acid (50)	90	90
Arachidonic acid (200)*	95	N.D.[b]
Sodium arachidonate (50)	90	90
Sodium arachidonate (200)*	95	N.D.
5,8,11,14-eicosatetraynoic acid (50)	90	90
5,8,11,14-eicosatetraynoic acid (200)*	95	95
5,8,11-eicosatriynoic acid (50)	90	N.D.
7,7-dimethyleicosadienoic acid (50)	25	N.D.
5,6-dehydro-arachidonic acid (50)	0	N.D.
Oleic acid (10)	0	N.D.
Oleic acid (32)	0	N.D.
Oleic acid (50)	25	N.D.
Sodium palmitate (32)	0	N.D.
Sodium palmitate (50)	0	N.D.
Myristic acid (10)	0	N.D.
Myristic acid (50)	0	N.D.

[a]Confluent monolayers of human fibroblasts grown in 96 well Micro-test II dishes were incubated with growth medium containing 1% or 10%* fetal calf serum in the presence or absence of test compound at the indicated concentration for 6 hr. The cells were then washed with 200 μl of growth medium containing 10% fetal calf serum and incubated with a serially diluted (two-fold) preparations of interferon for 2 hr. The interferon-treated cells were then challenged with Vesicular Stomatitis virus at an m.o.i. of 1. The % inhibition of antiviral state by the compounds tested is calculated by the following formula:

$$100-\left(\frac{\text{amount interferon conferring 50\% protection on control cells}}{\text{amount interferon conferring 50\% protection on cells pre-treated with test compound}}\times 100\right)$$

Each value represents the average of duplicate experiments.

[b]N.D. = not determined.

TABLE 2

Effect of Cell Washing on the Fatty Acid-Mediated Inhibition of the Interferon-Induced Antiviral State[a]

Compound (50 μM)	% Inhibition of α interferon-induced antiviral state		
	no wash	*1 wash*	*2 washes*
Arachidonic acid	93	93	93
5,8,11,14-eicosatetraynoic acid	93	93	93

[a]Monolayers of human fibroblasts in 96 well Micro-test II dishes were incubated with growth medium containing 1% fetal calf serum and 50 μM of each fatty acid for 6 hr. The fatty acid was removed by washing with 200 μl of growth medium containing 10% fetal calf serum for the number of times indicated. The cells were then incubated with 200 μl of a two-fold serially diluted interferon preparation (1000 I.U./ml) for 1 hr at 37°C, after which Vesicular Stomatitis virus was added to the cells at an m.o.i. of 1. The inhibition of the interferon-induced antiviral state was expressed as % inhibition compared to its control, and was determined as described in the legend to Table 1.

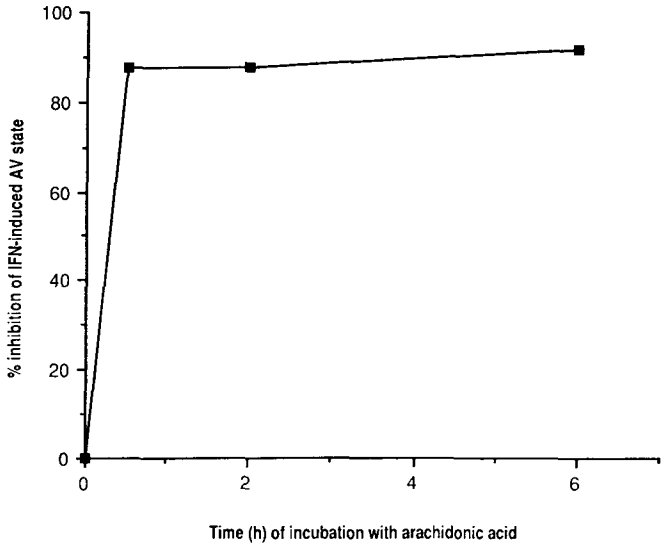

FIG. 1. Kinetics of the inhibition of the interferon-induced antiviral (AV) state by arachidonic acid in human fibroblasts. Human fibroblasts grown in 96 well Micro-test II plates were incubated with growth medium containing 1% fetal calf serum and 50 μM arachidonic acid for the times indicated. The cells were then assayed for the inhibition of the human α interferon-induced antiviral state.

tions, none of the three fatty acids were found to be toxic to the cells. Other compounds such as myristic acid, oleic acid, sodium palmitate, 7,7-dimethyleicosadienoic and 5,6-dehydroarachidonic acid did not inhibit or minimally affected the antiviral action of human α interferon (Table 1). To determine whether the inhibitory effect of these fatty acids were due to a direct interaction with the interferon ligand itself, the cells were washed with growth medium to remove as much of the fatty acid as possible before incubating the cells with interferon. The % inhibition was not found to be significantly affected by the number of washes performed (none, one or two) (Table 2), indicating that the effects of these fatty acids were on the cells *per se*. Inhibition of the antiviral action of interferon by arachidonic acid was rapidly established with maximum inhibition observed 20 min after the exposure of the cells to the fatty acids (Fig. 1). The inhibition was reversible with time (50% in 2 hr and completely by 16 hr) following the removal of fatty acids from the cells.

Cyclooxygenase, lipoxygenases and antiviral action. Cellular 5-, 12- and 15-lipoxygenase activities were measured in human fibroblasts treated with each of the substances listed in Table 3. Arachidonic, 5,8,11,14-eicosatetraynoic, 5,8,11-eicosatriynoic, 7,7-dimethyleicosadienoic and 5,6-dehydroarachidonic acids, inhibited cyclooxygenase activity of human fibroblasts by 44-97%. All these compounds, except for 7,7-dimethyleicosadienoic and 5,6-dehydroacrachidonic acids, also partially inhibited 15-lipoxygenase activity by 8-49% in human fibroblasts. However, none of the compounds tested inhibited 5- or 12-lipoxygenases and, in some cases, enhanced the activities of these enzymes. Of these compounds, only arachidonic, 5,8,11,14-eicosatetraynoic and 5,8,11-eicosatriynoic acids significantly inhibited the antiviral action of interferon (Table 3). The cyclooxygenase inhibitors indomethacin (300 μM) and oxyphenylbutazone (50 μM) did not inhibit the antiviral action of interferon in human

TABLE 3

Cellular Cyclooxygenase and Lipoxygenase Activities and the Antiviral Action of Interferon[a]

Compound μM	% Inhibition of activities for: Cyclooxygenase	15- Lipoxygenase[b]	12- Lipoxygenase[b]	5- Lipoxygenase[b]	% Inhibition of the antiviral action of human α interferon
Arachidonic acid (50)	76	14	+90	+85	90
5,8,11,14-Eicosatetraynoic acid	97	49	0	0	90
5,8,11-Eicosatriynoic acid (50)	64	8	+79	+78	90
7,7-Dimethyleicosadienoic acid (50)	44	0	+138	+86	25
5,6-Dehydro-arachidonic acid (50)	78	0	0	0	0
Indomethacin (50)	91	42	+64	+81	0
Indomethacin (300)	N.D.[c]	N.D.	N.D.	N.D.	0
Oxyphenylbutazone (50)	N.D.	N.D.	N.D.	N.D.	0

[a]Human fibroblasts were grown to confluency (6×10^6 cells per dish) in 10 cm diameter tissue culture plastic tissue culture dishes. The cells were then incubated with growth medium containing 1% fetal calf serum and each test compound at the indicated concentration for 6 hr. The monolayer was washed twice with 10 ml Tris-HCl buffer pH 7.4 and 3 ml of a solution containing 10 μM 1-^{14}C arachidonic acid from Amersham and 5 μM calcium ionophore A23187 from Sigma in Tris-HCl buffer was added to each dish. The cells were incubated at 37°C for 20 min after which the lipid soluble metabolites were extracted and analyzed by HPLC as described in the text. Each value in the table represents the average of duplicates. The antiviral activity of interferon was done separately in parallel experiments.

[b]Values with positive sign indicate a percentage increase instead of percentage inhibition of enzyme activity.

[c]N.D. = not determined.

fibroblasts or in mouse L cells even though at 50 μM, indomethacin inhibited cellular cyclooxygenase activity by 91%.

Effects on protein and RNA synthesis. Treatment of cells with 50 μM of arachidonic acid or its analogues resulted in minimal or no inhibition of cellular protein and RNA synthesis in cells (corrected for uptake of label) treated with these fatty acids, whereas the antiviral action of interferon was inhibited by 90%. Actinomycin D and cycloheximide, known inhibitors of cellular RNA and protein synthesis, inhibited RNA synthesis by 95% and protein synthesis by 89%, respectively. As expected, the antiviral action of interferon was inhibited by 99% with actinomycin D and 60% by cycloheximide.

Effect on interferon-induced gene transcription and 2'-5')-oligoadenylate synthetase. Induction of 6-16 RNA and (2'-5')-oligoadenylate synthetase activity are some of the know intercellular effects of interferon treatment. When cells were incubated with different concentrations of human α interferon for 2 hr, the transcription of the 6-16 gene increased dose-dependently up to 50 I.U./ml interferon (Fig. 2) with no further increases in the accumulation of this RNA when the cells were incubated with higher concentrations of interferon (more than 100 I.U./ml; Fig. 2). When human fibroblasts are pre-incubated with either 50 μM or arachidonic or 5,8,11,14-eicosatetraynoic acids in the presence of 1% fetal cal serum for 6 hr and then subsequently incubated with 10 I.U./ml of human α interferon for 2 hr, the accumulation of the interferon-induced 6-16 RNA was strongly inhibited (Fig. 3; compare lane 2 with lanes 4 and 6). Notably, the size of the interferon-induced 6-16 RNA is slightly larger than the 6-16 RNA of the uninduced cells. Whether this is due to differential splicing, polyadenylation or a newly induced RNA containing homologous 6-16 RNA sequences remains to be investigated. The level of the α-actin RNA in the fatty acid-treated fibroblasts was not affected when compared to that of control cells. In a separate experiment conducted under identical conditions, the induction of the interferon-induced (2'-5')-oligoadenylate synthetase activity was inhibited by ≥ 90% in the arachidonic (32 μM) or 5,8,11,14-eicosatetraynoic (32 μM) acid-treated cells. No inhibition in the induction of this enzyme was observed in human fibroblasts treated with 32 μM myristic acid or sodium palmitate.

Interferon and receptor binding sites. The Scatchard plot data shown in Figure 4 is representative of several such binding studies performed. Treatment of human fibroblasts with sodium arachidonate reduced the number

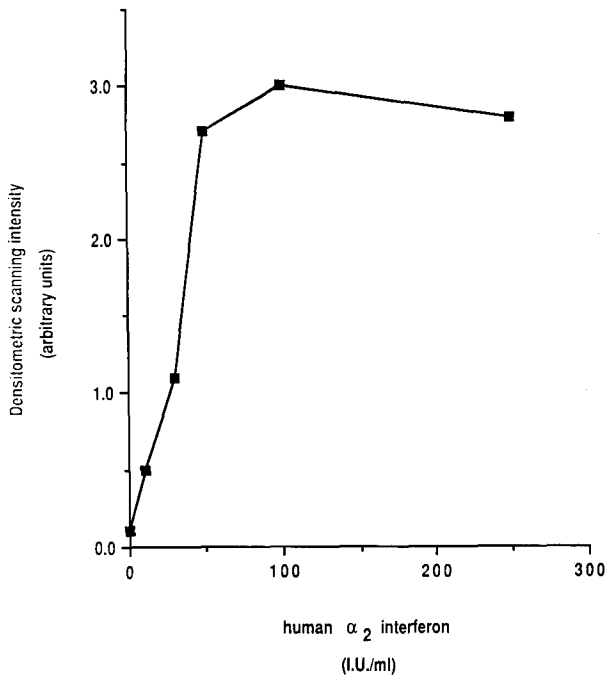

FIG. 2. Induction of 6-16 RNA in human fibroblasts incubated with increasing concentrations of human α interferon.

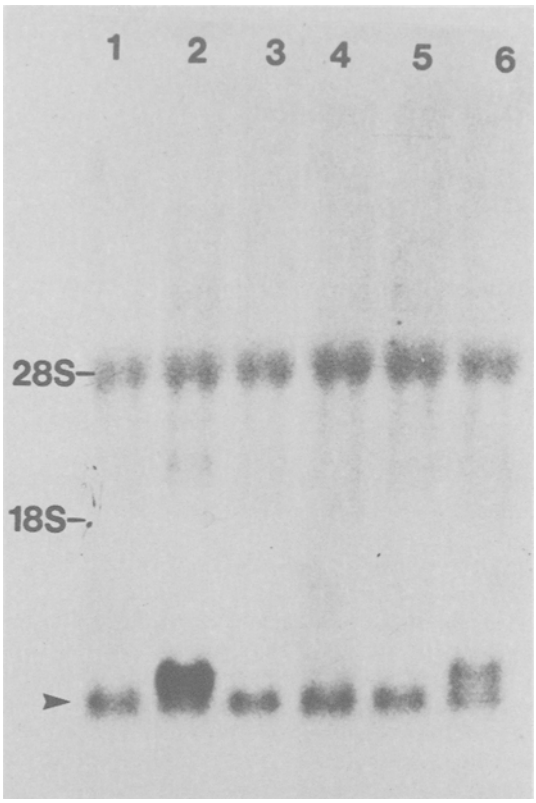

FIG. 3. Effect of arachidonic acid and 5,8,11,14-eicosatetraynoic acid on interferon-induced transcription of the 6-16 gene. Normal human fibroblasts were incubated for 6 hr with growth medium containing 1% fetal calf serum (lanes 1 and 2) in the presence of either 50 μM 5,8,11,14-eicosatetraynoic acid (lanes 3 and 4) or 30 μM arachidonic acid (lanes 5 and 6). Following this, the cells were then incubated for 2 hr with growth medium containing 1% fetal calf serum (lanes 1, 3 and 5) or 10 I.U./ml of human α interferon (lanes 2, 4 and 6). A total 20 μg of RNA obtained from each treatment was electrophoresed on formaldehyde-agarose gels and the Northern blots hybridized to ^{32}P oligolabelled 6-16 cDNA. The position of the 6-16 messenger RNA is indicated by➤.

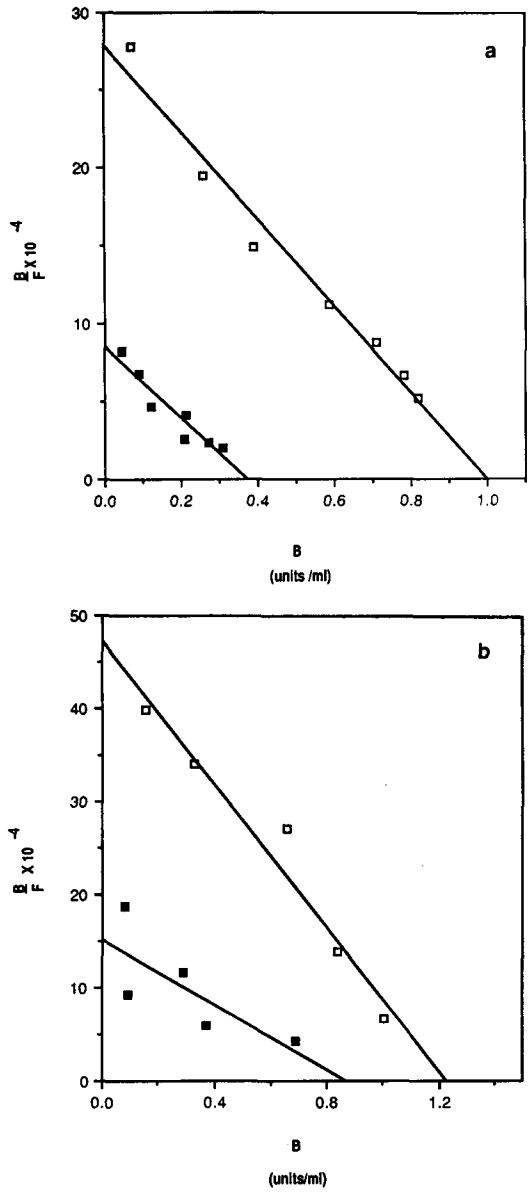

FIG. 4. Scatchard plots of human α interferon binding to their receptors in human fibroblasts. B and F are concentrations of bound and free ^{125}I-labelled human α_{2b} interferon, respectively. Human cells were treated with growth medium containing (a) 10% fetal calf serum and 200 μM sodium archidonate (■) or 10% fetal calf serum (□); and (b) with 10% fetal calf serum and 200 μM 5,8,11,14-eicosatetraynoic acid (■) or 10% fetal calf serum (□). The cells were washed once with 4 ml of growth medium containing 10% fetal calf serum to remove the fatty acid before they were used to measure the binding of ^{125}I-labelled human α interferon to their receptors.

of α interferon receptors in human fibroblasts from 2100 to 800 receptor binding sites per fibroblast (Fig. 4a). Similar observations were made with arachidonic acid. In parallel experiments, both sodium arachidonate and arachidonic acid inhibited the interferon-induced antiviral activity by $\geq$ 90%. Both arachidonic acid and sodium arachidonate had minimal effects on the K_d (1.4×10^{-10}M) for interferon binding in the fatty acid treated fibroblasts and their controls. We also measured the effect of one of the arachidonic acid analogues, viz. 5,8,11,14-eicosatetraynoic, on the binding of human α interferon to human fibroblasts and found the number of interferon receptors was reduced from 1900 to 1300 per fibroblast (Fig. 4b), while the antiviral action of interferon was inhibited by 93%. In all these experiments, no cytotoxicity was observed in the fatty acid treated cells as tested by procedures described in the experimental section. The binding of ^{125}I-labelled interferon was also performed in growth medium containing 1% fetal calf serum. In this case an arachidonic acid concentration of 50 μM reduced the binding of interferon by about 40%. The binding of the labelled interferon was preferably performed in growth medium containing 10% fetal calf serum because the percentage of measurable interferon binding to specific sites in fibroblasts is higher (90%) than in fibroblasts maintained in growth medium containing 1% fetal calf serum. In experiments where there was no inhibition, no difference in binding was measurable between the fatty acid-treated cells and their controls.

DISCUSSION

Arachidonic acid (5,8,11,14-eicosatetraenoic acid), sodium arachidonate, 5,8,11,14-eicosatetraynoic acid and 5,8,11-eicosatriynoic acid suppressed the interferon-induced antiviral state in human fibroblasts incubated with human interferons and in mouse L or A_9 cells incubated with mouse interferon. Two of the five C_{20} fatty acids tested (7,7-dimethyleicosadienoic and dehydroarachidonic acid) as well as oleic and myristic acids and sodium palmitate, had either no effect or only a minimal effect on interferon induction of the antiviral state, suggesting some degree of specificity (Table 1). Inhibition was dose-dependent and reversible with time upon the removal of the fatty acid from cells (Table 1 and Fig. 1).

Since all the substances used in this study are known to be sustrates or inhibitors of cyclooxygenase and 5-, 12- and 15-lipoxygenases (49-51), their effects on these enzymes in human fibroblasts was measured. Indomethacin inhibited cyclooxygenase in human fibroblasts by 91% and 15-lipoxygenases by 42%, but did not affect the antiviral action of a human α interferon. Likewise, dimethyleicosadienoic and dehydroarachidonci acid inhibited cyclooxygenase and had little effect on the antiviral action of interferon. Arachidonic and 5,8,11,14-eicosatetraynoic acids strongly suppressed the antiviral action of human interferon and partially inhibited cyclooxygenase and 15-lipoxygenase. On the other hand, 5,8,11-eicosatriynoic acid, which strongly suppressed the antiviral action of human ainterferon, did not inhibit 5-, 12- and 15-lipoxygenases. There is an apparent lack of correlation between the degree by which these compounds inhibited the enzymes or acrachidonic metabolism and the extent to which they suppressed the antiviral action of interferon (Table 3). The results of these analyses suggest that the enzymes of arachidonic acid metabolism are not likely to be responsible for mediating the effect of arachidonic acid itself on interferon action. Another explanation of how arachidonic acid and its analogues can inhibit the antiviral action of interferon is given below.

As arachidonic acid and its analogues are hydrophobic, they may inhibit the antiviral action of interferon at the cell membrane level. This possibility was examined by studying the effect of fatty acids on the binding of ^{125}I-labelled α interferon to human fibroblasts. We found that arachidonate and its analogues inhibited interferon action by reducing about two-fold the number of interferon receptors available for ligand binding. Epstein *et al.* (41) have reported a 30% reduction of interferon receptors in primary human fibroblasts disomic for chromosome 21, as compared to primary human fibroblasts trisomic for chromosome 21. The sensitivity of human diploid fibroblasts to interferon was about 67% to 93% less than that for trisomic 21 fibroblasts. Therefore, a 30% decrease in interferon receptor number has been correlated with a 67-93% decrease in antiviral response to interferon. Accordingly, the 32% to 62% decrease in the number of interferon receptors measured in fatty acid-treated human fibroblasts (from our binding studies) should be sufficient to account for most of the observed inhibition of 83-90% of the antiviral action of interferon by these fatty acids. Since the binding of interferon to its receptor is affected, subsequent molecular and biochemical events induced by interferon should also be inhibited. As expected, the induction of transcription of one of the interferon-induced genes, the 6-16 gene (Fig. 3) and the induction of (2'-5')-oligoadenylate synthetase by interferon were both strongly inhibited by arachidonic acid or 5,8,11,14-eicosatetraynoic acid.

The inhibition of the antiviral action of interferon by arachidonic acid or its analogues is dependent on the concentrations of the fatty acid as well as the concentration of fetal calf serum in the growth medium used to treat the cells. For example, the concentration of fatty acid was increased to 200 μM in growth medium containing 10% a fetal calf serum to obtain the same degree of inhibition observed with 50 μM of fatty acid in growth medium containing 1% fetal calf serum (Table 1), suggesting that lipid-binding proteins in fetal calf serum are carriers for these fatty acids. Arachidonic acid inhibition of interferon action is therefore dependent not only on the concentration of fatty acid but also on the concentration of serum. The abundance of lipid-binding proteins in serum and their functions in fatty acid uptake, transport and detoxification have already been described (52-54), although the physiological role(s) of these functions remains unresolved.

Several observations suggest that arachidonic acid and certain cytokines reciprocally require one another's production and release in various types of cells. For example, arachidonic acid has a putative second message function in the induction of γ interferon production (55-57), and metabolites of arachidonic acid have been shown to regulate the transcription of tumor necrosis factor in HL—60 promyelocytic leukemic cells stimulated by phorbol esters (16). On the other hand, interferon has been reported to affect the release of arachidonic acid from cells (28-33) and has also been shown to induce changes in cell membrane fluidity (58). We have also observed changes in the release of arachidonates from interferon treated human fibroblasts (S. Kowalski and Y.H. Tan, unpublished data), and are investigating these changes in the connection with the reported interferon-induced changes in the metabolism of polyenoic phospholipids and diacylglycerols (35) and in the transient changes in the concentration of cellular diacylglycerol (5-7). These reports and the present finding that arachidonic acid inhibits the antiviral action of interferon indicate the importance of examining the role of arachidonic acid in the context of regulating interferon action in cells. As arachidonic acid is a common component of serum and cells, its effect on interferon receptor availability suggests that it is a potential factor in modulating the interaction between interferon and cells.

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Identification of Carrot Inositol Phospholipids by Fast Atom Bombardment Mass Spectrometry

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Inositol phospholipids from carrot cell membranes grown in suspension culture were purified by thin-layer chromatography (TLC) or column chromatography and tentatively identified by co-migration on TLC with animal inositol phospholipid standards. For more rigorous chemical characterization, carrot inositol phospholipids were then analyzed by negative ion fast atom bombardment mass spectrometry (FABMS). One phosphatidylinositol (PI), two lysophosphatidylinositols (LPI), and one phosphatidylinositol monophosphate (PIP) were identified in the carrot samples by the observation of ions $[M-H]^-$ and numerous fragment ions in the negative FAB mass spectra. MS/MS analyses were carried out to obtain further structural information of these phospholipids using a double-focusing mass spectrometer in which the magnetic sector (B) and the electrostatic analyzer (E) were scanned at a constant ratio (B/E). These B/E linked scans provided fragment ions of selected precursor ions while eliminating matrix and other contaminating ions. No molecular ions were detected for lysophosphatidylinositol monophosphate (LPIP) or phosphatidylinositol bisphosphate (PIP_2), but fragment ions corresponding to these structures were observed. The primary fatty acids present in the carrot inositol phospholipids were linoleic (18:2) and palmitic (16:0) acids, whereas animal lipids contained arachidonic (20:4), stearic (18:0), linoleic, and palmitic acids. The only phosphatidylinositol found in carrot cells was palmitoyl linoleoyl PI.
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The inositol phospholipids, phosphatidylinositol monophosphate (PIP), and phosphatidylinositol bisphosphate (PIP_2) serve as sources of second messengers during signal transduction in animal cells (1,2). Studies are in progress to determine whether these phospholipids also serve as second messengers in plants (3–5). In order to understand the functions of these compounds in plant cells, it is necessary to first determine which inositol phospholipids are present in this system.

Inositol phospholipids, labelled with [^{32}-P]- or [^{3}H]inositol, have been purified from whole plants (6–8) and tissue culture cells (9–13) using thin-layer chromatography (TLC), high performance liquid chromatography (14), neomycin affinity columns (15), and other column chromatographic methods. Chemical identification of these compounds has been limited to co-migration with standards (3). Because inositol lysophospholipids are prevalent in plants and often co-migrate with inositol lipids and chromatography standards, and because inositol is metabolized in plants to non-lipid products such as glucuronic acid, polysaccharides, and glycoproteins, radiolabelled compounds purified by chromatography are not necessarily inositol phospholipids. Since precise identification of phosphoinositides was required for our physiological studies, we used mass spectrometry (MS) in this investigation to analyze inositol phospholipids from carrot cells grown in suspension culture.

Because the sensitivity of mass spectrometry permits the analysis of microgram or smaller quantities of samples, this technique was selected for the chemical analysis of carrot inositol phospholipids. Underivatized phospholipids have been analyzed by thermospray liquid chromatography-mass spectrometry (16), Californium-252 plasma desorption mass spectrometry (17), desorption chemical ionization (18), laser desorption (19), field desorption (20), and fast atom bombardment mass spectrometry (FABMS) (21–24). In addition, tandem mass spectrometry with collisional activation following FAB ionization has been shown to be useful in the structural analysis of phospholipids and phospholipid mixtures (23,24).

While various animal and bacterial phospholipids have been previously analyzed by mass spectrometry, few plant inositol phospholipids have been characterized by this technique. Following the approach of Jensen and co-workers (23), who first analyzed a phosphatidylinositol (PI) from soybeans using negative ion FAB and MS/MS, we report the identification of a series of carrot cell inositol phospholipids using negative ion FAB mass spectrometry, collisional activation, and B/E linked scanning.

EXPERIMENTAL PROCEDURES

Preparation of phosphatidylinositols. Carrot cells were grown in suspension culture according to previously published procedures (25), and 5 g were extracted according to the method of Boss and Massel (9). Lipids were then dried *in vacuo* and stored under nitrogen at −15°C. The lipid extract was reconstituted in 1.0 ml $CHCl_3/CH_3OH/NH_3$(15M aq.)/H_2O (90:90:7:22, v/v/v/v) (Solvent A) and loaded onto a silicic acid column which had been washed in petroleum ether and equilibrated in $CHCl_3/CH_3OH$ (1:1, v/v) (Solvent B). A small amount of [^{3}H]inositol-labelled lipids were mixed into the lipid extract prior to loading. The silicic acid column was 11 cm in height and 2.5 cm in width. The column was eluted with two volumes of Solvent B to remove the neutral lipids. The column was then eluted with six volumes of Solvent A. Fractions were collected in 125-ml, round bottom flasks and assayed for radioactivity by scintillation counting. Fractions containing tritium

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Abbreviations: FAB, fast atom bombardment; FABMS, fast atom bombardment mass spectrometry; LPA, lysophosphatidic acid; LPI, lysophosphatidylinositol; LPIP, lysophosphatidylinositol monophosphate; MS, mass spectrometry; PI, phosphatidyinositol; PIP, phosphatidylinositol monophosphate; PIP_2, phosphatidylinositol bisphosphate; and TLC, thin-layer chromatography.

were dried using a rotary evaporator and stored under nitrogen at −15°C.

Integrity of the lipid fractions was checked by TLC. Lipids were spotted and developed in Solvent A for 2 hr on LK5D TLC plates (Whatman, Clifton, NJ) which had been impregnated with potassium oxalate and oven dried at 110°C for 2 hr. Each fraction was found to contain a distinct phosphoinositide which co-migrated with one lipid from a [^{3}H]inositol-labelled whole cell extract standard run on the same plate. All of the phosphoinositides, PI, LPI, PIP, LPIP, and PIP_2, were accounted for in this manner. Soybean PI and LPI, and bovine brain PI, PIP, and PIP_2 were purchased from Sigma Chemical Company (St. Louis, MO) and used as reference standards.

In some instances, the lipids were reconstituted in $CHCl_3/CH_3OH$ (3:1, v/v) and developed on TLC plates as described above. The lipids were scraped from the plates and eluted from the silica gel with the developing solvent. In order to determine the relative quantities of the inositol phospholipids, carrot cells were labeled for 18 hr with [^{3}H]inositol, then the [^{3}H]inositol-labeled lipids were purified by TLC and scanned for radioactivity. The details of this procedure have been published elsewhere (3). Following purification by TLC, methyl esters of the acyl groups in the inositol phospholipids were prepared and the derivatized fatty acids were identified by gas chromatography as described by Trumbo and co-workers (26).

Mass spectrometry. Negative ion FAB mass spectra were obtained using a JEOL (Tokyo, Japan) JMS-HX110HF double focusing mass spectrometer, which was equipped with a JMA-DA5000 data system. During the acquisition of each mass spectrum, 7–10 scans were acquired and averaged by the data system. Calibration was carried out using sodium iodide in glycerol. The accelerating voltage was 10 keV, and the resolving power was either 1,000 or 3,000.

Samples were dissolved in Solvent A to give concentrations of 1–5 μg/μl. For each FAB analysis, the sample solution (1 μl) was mixed with the matrix, triethanolamine (1 μl), on the FAB probe tip. Xenon gas at 6 keV was used for FAB ionization.

B/E linked scanning is an MS/MS technique in which the magnetic field (B) is scanned in a constant ratio to the electrostatic analyzer voltage (E). In order to obtain B/E linked scans, precursor ions were formed by FAB, and fragmentation was enhanced by collisional activation using helium gas in a collision cell in the first field-free region. The helium gas pressure was adjusted to attenuate the precursor ion abundance 70%. Mass spectra were recorded at constant B/E by the DA-5000 data system.

RESULTS AND DISCUSSION

Phosphatidylinositol (PI). Analysis by TLC indicated that the most abundant inositol phospholipid isolated from carrot cell membranes was phosphatidylinositol (PI). The fraction corresponding to PI was scraped from the TLC plate, extracted from the silica gel, and analyzed by FAB mass spectrometry. The negative ion FAB mass spectrum of carrot PI is presented in Figure 1. In contrast to soybean phosphatidylinositol, which contained mixtures of linoleoyl, palmitoyl, oleoyl, and linolenoyl moieties (23), carrot cells were found to contain only one PI, palmitoyl linoleoyl phosphatidylinositol. This compound formed an abundant deprotonated molecule, $[M-H]^-$, that was detected at *m/z* 833 (Fig. 1).

The structures of the palmitoyl and linoleoyl groups were determined independently using MS/MS and gas chromatography. MS/MS identification of the two acyl groups was carried out by collisional activation of the ions at *m/z* 255 and *m/z* 279 in the negative ion FAB mass spectrum of PI, followed by B/E linked scanning of the resulting fragment ions. The B/E linked scan of *m/z* 255 following collisional activation contained a series of fragment ions differing by 14 mass units (the mass of a methylene group) formed by charge-remote fragmentation of a saturated fatty acid. This MS/MS spectrum was almost identical to the collisionally activated mass spectrum of palmitate obtained by Jensen and Gross (24), except that the mass resolution was much higher. Collisional activation and B/E linked scanning of the ion at *m/z* 279 resulted in a unit resolution mass spectrum similar to the lower resolution MS/MS spectrum of linoleate published by Jensen and coworkers (23). In separate experiments, methanolysis of PI produced methyl esters of palmitic acid and linoleic acid that were identified by gas chromatography.

Although abundant fragment ions appeared to be present in the FAB mass spectrum of PI, B/E linked scanning with collisional activation of the deprotonated molecule was carried out to eliminate matrix or other contaminating ions and confirm fragmentation pathways. In the B/E linked scan of $[M-H]^-$ in Figure 1, fragment ions corresponding to palmitate and linoleate were detected at *m/z* 255 and *m/z* 279, and other fragment ions corresponding to elimination of linoleic acid and palmitic acid were observed at *m/z* 553 and *m/z* 577, respectively. The fragment ion at *m/z* 299 was formed by elimination of both acyl groups, while elimination of both fatty acids (or the two acyl groups plus two hydrogen atoms) produced the ion at *m/z* 297. Loss of inositol from the deprotonated molecule, $[M-H]^-$, formed palmitoyl linoleoyl phosphatidic acid at *m/z* 671. In addition, dehydrated inositol phosphate was detected at *m/z* 241, and dehydrated glycerophosphate ($C_3H_6PO_5$) was observed at *m/z* 153. Based on the mass spectra in Figure 1, carrot cell membranes were found to contain only one PI, palmitoyl linoleoyl PI.

Except for the identification of the fragment ions at *m/z* 153 and *m/z* 671, the preceding fragmentation pathways were also described by Jensen *et al.* (23), for PI from soybeans. Although Jensen *et al.* (23) predicted that both fragment ions at *m/z* 299 and *m/z* 297 should be formed from deprotonated PI, these ions could not be resolved by their MS/MS system. Both fragment ions are clearly present and resolved in our B/E linked scan of PI (Fig. 1).

The structure of PI given in Figure 1 shows the palmitoyl group in the *sn*-1 position and linoleoyl group in the *sn*-2 position. In phospholipids, saturated fatty acids are found primarily in the *sn*-1 position, while unsaturated fatty acids are found in the *sn*-2 position (27). This is true for both plant and animal phospholipids. The positions of the palmitoyl and linoleoyl

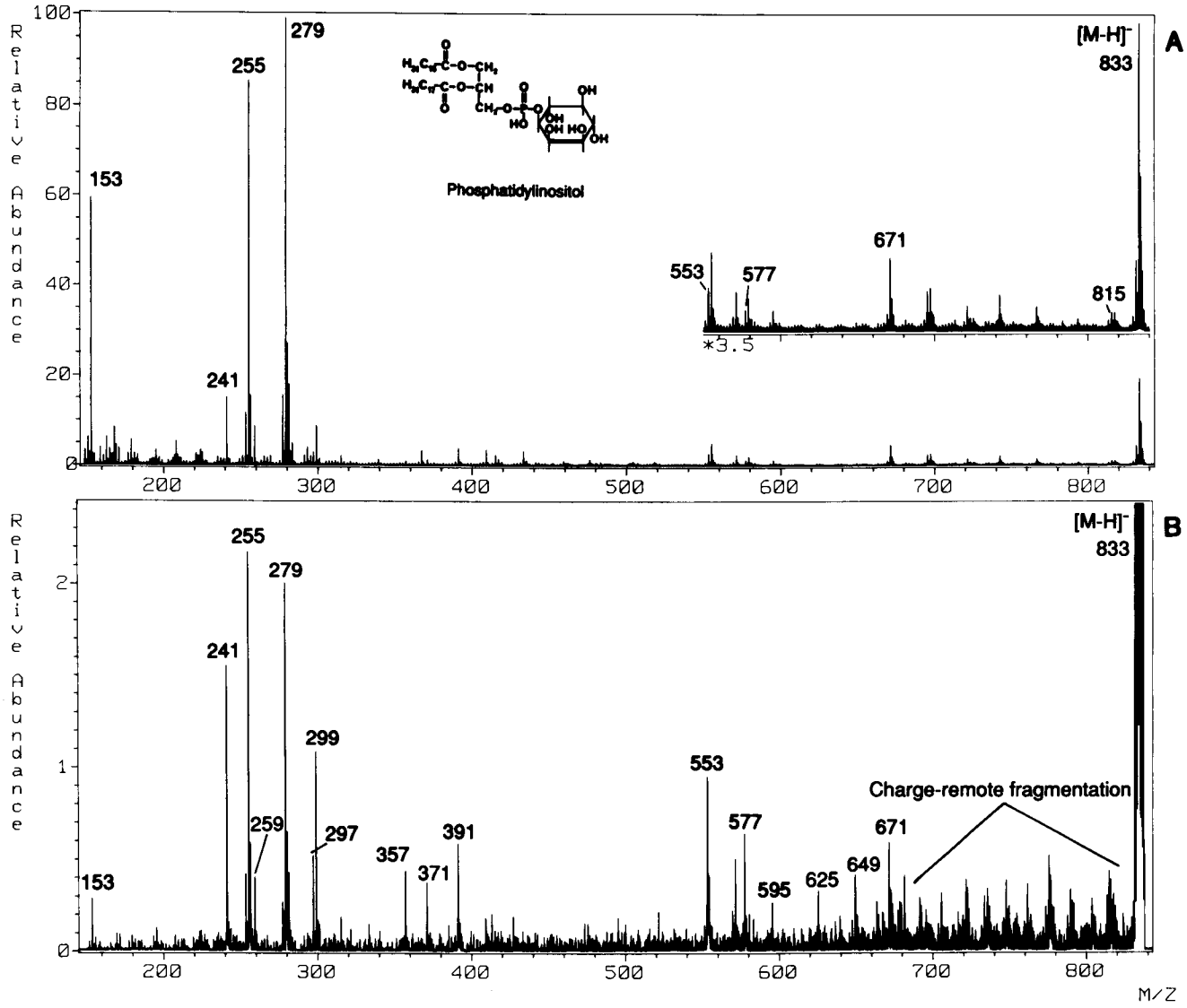

FIG. 1. (A) Negative ion FAB mass spectrum of phosphatidylinositol (PI) from carrot cells grown in suspension culture. (B) MS/MS analysis of the $[M-H]^-$ ion at *m/z* 833 using B/E linked scanning with collisional activation.

groups were verified by comparison with soybean palmitoyl linoleoyl phosphatidylinositol (PI), a commercially available standard in which palmitoyl group is located in the *sn*-1 position and linoleoyl group is in the *sn*-2 position. Besides co-migrating on TLC plates, carrot PI and the soybean PI standard produced identical negative ion FAB mass spectra and identical MS/MS spectra of the deprotonated molecule at *m/z* 833. Further MS/MS comparisons are discussed below.

Following the model of Jensen and Gross (24), who were able to assign the positions of the two fatty acids in phosphatidylcholine using MS/MS combined with FAB desorption, we found that the positions of the fatty acids in inositol phospholipid could be assigned by carrying out MS/MS analysis of the corresponding phosphatidic acid. In the negative ion FAB mass spectra of carrot PI (Fig. 1) and the soybean PI standard (data not shown), the fragment ion corresponding to palmitoyl linoleoyl phosphatidic acid (or loss of inositol, $[M-C_6H_{11}O_5]^-$) was detected at *m/z* 671. B/E linked scanning of *m/z* 671 following collisional activation produced two carboxylate fragments at *m/z* 255 and *m/z* 279 corresponding to palmitate and linoleate (data not shown). The MS/MS spectra of carrot PI and soybean PI were indistinguishable. The palmitate ion from the *sn*-1 position was approximately twice as abundant as the linoleate ion arising from the *sn*-2 position.

In order to confirm whether or not this MS/MS method might be a general technique for assigning the positions of fatty acids in inositol phospholipids, another standard PI, stearoyl arachidonoyl phosphatidylinositol from bovine brain, was analyzed. Bovine brain PI contains stearic acid at the sn-1 position and arachidonic acid at the *sn*-2 position. As expected, the B/E linked scan of $[M-C_6H_{11}O_5]^-$ from bovine brain PI produced carboxylate fragments in which the ion arising from the *sn*-1 position was over two-fold more abundant than the carboxylate ion from the *sn*-2 position. Based on the data discussed above, MS/MS analysis of phosphatidic acid from inositol phospholipids

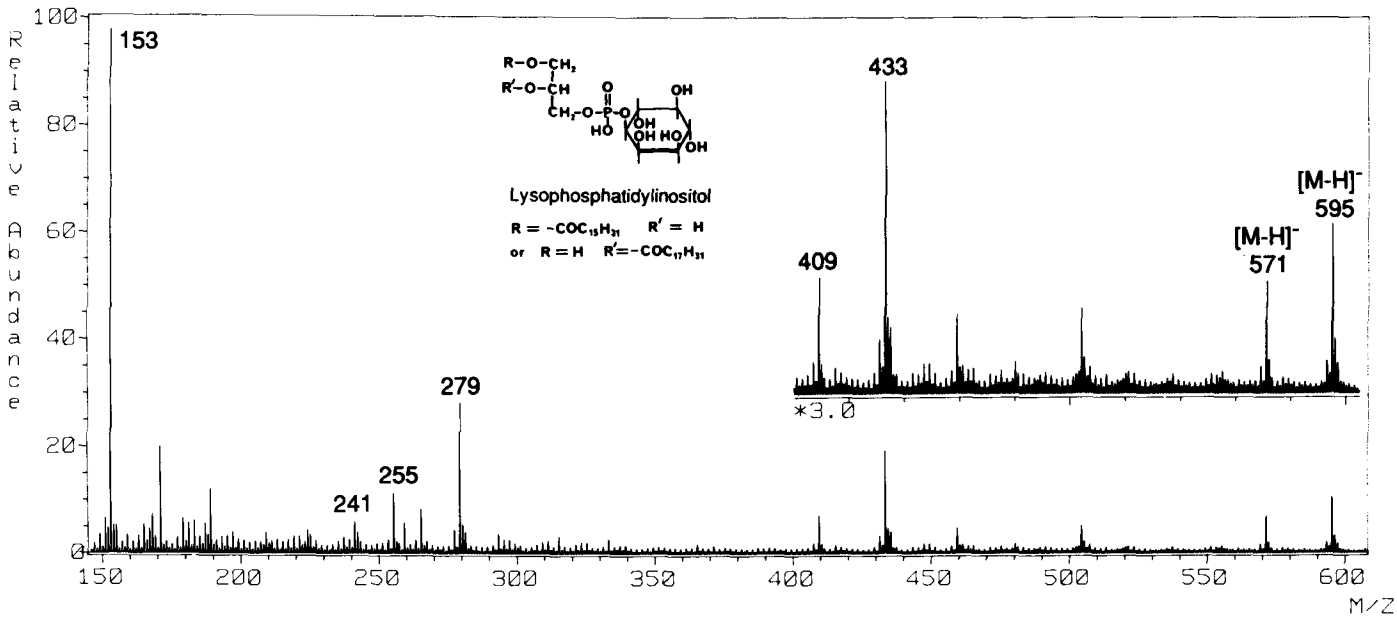

FIG. 2. Negative ion FAB mass spectrum of carrot cell lysophosphatidylinositol (LPI).

should provide carboxylate fragment ions in a ratio that indicates the position of the acyl group.

Lysophosphatidylinositol (LPI). Following purification by TLC and comparison to the migration of animal standards under identical conditions, the TLC spot corresponding to carrot lysophosphatidylinositol (LPI) was extracted and aliquots were analyzed either by mass spectrometry or gas chromatography. Gas chromatographic analysis of the fatty acid methyl esters formed by methanolysis of LPI indicated that esters of only two fatty acids were present, palmitic acid and linoleic acid. Analysis of intact LPI using negative ion FABMS showed a mixture of two LPI's based on the $[M-H]^-$ ions observed at *m/z* 571 and 595 (Fig. 2). Because this mass spectrum contained a mixture of fragment ions from two LPI compounds, B/E linked scanning of each deprotonated LPI molecule was carried out sequentially in order to obtain mass spectra containing fragment ions originating from only one of the LPI species at a time (Fig. 3).

The B/E linked scan of the deprotonated LPI molecule at *m/z* 571 contained fragment ions at *m/z* 255 and 315, corresponding to palmitate (16:0) and loss of palmitic acid (inositol glycerophosphate), respectively (Fig. 3). Fragment ions were also detected, confirming the presence of the inositol moiety. Specifically, dehydrated inositol phosphate was observed at *m/z* 241, and palmitoyl lysophosphatidic acid, which was formed by loss of inositol, was recorded at *m/z* 409. In addition, dehydrated glycerophosphate was detected at *m/z* 153. A series of fragment ions differing by 14 mass units (the mass of a methylene group) were detected in the region between approximately 400-550 mass units. These ions represent charge-remote fragmentation in the palmitoyl group (23). Overall, the mass spectrum in Figure 3 confirms the identity of this compound as palmitoyl LPI.

The other component of this mixture was identified as linoleoyl LPI based on the B/E linked scan of the deprotonated molecule at *m/z* 595 (Fig. 3). Fragment ions of linoleoyl LPI were detected that characterized both the fatty acid and the inositol phosphate groups. These fragment ions included *m/z* 279, *m/z* 315, *m/z* 241, *m/z* 153 and *m/z* 433, corresponding to linoleate (18:2), inositol glycerophosphate (loss of linoleic acid), dehydrated inositol phosphate, dehydrated glycerophosphate and linoleoyl lysophosphatidic acid, respectively. The position of the fatty acid in LPI (and the fatty acids in PIP below) was assumed to be identical to that of PI and was not determined experimentally.

Phosphatidylinositol monophosphate (PIP). Because a relatively small quantity of PIP was present in the carrot cell membrane extract as compared to PI or the two LPI's, it was necessary to purify PIP on a silicic acid column. PIP was initially identified based on the elution time during silicic acid column purification and migration on TLC compared to radiolabelled standards. Gas chromatography was used to analyze the methyl esters of fatty acids from purified PIP that had been subjected to methanolysis. Two fatty acid methyl esters were identified, which were derived from palmitic acid and linoleic acid. Next, the remaining intact PIP was analyzed by negative ion FABMS. In the mass spectrum shown in Figure 4, palmitoyl linoleoyl PIP was identified by the ions, $[M-H]^-$, $[M-2H+Na]^-$, and $[M-2H+K]^-$, that were observed at *m/z* 913, 935, and 951. Another similar set of ions was detected at *m/z* 849, 871, and 887. Because these ions were not significant in the B/E linked scans of the ions at *m/z* 913 (Fig. 4), *m/z* 935, or *m/z* 951 (data not shown), they were probably contaminants of PIP isolated by chromatography instead of fragment ions of PIP. The structures of these ions is under investigation.

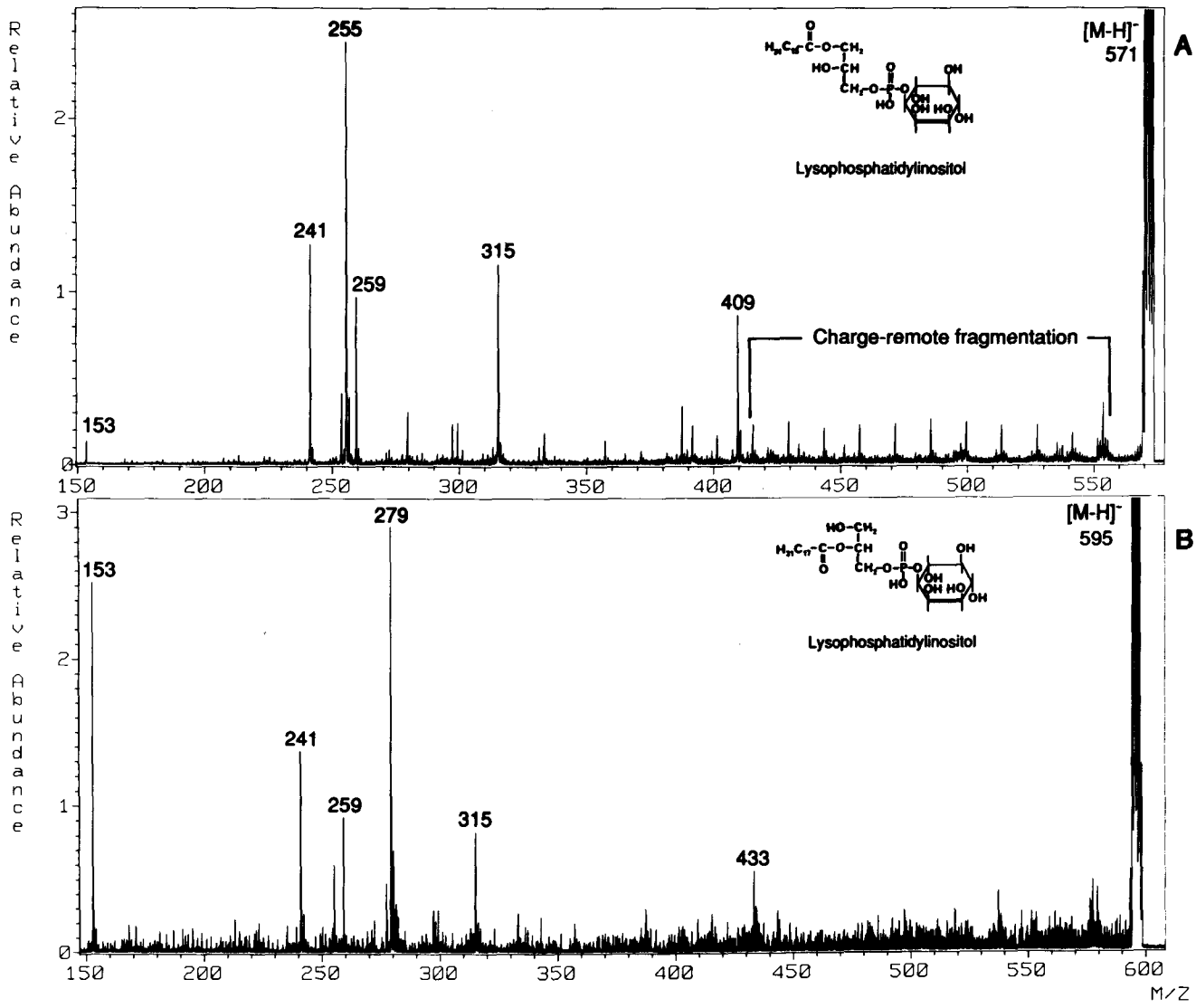

FIG. 3. B/E linked scan of the $[M-H]^-$ ion at (A) *m/z* 571; and (B) *m/z* 595 in the negative ion FAB mass spectrum of carrot cell LPI.

The B/E linked scan of *m/z* 913, the $[M-H]^-$ ion of PIP, was obtained and is shown in Figure 4. Fragment ions were observed at *m/z* 409 and 433, corresponding to palmitoyl lysophosphatidic acid (LPA) and linoleoyl LPA. The LPA ion at *m/z* 433 was more than twice as abundant as the corresponding ion at *m/z* 409 and might be indicative of the location of the acyl groups on the glycerol chain. It appears that the most abundant LPA fragment ion might correspond to LPA which is acylated at the *sn*-2 position. However, analysis of more PIP species must be carried out in order to confirm this hypothesis. The anions, $[M-409]^-$ and $[M-433]^-$, which weighed 504 and 480 mass units, respectively, were also observed but at lower relative abundance. These complementary fragment ions to lysophosphatidic acid were probably formed by gas phase rearrangement resulting in attachment of a palmitoyl group or a linoleoyl group to inositolphosphate followed by elimination of neutral LPA.

In addition, palmitoyl LPI and linoleoyl LPI were detected at *m/z* 571 and 595, and palmitate and linoleate were observed at *m/z* 255 and 279. The carboxylate ions at *m/z* 255 and 279 in the B/E linked scan of PIP (Fig. 4) were much lower in abundance than those in B/E linked scan of PI (Fig. 1). This might be the result of charge localization on the phosphate group located on carbon 4 of PIP instead of the phosphate on carbon 1 as in PI.

Lysophosphatidylinositol monophosphate (LPIP). When the region corresponding to lysophosphatidylinositol (LPIP) was scraped from TLC plates, the adsorbent was eluted, the lipids were analyzed by mass spectrometry, lipid ions were detected in low abundance relative to the matrix ions. Although the deprotonated molecule of LPIP could not be detected above the chemical noise, possible fragment ions of LPIP were observed at *m/z* 255 (palmitate), *m/z* 409 (palmitoyl lysophosphatidic acid), *m/z* 571 (palmitoyl LPI),

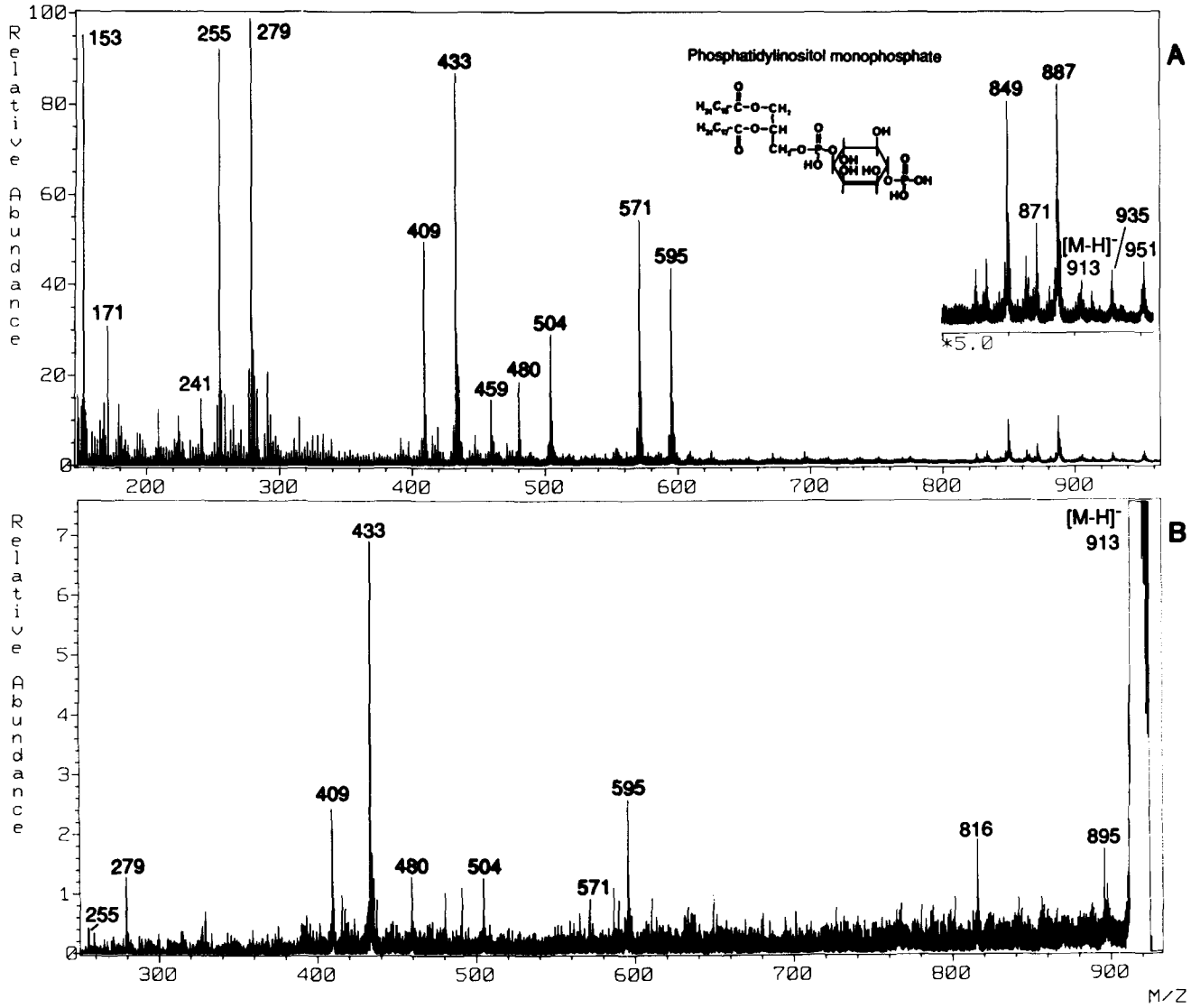

FIG. 4. (A) Negative ion FAB mass spectrum of carrot cell phosphatidylinositol monophosphate (PIP). (B) B/E linked scan of the $[M-H]^-$ ion at *m/z* 913 in the negative ion FAB mass spectrum of carrot cell PIP.

and *m/z* 595 (linoleoyl LPI) (data not shown). In order to obtain larger quantities of LPIP for analysis, silicic acid columns were used for purification instead of TLC plates, but still no molecular ions of LPIP could be detected. Investigations are in progress to obtain sufficient quantities of purified LPIP for mass spectrometric analysis.

Phosphatidylinositol bisphosphate (PIP_2). After purification by TLC, the spot corresponding to phosphatidylinositol-4,5-bisphosphate (PIP_2) was scraped off the TLC plate, extracted, and analyzed by negative ion FAB mass spectrometry. No PIP_2 ions were detected. Next, silicic acid column chromatography was used unsuccessfully to purify greater quantities of carrot PIP_2 for analysis. Because no ions corresponding to PIP_2 were observed by mass spectrometry in samples purified by TLC or silicic acid column chromatography, approximately 2 μg of arachidonoyl stearoyl PIP_2 from bovine brain was analyzed as a standard to determine if molecular ions for this group of compounds could be observed by negative ion FABMS. Two abundant deprotonated molecules, $[M-H]^-$, were observed at *m/z* 1047 and *m/z* 1071 (data not shown), which demonstrated that negative ion FABMS was suitable for analysis of PIP_2. While PIP_2 is relatively abundant in bovine brain, it is not abundant in plant cells. Thus, as in the case of LPIP above, experiments are continuing to isolate enough PIP_2 for analysis by mass spectrometry.

Standard methods of phosphatidylinositol purification, TLC and silicic acid column chromatography, yielded at least partially purified mixtures of carrot cell PI, LPI, PIP, LPIP, and PIP_2. These phospholipids were initially identified by comparison with standards during chromatography. Because mixtures of phospholipids were sometimes obtained using chromatography, the MS/MS technique of B/E linked scanning with collisional activation greatly facilitated the

identification of individual components of the mixture by providing fragment ion mass spectra of selected ion precursors. For example, there were two LPI species which were not resolved by chromatography, but were distinguished by MS using B/E linked scanning. B/E linked scanning also helped eliminate matrix ions that might have complicated interpretation of the fragmentation patterns. Finally, MS/MS was applied to the structural analysis phosphatidylinositol and was used to assign the positions of the two fatty acids. By carrying out B/E linked scans of the fragment ion corresponding to loss of neutral inositol, $[M-C_6H_{11}O_5]^-$ (phosphatidic acid), two carboxylate fragments were detected. The carboxylate anion derived from the *sn*-1 position was approximately twice as abundant as the anion derived from the *sn*-2 position. This appears to be a general observation.

In the negative ion FAB mass spectra of phospholipids from carrot cell membranes, deprotonated molecules were observed for PI, LPI, and PIP. B/E linked scans of the $[M-H]^-$ precursors confirmed the structures of PI, LPI, and PIP. For example, all of these compounds contained palmitoyl and/or linoleoyl groups, based on fragment ions at *m/z* 255 (palmitate), *m/z* 279 (linoleate), $[M-H-256]^-$ (loss of palmitic acid), and $[M-H-280]^-$ (loss of linoleic acid). In contrast to soybean (23) or animal inositol phospholipids, no other fatty acid acyl groups were detected in the carrot cell inositol phospholipids. Other ions which were commonly observed in the B/E linked scans were *m/z* 409 or 433 (lysophosphatidic acid), *m/z* 315 (inositol glycerophosphate), *m/z* 241 (dehydrated inositol phosphate), and *m/z* 153 (glycerophosphate). These fragment ions provided structural confirmation of the presence of the inositol ring, the phosphate group, the glycerol backbone, and the identity of the fatty acids. Finally, negative ion FABMS has been used to confirm the structures of carrot cell phosphatidylinositols, which had been previously only identified by chromatography.

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Contrasting Effects of Water-Soluble and Water-Insoluble Dietary Fibers on Bile Acid Conjugation and Taurine Metabolism in the Rat

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The effect of the type of dietary fiber on the bile acid and taurine metabolism was examined in rats. Diets containing 10% of various water-soluble fibers (citrus pectin, konjak mannan, guar gum) as compared to a fiber-free diet increased biliary excretion of total bile acids. In contrast, water-insoluble dietary fibers (cellulose, corn bran, chitin; 10% in the diets) as well as cholestyramine (5% in the diet) considerably decreased bile acid excretion. Water-soluble dietary fiber-mediated increases in bile acid excretion were totally attributable to increases in glycine-conjugates. Thus, these fibers greatly increased the bile acid glycine-to-taurine ratio (G/T). Excretion of glycine conjugates decreased more than that of taurine conjugates in rats fed various water-insoluble dietary fibers. As a result, G/T in rats fed water-insoluble fibers was significantly lowered as compared to G/T in animals fed a fiber-free diet. Cholestyramine did not affect the G/T ratio of bile acids. Fecal bile acid excretion and the activities of hepatic cholesterol 7α-hydroxylase (EC 1.14.13.17) in rats fed various water-soluble dietary fibers approximately doubled as compared to the respective values for rats fed a fiber-free diet. Whereas cholestyramine greatly increased these parameters, water-insoluble fibers did not significantly affect them. Various water-soluble fibers decreased hepatic concentration and urinary excretion of taurine as well as the activity of hepatic cysteine dioxygenase (EC 1.13.11.20). In contrast, water-insoluble fibers considerably increased hepatic taurine concentrations and enzyme activities. The parameters for taurine metabolism were unaffected by cholestyramine. It was suggested that the types of dietary fiber affected hepatic taurine synthesis and thus modified bile acid glycine/taurine ratios.
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A predominant portion of bile acids in mammalian species is conjugated with glycine or taurine before being excreted via the bile into the intestinal lumen (1-3). The ratio of glycine-to-taurine conjugated bile acids varies from one species to another (1,2,4,5). Although the rat has long been regarded as an exclusive taurine conjugator, there is some evidence (6,7) which indicates that this species can synthesize considerable amounts of glycine conjugated bile acids when the availability of taurine is limited. We have previously demonstrated a marked increase in the biliary excretion of glycine conjugated bile acids in rats fed pectin (8). This raises the interesting possibility that dietary pectin affects hepatic taurine metabolism, and thus modulates bile acid conjugation with glycine and taurine. In this context, in the present study we examined the effect of the type of dietary fiber, both water-soluble and water-insoluble, on bile acid conjugation and taurine metabolism in the rat.

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Abbreviations: EDTA, ethylenediaminetetraacetic acid; G/T, glycine-to-taurine ratio HPLC, high performance liquid chromatography; NADP, NAD phosphate.

MATERIALS AND METHODS

Animals. Male Wistar rats, obtained from Imamichi Institute of Animal Reproduction, Ibaraki, at 4 wk of age were used throughout the present study. The animals were housed individually in stainless steel mesh cages and were fed purified diets free from fiber sources or containing 10% (w/w) of defined types of dietary fiber. The composition of the purified experimental diet was (in wt %):casein, 20; corn oil, 5; corn starch, 15; mineral mixture, 3.5; vitamin mixture, 1; choline bitartrate, 0.2; and sucrose, up to 100. Dietary fibers were added to the experimental diet at the expense of sucrose. The compositions of mineral and vitamin mixtures were the same as described elsewhere (9). In the first experiment (Exp. 1) where the effects of water-soluble dietary fibers (citrus pectin, konjak mannan, and guar gum) and cholestyramine (5%, w/w) were examined, the animals were transferred to metabolic cages at day 15 of the feeding period and urine was collected for 2 days to measure taurine excretion. During this period, animals had continued free access to the experimental diet. The animals were then again transferred to and housed in the usual stainless steel mesh cages for the rest of the experimental period. In the second experiment (Expt. 2), the effects of water-insoluble dietary fibers (cellulose, corn bran, and chitin) were examined. Rats were fed experimental diets for 28-29 and 27-28 days for Expt. 1 and 2, respectively. Body weights of animals at the initiation of the experimental feeding period were 85-107 g and 108-125 g for Expt. 1 and 2, respectively. At the end of the experimental period, rats were anesthetized by intraperitoneal injection of Nembutal (50 mg/kg), and bile ducts were cannulated with PE-10 tubing at 8:30 a.m. to 10:30 a.m. (10). Bile was drained for 2 hr in a pre-weighed test tube cooled in ice. The rate of bile flow during this period was determined gravimetrically. Feces were collected for 2 days before termination of the experiments.

Assays for hepatic cholesterol 7α-hydroxylase and cysteine dioxygenase. After 2 hr of biliary drainage, rats were bled from the inferior vena cava and livers were quickly excised. About 3 g of each liver was homogenized in 20 mL of 0.25M sucrose, and microsomes were prepared according to the method of Kamath and Narayan (11). Microsomal cholesterol 7α-hydroxylase (EC 1.14.13.17) activities were assayed according to the method of Ogishima and Okuda (12) with the slight modifications described below. The mixture (0.4 mL)

containing 0.1 mM EDTA, 20 mM cysteamine, 5 mM $MgCl_2$, 8 mM glucose 6-phosphate, 1 mM NADP, 1 unit of glucose 6-phosphate dehydrogenase and microsomes (0.4–0.6 mg protein) in 100 mM potassium phosphate buffer (pH 7.4) was incubated at 37°C for 30 min. The enzyme reaction was terminated by the addition of 0.05 mL 5% (w/v) sodium cholate. Cholesterol oxidase (0.5 unit) dissolved in 0.02 mL 0.1M phosphate buffer (pH 7.4) was then added and the mixture was incubated at 37°C for an additional 60 min to convert 7α-hydroxycholesterol to 7α-hydroxy-4-cholestene-3-one. The product was extracted with hexane and assayed by normal phase HPLC using a Finepak SIL column (4.6 × 250 mm, JASCO Co., Ltd., Tokyo, Japan) under the conditions described elsewhere (12). Cysteine dioxygenase (EC 1.13.11.20) activity was assayed in the 15000 × g supernatant fraction of liver homogenates by the method of Sakakibara *et al.* (13) after anaerobic activation with the following modifications. The mixture (1.5 mL) in a test tube containing 10 mM cysteine and 1.3–1.8 mg enzyme protein in 0.1M glycine buffer (pH 9.0) was placed under a humidified gentle stream of nitrogen and incubated at 37°C for 30 min. One-mL aliquots of the mixture were then transferred to a 20 mL Erlenmeyer flask and incubated at 37°C for 20 min under air with shaking (100 strokes/min). The enzyme reaction was terminated by pouring the mixture into ice-cold 30% perchloric acid (0.25 mL). The denatured protein was sedimented by centrifugation, and the supernatant was neutralized with 3M K_2CO_3. The precipitate formed was removed by centrifugation, and the supernatant was applied onto a small column (0.5 × 4 cm) of Dowex 50W-X8 (100–200 mesh, H^+-form). The column was washed with 4 mL of water. Cysteine sulfinic acid, an enzyme product of cysteine dioxygenase, in the eluate was derivatized with *o*-phthalaldehyde (14) and analyzed by reversed phase HPLC using a Finepak SIL $C_{18}S$ column (4.6 × 150 mm, JASCO) with a mobile phase of acetonitrile/water (30:70, v/v; 25 mM sodium phosphate buffer, pH 6.0) at a flow rate of 0.4 mL/min and was detected with a fluorometer at 395 nm (excitation) and 455 nm (emission).

Analysis for bile acids. Biliary bile acids were extracted and purified using a commercial octadecylsilyl silica column (Sep-Pak C_{18}, Waters Associate, Milford, MA). Bile acids were fractionated according to the mode of conjugation using a 0.5 × 1.8 cm column of piperidinohydroxydextran gel (Shimadzu Corp., Kyoto, Japan) and quantified by gas-liquid chromatography as described previously (9,15). Lithocholic, deoxycholic, chenodeoxycholic, cholic and β-muricholic acids were well resolved by gas-liquid chromatography. However, there was poor separation between hyodeoxycholic and ursodeoxycholic acid. Also, α-muricholic, ω-muricholic and 7-ketodeoxycholic acids co-migrated and were not separated well on the chromatogram. Fecal acidic and neutral steroids were also determined by gas-liquid chromatography (8,10,15).

Analyses for taurine and glutathione. Protein-free samples of liver and serum were prepared as described previously (16). Taurine in the sample was purified using a cation/anion exchange column prepared by layering 0.5 × 2 cm of Dowex 50W-X8 (100–200 mesh, H^+-form) over 0.5 × 2 cm of Dowex 2-X8 (200–400 mesh, Cl^--form) (17). Three-mL eluates were lyophilized to remove Cl^- ions, which interfere with the subsequent derivatization of taurine with *o*-phthalaldehyde (18), and were redissolved in 2–4 mL of water. Hepatic and serum taurine derivatized with *o*-phthalaldehyde was analyzed by HPLC using a Finepak SIL $C_{18}S$ column and a mobile phase of acetonitrile/water (70:30, v/v; 12.5 mM sodium phosphate buffer, pH, 6.0) at a flow rate of 0.4 mL/min as described elsewhere (14). Taurine in the urine sample, when analyzed similarly as described above, was found to be degraded in the process of lyophilization. Therefore, clean-up of the sample was carried out using a Dowex 50W-X8 column (0.5 × 4 cm) and the effluent was directly analyzed by HPLC as described about using a modified mobile phase (acetonitrile/water, 20:80, v/v; 50 mM sodium phosphate buffer, pH 6.0). Hepatic glutathione in the deproteinized sample was assayed enzymatically as described elsewhere (19).

Materials. Dietary fibers were obtained from the following sources: citrus pectin (a polysaccharide in which D-galacturonic acid is a principal constituent) from Wako Chemicals, Osaka, Japan; konjak mannan (a polymer of mannose with glucose in the side chains) from Japan Konjak Association, Gunmma, Japan; guar gum (a polymer of mannose with galactose in the side chains) from Nissin Seifun Co., Tokyo, Japan; cellulose (a long linear polymer of 1,4-linked glucose units) from Asahi Chemical Industry Co., Tokyo, Japan; corn bran containing 85% neutral detergent fiber (60% hemicellulose and 25% cellulose) from Japan Maize Products Co. Ltd., Tokyo, Japan; and chitin (a polymer of *N*-acetyl-D-galactosamin) from NFI Laboratories, Yaizu, Japan. Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, glutathione reductase and NADP were purchased from Oriental Yeast Co., Tokyo, Japan. Cholesterol oxidase was from Toyobo Co., Osaka, Japan. The 7α-hydroxycholesterol was prepared from 7-ketocholesterol (Green Cross Co., Tokyo, Japan) by the method of Malinow *et al.* (20). *o*-Phthalaldehyde was from Nacalai Tesque Inc., Kyoto, Japan. Other chemicals used were of reagent grade.

Statistical analysis. Values were analyzed by one-way analysis of variance, and differences of means were inspected using Duncan's multiple range test (21). Data in each table are presented as means ± SE of 7 or 8 rats.

RESULTS

As shown in Table 1, the body weights of rats fed guar gum were considerably less than those fed a fiber-free diet (Expt. 1). Retardation of growth was apparently due to reduced consumption of the experimental diet in these animals (12.8 ± 0.3 g/day and 18.4 ± 0.6 g/day for rats fed guar gum and the fiber-free diet, respectively). The liver weights of rats fed guar gum were also considerably less than those fed a fiber-free diet. The body and liver weights of animals fed other types of dietary fiber, both water-soluble and water-insoluble, as well as cholestyramine, were approximately the same as those of rats fed a fiber-free diet. Various water-soluble dietary fibers not only enhanced bile flow

TABLE 1

Effects of Type of Dietary Fiber on Concentration and Excretion of Biliary Bile Acids

	Expt. 1				
	Fiber-free	Pectin	Konjak Mannan	Guar gum	Cholestyramine
Body weight (g)	314 ± 9[a,b]	294 ± 5[b]	303 ± 4[b]	234 ± 4	317 ± 5[b]
Liver weight (g/100 g bw)	5.58 ± 0.17[b]	5.09 ± 0.11	5.14 ± 0.11	4.70 ± 0.13	5.38 ± 0.08[b]
Bile flow (mL/hr)	0.90 ± 0.05	1.02 ± 0.11	1.52 ± 0.12[c,d,e]	1.35 ± 0.10[c,d]	0.74 ± 0.04
Bile acid					
Concentration (mg/mL)					
Total	5.72 ± 0.53	9.56 ± 1.51[d]	10.8 ± 0.7[c,d]	9.88 ± 1.12[d]	3.94 ± 0.31
Non-conjugated	0.14 ± 0.02	0.39 ± 0.06	0.86 ± 0.33[c,d]	0.93 ± 0.14[c,d]	0.07 ± 0.01
Glycine conjugated	1.26 ± 0.25	7.40 ± 0.89[c,d]	8.43 ± 0.68[c,d]	7.91 ± 1.02[c,d]	0.99 ± 0.24
Taurine conjugated	4.33 ± 0.61	1.77 ± 0.66[c]	1.47 ± 0.20[c]	1.04 ± 0.07[c,d]	2.89 ± 0.27
Excretion (mg/hr)					
Total	5.08 ± 0.37	8.73 ± 0.38[d]	16.1 ± 1.3[c,d,e]	13.4 ± 1.6[c,d,e]	2.86 ± 0.20
Non-conjugated	0.13 ± 0.02	0.38 ± 0.07	1.48 ± 0.68[d]	1.32 ± 0.25	0.05 ± 0.01
Glycine conjugated	1.18 ± 0.27	6.93 ± 0.37[c,d]	12.5 ± 0.8[c,d,e]	10.6 ± 1.3[c,d,e]	0.74 ± 0.19
Taurine conjugated	3.78 ± 0.41	1.42 ± 0.25[c]	2.18 ± 0.26[c]	1.43 ± 0.15[c]	2.07 ± 0.08[c]
G/T	0.353 ± 0.101	5.75 ± 0.84[c,d]	6.34 ± 1.01[c,d]	7.49 ± 0.70[c,d]	0.368 ± 0.10

	Expt. 2			
	Fiber-free	Cellulose	Corn bran	Chitin
Body weight (g)	335 ± 7	344 ± 4	336 ± 6	330 ± 4
Liver weight (g/100 b bw)	5.31 ± 0.08	5.10 ± 0.05	5.22 ± 0.09	5.15 ± 0.07
Bile flow (mL/hr)	0.86 ± 0.13	0.74 ± 0.04	0.68 ± 0.05	0.90 ± 0.05
Bile acid				
Concentration (mg/mL)				
Total	7.66 ± 0.73	5.16 ± 0.32	6.40 ± 0.93	5.81 ± 0.42
Non-conjugated	0.17 ± 0.04	0.05 ± 0.01[c]	0.04 ± 0.00[c]	0.11 ± 0.02
Glycine conjugated	2.02 ± 0.09	0.69 ± 0.14[c]	0.43 ± 0.08[c]	0.89 ± 0.16[c]
Taurine conjugated	5.47 ± 0.66	4.41 ± 0.42	5.94 ± 0.94	4.81 ± 0.49
Excretion (mg/hr)				
Total	6.23 ± 0.63	3.81 ± 0.27[c]	4.13 ± 0.46[c]	5.12 ± 0.24
Non-conjugated	0.16 ± 0.06	0.04 ± 0.01[c]	0.02 ± 0.00[c]	0.10 ± 0.02
Glycine conjugated	1.69 ± 0.21	0.52 ± 0.12[c]	0.29 ± 0.07[c]	0.80 ± 0.14[c,f]
Taurine conjugated	4.38 ± 0.42	3.25 ± 0.32	3.82 ± 0.46	4.22 ± 0.30
G/T	0.384 ± 0.034	0.181 ± 0.053[c]	0.086 ± 0.022[c]	0.202 ± 0.045[c]

[a]Means ± SE of 7 or 8 rats.
[b]Significantly different from rats fed guar gum at $p<0.05$.
[c]Significantly different from rats fed a fiber-free diet at $p<0.05$.
[d]Significantly different from rats fed cholestyramine at $p<0.05$.
[e]Significantly different from rats fed pectin at $p<0.05$.
[f]Significantly different from rats fed corn bran at $p<0.05$.

but also increased bile acid concentration in the bile (Expt. 1). As a consequence, hourly excretion of bile acids increased in all groups of rats fed various water-soluble dietary fibers. The increases were due to increases in glycine conjugates. By contrast, biliary concentrations and excretion of taurine conjugates were significantly decreased by the three types of water-soluble dietary fibers. Accordingly, glycine conjugated bile acids became prominent (80%) in biliary bile acids of rats fed various water-soluble dietary fibers, and glycine-to-taurine ratios in these animals were increased 15–25 times above the value found for animals fed a fiber-free diet. On the other hand, cholestyramine decreased the biliary bile acid concentration and excretion considerably but insignificantly. The resin did not affect the glycine-to-taurine ratio of bile acids. Various water-insoluble dietary fibers tended to decrease the biliary bile acid concentration. As cellulose and corn bran tended to decrease also the bile flow of animals, the hourly excretion of bile acids in rats fed these dietary fibers became significantly lower than in animals fed a fiber-free diet (Expt 2.). All three types of water-insoluble dietary fiber significantly reduced the concentration and excretion of glycine conjugated bile acids. The extent of reduction was most prominent with rats fed corn bran. On the other hand, the water-insoluble dietary fibers did not significantly modify the biliary concentration and excretion of taurine conjugates. As a result, the bile acid glycine-to-taurine ratios significantly decreased in rats fed the various types of water-insoluble dietary fiber.

Biliary bile acid composition of glycine and taurine conjugated fractions resembled each other for the various dietary treatments (data not shown). Lithocholic acid was not detectable in the biliary bile acids. Although 7-ketodeoxycholic acid (a bile acid of the cholic acid group) co-migrated with α- and ω-muricholic acids (bile acids of the chenodeoxycholic acid group), under

the conditions used for gas-liquid chromatography, the amount of this bile acid appeared to be minimal (22). The cholic acid group (deoxycholic and cholic acids) vs chenodeoxycholic acid group (chenodeoxycholic, hyodeoxycholic, ursodeoxycholic, α-muricholic, β-muricholic and ω-muricholic acids) ratios for rats fed a fiber-free diet were 0.455 ± 0.052 and 0.438 ± 0.053 for Expts. 1 and 2, respectively. The ratios for rats fed cholestyramine (0.850 ± 0.022) and corn bran (0.777 ± 0.064) were significantly higher than for the animals fed a fiber-free diet. Other dietary fibers did not modify this parameter (data not shown).

Table 2 shows the hepatic taurine, glutathione and serum taurine concentrations as well as hepatic cysteine dioxygenase activities at time of sacrifice (28–29 and 27–28 days of the feeding period for Expts. 1 and 2, respectively). In Expt. 1, daily taurine excretion into the urine collected during the 15–17 days of the feeding period was also determined. Although a difference of hepatic taurine concentration between rats fed guar gum and rats fed a fiber-free diet was statistically insignificant, the parameters decreased considerably in all groups of rats fed different types of water-soluble dietary fiber (Expt. 1). The value for rats fed cholestyramine was approximately the same as that for animals fed a fiber-free diet. Hepatic glutathione concentrations in rats fed konjak mannan and guar gum were insignificantly but considerably higher than found in animals fed a fiber-free diet. On the other hand, among the five groups of rats, this parameter was lowest in rats fed pectin. Urinary taurine excretion in rats fed various types of water-soluble dietary fiber decreased to about one-half of that in rats fed a fiber-free diet, while cholestyramine feeding did not affect urinary taurine excretion. Various types of water-soluble dietary fiber severely depressed the activities of hepatic cysteine dioxygenase, a rate-limiting enzyme in hepatic taurine synthesis (23). The enzyme activity in rats fed cholestyramine was similar to that in animals fed a fiber-free diet. Hepatic taurine concentrations in all groups of rats fed various water-insoluble dietary fibers were significantly higher than were observed in animals fed a fiber-free diet (Expt. 2). The response was most pronounced with rats fed corn bran. Hepatic glutathione concentration in rats fed chitin was significantly higher than in the other three groups. The activity of cysteine dioxygenase in rats fed chitin was significantly higher than in rats fed a fiber-free diet. Although the differences were statistically insignificant, the values for rats fed cellulose and corn bran were also considerably higher than those in animals fed a fiber-free diet. Various dietary fibers, both water-soluble and water-insoluble, as well as cholestyramine, did not affect serum taurine concentrations.

Since three types of water-soluble dietary fiber approximately doubled the weight of feces excreted without influencing the concentration of bile acids, the daily excretion of bile acids also approximately doubled (Table 3, Expt. 1). As expected, cholestyramine feeding increased fecal bile acid concentration and excretion. The fecal concentrations of bile acids in rats fed cellulose, corn bran and chitin were much lower than those observed in rats fed a fiber-free diet. As the daily excretion of bile acids in rats fed these water-insoluble fibers was approximately the same as that in rats fed a fiber-free diet, the decreases were apparently due to the dilution of bile acids by the increased volume of feces in these rats. Although the water-soluble dietary fibers, except for guar gum and cholestyramine, slightly decreased the fecal concentration of neutral steroids, the daily excretion of neutral steroids was apparently increased by these fibers and by cholestyramine (Expt. 1). As in the case of bile acids, three types of water-insoluble dietary fiber significantly reduced fecal neutral steroid concentrations. Cellulose and chitin, but not corn bran, also decreased the daily neutral steroid excretions slightly (Expt. 2).

TABLE 2

Effects of Type of Dietary Fiber on Taurine Metabolism

Dietary fibers	Hepatic taurine (μmol/g)	Hepatic glutathione (μmol/g)	Serum taurine (μmol/dl)	Urinary taurine (μmol/day)	Cysteine dioxygenase (nmol/min/mg)
Expt. 1					
Fiber-free	1.91 ± 0.16[a]	3.95 ± 0.35	23.7 ± 2.2	0.789 ± 0.137	7.95 ± 1.53
Pectin	1.26 ± 0.12[b]	3.28 ± 0.33[c]	17.2 ± 1.8	0.297 ± 0.068[b,c]	1.82 ± 0.39[b,c]
Konjak Mannan	1.14 ± 0.11[b,c]	5.21 ± 0.35[d]	18.2 ± 1.6	0.328 ± 0.074[b,c]	1.85 ± 0.37[b,c]
Guar gum	1.44 ± 0.04	4.76 ± 0.44[d]	20.1 ± 1.6	0.376 ± 0.037[b,c]	1.76 ± 0.39[b,c]
Cholestyramine	1.78 ± 0.22	5.60 ± 0.31[b]	18.0 ± 1.3	0.788 ± 0.098	10.2 ± 1.0
Expt. 2					
Fiber-free	2.47 ± 0.17	5.08 ± 0.20[e]	26.0 ± 1.2	—	5.22 ± 0.98
Cellulose	3.29 ± 0.17[b,f]	5.62 ± 0.18[e]	26.6 ± 1.9	—	7.12 ± 0.76[e]
Corn bran	5.50 ± 0.56[b]	5.48 ± 0.31[e]	26.5 ± 1.5	—	8.49 ± 1.28
Chitin	3.86 ± 0.25[b]	6.65 ± 0.16	25.1 ± 1.0	—	12.4 ± 1.6[b]

[a]Means ± SE of 7 or 8 rats.
[b]Significantly different from rats fed a fiber-free diet at $p<0.05$.
[c]Significantly different from rats fed cholestyramine at $p<0.05$.
[d]Significantly different from rats fed pectin at $p<0.05$.
[e]Significantly different from rats fed chitin at $p<0.05$.
[f]Significantly different from rats fed corn bran at $p<0.05$.

TABLE 3

Effects of Type of Dietary Fiber on Fecal Steroid Excretion and Activities of Hepatic Cholesterol 7α-hydroxylase

Dietary fibers	Feces weight (g/day)	Acidic steroid		Neutral steroid		Cholesterol 7α-hydroxylase (pmol/min/mg)
		Concentration (mg/g)	Excretion (mg/day)	Concentration (mg/g)	Excretion (mg/day)	
Expt. 1						
Fiber-free	0.41 ± 0.03[a]	8.38 ± 0.75[b]	3.27 ± 0.36	18.5 ± 1.4[b]	7.40 ± 0.59	29.3 ± 2.6[b]
Pectin	0.73 ± 0.03[c,b]	10.0 ± 0.8[b]	7.27 ± 0.53[b,c]	14.4 ± 0.7[c]	10.5 ± 0.7[b]	65.7 ± 5.6[b]
Konjak Mannan	0.71 ± 0.06[a,b]	8.50 ± 0.43[b]	6.03 ± 0.58[b,c]	16.3 ± 0.5[b]	11.7 ± 1.2[c]	78.7 ± 5.6[b,c]
Guar gum	0.65 ± 0.05[c,b]	10.8 ± 0.9[b]	7.28 ± 0.60[b,c]	19.0 ± 0.6[b,d]	12.3 ± 0.8[c]	74.4 ± 11.1[b,c]
Cholestyramine	1.10 ± 0.03[c]	16.2 ± 0.6	17.8 ± 0.70[c]	12.9 ± 0.4	14.1 ± 0.5[c]	188 ± 18[c]
Expt. 2						
Fiber-free	0.42 ± 0.03	9.90 ± 0.50	4.10 ± 0.31	11.8 ± 0.6	4.81 ± 0.21	40.6 ± 3.6
Cellulose	2.83 ± 0.08[c]	1.43 ± 0.11[c]	4.08 ± 0.41	1.18 ± 0.05[c]	3.35 ± 0.15[c,e]	47.3 ± 4.7
Corn bran	2.76 ± 0.07[c]	1.47 ± 0.10[c]	4.05 ± 0.24	1.76 ± 0.08[c]	4.84 ± 0.22	60.8 ± 3.5[c]
Chitin	3.03 ± 0.09[c]	1.06 ± 0.12[c]	3.17 ± 0.32	1.33 ± 0.06[c]	4.02 ± 0.19[c,e]	44.4 ± 4.0[e]

[a]Means ± of 7 or 8 rats.
[b]Significantly different from rats fed cholestyramine at $p<0.05$.
[c]Significantly different from rats fed a fiber-free diet at $p<0.05$.
[d]Significantly different from rats fed pectin at $p<0.05$.
[e]Significantly different from rats fed corn bran at $p<0.05$.

The activities of hepatic cholesterol 7α-hydroxylase, the rate-limiting enzyme of bile acid synthesis (24), more than doubled in rats fed various water-soluble dietary fibers. However, the types of water-soluble dietary fiber did not affect this value. The enzyme activity in rats fed cholestyramine was approximately 6-times higher than was observed in rats fed a fiber-free diet. Among the three types of water-insoluble dietary fiber, corn bran only slightly increased cholesterol 7α-hydroxylase activity.

DISCUSSION

We have previously demonstrated a large increase in glycine conjugation, and a concomitant decrease in taurine conjugation of biliary and luminal bile acids in rats fed a diet containing 10% pectin (8). The present study demonstrates that an increase in glycine conjugation of bile acids occurs in rats fed water-soluble dietary fibers, but not in animals fed water-insoluble dietary fibers (Table 1).

A significant portion of bile acids in rats is conjugated with taurine under most conditions (1,2,4,5). The specificity of the enzyme which catalyzes bile acid conjugation with amino acids (25) and active taurine synthesis (26,27) both contribute to this process. However, there is some evidence (6,7) that the rat synthesizes a considerable amount of glycine conjugated bile acids when the availability of taurine is limited. Reciprocal relationships between bile acid glycine-to-taurine ratios (Table 1) and hepatic taurine concentrations (Table 2) in rats fed various types of dietary fiber support the view that the availability of taurine in the liver is responsible for the effect of dietary fiber on the distribution of bile acid between glycine and taurine conjugates.

The amount of taurine in tissue can be modified by changes in several metabolic processes. These include the rate of synthesis and the availability of sulfur amino acids as the precursors as well as transport from other tissues (28,29). As sulfur amino acids are toxic (30,31), availability of cysteine as precursor for hepatic taurine synthesis is probably dependent on the larger pool of glutathione (23,30,32-34). In the present study, alterations in hepatic taurine concentrations in rats fed various types of water-soluble as well as water-insoluble dietary fiber did not necessarily parallel hepatic glutathione concentrations. Thus, the availability of sulfur amino acids could not account for changes in the concentration of hepatic taurine and thus of bile acid conjugation in rats fed various types of dietary fiber. Since the various dietary fibers did not alter serum taurine concentration, it is unlikely that the types of dietary fiber affected hepatic taurine uptake and thus modulated tissue taurine concentration. Alternatively, the close relationship between the activities of hepatic cysteine dioxygenase and hepatic taurine concentrations (Table 2) suggests that different types of dietary fiber affect hepatic taurine synthesis and thus modulate bile acid conjugation. Decreased urinary taurine excretions in rats fed various water-soluble dietary fibers may be the reflection of decreased taurine synthesis in the liver (23,28).

The mechanism(s), by which water-soluble and water-insoluble dietary fibers causes different responses in hepatic taurine synthesis and concentration, and thus in bile acid conjugation, is not clear at present. As water-soluble and water-insoluble dietary fibers caused contrasting effects on the rates of biliary bile acid excretion, it is plausible that differences in the quantitative aspects of bile acid metabolism in rats fed different fibers in some way affected hepatic taurine metabolism and thus modified bile acid glycine-to-taurine ratios. However, in spite of the fact that cholestyramine insignificantly but considerably decreased biliary bile acid excretion, as did the water-insoluble dietary fibers, the resin did not modify the bile acid glycine-to-taurine ratio. Thus, differences in the bile acid metabo-

lism alone do not account for different responses in bile acid conjugation in rats fed the different types of dietary fiber.

Modification of fecal bile acid excretion is presumed to reflect alteration of hepatic bile acid synthesis (35). Although analyses of fecal bile acids were done with only one fecal collection, the observed responses of fecal bile acid excretion to different fibers and cholestyramine generally paralleled hepatic cholesterol 7α-hydroxylase activities. It is apparent that the alterations of biliary bile acid output during the 2-hour bile collection, induced by dietary fiber and cholestyramine, do not necessarily reflect different rates of hepatic bile acid synthesis. The observation with rats fed cholestyramine supports this idea (Tables 1 and 3). It has been observed that an increased bile acid pool in the enterohepatic circulation system is associated with increased biliary bile acid output in the rat (8,15,36). We have previously observed that dietary pectin not only enhanced the biliary bile acid excretion but also increased the luminal pool of bile acids (8). Thus, it is likely that the types of dietary fiber affect the bile acid pool in the enterohepatic circulation system, and thus modify biliary bile acid excretion. A longer period of bile collection or analysis of luminal bile acids may provide a more definitive conclusion with regard to the effects of different fibers on the bile acid pool in the rat.

In conclusion, types of dietary fiber modified the partition of bile acids between glycine and taurine conjugation. Alterations in hepatic concentrations of taurine, through changes in the activity of hepatic cysteine dioxygenase and thus in taurine synthesis, are most likely responsible for this modification.

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METHODS

LCAT Inhibitors Interfere with the Enzymatic Determination of Cholesterol and Triglycerides

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5,5-Dithio*bis*-(2-nitrobenzoic acid) (DTNB) and *p*-chloromercuriphenylsulfonic acid (PCMPS) are well-known sulfhydryl inhibitors that are used to inhibit lecithin: cholesterol acyltransferase (LCAT). They were each found to interfere with the enzymatic assays of cholesterol and triglycerides. DTNB falsely reduced the measured plasma cholesterol content, and falsely increased triglyceride readings. The interference with the triglyceride assay could be largely prevented by blanking for glycerol. PCMPS had only a slight effect on the cholesterol assay, but falsely lowered the triglyceride readings to a great extent, even with glycerol-blanking. Thus, these inhibitors should be avoided when plasma samples are to be enzymatically analyzed for cholesterol or triglycerides.
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Lecithin:cholesterol acyltransferase (LCAT, EC 2.3.1.43) is an enzyme which catalyzes the transfer of fatty acids from lecithin to cholesterol, a process thought to take place on the surface of plasma lipoproteins (1). 5,5-Dithio*bis*-(2-nitrobenzoic acid) (DTNB) and *p*-chloromercuriphenylsulfonic acid (PCMPS) are sulfhydryl inhibitors of LCAT (2), and are often added to plasma samples to prevent *ex vivo* changes in the cholesteryl ester content of lipoproteins. These same plasma samples may then be analyzed for total cholesterol and triglyceride levels.

In preparing lipoproteins for analysis by gel filtration according to Rudel *et al.* (3), we found that the addition of DTNB to blood samples seemed to alter both the apparent cholesterol and triglyceride levels in the samples, but in opposite directions. Therefore, we have systematically studied the effects of DTNB (and PCMPS) on measured cholesterol and triglyceride levels as they are determined enzymatically.

METHODS

Fasting plasma samples (EDTA, 1 mg/mL) were obtained from hyperlipidemic patients and from normal volunteers. Two pools were prepared: a "high pool" (cholesterol 315 mg/dL and triglyceride 317 mg/dL) and a "low pool" (cholesterol 196 mg/dL and triglyceride 109 mg/dL). Increasing amounts of saline or a solution containing DTNB were added to one-mL aliquots from each of these pools which were then analyzed in triplicate. The DTNB solution contained the following: EDTA (0.12 M), DTNB (40 mM), and NaN_3 (61.5 mM), and when added to whole blood (0.25 mL/mL blood) produced a DTNB concentration of 0.4 mg/mL (3). The final concentration of DTNB in plasma would be approximately twice that amount (or 0.8 mg/mL) since DTNB is excluded from red blood cells. To achieve typical plasma levels, 50 μL of the DTNB solution would need to be added to one mL of plasma. Hence, in this experiment, the effects of 0-100 μL/mL of DTNB were tested.

In an experiment to test the effects of DTNB on triglyceride analyses when using a glycerol blank, four pools of plasma were prepared with triglyceride levels ranging from 73 to 330 mg/dL. These were analyzed for triglycerides after the addition of 50 μL of either saline or DTNB. To test the effects of PCMPS, three pools of plasma were prepared with or without PCMPS at a final concentration of 2 mM. The triglyceride contents of these pools were 88, 180 and 269 mg/dL.

Cholesterol and triglyceride levels were analyzed by the enzymatic methods of Allain *et al.* (4) and Bucolo and David (5), respectively, on an ABA 200 Bichromatic Analyzer (Abbott Diagnostics, Irving, Texas) (6) using reagents provided by Abbott (cholesterol, #6095; triglyceride, #6097). The triglyceride assays were run with and without glycerol-blanking using Abbott triglyceride reagents which omitted the lipase (#6087-03).

RESULTS

Increasing amounts of DTNB were added to plasma samples containing high and low concentrations of cholesterol and triglycerides. The DTNB consistently lowered the measured cholesterol levels (Fig. 1) and raised the reported triglyceride levels (Fig. 2). Table 1 shows the absolute and percentage changes in both of these analytes which resulted when recommended working concentrations of DTNB (0.8 mg/mL plasma) were added to the high and low plasma pools.

For the cholesterol assay, the absolute changes caused by the addition of DTNB were different for the high and low pools, but the percentage reductions were similar (11-13%) in both pools. DTNB falsely increased the triglyceride readings by the same absolute amount (68-69 mg/dL) in both the high and low triglyceride pools.

TABLE 1

The Influence of DTNB on the Enzymatic Analysis of Plasma Cholesterol and Triglyceride[a] (mg/dL)

	Cholesterol		Triglyceride	
	Low pool	High pool	Low pool	High pool
Control	189 ± 3	307 ± 9	105 ± 1	289 ± 4
DTNB	165 ± 6	274 ± 5	174 ± 3	357 ± 5
Absolute change	−24	−33	+69	+68
% Change	−13%	−11%	+66%	+24%

[a]Fifty μL of a DTNB solution (15.8 mg/mL) or a saline control was added to 1 mL plasma samples and analyzed in triplicate.

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Abbreviations: DTNB, 5,5-dithio*bis*-(2-nitrobenzoic acid); PCMPS, *p*-chloromercuriphenylsulfonic acid; LCAT, lecithin:cholesterol acyltransferase.

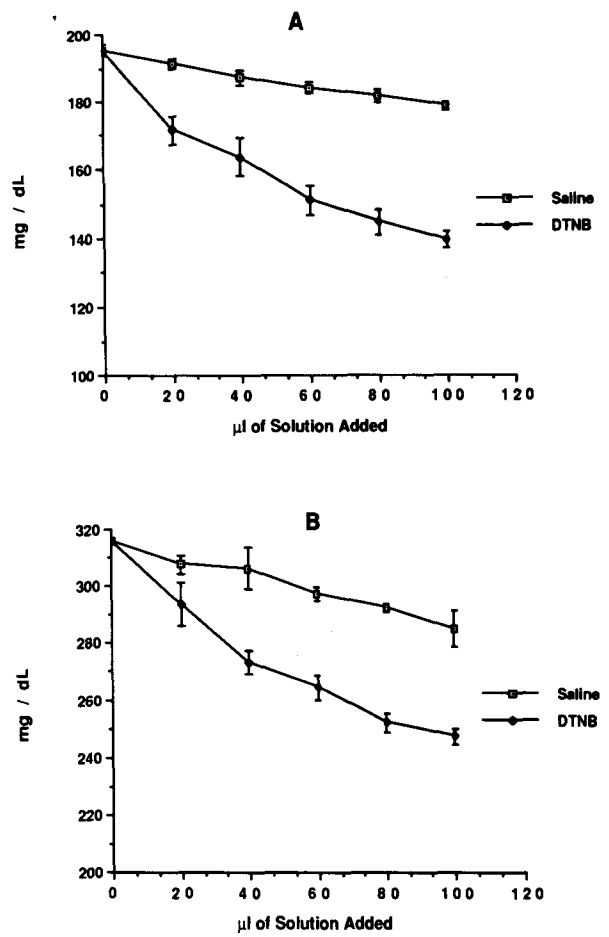

FIG. 1. Effect of DTNB on cholesterol measurements: A, low pool; B, high pool. The indicated amounts of saline or DTNB solutions were added to 1-mL plasma samples which were then assayed for cholesterol. Each point represents the mean of triplicate determinations (±S.D.).

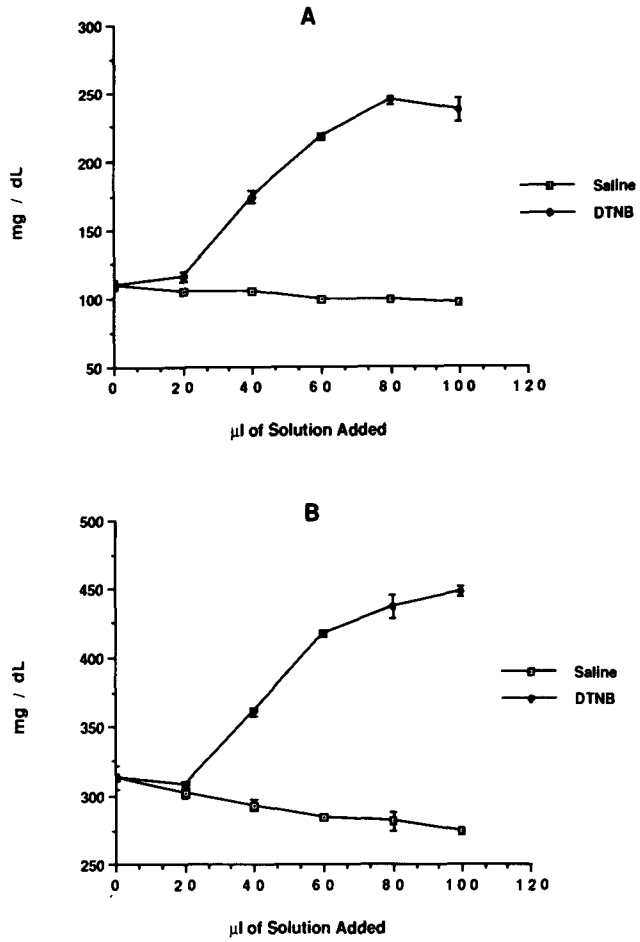

FIG. 2. Effect of DTNB on triglyceride measurements: A, low pool; B, high pool. The indicated amounts of saline or DTNB solutions were added to 1 mL-plasma samples which were then assayed for triglycerides. Each point represents the mean triplicate determinations (±S.D.).

TABLE 2

Effects of Glycerol-Blanking on the DTNB-Induced Interference with Enzymatic Triglyceride Assay

	Pool 1	Pool 2	Pool 3	Pool 4
Control-blanked	73 ± 0.4	160 ± 1	240 ± 1	330 ± 4
DTNB-blanked	83 ± 2[a]	175 ± 3[a]	254 ± 4[a]	350 ± 6[a]
DTNB-unblanked	178 ± 1[a]	293 ± 1[a]	392 ± 4[a]	499 ± 5[a]

[a]Value significantly different from the value about it, $p < 0.05$.

TABLE 3

Effects of PCMPS (2 mM) on Enzymatic Cholesterol and Triglyceride Analyses

	Pool 1	Pool 2	Pool 3
Cholesterol			
Control	60 ± 0.5	116 ± 0.5	174 ± 0.8
PCMPS	62 ± 0.5	119 ± 0.0	177 ± 0.5
Triglyceride[a]			
Control	88 ± 0.4	180 ± 1	269 ± 1
PCMPS	20 ± 1.0[b]	38 ± 1[b]	64 ± 2[b]

[a]Measured with glycerol-blanking.

[b]Value significantly different than control, $p < 0.01$.

Glycerol-blanking the triglyceride assay largely, but not completely, removed the adverse influence of DTNB (Table 2). Even with blanking, DTNB still caused a significant 10 to 20 mg/dL (14 to 6%) increase in triglyceride readings compared to control, glycerol-blanked samples.

The addition of PCMPS (Table 3) increased cholesterol readings only slightly (2-3 mg/dL), but it had a marked influence on triglyceride readings lowering them by 76 to 78%, even with glycerol-blanking.

DISCUSSION

DTNB and PCMPS are inhibitors of LCAT which are commonly added to plasma samples. Frequently the cholesterol and triglyceride levels in these same plasma samples are determined using enzymatic methods. We report here that both of these agents, in concentrations commonly used in plasma samples (7-10), interfere with a common enzymatic assay used for determination of these lipids. DTNB falsely reduced the cholesterol, and falsely increased the triglyceride readings. PCMPS only minimally affected the cholesterol assay, but markedly lowered the triglyceride results. For DTNB, the reduction in cholesterol was proportional to the mass of

cholesterol present, whereas the increase in triglyceride reading was constant and unrelated to the amount of triglyceride present. For PCMPS, the decrease in triglycerides was proportional to the triglyceride present in the assay.

In order to understand the possible mechanisms by which DTNB (the more commonly used LCAT inhibitor) affected these reactions, the specific steps of the enzymatic procedures must be understood. In the cholesterol assay (4), plasma cholesteryl esters are first hydrolyzed to free cholesterol by cholesterol ester hydrolase. The free cholesterol produced is oxidized by cholesterol oxidase to cholest-4-en-3-one with simultaneous production of hydrogen peroxide which oxidatively couples with 4-aminoantipyrine and phenol in the presence of peroxidase to yield a quinoneimine dye with an absorption maximum at 500 nm. The intensity of color produced is directly proportional to the total cholesterol content of the sample. DTNB may interfere with this assay by reacting with the hydrogen peroxide produced in the cholesterol oxidase reaction. This side reaction would reduce the amount of H_2O_2 available for oxidative coupling with 4-aminoantipyrine and phenol, reducing the amount of quinoneinmine dye produced, thus reducing the observed cholesterol content. This would be consistent with our observation that DTNB caused the same proportional decrease in cholesterol levels in the two pools studied.

The triglyceride assay employs a microbial lipase to completely hydrolyze triglycerides to free glycerol and free fatty acids. The liberated glycerol is then converted, by glycerol kinase in the presence of ATP, to glycerol-1-phosphate plus ADP. Pyruvate kinase, in the presence of ADP and phosphoenolpyruvate, produces ATP plus pyruvate which is then acted upon by lactate dehydrogenase in the presence of NADH to produce lactate plus NAD^+. The disappearance of NADH observed at 340 nm is a stoichiometric measure of the glycerol present, and thus, the triglyceride content of the sample. DTNB may affect this reaction sequence by reacting directly with NADH to produce NAD^+ and a sulfide compound. This would cause NADH levels to decrease more rapidly than they should causing falsely high triglyceride levels to be reported. This explanation fits with our observation that the increase in triglyceride levels was constant and not related to the mass of triglyceride present. It also is consistent with the observed (near) normalization of triglyceride readings by glycerol-blanking. The fact that glycerol-blanking did not completely correct the interference suggests that DTNB may affect more than one step in the triglyceride reaction.

In conclusion, it has been shown that at least two LCAT inhibitors adversely affect common enzymatic methods used for lipid analysis. As alternatives to the Allain *et al.* procedure (4), non-enzymatic, colorimetric methods such as the Abell *et al.* (11) or the *o*-phthalaldehyde method of Rudel and Morris (12) are not influenced by DTNB and may be used. The DTNB interference with the triglyceride assay was largely, but not completely, removed by glycerol-blanking. PCMPS only minimally affected the enzymatic determination of cholesterol, but it interfered markedly with the triglyceride assay even after glycerol-blanking. Thus, neither DTNB nor PCMPS should be present in the sample during triglyceride analysis by this enzymatic method.

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Purification of Plasmalogens using *Rhizopus delemar* Lipase and *Naja naja naja* Phospholipase A_2

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Bovine heart ChoGpl (choline glycerophospholipid) and bovine brain EtnGpl (ethanolamine glycerophospholipid) contain diacyl, alkenylacyl and alkylacyl analogs. Purification of plasmalogens was achieved using *R. delemar* lipase and *N. naja naja* phospholipase A_2 digestion. The *R. delemar* lipase hydrolyzes the acyl bond at the 1-position of 1,2-diacyl glycerophospholipids. The *N. naja naja* phospholipase A_2 has greater activity with diacyl and alkylacyl than with alkenylacyl glycerophospholipids. These enzymes were mainly used to remove diacyl and alkylacyl analogs respectively. When the diacyl types were removed by double incubation with *R. delemar lipase,* the plasmalogen content was 94.2% ± 0.21% (mean ± S.E.M., n = 4) for PlsCho (plasmenylcholine) and 94.9% ± 0.19% (mean ± S.E.M., n = 3) for PlsEtn (plasmenylethanolamine). Recoveries were 74% and 88% respectively. These partially purified plasmalogens were treated with *N. naja naja* phospholipase A_2. Finally, 97.7% ± 0.24% (mean ± S.E.M., n = 4) and 98.8% ± 0.27% (mean ± S.E.M., n = 3) pure plasmalogens were obtained for PlsCho and PlsEtn respectively. Plasmalogens were recovered in an overall yield of 7.7% ± 0.7% (mean ± S.E.M., n = 4) and 10.2% ± 1.2% (mean ± S.E.M., n = 3) for PlsCho and PlsEtn.
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Plasmalogens, 1-alk-1′-enyl-2-acyl-*sn*-glycero-3-phospholipids, are major phospholipid constituents of mammalian membranes (1). They are widely distributed in nature and are found admixed with 1,2-diacyl and 1-alkyl-2-acyl analogs. Plasmalogens are hydrolyzed more slowly than diacylglycerophospholipids with snake venom phospholipase A_2 (2), *Clostridium welchii* phospholipase C (3) and cabbage phospholipase D (4). Diacylglycerophospholipids are also hydrolyzed faster than plasmalogens by very mild alkaline hydrolysis (5,6). Many attempts have been made to purify plasmalogens using these enzymic and chemical reactions (2–6). However, these purification methods have disadvantages of either low yield, low efficiency or nongenerality for both PlsCho and PlsEtn. Another purification procedure employs highly purified lipase from porcine pancreas (7). The method is based on the selective deacylation by lipase action at the 1-position of the diacylglycerophospholipids (8,9). Free fatty acids (FFA) and lysoglycerophospholipids were removed by column chromatography and reincubated. Plasmalogen samples were obtained with purities of 98% and recoveries of PlsCho and PlsEtn of 73% and 74%, respectively (7). *Rhizopus arrhizus* and *Rhizopus delemar* lipases also hydrolyze acyl groups at the 1-position of diacylglycerophospholipids. These enzymes have also been used for the purification of plasmalogens (10,11).

The contents of alkylacylglycerophospholipids among bovine heart ChoGpl and brain EtnGpl have been reported. The ratio ranges from 3.2% to 4.3% for PakCho (1-alkyl-2-acyl-*sn*-glycero-3-phosphocholine) and 3.5% for PakEtn (1-alkyl-2-acyl-*sn*-glycero-3-phosphoethanolamine) (12–16). For highly pure preparations of plasmalogens, the removal of alkylacyl analogs is important. However, no previous purification methods have mentioned the hydrolysis of these phospholipids. The *Naja naja naja* phospholipase A_2 has greater activity with alkylacyl than alkenylacyl analogs (17). Therefore, partially purified plasmalogens obtained by repeated incubation with *R. delemar* lipase were further treated with *N. naja naja* phospholipase A_2. The purpose of the present paper is to describe optimal conditions and kinetic parameters of enzymes used for the purification of plasmalogens.

Thin-layer chromatography. TLC plates (20 × 20 cm) for analytical TLC were activated for 1 hr at 110°C. Aliquots from reaction mixtures were extracted with 4 vol of chloroform/methanol (2:1, v/v), dried under nitrogen and chromatographed on TLC. The solvent system for the first dimension was chloroform/methanol/NH_4OH (65:25:4, v/v/v), and the solvent system for the second dimension was chloroform/acetone/methanol/acetic acid/water (75:30:15:15:8.5, by vol) (18). The TLC plates were exposed to HCl for 3 min between the developments in the first and second dimension. Spots were visualized with I_2 vapor and the phosphorus content of the factors was determined (19).

Total FFA were separated with petroleum ether/diethyl ether/acetic acid (110:90:4, v/v/v) as the developing solvent. The FFA band was visualized with 2-*p*-toluidinylnaphthalene-6-sulfonate under ultraviolet light (20).

Gas-liquid chromatography. The FFA band was scraped into screw-cap test tubes, and esterified in 3.0 mL of 0.38M H_2SO_4 in methanol/toluene (1:1, v/v) at 60°C for 4 hr. The fatty acid methyl esters were quantified by GLC with a Shimadzu GC-8A gas chromatograph using heptadecanoic acid as an internal standard. A Supelco SP-2330 capillary column (0.32 mm ID × 30 m) was used with N_2 as the carrier gas and an operating temperature of 190°C. Peak areas were calculated with a Nelson Analytical 760 intelligent interface and Nelson Analytical software (Cupertino, California).

High performance liquid chromatography. The preparation of individual lipids after enzymic hydrolysis of ChoGpl and EtnGpl was performed by HPLC. A Beckman Model 344 HPLC system with two Beckman Model 114 M pumps was used. The injection port was a Rheodyne Model 7125. All separations were performed on Dupont Zorbax SIL (25 cm × 21.2 mm I.D.) semipreparative column which was maintained at 34°C with a Jones chromatography column heating block (Columbus, Ohio). Elution was monitored at 205 nm with a ISCO

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Abbreviations: ChoGpl, choline glycerophospholipid, 1,2-diradyl-*sn*-glycero-3-phosphocholine; EtnGpl, ethanolamine glycerophospholipid, 1,2-diradyl-*sn*-glycero-3-phosphoethanolamine; PlsCho, plasmenylcholine, 1-alk-1′-enyl-2-acyl-*sn*-glycero-3-phosphocholine; PlsEtn, plasmenylethanolamine, 1-alk-1′-enyl-2-acyl-*sn*-glycero-3-phosphoethanolamine; PakCho, phosphalkanylcholine, 1-alkyl-2-acyl-*sn*-glycero-3-phosphocholine; PakEtn, phosphalkanylethanolamine, 1-alkyl-2-acyl-*sn*-glycero-3-phosphoethanolamine.

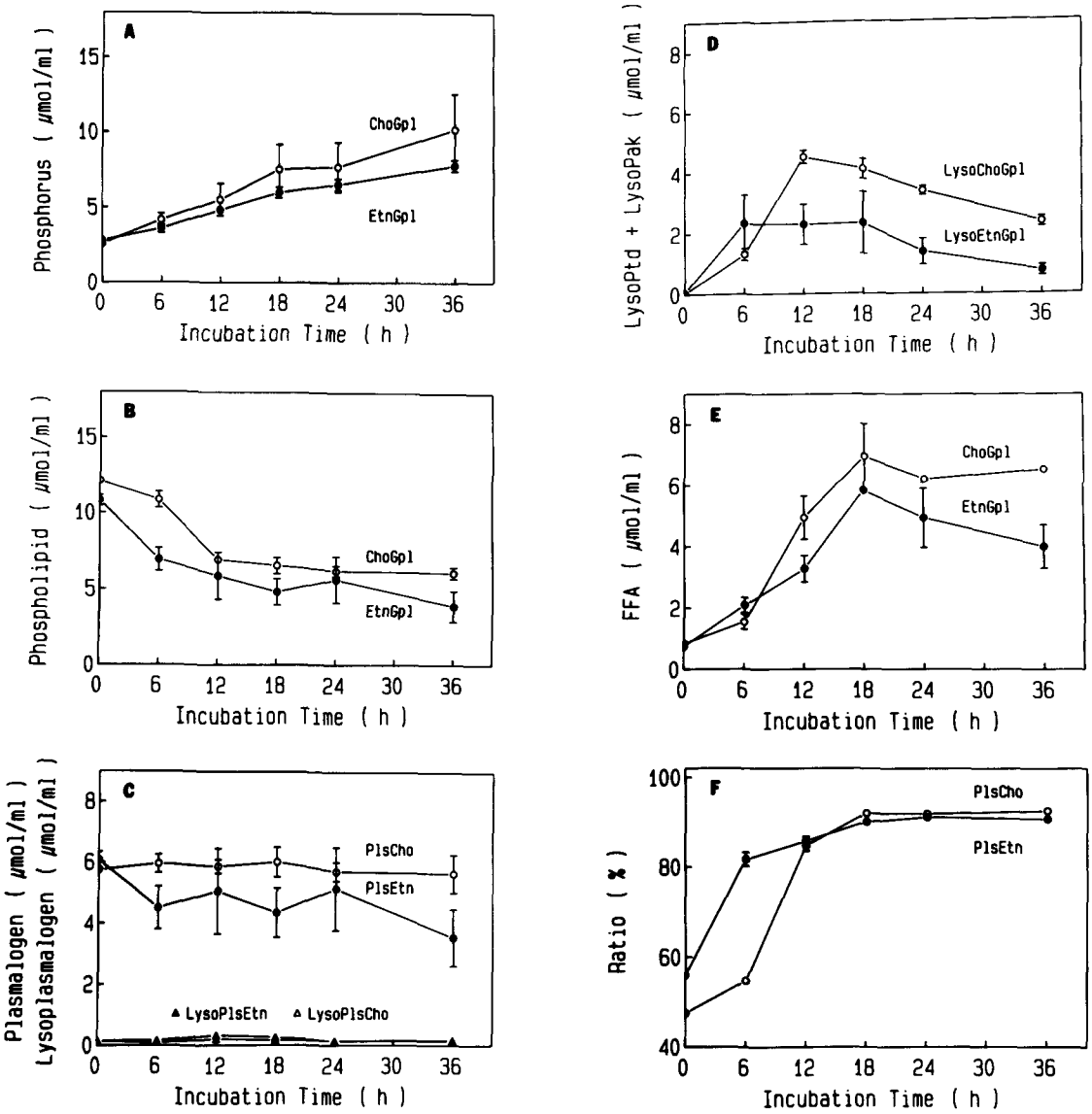

FIG. 1. The time course for the digestion of ChoGpl and EtnGpl with *R. delemar* lipase. Lipid (13 mM for ChoGpl and 12 mM for EtnGpl) was incubated at room temperature with 1% Triton X-100 for ChoGpl or 0.2% Triton X-100 for EtnGpl, 5 mM $CaCl_2$, 0.5% fatty acid free bovine serum albumin and 8 mg/mL *R. delemar* lipase in 0.1 M MOPS buffer, pH 7.0. Panels A–F show the amounts of aqueous phase phosphorus (A), total phospholipid (B), plasmalogen and lysoplasmalogen (C), lysoChoGpl and lysoEtnGpl except lysoplasmalogen (D), total FFA (E) and the proportion of plasmalogen in the phospholipids (F).

Model V[4] variable-wavelength detector. Solvent A was hexane/2-propanol (3:2, v/v), and the water-containing solvent B was made by combining 55 mL water with solvent A to a final volume of 1000 mL. A flow-rate of 36 mL/min was maintained throughout the separation. The initial solvent was a mixture of solvents A and B (55:45, v/v). For the separation of ChoGpl, at 5 min a gradient from 45% B to 76% B was initiated, at 14 min solvent B was then increased to 100%, and at 45 min the solvents were returned to the initial ratio. For the separation of EtnGpl, at 15 min solvent B was increased to 76% and at 45 min the solvents were returned to a 55:45 (v/v) ratio. All gradients were changed over a 1-min period. EtnGpl emerged at 8.0 min and ChoGpl at 24.5 min.

Purification of plasmalogen with R. delemar *lipase.* Commercial ChoGpl and EtnGpl were used as starting materials. Lipid (10–15 μmol/mL) was incubated for 18 hr with 1% Triton X-100 for ChoGpl or 0.2% Triton X-100 for EtnGpl, 5 mM $CaCl_2$, 0.5% fatty acid free bovine serum albumin and 8 mg/mL *R. delemar* lipase in 0.1 M MOPS buffer, pH 7.0, at room temperature. After sonication of lipid with the buffer including Triton X-100 and $CaCl_2$, other constituents were added. The total volume of the reaction mixture was 8 mL. The reaction was performed under nitrogen.

The reaction was terminated by the addition of 4 vol of chloroform/methanol (2:1, v/v). The aqueous phase was extracted once more with the same volume of chloroform/methanol (2:1, v/v). The combined lower phases were then taken to dryness under reduced pressure. The remaining lipids were incubated again with *R. delemar* lipase for 9 hr as described above. The termination of the reaction, the

METHODS

TABLE 1

The Content and Yield of Plasmalogens After Individual Steps of Purification[a]

	PlsCho		PlsEtn	
	Content (%)	Yield (%)	Content (%)	Yield (%)
Starting material	48.8 ± 0.10 (n = 4)		57.1 ± 0.14 (n = 4)	
First incubation with *R. delemar* lipase	92.6 ± 0.11 (n = 4)	84.6 ± 3.50 (n = 4)	92.8 ± 0.77 (n = 4)	96.5 ± 2.44 (n = 4)
Second incubation with *R. delemar* lipase	94.2 ± 0.21 (n = 4)	73.9 ± 5.20 (n = 4)	94.9 ± 0.19 (n = 3)	87.6 ± 5.04 (n = 3)
Final purified plasmalogen	97.7 ± 0.24 (n = 4)	7.74 ± 0.78 (n = 4)	98.8 ± 0.27 (n = 3)	10.2 ± 1.15 (n = 3)

[a]Values are expressed as mean ± S.E.M.

extraction of lipids and the evaporation of solvents were performed by the same procedures as the first incubation. The remaining lipids were then subjected to *N. naja naja* phospholipase A_2 treatment.

Purification of plasmalogens with N. naja naja *phospholipase* A_2. Partially purified plasmalogens were incubated for 3 hr with 0.1% Triton X-100, 5 mM $CaCl_2$, 0.5% fatty acid free bovine serum albumin and 0.5 μg/mL *N. naja naja* phospholipase A_2 for PlsCho or 5 μg/mL for PlsEtn in 0.1 M MOPS buffer, pH 7.4, at room temperature. The phospholipid concentration was about 2 mM, and total volumes of the reaction mixture were about 21.8 mL and 3.8 mL for Cho Gpl and EtnGpl, respectively. After sonication of the lipids with buffer solution including Triton X-100 and $CaCl_2$, the remaining constituents were mixed. The reaction was performed under nitrogen.

RESULTS

The time course of the hydrolysis of ChoGpl and EtnGpl by *R. delemar* lipase is shown in Figure 1. The reaction mixture at each time period was extracted with 4 vol of chloroform/methanol (2:1, v/v). The phosphorus content of the aqueous phase was assayed. The organic phase was dried under nitrogen and subjected to two-dimensional reaction TLC (18). The total ChoGpl and EtnGpl contents decreased with the concomitant increase of monoacylglycerophospholipids, while neither PlsCho nor PlsEtn changed in their concentration. Consequently, a plasmalogen proportion of more than 90% was obtained after 16 hr incubation. This may be due to the specific hydrolysis of diacylglycerophospholipids by the phospholipase A_1 activity of *R. delemar* lipase (10). The decrease in the level of monoacylglycerophospholipids after 12-hr incubation and the gradual increase of water-soluble phosphorus suggested that some lysophospholipase-like activity was also present. Total FFA levels increased corresponding to the decrease in phospholipids. The lysoPlsEtn and lysoPlsCho which were present at a low concentration did not show any changes during the entire time course.

The plasmalogen contents were 92.6% ± 0.11% (mean ± S.E.M., n = 4) for PlsCho and 92.8% ± 0.77% (mean ± S.E.M., n = 3) for PlsEtn after the first incubation with *R. delemar* lipase, while the plasmalogen proportion increased to 94.2% ± 0.21% (mean ± S.E.M., n = 4) for PlsCho and 94.9% ± 0.19% (mean ± S.E.M., n = 3) for PlsEtn after the second incubation (Table 1). The remainder was mostly alkylacyl types with some diacyl types.

The effects of variation of *N. naja naja* phospholipase A_2 on the ratio of plasmalogens and the extent of plasmalogen hydrolysis are demonstrated in Figure 2, A and B. The effects of incubation time were also examined (data not shown). A plasmalogen purity of more than 97% was obtained by incubation for 3 hr with 0.5 μg protein/mL phospholipase A_2 for PlsCho or 5.0 μg protein/mL for PlsEtn. However, a large quantity of plasmalogens was lost during this reaction. Approximately 95% of the PlsCho and 90% of the PlsEtn were hydrolyzed because phospholipase A_2 of *N. naja naja* also has activity with plasmalogens.

The phosphorus contents of plasmalogens obtained from analytical TLC plates were more than 150 nmol. The lowest limit for positive detection of phosphorus is about 1 nmol, and therefore, the assay method employed in this study was sufficient to assess the purity of plasmalogens. The proportion and yield of plasmalogens at each step are summarized in Table 1. The final PlsCho was 97.7% ± 0.24% (mean ± S.E.M., n = 3) pure with a yield of 7.74% ± 0.78% (mean ± S.E.M., n = 4), and the PlsEtn was 98.8% ± 0.27% (mean ± S.E.M., n = 3) pure with a yield of 10.17% ± 1.15% (mean ± S.E.M., n = 3).

DISCUSSION

A previous method for the preparation of plasmalogen used highly purified pancreatic lipase with selective deacylation at the 1-position of the diacylglycerophospholipids (7). In order to achieve complete hydrolysis of diacylglycerophospholipids, reincubation with the same enzyme was necessary after removing FFA and lysoproducts with a Florisil column. This procedure is time-consuming for routine work. The authors also reported that 98%-pure plasmalogens were obtained starting with bovine heart ChoGpl and bovine brain EtnGpl, although calculations from the contents of PakCho and PakEtn in these sources (12–16) show that the maximum purity of

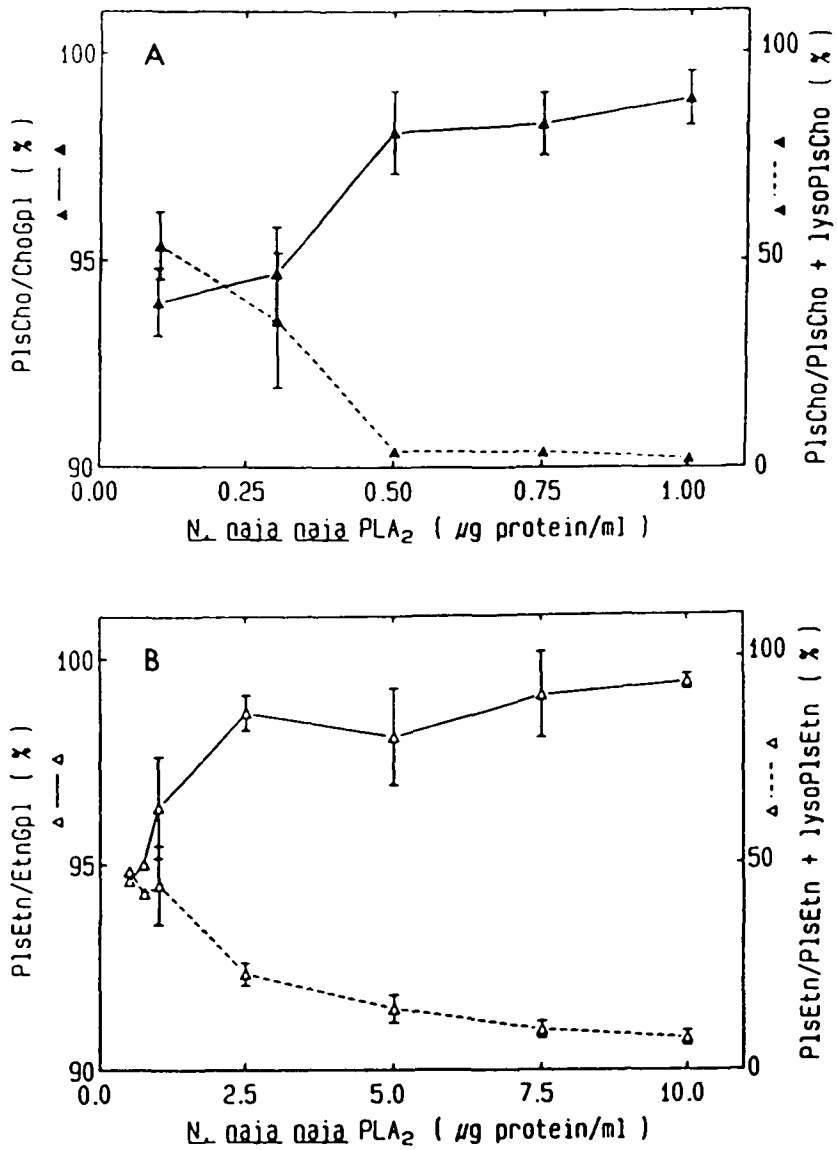

FIG. 2. The effects of variation of *N. naja naja* phospholipase A_2 on the proportion of plasmalogen in the phospholipids and the extent of plasmalogen hydrolysis. Partially purified plasmalogens (2 mM), PlsCho(A) and PlsEtn(B), were incubated for 3 hr at room temperature with 0.1% Triton X-100, 5 mM $CaCl_2$, 0.5% fatty acid free bovine serum albumin and the indicated amount of *N. naja naja* phospholipase A_2 in 0.1 M MOPS buffer, pH 7.4 at room temperature.

plasmalogens would be 94.0% for PlsCho and 95.2% for PlsEtn after complete hydrolysis of diacylglycerophospholipids.

In other studies, *R. arrhizus* and *R. delemar* lipases, which also have phospholipase A_1 activity, were used for the purification of plasmalogens (10,11). These enzymes are commercially available and inexpensive. Previously, this laboratory has obtained 93% pure plasmalogen using *R. delemar* lipase with more than 80% recovery (10). Lysophospholipase-like activity was found in the crude enzyme preparation but apparently did not disturb the progress of purification. In order to complete the hydrolysis of diacyl analogs in this study, double incubation with *R. delemar* lipase was performed by a modification of the previous method (10).

Plasmalogens are poor substrates for *N. naja naja* phospholipase A_2. The substrate specificity ratios were 100, 36.4 and 94.8 for diacyl-, alkenylacyl- and alkylacyl-Gro*P*Cho, respectively (17). The purification method using only *N. naja naja* phospholipase A_2 was not efficient (2), but this method coupled with *R. delemar* lipase treatment became quite effective. Previously described methods for the purification of plasmalogens had attempted to hydrolyze only the diacyl analog but not the alkylacyl analog. In order to obtain highly pure preparations of plasmalogens, it is important to remove the alkylacylglycerophospholipids. *N. naja naja* phospholipase A_2 can be used as a valuable tool to remove alkylacyl analogs. The ratio of alkenylacyl analog to alkylacyl analog is so high in the reaction mixture with phospholipase A_2

that a large quantity of plasmalogen is wasted in spite of the higher substrate specificity for the alkylacyl analog. Therefore, in order to obtain better yields, the partially purified plasmalogen preparations should be prepared as pure as possible using the *R. delemar* lipase.

In the present study, we also used a semi-preparative HPLC method for the separation of lipids. HPLC is a rapid procedure and convenient for routine work.

Plasmalogens may be degraded by receptor-mediated processes during signal transduction (21,22). Therefore, the enzymes hydrolyzing plasmalogens may be very important metabolically. Spectrophotometric- and fluorometric-coupled enzyme assay procedures have been developed using auxiliary enzymes (23–27). Therefore, purified plasmalogens and their metabolites are needed as substrates for plasmalogen hydrolyzing enzymes. Quantitative analyses of subclasses of phospholipids have been developed (14,16,28). Our purification procedure will also offer highly pure plasmalogen standards.

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Chromatographic Resolution of Chiral Diacylglycerol Derivatives: Potential in the Stereospecific Analysis of Triacyl-*sn*-glycerols

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Diacylglycerols have been separated as their (*S*)-(+)-or (*R*)-(-)-1-(1-naphthyl)ethyl urethanes by high performance liquid chromatography (HPLC) on a column of silica gel with 0.5% 2-propanol in hexane as the mobile phase. The elution order of components derivatized with the (*S*)-form of the reagent was 1,3-, followed by 1,2-, and finally 2,3-diacyl-*sn*-glycerols. The elution order of 1,2- and 2,3-diastereomers was reversed when the (*R*)-form of 1-(1-naphthyl)ethyl isocyanate was used for derivatization. Single-acid 1,2- and 2,3-diastereomers were separated to the baseline with a resolution factor from 5.2–5.7, and the resolution factor between 1,3- and 1,2- or 2,3-diacyl-*sn*-glycerol derivatives was more than 23. Molecular species of single-acid diacylglycerol derivatives were separated in the sequence 18:1 < 18:0 < 18:2 <16.0.

In order to assess this methodology as part of a procedure for the stereospecific analysis of triacyl-*sn*-glycerols, we prepared diacyl-*rac*-glycerols from maize oil, evening primrose oil and egg yolk triacylglycerols by partial hydrolysis with ethyl magnesium bromide. The 1,3-, 1,2- and 2,3-diacyl-*sn*-glycerols as (*S*)-(+)-1-(1-naphthyl)ethyl urethanes were isolated and their fatty acid compositions were determined. Although this only permitted an indirect determination of the compositions of positions *sn*-1, -2 and -3, it was sufficient to indicate the potential of the methodology because results comparable to those published earlier were achieved.

Lipids 25, 349–353 (1990).

Isocyanates form urea derivatives by reaction with amines and urethanes (or "carbamates") by reaction with alcohols. Chiral urethanes have been shown to be particularly well suited to the resolution of diastereomers of various kinds (1), and chiral mono- and dialkylglycerols have been resolved in this form by high performance liquid chromatography (HPLC) on a column of silica gel (2). However, single-acid diacyl-*sn*-glycerols derivatized with (*S*)-(+)-1-(1-naphthyl)ethyl isocyanate were only partially separated into 1,2- and 2,3-diastereomeric forms under the conditions tried (2). Takagi and Itabashi (3,4) were able to demonstrate a complete resolution of single-acid diacyl-*sn*-glycerols as 3,5-dinitrophenyl urethanes by HPLC with a chiral stationary phase, but the separation was greatly complicated if more than one fatty acid was present.

We have now shown that diacyl-*sn*-glycerols, as their (*S*)- or (*R*)-1-(1-naphthyl)ethyl urethanes, can be well resolved by HPLC on a column of silica gel, and have investigated the potential of the method in the stereospecific analysis of triacyl-*sn*-glycerols.

MATERIALS AND METHODS

Standards, reagents and lipid samples. Monoacid 1,3- and 1,2-diacyl-*rac*-glycerols of palmitic, stearic and oleic acid and *L*-phosphatidylcholines containing stearic, oleic or linoleic acid were purchased from the Sigma Chemical Co (Poole, U.K.). Phospholipase C (EC 3.1.4.3) type I from *Clostridium perfringens* (15 units/mg protein) was also from Sigma, as were most of the remaining reagents. (*R*)-(-)- and (*S*)-(+)-1-(1-naphthyl)ethyl isocyanate were obtained from Aldrich Chemical Co. (Gillingham, U.K.). The solvents used were HPLC grade and were obtained from Fisons Ltd. (Poole, U.K.). 1,2-Diacyl-*sn*-glycerols were prepared from the phosphatidylcholines by hydrolysis with phospholipase C (5).

Evening primrose oil (Efamol™) (*Oenothera biennis*) was obtained from a local health food shop. Maize oil (*Zea mays* L.) and eggs (*Gallus gallus*) were bought from a local super market. Lipids were extracted from the yolk of an egg with chloroform/methanol (2:1, v/v). Lipid samples were first purified on short Florisil™ columns, eluted with hexane/diethyl ether (4:1, v/v), after which the triacylglycerol fractions were obtained by preparative HPLC on a silica column (Spherisorb™ S5W, 25 cm × 8 mm i.d.) with 2% tetrahydrofuran in hexane as the eluent at a flow-rate of 2 ml/min, and with refractive index detection (Knauer, Oberursel/Taunus, FRG).

Partial hydrolysis of triacylglycerols. Triacylglycerols (40 mg) were dissolved in dry diethyl ether (2 ml), freshly prepared 0.5 M ethyl magnesium bromide in dry diethyl ether (1 ml) was added, and the mixture was shaken for 1 min before glacial acetic acid (50 μl) and water (2 ml) were added to stop the reaction (6). The ether layer was washed with 2% (w/v) potassium bicarbonate solution and water, and was dried over anhydrous sodium sulfate. After evaporating the solvent, the diacylglycerols were isolated rapidly by preparative HPLC with hexane/tetrahydrofuran/2-propanol (100:0.3:1.5, v/v/v) as the mobile phase.

Preparation of 1-(1-naphthyl)ethyl urethane derivatives. Diacylglycerols (1–2 mg) were dissolved in dry toluene (300 μl) and were reacted with the (*R*)- or (*S*)-forms of 1-(1-naphthyl)ethyl isocyanate (10 μl) in the presence of 4-pyrrolidinopyridine (approx. 10 μmole) overnight at 50°C. The products were extracted with hexane/diethyl ether (1:1, v/v) and washed with 2 M HCl and water. The organic layer was taken to dryness under a stream of nitrogen and the sample was purified on a short column of Florisil™ eluted with diethyl ether.

HPLC. HPLC separation of the diastereomeric diacylglycerol derivatives was carried out using a Spectra-

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Abbreviations: GC, gas chromatography; HPLC, high performance liquid chromatography; LC-MS, liquid chromatography-mass spectroscopy.

Physics (St. Albans, U.K.) Model 8770 isocratic pump together with a Pye Unicam (Cambridge, U.K.) PU 4025 UV-detector and a Spectra-Physics Model 4270 integrator. For optimum resolution, two columns of silica gel (Hypersil™ 3μ, 25 cm × 4.6 mm i.d.) in series were utilized with 0.5% (v/v) 2-propanol in hexane as mobile phase at a flow rate of 0.8 ml/min. The sample was dissolved in the minimum volume of 1,2-dichloroethane. Detection was at 280 nm.

Fatty acid analysis. Fatty acid methyl esters from the derivatized diacylglycerols were prepared by acid-catalyzed transesterification with 1% sulfuric acid in methanol at 50°C overnight in a stoppered test-tube, with methyl nonadecanoate added as an internal standard (7). A Carlo Erba Model 4130 capillary gas chromatograph equipped with a split/splitless injection system was used for fatty acid analyses with a fused silica column coated with CP-Sil 84 (25 m × 0.22 mm i.d., d_f = 0.2 μm; Chrompack UK Ltd., London, U.K. The temperature of the oven was maintained at 145°C for 5 min, then it was raised at 2°C/min to 180°C. Hydrogen was the carrier gas.

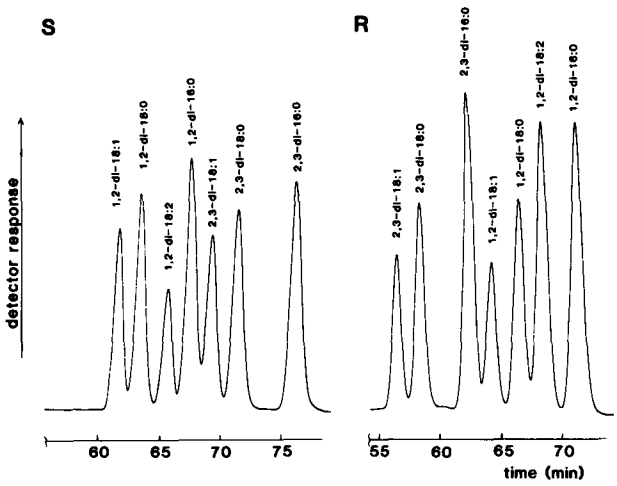

FIG. 1. Separation of a standard mixture of single-acid 1,2- and 2,3-diacyl-*sn*-glycerols in the form of the chiral 1-(1-naphthyl)ethyl urethane derivatives by HPLC (conditions as in the text). S: (*S*)-(+)-1-(1-naphthyl)ethyl urethanes. R: (*R*)-(-)-1-(1-naphthyl)ethyl urethanes.

RESULTS AND DISCUSSION

Diacyl-*sn*-glycerols were found to react completely with the (*R*)- or (*S*)-forms of 1-(1-naphthyl)ethyl isocyanate under the conditions described to give diacyl-*sn*-glycerol 1-(1-naphthyl)ethyl urethanes. No acyl migration or racemization of the components were detected, since pure 1,2-diacyl-*sn*-glycerols, obtained from "natural" *L*-phosphatidylcholines on hydrolysis with phospholipase C gave single chromatographic peaks on HPLC analysis. Higher temperatures and shorter reaction times were used by others with no apparent isomerization (8).

Diastereomeric diacyl-*sn*-glycerol 1-(1-naphthyl)ethyl urethanes were well separated on a single column (25 cm) of silica gel (5 μm particle size), but much better resolution (31,000–34,000 theoretical plates) of mixtures was obtained with two 25 cm columns of silica gel (3 μm) in series and isocratic elution with 0.5% 2-propanol in hexane. The compounds were detected by UV spectrophotometry at 280 nm, which was sensitive down to the nanomole level; fluorescence detection would permit even higher sensitivity (8).

The separations of mixtures of both saturated and unsaturated monoacid diacyl-*sn*-glycerols derivatized with either (*S*)-(+)- or (*R*)-(-)-1-(1-naphthyl)ethyl isocyanate are shown in Figure 1. The standard was prepared by derivatizing synthetic single-acid (16:0, 18:0 and 18:1) *rac*-diacylglycerols and 1,2-dilinoleoyl-*sn*-glycerol, prepared from *L*-dilinoleoylphosphatidylcholine—they were then mixed in approximately equimolar amounts. Peaks were identified first by coinjecting single-acid 1,3-diacyl-*sn*-glycerols and 1,2-diacyl-*rac*-glycerol derivatives. The 1,2-isomers were distinguished with 1,2-diacyl-*sn*-glycerols prepared from *L*-phosphatidylcholines by phospholipase C hydrolysis. Diacyl-*sn*-glycerols derivatized with (*S*)-(+)-1-(1-naphthyl)ethyl isocyanate eluted in the order 1,3-followed by 1,2-, and finally 2,3-isomers. When the (*R*)-form of 1-(1-naphthyl)ethyl isocyanate was used for derivatization, the elution order changed so that 2,3-isomers eluted before 1,2-isomers. The 1,3-isomers are not shown in Figure 1, but they eluted in approximately half of the time needed for 1,2- and 2,3-isomers. Molecular species of the single-acid diacyl-*sn*-glycerol derivatives studied were generally well resolved, although the order of elution is rather puzzling, 18:1 < 18:0 < 18:2 < 16:0, i.e. it is neither that expected for "normal" nor for reversed-phase partition chromatography. Mixed-acid diacylglycerol derivatives were not available for study, although this would certainly be desirable.

The resolution factors (R) between pairs of 1,2- and 2,3-diastereomers calculated from Figure 1 were 5.2–5.3 for the (*S*)- and 5.6–5.7 for the (*R*)-derivatives. This resolution is more than twice that achieved with diacylglycerol 3,4-dinitrophenyl urethanes on chiral stationary phases (3), although it must be admitted that faster elution times were utilized in the latter work. The R value for components differing by four carbon atoms, e.g., 1,2-dipalmitoyl- and 1,2-distearoyl-*sn*-glycerol was 2.8 when derivatized with the (*S*)-form of the reagent and 3.0 when derivatized with the (*R*)-form. In addition, the (*S*)-derivatives of 1,2-dioleoyl-*sn*-glycerol and 1,2-dilinoleoyl-*sn*-glycerol were separated with an R value of 2.9. The R value for 1,3- and 1,2-dipalmitoyl-*sn*-glycerols derivatized with (*S*)-(+)-1-(1-naphthyl)ethyl isocyanate was 23.6, and that for 1,3- and 2,3-dipalmitoyl-*sn*-glycerols was 28.5.

A key pair in the standard mixture derivatized with (*S*)-(+)-1-(1-naphthyl)ethyl isocyanate is 1,2-dipalmitoyl- and 2,3-dioleoyl-*sn*-glycerol, as this marks a division between the 1,2- and 2,3-isomers with the common range of fatty acids studied here. The resolution factor was 1.1 under the elution conditions described. When the derivatives were prepared with (*R*)-(-)-1-(1-naphthyl)ethyl isocyanate, the R value for the same pair increased to a value of 9.6. The use of both the (*R*)- and (*S*)-forms of the 1-(1-naphthyl)ethyl urethane derivatives gives a substantial advantage in that such components (i.e., a 1,2-isomer and a 2,3-isomer)

which are poorly separated in one form will be much better resolved with the other; the order of elution of 1,2- and 2,3-isomer groups will be reversed, but the order of elution of single molecular species within each group remains the same. With the key pair illustrated, the two components least well resolved become those best separated in going from the (S)- to the (R)- derivatives.

With this elution system, reproducible retention times were difficult to obtain and usually increased during the day, as is evident from the time scales of the figures. This variability was reduced greatly by leaving a slow flow (0.4 ml/min) of mobile phase through the columns overnight, when this was practicable. Difficulties with retention times have been reported earlier with silica gel-based separation systems (2,4). The problem may be due to the changes in the hydration state of the silica gel or in the degree of adsorption of the more polar component of the mobile phase. This meant that peaks could not be identified by retention times only. The elution system was also very sensitive to the 2-propanol concentration; when the amount of 2-propanol was increased, the retention times became shorter but resolution diminished; and when the concentration of 2-propanol was decreased, the elution time increased and chromatographic peaks tended to tail. Nonetheless, under the latter conditions, base-line separation of all the 1,2- and 2,3-diacyl-*sn*-glycerols in the standard mixture could sometimes be achieved but with elution times of more than 2 hr.

During this work, (-)-menthyl chloroformate and (*R*)-(-)-2-phenylpropionic acid were also used to derivatize diacylglycerols, but no resolution of diastereomers was obtained. Unlike 1-(1-naphthyl)ethyl urethanes, they do not have a hydrogen attached to a nitrogen atom which is capable of forming hydrogen bonds. This is believed to be the reason for the efficacy of urethane derivatives in the chromatographic resolution of diastereomers (1). We have yet to study the properties of these derivatives on a column containing a chiral phase in a systematic manner. It also appears that an aliphatic alcohol is an essential component of the mobile phase; other modifier solvents tested gave little or no resolution. This may explain why Michelsen *et al.* (2) were not able to achieve the separations shown here; indeed, they reported that inferior separations were obtained with the addition of isopropanol as a modifier.

A key step in the stereospecific analysis of triacyl-*sn*-glycerols, i.e., for the determination of the fatty acid compositions of positions *sn*-1, -2 and -3, is the preparation by partial hydrolysis of a mixture of 1,2- and 2,3-diacyl-*sn*-glycerols, which must then be converted to phospholipids for hydrolysis with stereospecific phospholipases (9,10). There would obviously be advantages in speed, convenience and possibly accuracy if the resolution of the chiral diacylglycerols could be accomplished by an HPLC method, especially if this gave information simultaneously on the composition of molecular species.

In order to assess the potential of the HPLC methodology described above for this purpose, diacylglycerols were obtained by partial hydrolysis of maize oil triacylglycerols with ethyl magnesium bromide, and they were converted to the (*S*)-(+)-1-(1-naphthyl)ethyl urethanes and separated as shown in Figure 2. Diacylglycerols prepared from evening primrose oil and egg yolk triacylglycerols were analyzed in the same way. The 1,3-, 1,2- and 2,3-diacyl-*sn*-glycerol groups of peaks were well separated in general, except for the egg yolk sample where there was a small amount of overlap between the 1,2- and 2,3-isomers. The components seen at the start of the chromatogram are from the derivatizing reagent. In order to identify each of the diacylglycerol fractions, they were collected and transesterified (base-catalyzed transesterification gave interfering peaks derived from the naphthylethyl moiety) for fatty acid analysis by gas chromatography

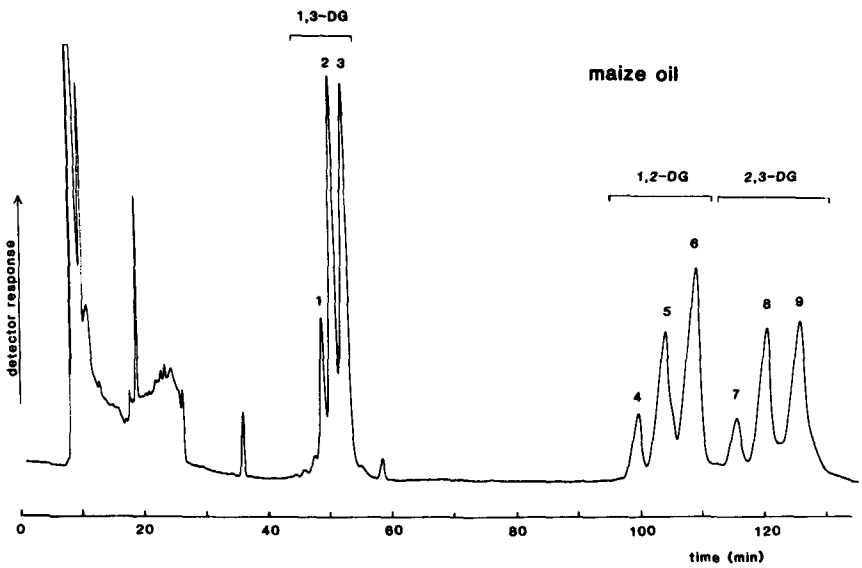

FIG. 2. Separation of diacyl-*sn*-glycerols, formed by partial hydrolysis of maize oil with ethyl magnesium bromide, in the form of the (*S*)-(+)-1-(1-naphthyl)ethyl urethane derivatives (conditions as in text). Abbreviation: DG, diacyl-*sn*-glycerol.

TABLE 1

Fatty Acid Compositions (mole %) of Diacyl-*sn*-Glycerol (*S*)-(+)-1-(1-Naphthyl)Ethyl Urethane Fractions from Maize Oil[a]

Fraction no.	Fatty acids				
	16:0	18:0	18:1	18:2	18:3 (n-3)
1	5.3	9.9	66.2	16.6	2.0
2	15.2	4.5	41.4	35.9	3.0
3	16.0	2.0	4.0	78.0	—
4	11.5	6.0	43.4	38.4	0.8
5	5.9	5.1	46.6	42.3	—
6	11.8	2.6	7.4	78.2	—
7	8.3	4.9	47.5	37.0	2.3
8	3.4	4.4	47.6	44.7	—
9	8.0	2.3	4.9	84.4	0.3

[a]Fraction numbers refer to the peaks in Figure 2.

(GC); the results for maize oil are listed in Table 1. The main component of each of the three 1,3-diacyl-*sn*-glycerol peaks consists predominantly of 18:1-18:1, 18:1-18:2 (position not specified) and 18:2-18:2, respectively, although many other components are obviously present. Similarly, each of the three peaks from the 1,2- and 2,3-diacyl-*sn*-glycerols must contain a mixture of molecular species. Peaks 4 and 5, together with 7 and 8, have rather similar compositions, and must be mainly oleoyllinoleoyl species of various kinds. As there is more linoleic acid in position 2 than in the other position, it is tempting to suggest, from the relative abundances of the components, that peak 4 is predominantly the 1-linoleoyl-2-oleoyl-*sn*-glycerol species and peak 5 the 1-oleoyl-2-linoleoyl-*sn*-glycerol species. Other procedures such as mass spectrometry or lipase digestion will be required for confirmation. Peak 6 is mainly the dilinoleoyl species. Peaks 7 to 9 are the respective diastereomers. Further work will be necessary to identify the minor species. Kuksis *et al.* (11) have resolved and identified diacylglycerols, prepared from corn oil, by HPLC-mass spectrometry (MS) on a chiral column in the form of the dinitrophenylurethanes.

The distribution of fatty acids in positions *sn*-1, 2 and 3 of the triacyl-*sn*-glycerols were determined indirectly from the compositions of the combined 1,3-, 1,2- and 2,3-diacyl-*sn*-glycerol fractions. The results for maize and evening primrose oils and for egg yolk are presented in Table 2. For example, the composition of fatty acids in position *sn*-1 were calculated for each component by subtracting twice its concentration in the 2,3-diacyl-*sn*-glycerols from three times its concentration in the intact triacyl-*sn*-glycerols. The data for the other positions were calculated in a similar way. The results obtained here are very similar to others published earlier for maize oil (12), evening primrose oil (13) and egg yolk (14) triacyl-*sn*-glycerols. Perhaps the greatest difference is in position *sn*-2 of egg yolk, where somewhat more palmitic acid was found than in previous analyses. In this instance, separation of the 1,2- and 2,3-diacyl-*sn*-glycerol derivatives was not quite complete, contributing to the error. Also, the partial hydrolysis procedure tends to give greater errors with this position (6).

It would obviously be desirable to determine the compositions of the various positions by a direct method, for example, by hydrolysis of each fraction with an

TABLE 2

Compositions of Fatty Acids (mole %) in Positions *sn*-1, -2 and -3 of the Triacyl-sn-Glycerols of Maize Oil, Evening Primrose Oil and Egg Yolk

	Fatty acid					
	16:0	16:1	18:0	18:1	18:2	18:3
Maize oil						
position *sn*-1[a]	15.3	—	0.6	34.9	48.2	1.0[d]
2[b]	0.5	—		33.7	62.9	2.8
3[c]	10.2	—	0.3	35.1	51.5	2.8
Evening primrose oil						
position *sn*- 1	10.7	—	2.8	9.3	70.0	7.2[e]
2	2.6	—	0.3	7.0	79.4	10.7
3	8.2	—	2.6	11.1	67.2	10.9
Egg yolk						
position *sn*- 1	65.0	6.7	3.2	21.8	3.3	—
2	11.3	3.6	2.0	55.9	27.3	—
3	16.3	8.1	5.2	67.6	2.8	—

Data were calculated from the composition of each fatty acid in the triacylglycerols and those in the 2,3-,1,3- and 1,2-diacyl-*sn*-glycerol (*S*)-(+)-1-(1-naphthyl)ethyl urethane derivatives, respectively.
[a]3 × TG - 2 × 2,3-DG.
[b]3 × TG - 2 × 1,3-DG.
[c]3 × TG - 2 × 1,2-DG.
[d]18:3(n-3).
[e]18:3(n-6).

enzyme such as the triacylglycerol lipase from *Rhizopus arrhizus* and analysis of the products. A further improvement might be obtained by derivatizing the products of ethyl magnesium bromide hydrolysis of triacylglycerols immediately, rather than after isolating a diacylglycerol fraction as this would limit opportunities for acyl migration. HPLC linked to mass spectrometry would give much more information on the composition of the fractions, especially if both the (*R*)- and (*S*)-forms of the urethane derivatives of the diacyl-*sn*-glycerols were utilized. Michelsen *et al.* (2) have published the mass spectrum of a derivative of this kind prepared from 1,2-dilauroylglycerol. Kuksis *et al.* (11) have utilized LC-MS to identify nitrourethane derivatives of enantiomeric diacylglycerols. LC-MS would be of great value with triacyl-*sn*-glycerols containing a wide range of fatty acids such as milk fat or fish oils.

Further improvements in this elution system may be possible, for example, by modifying the mobile phase. The present system makes use of conventional silica gel columns and reagents that are readily available commercially. Obviously much remains to be done, but the potential of this approach to the resolution of chiral diacylglycerol derivatives in the stereospecific analysis of triacyl-*sn*-glycerols appears clear.

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COMMUNICATIONS

Δ6 Desaturase in Brain and Liver During Development and Aging

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Δ6 Desaturase was measured in the mouse brain and liver using linoleic acid as substrate. During pre- and postnatal development, Δ6 desaturase in brain decreased dramatically (12-fold) up to postnatal day 21 and remained nearly constant thereafter. In liver, the activity increased approximately 9-fold between day 3 before birth and day 7 after birth. Then it decreased slightly up to weaning and was approximately constant up to 4 mo. From then on, Δ6 desaturase decreased with age (40% between 4 and 17 mo).
Lipids 25, 354-356 (1990).

Brain is one of the tissues containing high amounts of lipids which, in turn, play a role in modulating the structure, fluidity and function of brain membranes (1-3). Brain lipids contain polyunsaturated fatty acids derived from dietary essential linoleic and α-linolenic acids. More than one-third of the brain fatty acids are polyunsaturated with a prevalence of acids containing very long chains (mainly arachidonic acid, 20:4 n-6, and cervonic acid, 22:6 n-3). In fact, brain cells and organelles contain only trace amounts of linoleic acid and α-linolenic acid (4-6). Thus, either these fatty acids are rapidly and completely transformed into the longer chain fatty acids after crossing the blood brain barrier, or the fatty acids essential for the brain are in fact the very long chain fatty acids which are either synthesized in the liver or are provided with the diet. *In vitro* studies, using dissociated fetal brain cells in culture, have shown that differentiation, multiplication of the cells, and release of neuromediators are effective only if the cells are grown in the presence of 20:4 n-6 and 22:6 n-3 (7,8). Essential fatty acid deficiency is known to have dramatic effects on various organs (9-11).

During development, it is essential to provide brain cells with polyunsaturated fatty acids. Otherwise, irreparable damage can occur. Polyunsaturated fatty acid requirements during development are particularly critical because the turnover of brain membranes is quite slow—more than one year for myelin fatty acids (12) and because renewal of neurons and oligodendrocytes is minimal. It has been hypothesized that one aspect of aging could be the reduced activity of Δ6 desaturase which would impede membrane renewal (13).

Studies on desaturase, though numerous in liver but rare in brain, have generally been limited to one tissue at only one or two time points. Unfortunately, only few studies have been undertaken to follow Δ6 desaturase activity during development, and nothing is known in regard to Δ6 desaturase activity in brain during aging. In a study that focussed on early development (up to 30 days after birth in the rat), Strouve-Vallet and Pascaud (14) found that Δ6 desaturase activity in rat liver microsomes remained constant, but dramatically decreased in brain microsomes. Cook (15) obtained similar results during early development in rat brain homogenate, but reported markedly increased activity in liver from day 4 up to day 32 after birth. Purvis *et al.* (16) found that desaturase activities in brain microsomes of newborn pigs at 60 days gestation and at 5 wk post-partum were nearly identical. In contrast, Δ6 desaturase activity was approximately 3-fold higher in the liver. Sanders and Rana (17) found that the activity in 21-day fetuses was approximately 3-times higher in brain than in liver. In adult and fetal human liver, Δ6 desaturase is the rate limiting step of arachidonic synthesis (18). Interestingly, Δ6 desaturase has been reported to peak 3 days after surgery in regenerating rat liver (19).

As polyunsaturated fatty acids appear especially important to maintain functional structures in brain (20-23), this work was undertaken to determine Δ6 desaturase activity in both brain and liver during development and aging. The mouse was chosen as model because it is known that desaturating activity in mice is lower than that in rats (24,25) and thus is closer to that in humans, taking into account the ratio of very-long-chain polyunsaturated fatty acids to their precursors in the blood and liver.

MATERIALS AND METHODS

Mice were bred in our laboratory (C3H-SWV strain; similar results were obtained with Swiss strain) and fed standard chow (Iffa-Credo, l'Arbresle, France). As young animals were used prior to weaning, all animals had free access to chow. Animals were killed by decapitation. Livers and brains were immediately excised, rinsed with ice-cold physiological saline, blotted, and homogenized in a Potter apparatus.

Incubations were performed essentially as described by Cook (15) and as modified by Blond *et al.* (26), Blond and Lemarchal (27), and Narce and Poisson (28). Briefly, tissues were homogenized in 0.25 M saccharose, 0.05 M phosphate, and 2 mM glutathione, pH 7.4 (2 g fresh weight tissue/5 mL buffer). Homogenates were centrifuged for 15 min at 12000 × *g* in a Sorvall centrifuge, supernatant was carefully taken up, and in case of liver homogenates diluted two-fold with buffer. Protein was measured according to Lowry *et al.* (29). Incubation media (2 mL) contained variable amounts of protein, Na_2HPO_4 (50 mM), ATP (7.5 mM), $MgCl_2$ (3.8 mM), NADPH (0.2 mM), NADH (0.5 mM), CoA (0.2 mM), and [1-^{14}C]linoleic acid (100 nmol, 2 μCi, 20 μL). After thirty minutes, incubations were stopped by addition of 0.5 N KOH in ethanol. After adding unlabelled fatty acids (commercial grade) as carriers (30 μg each of 18:2 n-6, 18:3 n-6, 20:3 n-6, 20:4 n-6, 20:5 n-3, and 22:6 n-3),

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Abbreviations: ATP, adenosine 5'-triphosphate; b.p., boiling point; CoA, coenzyme A; KOH, potassium hydroxide; NADH, reduced NAD (nicotinamide adenine dinudeotide; NADPH, reduced NADP (NAD phosphate).

lipids were saponified at 90° for 30 min. After acidification (0.4 mL 10 N HCl), fatty acids were extracted 2-times with 5 mL of hexane and were methylated with 14% boron trifluoride in methanol for one hour. Methyl esters were purified by thin-layer chromatography using petroleum hydrocarbon (b.p. 40'-60°)/diethyl ether (80:20, v/v) as developing solvent. Radioactive methyl esters were visualized by autoradiography, the fractions were extracted 2-times with 3 mL of petroleum hydrocarbon and 2-times with diethyl ether, the solvent was evaporated in a stream of nitrogen, and the lipids were further resolved according to their degree of unsaturation by argentation thin-layer chromatography. Silver nitrate impregnated silica gel plates (30% $AgNO_3$) were prepared by immersing silica gel plates into a 10% $AgNO_3$ solution in acetonitrile for 15 min and drying for 15 min at 100°. After development with petroleum hydrocarbon (b.p. 40-60°)/diethyl ether (50:50, v/v), fractions were visualized by autoradiography and by staining with 0.1% dichlorofluorescein, then were scraped off, and counted in a scintillation counter.

RESULTS AND DISCUSSION

With brain homogenate, Δ6 desaturase activity was linear up to 4 mg protein per mL incubation medium at all ages. With liver homogenate, it was linear up to 2 mg protein/mL up to 3 days after birth. Thereafter Δ6 desaturase activity was linear up to 1 mg/mL. Figure 1 shows Δ6 desaturase activity during development and aging in brain and in liver. In liver, the activity increased approximately 9-fold from day 18 at fetal age up to day 7 after birth. It decreased by 44% up to weaning and was constant then up to 4 mo. Interestingly, Δ6 desaturase activity decreased by 40% during aging. In brain, the activity dramatically decreased during early development: between day 18 of gestational age and weaning it was reduced about 14-fold, but remained nearly stable during adulthood and aging. These results are consistent with those of Purvis *et al.* (16) which showed that preterm desaturase activities in both pig liver and pig brain were lower than at term. They also are consistent with the data of Sanders and Rana, (17) that showed that activity in 21-day fetal rat brain is 3.4-fold higher than in liver, and those of Strouve-Vallet and Pascaud (14) for the 4-to-30 day postnatal period in both liver and brain, although the latter did not observe the day-7 peak in liver or any activity in brain after 21 days. In agreement with Cook (15) we found a decrease in brain activity during early development, but in disagreement with him we did not find any increased activity in the liver after the 4th day. Our results are also in agreement with those of Ravel *et al.* (30) who found that the elongation rate of linoleic acid in fetal rat liver was lower than in maternal liver. However, these authors found that there was no significant difference between liver Δ6 activity in the fetus and in the pregnant rat.

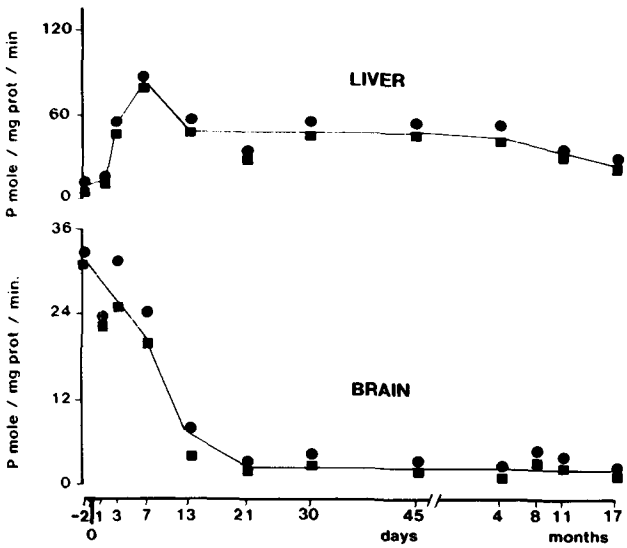

FIG. 1. Δ6 Desaturase during development and aging in mouse brain and liver homogenates. ●, total synthesis of polyunsaturated fatty acids from linoleic acid (mainly γ-linolenic acid, with small amounts of arachidonic acid and trace amounts of adrenic acid); ■, γ-linolenic acid synthesized from linoleic acid.

Δ6 Activity in brain is very high during early development up to 7 days after birth. This corresponds to the period of neuronal and glial multiplication, the latter event being at a peak at 3-5 days after birth in the mouse. Early brain development requires large quantities of polyunsaturated fatty acids for membrane synthesis. Interestingly, Δ6 activity does not peak during myelination, although myelin contains large amounts of polyunsaturated fatty acids. The same pattern was found for Δ9 desaturase activity (31). Thus, polyunsaturated fatty acids required for myelination are either accumulated in the oligodendrocytes before myelination or possibly are supplied through the blood stream. This is in contrast to the synthesis of saturated and monounsaturated long-chain and very-long-chain fatty acids which peaks during myelination and is impaired in neurological dysmyelinating mutants (32). Interestingly, chain lengthening of eicosapentaenoic acid (EPA) is less affected in these mutants than elongation of erucic and arachidic acids (33).

The question remains whether the residual Δ6 desaturase activity after day 21 is sufficient for synaptogenesis and, later on, to support the turnover of brain membranes. If it is not, the very-long-chain fatty acids would have to be synthesized by the liver. As liver synthesis decreases during aging this source may be insufficient.

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Mechanism of Lysophosphatidylcholine Accumulation in the Ischemic Canine Heart

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The metabolism of lysophosphatidylcholine (LPC) in non-ischemic and ischemic canine heart was investigated by *in vitro* enzyme analysis. Selected subcellular fractions were assayed for the LPC-producing enzyme phospholipase A and the LPC-eliminating enzymes LPC:acyl-CoA acyltransferase, LPC:LPC transacylase and lysophospholipase. The canine heart was found to contain all enzymes differing, however, in subcellular distribution and specific activity. Phospholipase A activity did not change significantly in any of the fractions prepared from the ischemic tissue of hearts rendered ischemic for 1, 3 or 5 hr when compared to non-ischemic tissue. Changes in the activity of the microsomal LPC:acyl-CoA acyltransferase over the course of 5 hr of ischemia were observed. Significant decreases in the activity of the cytosolic and microsomal lysophospholipases were detected especially after 3 and 5 hr of ischemia. Similarly, a decrease in the activity of the microsomal LPC:LPC transacylase was noted after 3 and 5 hr of ischemia. Our results suggest that impaired catabolism of LPC rather than an enhanced production of LPC is the principal mechanism for the increase in LPC levels in the ischemic canine heart.
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Lysophospholipids, in particular lysophosphatidylcholine (LPC), have been implicated as possible biochemical mediators of arrhythmogenesis during myocardial ischemia. This stems from the observation by Sobel *et al.* (1) and later reported by others (2-4) of elevated levels of LPC in the ischemic heart. These findings coupled with the ability of pathophysiological concentrations of LPC to elicit marked electrophysiological changes similar to those seen in myocardial ischemia *in vivo* (5,6) strongly suggest a role for increased LPC in the production of arrhythmias during ischemia.

LPC is a normal constituent of virtually all biological membranes (7). Due to its cytolytic properties, the level of LPC is by necessity maintained low and is under strict metabolic control. In the heart the majority of LPC is produced *via* hydrolysis of the parent phospholipid phosphatidylcholine (PC) by phospholipase A. This enzyme has been reported in several subcellular fractions including the cytosol (8), mitochondria and microsomes (9). Catabolism of LPC may proceed either by reacylation catalyzed by LPC:acyl-CoA acyltransferase, by transacylation catalyzed by LPC:LPC transacylase or by deacylation catalyzed by lysophospholipase. These enzyme activities have all been detected in the heart (10,11). However, the relative importance of each enzyme to LPC catabolism, in particular during ischemia, is unclear.

In this study, we have attempted to provide a possible explanation for the elevation of LPC levels in the ischemic canine heart based on differences in the activity of the LPC-metabolizing enzymes in various subcellular fractions from ischemic and non-ischemic tissue. This information may be useful in designing pharmacological interventions aimed at preventing increase in LPC during ischemia and thus protecting the heart from the deleterious effects of increased LPC.

MATERIALS AND METHODS

Materials. [1-^{14}C]Palmitoyl LPC (58.5 mCi/mmol) and [1-^{14}C]linoleoyl CoA (53.9 mCi/mmol) were obtained from New England Nuclear Corporation (Boston, MA). LPC and PC (pig liver) were purchased from Serdary Research Laboratories (Ontario, Canada). Linoleoyl CoA was purchased from Sigma Chemical Co. (St. Louis, MO). Thin-layer chromatography plates (SIL G-25) were the products of Brinkmann Instruments, Ltd. (Toronto, Canada). Evan's blue dye was obtained from Sigma Chemical Co. All other chemicals and solvents were of reagent grade and were purchased from Fisher Scientific (Ontario, Canada).

Surgery and preparation of subcellular fractions. Mongrel dogs of either sex (8-12 kg) were anesthesized with 30 mg/kg i.v. sodium pentobarbital and ventilated with room air using a Harvard respirator via a cuffed endotracheal tube. The heart was exposed by a left thoractomy and the left anterior descending coronary artery was dissected free 1-2 cm from the origin. Coronary artery ligation was performed by a two-stage procedure as described by Harris (12). In some dogs it was necessary to administer lidocaine 5 mg/kg i.v. to manage arrhythmias which developed within 30 min after the second stage of ligation. After 1, 3 or 5 hr, hearts were excised and placed in ice cold saline. In the early stages of this study, a modification of the procedure described by Romson *et al.* (13) was used to aid in the identification of the ischemic area. A cannula was inserted into the aorta above the coronary ostia and securely fastened with braided umbilical tape. After clearing the coronary vasculature with saline, sufficient Evan's blue dye (0.5% in saline) was injected to provide a clear demarcation of the ischemic zone of the heart.

Transmural sections of both the ischemic and non-ischemic areas were removed and cytosolic, mitochondrial and microsomal fractions were prepared by the method of Renooj and Snyder (14). The purity of the subcellular fractions was assessed by marker enzyme analysis. Succinate dehydrogenase (15) was used as the mitochondrial marker, whereas glucose-6-

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Abbreviations: ANOVA, analysis of variance; FA, fatty acid; LPC, lysophosphatidylcholine; PC, phosphatidylcholine.

phosphatase (16) was employed as the microsomal marker. The microsomal fraction was found to be contaminated with 7% of mitochondrial protein, while the mitochondrial fraction was found to contain 11% of microsomal material. The cytosolic fraction was determined to contain less than 3% of the mitochondrial and microsomal enzymes. The recovery of protein in both the cytosolic and microsomal fractions from normal and ischemic tissues is shown in Table 1. No appreciable change in protein distribution between subcellular fractions collected from non-ischemic and ischemic tissues was found at any time point. In all subsequent analyses, corrections for total protein were made. All fractions were stored at −80°C. Protein was measured by the procedure of Lowry *et al.* (17).

Preparation of labeled phosphatidylcholine substrate. Labeled phosphatidylcholine (PC) substrate ([^{3}H]*methyl* PC) was prepared biosynthetically using the procedure of Cao *et al.* (18). The PC obtained was subjected to mild acid hydrolysis (19) to destroy the alkenyl component. Briefly, lipid (approx. 4.0 μmol) was placed in test tubes and dissolved in 1.6 mL chloroform/methanol (5:11, v/v) to which were added 400 μL of 0.025 M $HgCl_2$ in 0.05 M HCl. The tubes were then incubated for 30 min at 37°C. Lipids were then extracted, subjected to thin-layer chromatography and re-extracted from the silica gel. The PC fraction obtained was chromatographically pure when compared with commercially available standards. Specific activity of labeled substrate was typically 8,000 dpm/nmol. The labeled substrate was stored at −20°C in chloroform.

Assay of phospholipase A activity. Cytosolic, mitochondrial and microsomal fractions from both non-ischemic and ischemic zones of the heart were assayed for phospholipase A activity by the procedure of Tam *et al.* (20). It has been previously shown that phospholipase A activity is dependent on the molecular species of its PC substrate (18). Therefore, we elected to use biosynthetically prepared substrate which had a varied composition of fatty acid residues to mimic more closely the endogenous substrate (18). In addition, we chose not to study the plasmalogen form of PC, thus only the diacyl form was used as substrate. Inclusion of 200 nmol of LPC per reaction assay was necessary to inhibit the lysophospholipase and allow for accumulation of the product (i.e., labeled LPC). Similar results were obtained with 0.2% deoxycholate as a lysophospholipase inhibitor. The reaction mixture contained 50 mM Tris-HCl (pH 8.5), 5 mM calcium chloride, 200 nmol LPC, 100 nmol labeled substrate (dispersed in water by sonication) and 250 μg of the required protein fraction in a final volume of 0.5 mL. Reaction blanks contained water in place of protein. All assays were performed in triplicate. The reaction mixtures were incubated at 37°C for 30 min, upon which 1.5 mL of chloroform/methanol (2:1, v/v) and 250 μL of water were added to each tube. The tubes were vortexed and spun in a table top centrifuge. Aliquots of the lower phase were drawn and the solvent removed under nitrogen. The residue was dissolved in a small amount of chloroform/methanol (2:1, v/v) and transferred to thin-layer chromatography plates along with carrier LPC. The plates were placed in chromatography tanks containing chloroform/methanol/water/acetic acid (75:60:8:10, v/v/v/v) and allowed to develop. The plates were then removed and briefly dried in an oven set at 110°C. Bands were visualized in iodine vapor and the LPC bands scraped into scintillation vials and then subjected to scintillation counting. Phospholipase A activity is expressed as nmol LPC formed/mg protein/hr.

Assay of lysophospholipase and LPC:LPC transacylase activities. Lysophospholipase was assayed in each fraction from ischemic and non-ischemic tissue using the method outlined by Severson and Fletcher (11). The reaction mixture (200 μL final volume) contained 50 mM sodium phosphate buffer (pH 7.5), 2 mM magnesium chloride, 100 μM [1-^{14}C]LPC (specific activity 2.27 μCi/μmol), and 50 μg protein. It has been previously demonstrated that rabbit microsomal lysophospholipase activity deviates from Michaelis-Menten kinetics at high substrate/protein ratios (10). Unlike the rabbit enzyme, the present canine microsomal preparation was not inhibited to any appreciable extent at the substrate/protein ratio used. Moreover, the substrate profiles were qualitatively similar between non-ischemic and ischemic microsomal lysophospholipase (data not shown). Reaction blanks contained water instead of protein. All analyses were performed in triplicate. The tubes were incubated for 15 min at 37°C after which 1.5 mL chloroform/methanol (2:1, v/v) and 550 μL water were added. The tubes were

TABLE 1

Recovery of Protein from Subcellular Fractions Prepared from Normal and Ischemic Tissues[a]

		Cytosolic	Microsomal	
1 hr	normal	23.6±1.6	1.24±0.10	n=3
	ischemic	23.6±2.4 (100%)[b]	1.26±0.11 (102%)	
3 hr	normal	22.6±0.8	1.06±0.08	n=5
	ischemic	23.1±1.1 (102%)	0.88±0.12 (80%)	
5 hr	normal	25.0±0.4	1.25±0.09	n=5
	ischemic	24.9±0.5 (99%)	0.97±0.09 (78%)	

[a]All values are expressed as mg protein per g wet weight (mean ± S.E.); n, number of experiments.

[b]Values in parentheses denote percent recovery of protein from ischemic tissue vs normal tissue.

vortexed and centrifuged as before. Aliquots were drawn from the lower phase and applied to thin-layer chromatography plates. The plates were developed in chloroform/methanol/water/acetic acid (70:30:4:2, v/v/v/v). After staining in iodine vapor the area corresponding to the neutral lipid fraction was scraped into scintillation vials and analyzed for radioactivity. Lysophospholipase activity is expressed as nmol fatty acid (FA) formed/mg protein/hr. LPC:LPC transacylase activity was assayed in a similar manner except that the PC band was analyzed for radioactivity. Since PC synthesized by LPC:LPC transacylase would have twice the specific activity of the LPC substrate, the radioactivity in the PC was divided by two to calculate reaction velocity. LPC:LPC transacylase activity is expressed as nmol PC formed/mg protein/hr.

Assay of LPC:acyl-CoA acyltransferase activity. The cytosolic and mitochondrial enzyme were assayed as described by Severson and Fletcher (11). The reaction mixture contained 100 μM linoleoyl-CoA, 100 μM [1-^{14}C]LPC (specific activity 2.27 μCi/μmol), 50 mM sodium phosphate buffer (pH 7.5), 2 mM magnesium chloride and 50 μg protein in a final volume of 200 μL. the reaction was carried out as described above for the LPC:LPC transacylase except the incubation temperature was 25°C. Microsomal activity was assayed using 5 μg protein and the incubation time was 2.5 min. In some experiments, [1-^{14}C]linoleoyl CoA (100 μM) was employed as the labeled substrate. LPC:acyl-CoA acyltransferase activity is expressed as nmol PC formed/mg protein/hr except for microsomal activity which is expressed as μmol/mg protein/hr.

Statistical analyses. Analysis of variance (randomized ANOVA) followed by Duncan's test or Student's t-test for paired and unpaired data were used where appropriate. Values are expressed as mean ± standard error (S.E.).

RESULTS

Subcellular distribution of LPC-metabolizing enzymes in the canine heart. There was no difference between the enzyme activities of the various subcellular fractions obtained from normal hearts and the non-ischemic region of hearts subjected to coronary artery ligation. Table 2 summarizes the results from the pooled data. It is clear that the microsomal fraction contained the highest specific activity of the enzymes assayed. The cytosolic fraction was completely devoid of any LPC:LPC transacylase activity. The LPC:LPC transacylase and lysophospholipase activities detected in the mitochondrial fraction were small relative to the activity in the microsomal fraction. When corrected for microsomal contamination, there was no detectable activity.

Comparison of LPC-metabolizing enzyme activities after various durations of ischemia in ischemic and non-ischemic regions. The percentage change in phospholipase A activity of the ischemic region with respect to the corresponding non-ischemic region after 1, 3, and 5 hr of ischemia is shown in Figure 1. Although there appeared to be a trend towards increased activity in the cytosolic fraction, especially after 5 hr of ischemia, no statistically significant difference in activity in either the cytosolic, mitochondrial and microsomal fractions was detected. Since there was no significant alteration in total phospholipase A activity during ischemia, no attempt was made to further differentiate between changes in phospholipase A_1 and A_2 activities.

A reduction in the microsomal LPC:acyl-CoA acyltransferase activity was observed at 3 hr of ischemia but a significant decrease in enzyme activity was detected only after 5 hr of ischemia ($p<0.01$). However, a time-dependent increase in the activity of this enzyme associated with the mitochondrial fraction from ischemic tissue was observed (Fig. 2). Mitochondria isolated from tissue rendered ischemic for 5 hr showed a 60% greater capacity to metabolize LPC by this route when compared to control tissue ($p<0.01$). There were no changes in cytosolic activity.

A time-dependent decrease in LPC:LPC transacylase activity associated with the microsomal fraction was found (Fig. 3). The reduction in LPC:LPC transacylase activity (54%) after 5 hr of ischemia was statistically significant ($p<0.01$). There was also a time-dependent reduction in the microsomal lysophospholipase activity attaining statistical significance at 5 hr of ischemia (Fig. 4). The extent of reduction in activity (55% after 5 hr) detected with this enzyme was similar to the microsomal LPC:LPC transacylase. A parallel

TABLE 2

Activity of LPC Metabolizing Enzymes in Various Subcellular Fractions from Normal and Non-Ischemic Regions of Canine Hearts[a]

	Cytosolic	Mitochondrial[b]	Microsomal
Phospholipase A (nmol LPC formed/mg protein/hr)	0.5±0.01 n=9	0.8±0.1 n=9	3.2± 0.3 n=9
LPC:acyl-CoA acyltransferase (nmol PC formed/mg protein/hr)	14 ±2 n=12	63 ±4 n=12	18.0± 1.0[c] n=13
LPC:LPC transacylase (nmol PC formed/mg protein/hr)	[d]	[d]	25 ± 2 n=18
Lysophospholipase (nmol FA formed/mg protein/hr)	25 ±1 n=32	[d]	424 ±26 n=33

[a]Enzyme activities were assayed as described in Materials and Methods. Values represent mean ± S.E.; n, number of experiments.
[b]Data were corrected for microsomal contamination.
[c]Data expressed as μmol PC formed/mg protein/hr.
[d]No detectable activity.

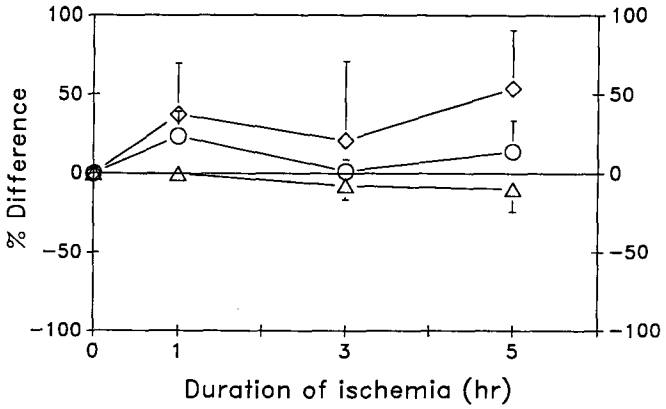

FIG. 1. The relationship between duration of ischemia and phospholipase A activity. Cytosolic (◇), mitochondrial (○) and microsomal (△) fractions of canine heart subjected to various durations of ischemia were assayed for enzyme activity as described in Materials and Methods. Values from the ischemic fractions are calculated as a percentage of the corresponding non-ischemic fractions. Each data point represents the mean ± S.E. of the percentage difference from four separate experiments. Enzyme activities in the non-ischemic region at 1, 3 and 5 hr are: 0.6, 0.4 and 0.4 nmol/mg protein/hr, respectively, for the cytosolic enzyme; 0.8, 0.9 and 0.8 nmol/mg protein/hr, respectively, for the mitochondrial enzyme; and 3.2, 3.4 and 2.8 nmol/mg protein/hr, respectively, for the microsomal enzyme. **p<0.01, ***p<0.001.

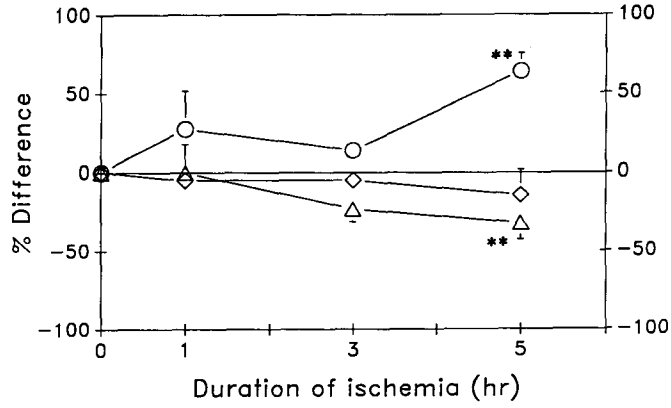

FIG. 2. The relationship between duration of ischemia and LPC:acyl-CoA acyltransferase activity. Data presentation and symbols are the same as in Figure 1. Enzyme activities in the non-ischemic region at 1, 3 and 5 hr are: 13, 14 and 12 nmol/mg protein/hr, respectively, for the cytosolic enzyme (n=4); 159, 177 and 135 nmol/mg protein/hr, respectively, for the mitochondrial enzyme (n=4); and 16.7 and 18.7 μmol/mg protein/hr (3 and 5 hr), respectively, for the microsomal enzyme (n=5).

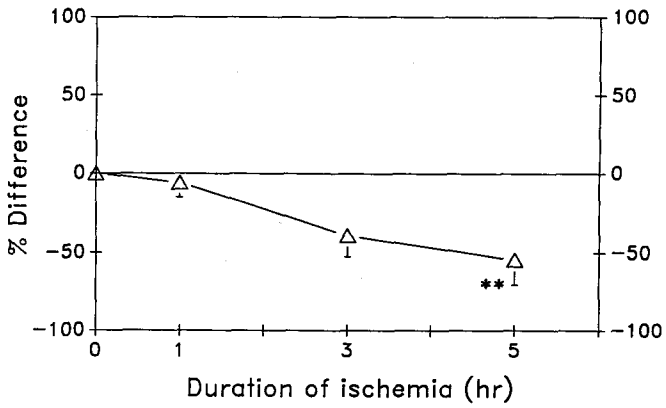

FIG. 3. The relationship between duration of ischemia and LPC:LPC transacylase activity. Data presentation and symbols are the same as in Figure 1. Enzyme activities in the non-ischemic region at 1, 3 and 5 hr are 25, 23 and 28 nmol/mg protein/hr, respectively, for the microsomal enzyme (n=6).

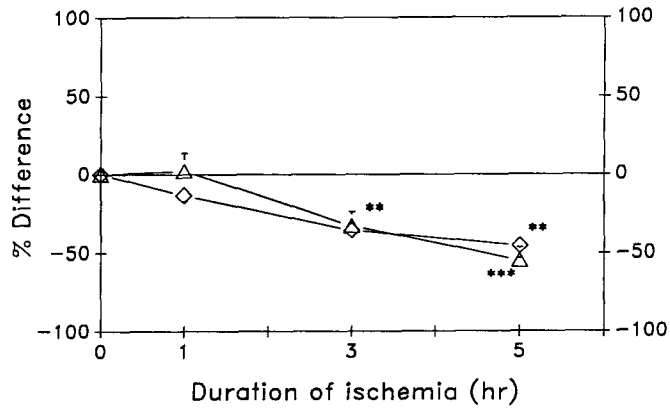

FIG. 4. The relationship between duration of ischemia and lysophospholipase activity. Data presentation and symbols are the same as in Figure 1. Enzyme activities in the non-ischemic region at 1, 3 and 5 hr are: 28 (n=7), 26 (n=8) and 25 (n=11) nmol/mg protein/hr, respectively, for the cytosolic enzyme; and 490 (n=7), 352 (n=8) and 436 (n=11) nmol/mg protein/hr, respectively, for the microsomal enzyme.

decrease in activity of the cytosolic enzyme was also observed attaining a level of significance at both 3 and 5 hr ($p<0.01$).

DISCUSSION

Although several studies have demonstrated an accumulation of LPC in the ischemic heart (1,2,21) and a temporal link between this accumulation and the occurrence of arrhythmias exists (3), little is known about the metabolism of LPC, especially during myocardial ischemia. We undertook this investigation to examine more fully the metabolism of LPC in the canine heart and to provide the evidence for a possible biochemical mechanism for its increase during ischemia.

Our results clearly show that the LPC-producing enzyme phospholipase A and the LPC-eliminating enzymes LPC:LPC transacylase, LPC:acyl-CoA acyltransferase and lysophospholipase are all present in the canine heart albeit with varying specific activities and intracellular distribution. The present findings are in general agreement with studies in canine (8,9) and rabbit heart (10), and isolated rat cardiac myocytes (11). However, several noteworthy differences exist. Unlike the rabbit heart (10), we did not detect any cytosolic LPC:LPC transacylase activity. This clearly indicates that the pattern of distribution of some of these enzymes is species-specific as documented previously (22). In addition, we were able to demonstrate substantial mitochondrial LPC:acyl-CoA acyltransferase activity which was present only at relatively low levels in the rabbit heart (10). Although this result may also be ascribed to a difference in model, it is more likely due to our use of linoleoyl-CoA as fatty acid donor instead of palmitoyl-CoA. Our use of linoleoyl-CoA was based on the recent demonstration by Arthur *et al.* (23) of a linoleoyl-CoA-specific LPC:acyl-CoA acyltransferase in mitochondria of the mammalian heart. The phospholipase A activity may be affected by our use of biosynthetically prepared PC. However, we feel that PC with varied fatty acid residues can mimic the endogenous substate *in vivo* more closely (18). The dispersion of the substrate in water by sonication may also affect the enzyme activity.

In order to probe differences in enzyme activity between ischemic and non-ischemic regions of canine heart, it is essential that the ischemic tissue is clearly differentiated from the non-ischemic or normally perfused tissue. To this end, we have injected Evan's blue dye retrogradely through the aorta to facilitate identification of the ischemic (i.e., nonstaining) portion of the left ventricle. We found this step to be necessary as the size and precise delineation of the ischemic area varied slightly from one preparation to the next possibly due, in part, to differences in the extent of vascularization by collateral vessels. The extra time required between excision of the heart and homogenization for the necessary manipulation had no effect on the activity of any of the enzymes studied. Since ischemic and non-ischemic samples were taken from the same heart, the problem of animal to animal variation was eliminated.

Since we did not observe any significant change in phospholipase A activity during ischemia, our data do not support increased production of LPC as a mechanism for the elevated levels of LPC in ischemic canine heart. It appears that impaired catabolism is a major factor in the accumulation of LPC in the ischemic canine heart. Hearts rendered ischemic for 5 hr have a significantly reduced capacity (50–60% reduction) to metabolize LPC by both the microsomal and cytosolic lysophospholipase, and the microsomal LPC:LPC transacylase. Significant reductions in the activity of the microsomal LPC:acyl-CoA acyltransferase during ischemia were also observed. Although the microsomal fraction has a higher specific activity for lysophospholipase than the cytosolic fraction, the cytosolic fraction contains greater total activity. However, the relative importance of the microsomal and cytosolic lysophospholipase in the regulation of LPC level in the ischemic heart remains undefined.

The decrease in the activity of the microsomal LPC:LPC transacylase and lysophospholipase was selective in that the microsomal phospholipase A and the LPC:acyl-CoA acyltransferase were not affected to the same extent. Long chain acylcarnitines have been shown to accumulate in ischemic cells (24). Gross *et al.* (25,26) have demonstrated that long chain acylcarnitines are competitive inhibitors of both the lysophospholipase and the LPC:LPC transacylase. The concentrations of the long chain acylcarnitines found in ischemic heart (24) are high enough to cause substantial inhibition of these enzymes. It has also been shown that the reduction of acylcarnitines by POCA attenuates the electrophysiological changes produced by hypoxia (27), suggesting an important role for acylcarnitines. The decrease in lysophospholipase and transacylase activities associated with the microsomal fraction might be the result of selective translocation of these enzymes to the cytosol while the cytosolic activities are masked by the accumulation of metabolites during ischemia. It is also possible that membrane damage during ischemia may affect the enzymes in the microsomal membrane. In addition, an alteration in intracellular pH, especially during ischemia, can affect the enzyme activities. Indeed, it has been demonstrated that membrane bound lysophospholipase showed marked sensitivity to reduced pH (10). Since the capacity for the elimination of LPC is reduced during ischemia, pharmacological intervention aimed at attenuating the factors that are involved in the alterations of LPC metabolism may militate against the accumulation of LPC during ischemia.

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Fatty Chain Compositon of Ether and Ester Glycerophospholipids in the Japanese Oyster *Crassostrea gigas* (Thunberg)

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The fatty chain compositions of 1-*O*-alk-1′-enyl-2-acyl, 1-*O*-alkyl-2-acyl, and 1,2-diacyl glycerophospholipids of the Japanese oyster *Crassostrea gigas* (Thunberg) were investigated. Major fatty chains in the *sn*-1 position of 1-alk-1′-enyl-2-acyl ethanolamine phospholipids (EPL) were 18:0 (64.7%) and 20:1 (11.1%). Major *sn*-1 chains of alkenylacyl choline phospholipids (CPL) were 18:0 (63.3%) and 16:0 (22.2%). In the case of 1-alkyl-2-acyl EPL, the predominant fatty chains in the *sn*-1 position were 18:0 (51.5%), 16:0 (16.0%) and 20:1 (12.5%); in the case of 1-alkyl-2-acyl CPL, the major *sn*-1 chains were 16:0 (44.0%) and 14:0 (23.4%). Saturated fatty chains were predominant in both EPL and CPL. Prominent fatty acids in the *sn*-2 position of the alkenylacyl EPL were 22:6n-3 (29.0%), 20:5n-3 (19.0%) and 22:2 NMID (non-methylene interrupted dienes, 16.6%) contributing to about 65% of the total fatty acids, while alkenylacyl CPL was rich in the saturated acids 16:0 (32.0%) and 18:0 (9.2%). In the alkylacyl EPL, 16:0, 18:1n-9, 18:0 and 16:1n-7 were prominent *sn*-2 fatty acids and accounted for 30.6%, 10.0%, 9.8%, and 8.3%, respectively. Polyunsaturated fatty acids were detected, but were present at extremely low percentages. Major *sn*-2 fatty acids in alkylacyl CPL were 16:0 (25.4%), 22:6n-3 (16.0%) and 20:5n-3 (8.4%). The major fatty acids of diacyl EPL were 20:5n-3 (22.3%), 16:0 (17.9%), and 18:0 (16.1%), and those of diacyl CPL were 16:0 (30.4%), 20:5n-3 (17.6%) and 18:1n-7 (7.4%).
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Since Feulgen and Voit (1) discovered plasmalogens in the protoplasm of certain animal tissues in 1924, much information has become available on the distribution of ether glycerophospholipids in marine invertebrates, although their biological significance has remained relatively obscure. Rapport and Alonzo (2) determined the fatty aldehydes derived from plasmalogens of sea urchin, starfish, whelk, squid, sea anemone, mussel, softshell clam, scallop, sea-cucumber, quahaug and horseshoe crab, and found a great variety of marine invertebrates to be rich in plasmalogens. Thompson and Lee (3) reported on the plasmalogen contents of the lamellibranch *Protothaca staminea* and tentacles of *Octopus dolfleini.* Marine worms (4), abalone (5), snails and bivalves (6) and sponges (7) have also been reported to contain significant amounts of plasmalogens. Dembitsky (8) carried out a comprehensive survey of the plasmalogen contents of marine invertebrates, and found that coelenterates, mollusks and echinoderms were particularly rich in plasmalogens.

Thompson and Lee (3) determined the 1-*O*-alkyl-2-acyl glycerophospholipid contents in *Katherina tunicata, Thais lamellosa, P. staminea* and *O. dolfleini.* Starfish (9), coelenterata, arthropoda, mollusks and tunicata (10) were also reported to contain significant amounts of the alkylacyl glycerophospholipids.

The analysis of the fatty acid compositions of oyster lipids has been carried out by several groups. Watanabe and Ackman (11) investigated the influence of dietary algal fatty acids on the composition of lipids in two species of oyster: American oyster *Crassostrea virginica* and European flat oyster *Ostrea edulis.* Takagi *et al.* (12) analyzed the fatty acid compositions of other species of bivalves, as well as of Japanese oyster *Crassostrea gigas,* by wall-coated open-tubular gas-liquid chromatography (GLC). Recently, Napolitano *et al.*(13) studied in detail the fatty acid profiles of all major lipid classes of 1- and 10-day-old larvae of European oyster *O. edulis* by capillary GLC. However, no information has been available on the particular fatty acids esterified in the *sn*-2 position of 1-*O*-alk-1′-enyl-2-acyl- and 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphoethanolamines (EPL) and *sn*-glycero-3-phosphocholines (CPL) in marine invertebrates. In the present study, the contents and fatty chain compositions of alkenylacyl, alkylacyl, and 1,2-diacyl EPL and CPL of the Japanese oyster were investigated in detail.

MATERIALS AND METHODS

Sample. Japanese oyster *Crassostrea gigas* (Thunberg), cultured in the Hiroshima Bay, Japan, was harvested in March 1988. The oysters were transported to the factory, where the total wet organic tissues of the oyster were removed, washed, frozen with a spiral-freezer at −50°C, and ice-glazed by means of dipping the frozen sample in water for about 1 min. The sample was put into a pouch of ethylene-vinyl acetate copolymer (20 cm × 15 cm in size, 60 μm in thickness), and the opening heat-sealed. The pouched sample was stored for one day at −20°C, then transported to the laboratory on dry-ice, and used immediately. Duplicate samples (groups 1 and 2, each involving 10–11 specimens) were prepared.

Lipid extraction and fractionation. The frozen samples were thawed, minced, and extracted with chloroform/methanol according to the Bligh and Dyer procedure (14). The respective ethanolamine- and choline-containing phospholipids (EPL and CPL) subclasses in the extracted lipids (total lipids, TL) were separated by column chromatography on silicic acid (2.5 cm i.d. × 50 cm; Silica Gel 60, 70–230 mesh; E. Merck, Darmstadt, FRG), using chloroform/methanol in different ratios (4:1, 3:2 and 1:4, v/v/v) as eluants according to the method of Hanahan *et al.* (15).

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Abbreviations: CI, chemical ionization; CPL, choline phospholipids; EPL, ethanolamine phospholipids; GLC, gas-liquid chromatography; CLPL, choline lysophospholipids; ELPL, ethanolamine lysophospholipids; MS, mass spectrometry; NMID, non-methylene interrupted diene; IPL, inositol phospholipids; SPL, serine phospholipids; PUFA, polyunsaturated fatty acids; TLC, thin-layer chromatography; TMS, trimethylsilyl; alkenylacyl-, 1-*O*-alk-1′-enyl-2-acyl-; alkylacyl-, 1-*O*-alkyl-2-acyl-; diacyl-, 1,2-diacyl-.

Determination of phospholipid class composition. Quantitative analysis of phospholipid classes was carried out by two-dimensional thin-layer chromatography (TLC) on silicic acid and a subsequent phosphorus determination; an aliquot of total lipids was spotted on a precoated Silica Gel G plate (0.25mm in thickness, E. Merck) and the plate was developed with chloroform/methanol/acetic acid/water (65:45:1:2, by vol) in the first direction. In the second direction, chloroform/methanol/water (65:25:4, v/v/v) was used as the solvent system. Lipids were visualized by exposing the plate to iodine vapor. Fractions corresponding to individual phospholipids were scraped off. Each fraction was extracted from the recovered Silica Gel G using a Bligh and Dyer procedure (14). The phosphorus content of the extracted phospholipids was determined spectrophotometrically according to the method of Bartlett (16).

Determination of alkenylacyl, alkylacyl, and diacyl chains. Each of the EPL and CPL fractions was hydrolyzed with phospholipase C (*Bacillus cereus*, Boehringer Mannheim, Mannheim, FRG) to obtain the corresponding 1,2-diradyl glycerols (17). These were purified by preparative TLC on silicic acid and then acetylated to obtain 1-*O*-alk-1′-enyl-2-acyl-3-acetylglycerol, 1-*O*-alkyl-2-acyl-3-acetylglycerol, and 1,2-diacyl-3-acetylglycerol (18). The acetylated derivatives of the phospholipid subclasses were separated from each other by preparative TLC on Silica Gel G; petroleum ether/diethyl ether/acetic acid (85:15:1, v/v/v) was used as a solvent system. The separated and purified acetylglycerol derivatives were saponified with 1 N KOH in ethanol at 85°C for 1 hr. The 1-*O*-alk-1′-enylglycerols and 1-*O*-alkylglycerols formed were extracted with diethyl ether and converted to their corresponding trimethylsilyl (TMS) ether derivatives, i.e. 1-*O*-alk-1′-enyl-2,3-TMS-glycerol and 1-*O*-alkyl-2,3-TMS-glycerol, respectively, by means of trimethylchlorosilane and hexamethyldisilazane (Tokyo Kasei Co. Ltd., Tokyo, Japan). Also, the fatty acids liberated upon saponification from the *sn*-1 and *sn*-2 positions of 1,2-diacylglycerol and those from the *sn*-2 positions of 1-*O*-alk-1′-enyl-2-acylglycerol and 1-*O*-alkyl-2-acylglycerol were extracted with diethyl ether and were, after methylation, analyzed by GLC under the conditions mentioned below.

Determination of alkenylacyl, alkylacyl, and diacyl EPL and CPL contents. The fatty acid contents of alkenylacyl, alkylacyl, and diacyl EPL and CPL in the total lipids were determined by GLC using 13:0 fatty acid as internal standard (19).

Gas-liquid chromatography and gas chromatography-mass spectrometry. The fatty chain compositions of 1-*O*-alk-1′-enyl-2,3-TMS-glycerol and 1-*O*-alkyl-2,3-TMS-glycerol derivatives were analyzed by GLC using a Shimadzu GC 8APF instrument (Shimadzu Seisakusho Co. Ltd., Kyoto, Japan) equipped with a SUPEL-COWAX-10 fused silica wall-coated open tubular column (30 m × 0.25 mm i.d., Supelco Japan Ltd., Tokyo) and a Shimadzu CLH 702 split injector (20). The injector and the column were held at 250°C and 210°C, respectively. The split ratio was 1:50. Helium was used as carrier gas at a constant inlet pressure of 1.5 kg/cm^2.

Fatty acid compositions of the phospholipids were analyzed by GLC using the same instrument. The analytical conditions used were similar to those for the analysis of the fatty chain compositions of TMS ether derivatives, except that the column temperature was 195°C.

Mass spectrometric analysis of the TMS ether derivatives of alkenylacyl EPL and alkylacyl CPL was carried out with a Shimadzu GC-MS 9020 DF instrument with a CI source to which the outlet of SUPEL-COWAX-10 column (30 m × 0.25 mm i.d.) was connected directly. The column and injector temperatures were 230°C and 250°C, respectively. The sample eluted from the GLC column was ionized in the CI mode by adding *iso*-butane as a reagent gas to the ion source at a pressure of about 1 Torr, using 200 eV electron beam energy, 200 μA emission current, 3 KV acceleration voltage, and a source temperature of 250°C. Data acquisition and processing was done with an on-line Shimadzu GC-MS 1100 data system.

RESULTS AND DISCUSSION

Lipid contents. The moisture contents and the lipid classes of the Japanese oyster samples are shown in Table 1. The TL, polar lipid (PL), and non-polar lipid (NL) contents of the sample were 2.05%, 0.55% and 1.50%, respectively, showing that PL accounts for about 27% of TL. The PL content was slightly less than that (38%) reported for American oyster harvested in February (21). This may be attributable to the differences in location and season of harvesting (22).

As shown in Table 2, the major phospholipid classes of the sample were CPL (54%) and EPL (30%), amounting to more than 80% of the PL; CLPL, ELPL, SPL and IPL were not present at significant concentrations.

Contents of alkenylacyl, alkylacyl, and diacyl EPL and CPL. The subclass compositions of EPL and CPL in the PL are shown in Table 3. Alkenylacyl EPL and CPL accounted for 59.8% of EPL and 7.2% of CPL corresponding to 96.9 mg/100 g total wet organic tissues and 21.2 mg/100 g total wet organic tissues, respectively. Of the phospholipids in the oysters used in the present study, diacyl CPL was the most prominent component at 83.1% (244 mg/100 g), while the content of diacyl EPL was lower than that of alkenylacyl EPL, although higher than that of alkylacyl EPL. Dembitsky and Vaskovsky (23) reported that there were seasonal variations of the plasmalogens in EPL and CPL, i.e., the plasmalogen contents in the oyster *Crassostrea gigas* analyzed were 54.5% of EPL and 12.9% of CPL, respectively, in winter, while the respective contents in summer were 81.1% of EPL and 6.7% of CPL (8). As has been presumed by Joseph (22), these seasonal changes in the EPL and CPL compositions might be related to the reproduction cycle and thus be a result of gonadal development or loss of gametes after spawning. Sampugna *et al.* (21) found that in the oyster *Crassostrea virginica* the glyceryl ether phospholipid content was 8.3% and that the plasmalogens accounted for 68% of the PL.

Identification of alkenyl and alkyl chains in EPL and CPL. Twelve well-resolved peaks appeared on the gas chromatograms of 1-*O*-alk-1′-enyl-2,3-TMS-glycerol

TABLE 1

Moisture and Lipid Contents of Japanese Oyster Samples (wt %)

	Group 1[a]	Group 2	Mean
Moisture	82.8	83.5	83.2
Total lipid	2.08	2.02	2.05
Polar lipid	0.55	0.54	0.55
Nonpolar lipid	1.53	1.48	1.50

[a]Each group consisted of 10–11 specimens.

TABLE 2

Compositions of Polar Lipids in Japanese Oyster Samples

Phospholipid class	
Choline phospholipids (CPL)	54.3[a] (294)[b]
Ethanolamine phospholipids (EPL)	29.9 (162)
Choline lysophospholipids (CLPL)	3.1 (17.0)
Ethanolamine lysophospholipids (ELPL)	4.3 (23.0)
Serine phospholipids (SPL)	1.9 (10.1)
Others	6.6 (35.8)

[a]Value is presented as % of recovered phosphorus.
[b]Figure in parentheses shows the content in mg/100 g total wet organic tissues.

derived from EPL, as shown in Figure 1. For the purpose of explaining peak assignments, typical mass spectra obtained on peaks No. 4 and No. 12 are shown in Figure 2A and 2B, respectively. Our previous study showed that 1-*O*-alk-1′-enyl-2,3-TMS ether derivatives yielded ion peaks at *m/z* 219 (base peak) due to loss of alkenyl group, and *m/z* 103, *m/z* 205 and *m/z* 235 on the *iso*-butane CI mass spectra (20). The spectrum of peak No. 4 showed a base peak at *m/z* 219, and the other characteristic fragment ion peaks were at *m/z* 103, *m/z* 205 and *m/z* 235. A peak at *m/z* 473 was considered attributable to $[M + 1]^+$ indicating the presence of the

TABLE 3

Alkenylacyl, Alkylacyl, and Diacyl EPL and CPL Contents of Japanese Oyster Lipids (%)[a]

Subclass	EPL	CPL
Alkenylacyl	59.8 (96.9)	7.2 (21.2)
Alkylacyl	7.5 (12.1)	9.8 (28.7)
Diacyl	32.7 (53.0)	83.1 (244)

[a]Figures in parentheses show the content in mg/100 g total wet organic tissues.

1-*O*-alk-1′-enyl-2,3-TMS ether derivatives having a molecular weight of 472. Under these experimental conditions, $(M^+ - 235)$ reflects the molecular weight of the 1-*O*-alk-1′-enyl chain, because *m/z* 235 is considered to be due to the loss of a part of an alkenyl group. The alk-1′-enyl chain of this TMS ether derivative, therefore, has a molecular weight of 237, which corresponds to that of a 17:0 alkenyl chain. Similarly, the component of peak No. 12 was identified as 1-*O*-(20:1)-2,3-TMS ether derivative, since the fragment ion peaks characteristic of the glycerol skeleton of this compound appeared at *m/z* 219, *m/z* 103 and *m/z* 235 on the mass spectrum and mass number unit $(M^+ - 235)$, i.e. 277 in this case, supports the identification of the alkenyl chain as 20:1. The other peaks shown in Figure 1 were identified in the same manner as follows: peak No. 1, 16:0, 2, *iso*-17:0; 3, *anteiso*-17:0; 5, *iso*-18:0; 6, *anteiso*-18:0; 7, 18:0; 8, 18:1; 9, 18:2; 10, 19:0; 11, 20:0.

A gas chromatogram of 1-*O*-alkyl-2,3-TMS ether derivatives from CPL is shown in Figure 3. The CI mass spectra of peaks 1 and 5 in the Figure are illustrated in Figure 4A and 4B, respectively. In our previous study, 1-*O*-alkyl-2,3-TMS ether derivatives yielded a $[M + 1]^+$ ion, indicating the molecular weight, and *m/z* 205, *m/z* 219 and *m/z* 103 ions, the latter 3 ions being derived from their glycerol backbone as in the case of 1-*O*-alk-1′-enyl-2,3-TMS ether derivatives, al-

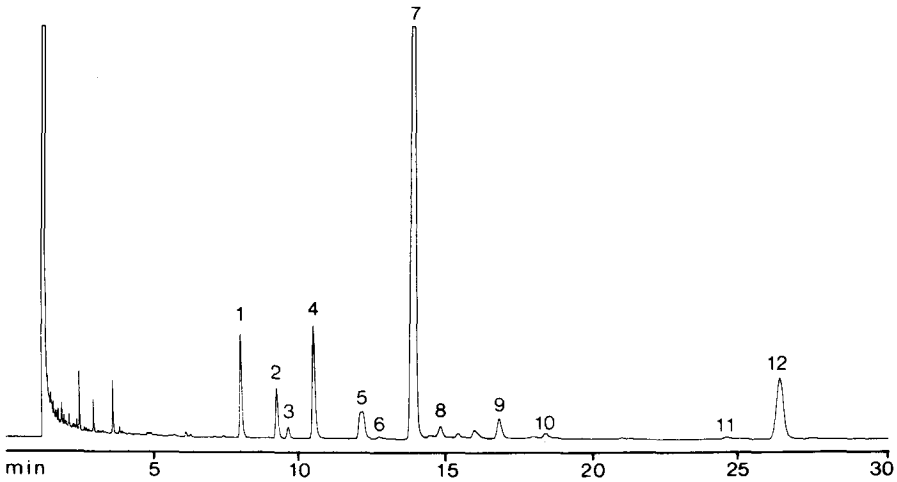

FIG. 1. A typical gas chromatogram of 1-*O*-alk-1′-enyl-2,3-TMS-glycerols derived from the ethanolamine phospholipids in the total wet organic tissues of Japanese oyster. See the text for peak assignments. Column: a SUPELCOWAX-10 fused silica wall-coated open-tubular, 30m × 0.25mm i.d. Temp. 210°C; Injection temp., 250°C; Detector, FID; Carrier gas, He (1.5Kg/cm²).

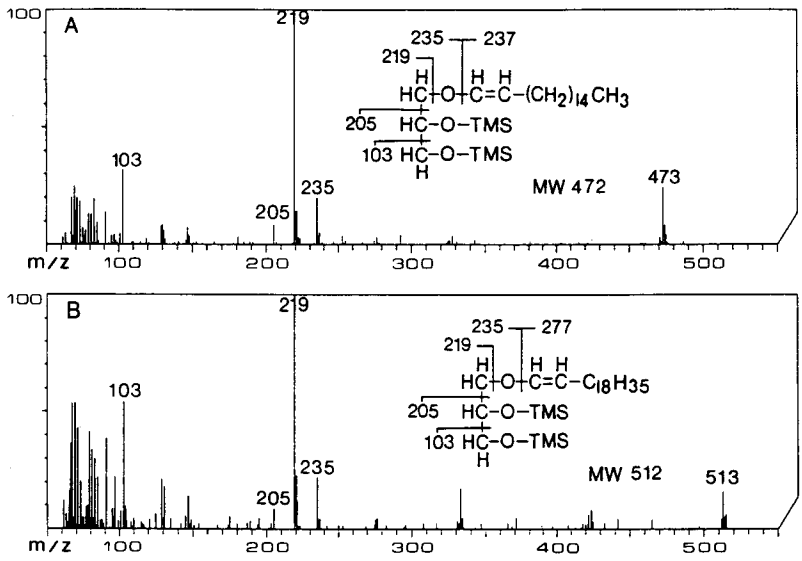

FIG. 2. CI (*iso*-butane) mass spectra of 1-*O*-alk-1′-enyl-2,3-TMS-glycerols derived from the ethanolamine phospholipids in the total wet organic tissues of Japanese oyster. (A), peak No. 4 in Fig. 1. (B), peak No. 12 in Fig. 1. GC/MS conditions: GC, a SUPELCOWAX-10 fused silica wall-coated open-tubular column, 30m × 0.25mm i.d., Temp., 230°C. Injection temp., 250°C. MS; 200 eV electron beam energy, 200 μA emission current, 3KV accelerating voltage, ion source temp., 250°C.

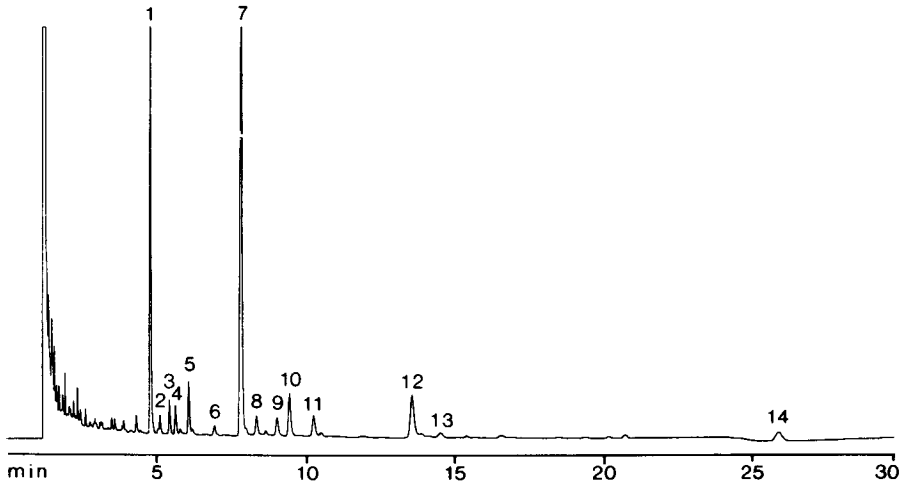

FIG. 3. A typical gas chromatogram of 1-*O*-alkyl-2,3-TMS-glycerols derived from the choline phospholipids in the total wet organic tissues of Japanese oyster. See the text for peak assignment. Conditions are the same as in Fig. 1.

though they did not yield a fragment ion of *m/z* 235. The peaks 1 and 5 shown in Figure 4A and 4B yielded the base peaks at *m/z* 433 and *m/z* 447 due to $[M + 1]^+$, respectively, indicating the molecular weights of the TMS ether derivatives. In addition, both peaks yielded 3 ion peaks at *m/z* 103, *m/z* 205 and *m/z* 219, but not at *m/z* 235. From these results, peaks No. 1 and 5 were identified as the TMS ether derivatives having 14:0 and 15:0 as alkyl chains, respectively. Similarly, the following results were obtained on alkyl chains of the components of peaks shown in Figure 3: peak No. 2, 14:1; 3, *iso*-15:0; 4, *anteiso*-15:0; 6, *iso*-16:0; 7, 16:0; 8, 16:1; 9, *iso*-17:0; 10, *anteiso*-17:0; 11, 17:0, 12, 18:0; 13, 18:1, 14, 20:1.

The 1-*O*-alk-1′-enyl-2,3-TMS-glycerols derived from EPL were identical to those derived from CPL, since

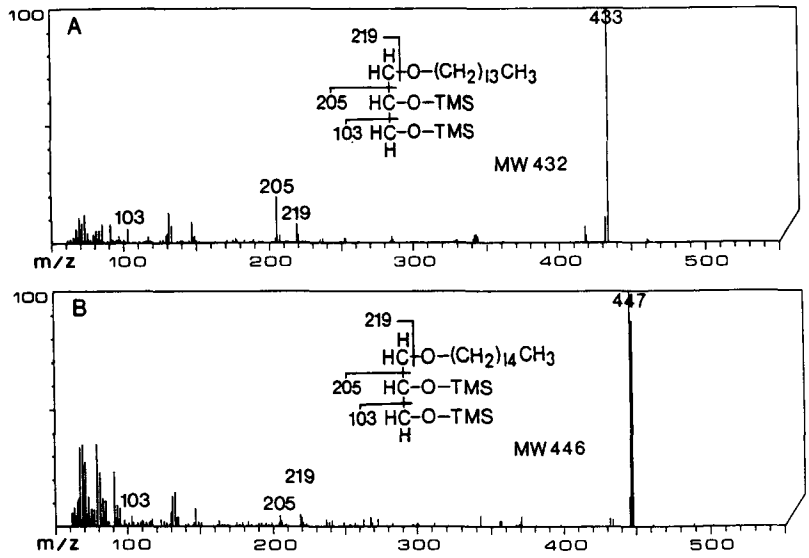

FIG. 4. CI (*iso*-butane) mass spectra of 1-*O*-alkyl-2,3-TMS-glycerols derived from the choline phospholipids in the total wet organic tissues of Japanese oyster. (A), peak No. 1 in Fig. 3. (B), peak No. 5 in Fig. 3. Conditions are the same as in Fig. 2.

alkenylacyl EPL are different only in their organic base moiety: either ethanolamine or choline. For the purpose of identifying alkenyl chains of CPL, therefore, the retention times of peaks of 1-*O*-alk-1′-enyl-2,3-TMS-glycerols of CPL on the gas chromatogram were compared with those of EPL of which the alkenyl chains had already been identified as mentioned above. Identification of the alkyl chain of EPL was carried out in the same manner.

Alkenyl and alkyl chain compositions of EPL and CPL. The fatty chain compositions in the *sn*-1 positions of glycerol moieties of EPL and CPL in the Japanese oyster are listed in Tables 4 and 5. The prominent alkenyl chains in EPL were as follows: 18:0 (64.7%), 20:1 (11.1%) and 17:0 (7.3%), while those of CPL were 18:0 (63.3%), 16:0 (22.2%) and 17:0 (10.8%). In the case of the alkyl chains: 18:0 (51.5%), 16:0 (16.0%), 20:1 (12.5%) and 17:0 (9.0%) were important in EPL, and 16:0 (44.0%), 14:0 (23.4%) and 18:0 (7.8%) in CPL. Snyder *et al.* (9) reported that the TL of a microsomal fraction of the digestive diverticulum of starfish consisted mainly of phospholipid, which was constituted mainly of 18:0 alkenylglycerol (79%), and that the prominent alkyl chains in the TL were 18:0 (39%), 16:0 (31%) and 20:1 (13%). Thompson and Lee (3) studied the alkyl chain compositions of the NL and PL fractions in *K. tunicata, T. lamellosa, P. staminea,* and *O. dolfleini,* and showed that the major alkyl chain components of both fractions were 16:0 and 18:0. Joh and Hata (6) investigated aldehyde compositions of the EPL and CPL in snails, including abalone, *Turbo cornutus,* and bivalves, the latter including *Spisular sachalinensis* and *Mactra sulcatria,* and postulated that the differences in aldehyde compositions between snails and bivalves came from the differences in their diets: the major fatty chains of the former were 18:0, 16:0 and 16:1, whereas they were 20:1, 18:0 and 16:0 in the latter. It is worth pointing out that the percentage of 20:1 dimethylacetal in the plasmalogen of bivalves was higher than that in the snails. In the case of the Japanese oyster, the major alkenyl chain components were similar to those of the marine invertebrates examined in a previous paper (6): 20:1 was present at a high level. Furthermore, the content of odd-numbered ether-linked chains such as 17:0 was higher than that reported previously (3,6). From the ether-linked chain compositions of EPL and CPL in the Japanese oyster, it becomes clear that CPL were rich in those with alkenyl chains longer in chain length than alkyl chains, and that EPL were rich in those composed of alkenyl and alkyl chains longer than in CPL.

Fatty acyl chain compositions of EPL and CPL. As shown in Tables 4 and 5, the prominent fatty acyl chains in the *sn*-2 positions of alkenylacyl EPL were 22:6n-3 (29.0%), 20:5n-3 (19.0%) and 22:2 NMID (16.6%), accounting for about 65% of the total fatty acids, while those of alkenylacyl CPL were rich in saturated fatty acids: 16:0 (32.0%), 18:0 (9.2%) and 14:0 (7.4%). However, the percentages of polyunsaturated fatty acid (PUFA) in alkylacyl EPL were found to be less than those in alkylacyl CPL. The fatty acyl chains in the *sn*-1 and *sn*-2 positions of diacyl EPL were mainly 20:5n-3 (22.3%), 16:0 (17.9%) and 18:0 (16.1%); those of diacyl CPL were 16:0 (30.4%), 20:5n-3 (17.6%) and 18:1n-7 (7.4%).

The fatty acids 20:2 and 22:2 NMIDs were first observed in two mollusks *Littorina littorea* and *Lunatia triseriata* (24), and their structures were characterized as 20:2(Δ5,11), 20:2(Δ5,13), 22:2(Δ7,13) and 22:2(Δ7,15) (25). Takagi *et al.* (12) also observed 20:2

TABLE 4

Fatty Chain Compositions of Ethanolamine Phospholipids in Japanese Oyster[a]

Fatty chain[b]	Subclass	Alkenylacyl		Alkylacyl		Diacyl
	Position	*sn*-1	*sn*-2	*sn*-1	*sn*-2	*sn*-1 + *sn*-2
14:0		—[c]	1.28 ± 0.44	—	7.07 ± 1.81	2.18 ± 1.76
15:0 *iso*		—	tr[d]	—	1.37 ± 0.26	tr
15:0 *anteiso*		—	tr	—	2.37 ± 0.51	tr
15:0		—	tr	—	3.53 ± 0.85	tr
16:0 *iso*		—	tr	—	2.00 ± 0.69	tr
16:0		4.90 ± 0.10	4.90 ± 0.10	16.0 ± 2.61	30.6 ± 2.89	17.9 ± 2.26
16:1n-7		—	1.60 ± 0.59	—	8.25 ± 0.69	1.18 ± 0.22
16:1n-5		—	tr	—	1.37 ± 0.50	tr
17:0 *iso*		3.60 ± 0.64	tr	—	tr	1.10 ± 0.10
17:0 *anteiso*		tr	—	2.67 ± 1.03	—	—
17:0		7.26 ± 0.09	tr	9.01 ± 0.71	2.27 ± 0.77	3.41 ± 0.26
17:1n-8		—	tr	—	1.34 ± 0.34	tr
18:0 *iso*		2.72 ± 0.86	—	1.94 ± 0.32	—	tr
18:0 *anteiso*		tr	—	—	—	—
18:0		64.7 ± 0.04	2.00 ± 0.98	51.5 ± 4.11	9.82 ± 1.55	16.1 ± 2.47
18:1n-9		1.42 ± 0.23	1.78 ± 0.40	2.62 ± 0.88	10.0 ± 1.38	1.85 ± 0.11
18:1n-7		—	tr	—	1.16 ± 0.15	4.11 ± 0.43
18:2n-6		2.38 ± 0.12	tr	3.75 ± 0.83	1.20 ± 0.39	tr
19:0		tr	—	—	—	—
18:4n-3		—	tr	—	—	tr
20:0		tr	—	—	—	tr
20:1n-11		11.1 ± 0.96	3.01 ± 0.21	12.5 ± 3.39	tr	tr
20:1n-9		—	tr	—	tr	1.26 ± 0.11
20:1n-7		—	tr	—	—	5.17 ± 0.70
20:2NMID(5,11)		—	—	—	—	tr
20:2NMID(5,13)		—	tr	—	1.36 ± 0.61	tr
20:4n-6		—	1.66 ± 0.07	—	—	2.83 ± 0.21
20:5n-3		—	19.0 ± 1.05	—	3.49 ± 1.47	22.3 ± 0.81
22:2NMID(7,13)		—	4.72 ± 0.39	—	—	1.00 ± 0.27
22:2NMID(7,15)		—	16.6 ± 2.38	—	2.54 ± 0.96	3.87 ± 0.74
21:5n-3		—	2.11 ± 0.36	—	4.72 ± 1.29	1.07 ± 0.28
22:5n-3		—	5.35 ± 0.45	—	—	tr
22:6n-3		—	29.0 ± 2.22	—	3.19 ± 0.90	6.79 ± 1.44
Others		1.93	6.04	—	2.32	7.86

[a]The data are presented as the mean ± standard deviation of 6 (2 groups × 3) determinations.

[b]The position of the double bond applies to fatty acyl chains.

[c]The '—' denotes not detected.

[d]The 'tr' denotes < 1.0%.

TABLE 5

Fatty Chain Compositions of Choline Phospholipids in Japanese Oyster[a]

Fatty chain[b]	Subclass	Alkenylacyl		Alkylacyl		Diacyl
	Position	*sn*-1	*sn*-2	*sn*-1	*sn*-2	*sn*-1 + *sn*-2
14:0		—[c]	7.40 ± 0.68	23.4 ± 2.13	6.59 ± 1.26	6.68 ± 0.96
14:1		—	—	1.32 ± 0.18	—	—
15:0 *iso*		—	1.40 ± 0.28	2.15 ± 0.15	tr[d]	tr
15:0 *anteiso*		—	2.09 ± 0.71	1.49 ± 0.29	1.27 ± 0.45	tr
15:0		—	3.75 ± 0.22	4.06 ± 0.23	2.82 ± 0.43	1.59 ± 0.11
16:0 *iso*		—	2.35 ± 0.85	tr	1.74 ± 0.23	tr
16:0		22.2 ± 6.23	32.0 ± 0.56	44.0 ± 1.92	25.4 ± 3.64	30.4 ± 1.08
16:1n-7		—	6.87 ± 1.58	2.10 ± 0.40	7.57 ± 0.67	4.10 ± 0.28
16:1n-5		—	1.47 ± 0.41	—	tr	tr
17:0 *iso*		—	tr	2.08 ± 0.19	tr	tr
17:0 *anteiso*		—	—	3.30 ± 1.18	—	—
17:0		10.8 ± 0.97	1.84 ± 0.42	3.10 ± 0.19	1.29 ± 0.32	1.34 ± 0.04
17:1n-8		—	1.03 ± 0.17	—	tr	tr
18:0 *iso*		1.04 ± 0.22	—	—	—	tr
18:0		63.3 ± 5.67	9.18 ± 1.76	7.79 ± 0.54	7.29 ± 0.69	4.30 ± 0.18
18:1n-9		—	8.71 ± 0.72	1.10 ± 0.22	6.86 ± 0.42	2.33 ± 0.09
18:1n-7		—	1.34 ± 0.48	—	tr	7.36 ± 0.61
18:2n-6		—	1.67 ± 0.43	—	1.76 ± 0.25	1.37 ± 0.08
18:4n-3		—	—	—	tr	2.79 ± 0.11
20:1n-11		2.73 ± 0.60	1.05 ± 0.69	3.21 ± 0.47	—	tr
20:1n-9		—	—	—	—	tr
20:1n-7		—	—	—	—	2.65 ± 0.15
20:2NMID(5,11)		—	—	—	—	1.31 ± 0.14
20:2NMID(5,13)		—	1.95 ± 0.58	—	2.15 ± 0.56	tr
20:4n-6		—	—	—	1.08 ± 0.11	2.06 ± 0.17
20:5n-3		—	3.09 ± 1.03	—	8.35 ± 0.65	17.6 ± 0.60
22:2NMID(7,13)		—	—	—	—	tr
22:2NMID(7,15)		—	1.09 ± 0.07	—	tr	1.44 ± 0.80
21:5n-3		—	6.84 ± 1.73	—	3.31 ± 0.76	1.20 ± 0.95
22:5n-3		—	—	—	1.54 ± 0.37	tr
22:6n-3		—	4.26 ± 1.74	—	16.0 ± 6.74	6.08 ± 0.50
Others		—	0.62	0.91	5.04	5.34

[a]The data are presented as the mean ± standard deviation of 6 (2 groups × 3) determinations.
[b]The position of the double bond applies to fatty acyl chains.
[c]The '—' denotes not detected.
[d]The 'tr' denotes < 1.0%.

and 22:2 NMIDs in TL of Japanese oyster, and reported that the content of 22:2(Δ7,15) was the highest of the NMID fatty acids. In the present study, 20:2 and 22:2 NMIDs were also found in the Japanese oyster, and 22:2(Δ7,15) in the *sn*-2 position of alkenylacyl EPL accounted for 16.6% of total fatty acids, which was the highest percentage among the EPL and CPL subclasses. From these results, most of the 22:2 NMID in the Japanese oyster is considered to occur in the alkenylacyl EPL. The total PUFA contents of EPL subclasses were higher than those corresponding CPL; particularly, total PUFA contents of alkenylacyl EPL were about 80%, which is the highest percentage in the EPL and CPL subclasses. The report of the fatty chain compositions in the *sn*-1 and *sn*-2 positions of alkenylacyl and alkylacyl EPL and CPL in marine invertebrates is the first comprehensive study, although those of alkenylacyl and alkylacyl phospholipids in bonito *Euthynnus pelamis* white muscle had been elucidated by Ohshima *et al.* (20).

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Palmitic Acid-Labeled Lipids Selectively Incorporated into Platelet Cytoskeleton During Aggregation

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Previous experiments showed that during the early stages (20–30 seconds) of aggregation induced by adenosine diphosphate (ADP, 2 μM) or thrombin (0.1 U/mL) of rabbit or human platelets prelabeled with [^{3}H]palmitic acid, labeled lipid became associated with the cytoskeleton isolated after lysis with 1% Triton X-100, 5 mM EGTA [ethylene glycol-*bis*-(β-aminoethyl ether)]-*N,N,N',N'*-tetra-acetic acid. The association appeared to be related to the number of sites of contact and was independent of the release of granule contents. We have now investigated the nature of the labeled lipids by thin-layer and column chromatography and found differences between the distribution of the label in intact platelets (both stimulated and unstimulated) and the isolated cytoskeletons. In both species, and with either ADP or thrombin as aggregating agent, 70–85% of the label in both intact platelets and in the cytoskeletons was in phospholipids. The distribution of label among the phospholipids in the cytoskeletons was similar to that in intact platelets except that the percentage of label in phosphatidylcholine was significantly higher in the cytoskeletons of human platelets than in the intact platelets, and the percentage of label in phosphatidylserine/phosphatidylinositol was significantly lower in the cytoskeletons of rabbit platelets and thrombin-aggregated human platelets than in intact platelets. The cytoskeletons contained a lower percentage of label in triacylglycerol, diacylglycerol, and cholesterol ester than the intact platelets. Contrary to a report in the literature, we found no evidence for the incorporation of diacylglycerol and palmitic acid into the cytoskeleton. Although intact rabbit platelets had more label in ceramide (6.7 ± 2.9%) than intact human platelets (1.5 ± 0.9%), platelets of both species exhibited a three- to four-fold enrichment of labeled ceramide in the cytoskeletons. Thus phospholipids and ceramide that are readily labeled with palmitic acid are selectively incorporated into the cytoskeleton during the initial stages of platelet aggregation.
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In an earlier study (1), we reported that during the early stages of platelet aggregation induced by thrombin or adenosine diphosphate (ADP), labeled lipid became associated with the cytoskeleton of platelets prelabeled with [^{3}H]palmitic acid, and that the extent of labeling appeared to be related to the number of sites of contact between platelets. This association was dependent on aggregation, and dissociation occurred upon deaggregation. The association was independent of the release of granule contents since it occurred to a similar extent with ADP (which does not cause release under the conditions used) or with thrombin. Several approaches were used (1) to establish that the labeled lipid associated with the cytoskeletons under these conditions of limited aggregation could not be attributed to contamination of the cytoskeletons with membrane fragments or non-specific trapping of labeled material.

Burn *et al.* (2) had observed α-actinin-lipid complexes in platelets prelabeled with [^{3}H]palmitic acid and they had concluded that diacylglycerol and certain fatty acids (such as palmitic acid) in a 1:1 molar ratio may take part in a connection involving α-actinin, the cytoskeleton and the platelet membrane. They reported that only 3% of the label in the platelets was present as phospholipid after 2 hr, whereas other investigators (3–6) and we (1) found much higher percentages (55–80%) of the label in the phospholipids of intact platelets at 2 hr and at shorter times as well. However, in our previous study, the distribution of label among the various lipids and phospholipids associated with the cytoskeleton of aggregated platelets was not examined.

Earlier investigators (4–6) examined the distribution of labeled palmitic acid among the lipids and phospholipids of intact human and rabbit platelets, but studies with isolated cytoskeletons were not done. A few groups of investigators have examined the lipid composition of platelet cytoskeletons or some aspects of it (7–9). Schick *et al.* (7) concluded from their analysis that the composition of cytoskeleton lipids, particularly the glycolipids and phospholipids, is specific and distinctly different from that in intact platelets.

We have now examined the distribution of label from [^{3}H]palmitic acid among the lipids in the cytoskeletons of rabbit and human platelets during the early stages of aggregation induced by low concentrations of ADP or thrombin, and compared this distribution with that in intact platelets.

MATERIALS AND METHODS

Materials. Lyophilized human fibrinogen was grade L from AB Kabi (Stockholm, Sweden)—it was dialyzed against 0.14 M NaCl before use. Bovine albumin was fraction V from ICN Immunobiologicals (Lisle, IL). Bovine thrombin was from Parke Davis (Detroit, MI). Apyrase (ATP diphosphohydrolase, EC 3.6.1.5) was prepared from potatoes by the method of Molnar and Lorand (10), and used as described previously (11). ADP, ethylene glycol-*bis*(β-aminoethyl ether) *N,N,N',N'*-tetra-acetic acid (EGTA), *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), leupeptin, benzamidine and lipids (standards, including ceramide types III and IV) were purchased from Sigma Chemical Co. (St.

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Abbreviations: ADP, adenosine diphosphate; EGTA, ethylene glycol-*bis*(β-aminoethyl ether) *N,N,N',N'*-tetra-acetic acid; HEPES, *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PGE_1, prostaglandin E_1; PI, phosphatidylinositol; PS, phosphatidylserine.

Louis, MO). Ceramide (natural mixture, bovine) was from Serdary, London, Ontario. Triton X-100 was molecular biology grade, ultra pure reagent (International Biotechnologies, New Haven, CT). Petroleum ether, boiling point 80–100°C, was from BDH Chemicals (Toronto, Ontario). Soluene-350 and Hionic-fluor were obtained from Packard Instrument (Mississauga, Ontario). [9,10-^{3}H]Palmitic acid (30–60 Ci/mmol), [1-^{14}C] palmitic acid (56 mCi/mmol), and Aquasol were from Du Pont, Canada, NEN Products, Lachine, Quebec.

Preparation of suspensions of platelets labeled with [^{3}H]palmitic acid. Suspensions of washed platelets from rabbits were prepared from blood anticoagulated with acid-citrate-dextrose according to the method of Ardlie *et al.* (12,13). They were labeled with [^{3}H]palmitic acid (5 μCi/mL; 0.04–0.083 μM; platelet count, approximately 500,000/μL) in the third washing fluid (modified Tyrode solution, pH 7.35, with no added Ca^{2+}, no albumin, but containing apyrase). In a few experiments, platelets were labeled with 3.5 μM [^{3}H]palmitic acid or 3.4 μM [^{14}C]palmitic acid. Unless otherwise stated, the incubation time with labeled palmitic acid at room temperature was 15 min. At the end of the incubation, 3.5% albumin solution was added to give a final albumin concentration of 0.35%. The platelets were recovered by centrifugation and resuspended for aggregation studies at a platelet count of 300,000/μL in a modified Tyrode-albumin solution containing 1.8 mM Ca^{2+}, pH 7.35, and apyrase.

Suspensions of human platelets were prepared from blood anticoagulated with acid-citrate-dextrose as described previously (11), except that prostaglandin E_1 (PGE_1) (3 μM) was included in both the washing solutions, and albumin was omitted from the second washing solution. The platelets in the second washing fluid, at a platelet count of 500,000/μL, were labeled with [^{3}H]palmitic acid as described above. Albumin was added and the platelets were recovered by centrifugation and resuspended as described above. At least 60 min elapsed from the end of the incubation until the platelets were stimulated to aggregate and the cytoskeletons prepared.

Aggregation and isolation of cytoskeletons. For aggregation studies, human fibrinogen (100 μg/mL) was added before the addition of ADP. Aggregation was studied at 37°C by recording light transmission through 1 mL samples of platelet suspension stirred at 1100 rpm in an aggregometer cuvette (Payton Associates, Scarborough, Ontario). Aggregation was measured as the percentage change in light transmission through the platelet suspension, with 0% defined as the lowest point of the light transmission curve after addition of the aggregating agent and 100% defined as light transmission after addition of Triton X-100-EGTA. The aggregating agents used were ADP and thrombin. The amounts of all materials added to the platelet suspensions are expressed as final concentrations after all additions.

Aggregation was stopped by the addition of a lysis solution containing 10% Triton X-100, 50 mM EGTA, leupeptin, and benzamidine, pH 7.35, to give final concentrations of 1% Triton, 5 mM EGTA, 0.5 mM leupeptin and 50 mM benzamidine. The material that is insoluble in this medium is commonly defined as cytoskeleton. Cytoskeletons were isolated as described by Phillips *et al.* (14) and Burn *et al.* (2). Briefly, the samples were stirred for 45 seconds, cooled in ice, transferred quantitatively to an Eppendorf centrifuge tube and centrifuged for 4 min at 12,000 × *g*. The supernatant was removed by suction and the pellet was washed twice without disturbing it with a modified Tyrode solution containing 1% Triton, 5 mM EGTA, but no Ca^{2+}. After a final wash in Ca^{2+}-free Tyrode solution, the tip of the centrifuge tube was cut into a liquid scintillation counting vial and the contents solubilized with 0.9 mL of Soluene-350. After the addition of 10 mL of Hionic-fluor, the amount of ^{3}H or ^{14}C in the samples (cytoskeletons) was determined and expressed as a percentage of the total counts per min (cpm) in the platelet pellet.

Lipid extraction. Intact platelets were extracted by a modification (15) of the method of Bligh and Dyer (16). Samples of platelet suspension (1 mL) were centrifuged in an Eppendorf tube, the supernatant was removed and 1 mL of modified Tyrode solution (no albumin) was added to the pellet, with mixing. The mixture was transferred to a 40-mL extraction tube with 3.75 mL of chloroform/methanol (1:2, v/v), allowed to stand for 1 hr, and 1.25 mL chloroform and 1.25 mL water were added with mixing after each addition. The mixture was centrifuged and the bottom layer was removed. Then 9.5 mL of chloroform/methanol/conc. HCl/water (3:4:0.5:0.1, v/v/v/v) was added to the upper phase. After mixing, 3.75 mL chloroform and 3.75 mL of water were added with mixing after each addition, and the mixture was centrifuged. The bottom layer was removed and pooled with the previous bottom layer. The pooled bottom layers were stored at −20°C overnight, solvents were removed under a stream of nitrogen and the residue dissolved in 50 μL of chloroform/methanol (1:1, v/v) or chloroform.

Cytoskeletons were extracted by adding to each sample of isolated cytoskeleton (from 1 mL of platelet suspension), 0.375 mL of chloroform/methanol (1:2, v/v) and by mixing with a vortex mixer. Ten samples were transferred to a 40 mL extraction tube and the procedure described for intact platelets was followed.

Thin-layer chromatography. Lipids were separated by one- and two-dimensional chromatography on thin-layer plates (TLC-plates precoated SIL G-50, 0.5 mm with fluorescent indicator, Brinkmann, Rexdale, Ontario). The solvent used for separation of phospholipids in one dimension was Solvent I—chloroform/methanol/acetic acid/water (90:40:12:2, v/v/v/v). For two-dimensional separation of neutral lipids and glycolipids from each other and from phospholipids, Solvent II—petroleum ether/diethyl ether/acetic acid (120:25:1.5, v/v/v)—was used in the first dimension and Solvent III—chloroform/methanol/acetic acid (95:5:5, v/v/v)—was used in the second dimension. Non-radioactive standards were run with the radioactive samples so that the positions of the various phospholipids could be visualized after exposure to UV light or iodine vapor. The standards were: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidic acid (PA), sphingomyelin, ceramide types III and IV, a natural mixture of ceramides, monoacylglycerol, diacylglyc-

erol, triacylglycerol and cholesterol ester. The average number of dpm in the samples of the lipid extracts applied to chromatographic plates was approximately 100,000 dpm for intact, unstimulated platelets and 30,000 dpm for 10 combined cytoskeletal samples from aggregated platelets. The areas of the silica gel containing the lipid fractions were scraped from the plates into liquid scintillation counting vials containing Aquasol.

Column chromatography. In some experiments, lipids of intact platelets and platelet cytoskeletons were initially separated by chromatography on a Unisil column (activated silicic acid, Clarkson Chemical Co., Williamsport, PA) (17). Lipid extracts (2 mL in chloroform) were added to 2 g of Unisil in chloroform in 1.5 cm diameter columns; neutral lipids were eluted with 40 mL of chloroform (fraction 1); ceramides, glycolipids and small amounts of acidic phospholipids were eluted with 120 mL of acetone/methanol (108:12, v/v) (fraction 2); and the phospholipids were eluted with 90 mL of methanol (fraction 3). The three fractions were then separated by two-dimensional chromatography as described above. The labeled lipids were identified by comparison of their positions with those of standards. In addition, fraction 2 was separated by one-dimensional thin-layer chromatography with chloroform/methanol (150:10, v/v) as solvent; in this system, ceramides are well separated from glycolipids and phospholipids.

Analysis of data. Means ± S.D. are shown. Paired t-tests were used to calculate the significance of differences. Differences were considered to be significant when $P < 0.05$.

RESULTS

Incorporation of labeled palmitic acid into platelets. As reported previously (1), [^{3}H]palmitic acid (0.04–0.083 μM) was rapidly incorporated into both rabbit and human platelets, reaching a maximum of about 45% in 15 min. Subsequent washing of the platelets with a medium containing 0.35% albumin reduced this to about 40%. When [^{3}H]palmitic acid and [^{14}C]palmitic acid were mixed (total molarity 3.5 μM), approximately 14% of each label was taken up under the conditions used. The labeling pattern of the lipids of intact platelets was determined following extraction of platelets removed from their suspending medium by centrifugation so that any radioactivity in the medium was not included. Preliminary experiments showed that after incubation with 0.04–0.083 μM [^{3}H]palmitic acid and resuspension of the platelets in fresh medium, the distribution of the label in unstimulated platelets among the lipid classes separated by two-dimensional thin-layer chromatography was similar to the distribution observed when a much higher concentration (3.5 μM) was used. In addition, the distribution was unchanged over the 1–2 hr during which samples were being aggregated for isolation of cytoskeletons.

For studies of the association of labeled lipid with the cytoskeleton, platelets were aggregated with 0.1 U/mL of thrombin or 2 μM ADP until a 20–25% change in light transmission had occurred (approximately 25 seconds). The amount of label incorporated into the cytoskeletons in this time varied from 2.0 to 7% of the total label that was in the intact platelets. In double label experiments in which a mixture of [^{3}H]palmitic acid and [^{14}C]palmitic acid was used (3.5 μM), the percentages of the labels that were incorporated into the cytoskeletons were the same and were similar to the percentages incorporated when [^{3}H]palmitic acid (0.04–0.083 μM) was used. These observations indicate that the incorporated labels did not represent degradation products of palmitic acid. The cytoskeletons of unstimulated platelets contained only 0.03% of the label in intact, unstimulated platelets (1), and this amount of radioactivity was insufficient, even when 20 samples were combined, to give reliable data.

Distribution of labeled palmitic acid among lipid classes. Table 1 shows the distribution of label among the various phospholipid classes separated by Solvent I. As reported by earlier investigators (6), the percentage of the label in PE was greater in intact (unstimulated) rabbit platelets than in human platelets. In both species, the distribution of label among the phospholipids in the cytoskeletons was similar to that in the intact platelets, except that the percentage of label in PC was significantly higher in the cytoskeletons of human platelets than in intact platelets, and the percentage of label in PS/PI in intact rabbit platelets (4.9 ± 3.9%) was significantly greater than in the cytoskeletons—2.1 ± 2.7% for thrombin and 0.7 ± 0.5% for ADP. With human platelets, there was a significant difference between the percentage of label in PS/PI in intact platelets as compared with the cytoskeletons of thrombin-aggregated platelets. The distribution of label among the phospholipid classes in the cytoskeletons of ADP-aggregated and thrombin-aggregated platelets was similar. Most of the label in phospholipids in both intact platelets and cytoskeletons was in PC. With human platelets, the percentage of the label running at the solvent front that appeared in the lipid extract of the cytoskeleton was smaller than the percentage in the extract from intact platelets. Therefore, the cytoskeletons appeared to be enriched in labeled PC.

Table 2 shows the distribution of label among the lipid classes separated by two-dimensional chromatography in Solvents II and III. As also shown in Table 1, most of the label was in phospholipids in both the intact platelets and in the cytoskeletons. The intact platelets also had appreciable percentages of label in fractions identified by standards as cholesterol ester, triacylglycerol, diacylglycerol, and free fatty acid (a total of approximately 20% for platelets from both species). However, the percentages in these lipids were considerably less in the cytoskeletons—rabbit platelets, 4.8% thrombin and 7.8% for ADP; human platelets, 8.7% for thrombin and 9.6% for ADP. The apparent enrichment of the cytoskeletons of human platelets with labeled phospholipids reflects the lower percentages of labeled triacylglycerol, diacylglycerol, free fatty acid and cholesterol ester in the cytoskeleton. Most noteworthy was the relatively large amount of label in triacylglycerol of intact platelets that did not appear in the cytoskeletons.

Effect of limited aggregation on labeled lipids of intact platelets. To determine whether stimulation of

TABLE 1

Distribution of Label (%) from [^{3}H]Palmitic Acid Among the Phospholipids Separated by Solvent I[a]

	Rabbit platelets			Human platelets		
Lipid	Intact (Unstimulated)	Cytoskeleton (Thrombin)	Cytoskeleton (ADP)	Intact (Unstimulated)	Cytoskeleton (Thrombin)	Cytoskeleton (ADP)
PC	49.8 ± 6.5	47.8 ± 6.1	54.5 ± 1.0	59.8 ± 2.9	74.6 ± 2.9[b]	69.5 ± 2.8[c]
PS/PI	4.9 ± 3.9	2.1 ± 2.7[d]	0.7 ± 0.5[c]	1.8 ± 0.8	1.2 ± 1.1[c]	1.0 ± 0.5
PE	12.3 ± 3.6	14.4 ± 5.6	12.3 ± 4.8	4.7 ± 0.9	3.9 ± 0.6[e]	3.9 ± 0.2
Sphingomyelin	3.5 ± 1.2	5.2 ± 1.4[c]	4.6 ± 0.3	6.0 ± 1.1	3.8 ± 0.6[f]	5.5 ± 2.0
Solvent front[g]	28.1 ± 5.3	27.9 ± 5.7	25.1 ± 5.0	26.7 ± 3.9	16.1 ± 3.3[b]	19.8 ± 1.6
Unidentified	1.4 ± 1.2 (n=8)	2.6 ± 1.9 (n=7)	2.7 ± 0.6 (n=3)	0.2 ± 0.1 (n=4)	0.2 ± 0.2 (n=4)	0.1 ± 0.0 (n=3)

[a]Values are means ± S.D. of the percentage of the label applied to the chromatography plate. Significance of difference compared with intact platelets:
[b]$P < 0.0025$.
[c]$P < 0.05$.
[d]$P < 0.001$.
[e]$P < 0.025$.
[f]$P < 0.005$.
[g]The solvent front contained label associated with triacylglycerol, diacylglycerol, monoacylglycerol, free fatty acid, cholesterol ester, ceramide, cardiolipin and cerebroside (5).

TABLE 2

Distribution of Label (%) from [^{3}H]Palmitic Acid Among the Lipids Separated in Two Dimensions with Solvents II and III[a]

	Rabbit platelets			Human platelets		
Lipid	Intact (Unstimulated)	Cytoskeleton (Thrombin)	Cytoskeleton (ADP)	Intact (Unstimulated)	Cytoskeleton (Thrombin)	Cytoskeleton (ADP)
Phospholipids	70.6 ± 7.9	75.4 ± 6.2	74.5 ± 7.7	77.1 ± 2.7	86.0 ± 2.5[b]	84.8 ± 5.9
Ceramide	6.7 ± 2.9	17.4 ± 5.9[c]	14.8 ± 4.6[d]	1.5 ± 0.8	4.1 ± 2.3[b]	4.6 ± 1.8[e]
Triacylglycerol	10.9 ± 5.4	1.2 ± 1.4[f]	1.9 ± 3.3[b]	7.6 ± 5.3	3.5 ± 2.2[e]	3.3 ± 4.4
Diacylglycerol	3.5 ± 2.8	1.5 ± 1.2	1.8 ± 2.5	1.5 ± 0.9	0.9 ± 0.3	1.0 ± 0.4
Monoacylglycerol	0.2 ± 0.1	0.4 ± 0.3	0.4 ± 0.5	0.1 ± 0.2	0.2 ± 0.1	0.1 ± 0.0
Free fatty acid	2.8 ± 1.5	1.5 ± 1.2[e]	3.6 ± 4.3	5.1 ± 7.6	2.5 ± 2.4	3.7 ± 3.9
Cholesterol ester	2.6 ± 2.3	0.6 ± 0.2[d]	0.5 ± 0.1[e]	5.2 ± 3.9	1.8 ± 0.4	1.7 ± 0.1
Unidentified	2.8 ± 2.3 (n=11)	2.0 ± 0.6 (n=8)	2.9 ± 1.5 (n=4)	1.9 ± 0.7 (n=4)	0.7 ± 0.3 (n=4)	0.6 ± 0.4 (n=3)

[a]Values are means ± S.D. of the percentages of the label applied to the chromatography plate. Significance of difference compared with intact platelets:
[b]$P < 0.025$.
[c]$P < 0.001$.
[d]$P < 0.01$.
[e]$P < 0.05$.
[f]$P < 0.002$.

the platelets per se results in changes in the distribution of the label from [^{3}H]palmitic acid among the various lipids, a suspension of rabbit platelets that had been labeled with [^{3}H]palmitic acid (3.5 μM) was divided into two parts. Samples from one half were aggregated with 0.1 U/mL of thrombin in the usual manner (20–25% change in light transmission) and control samples from the other half were treated with the solvent for thrombin (0.14 M NaCl). In four experiments, the percentage of label in the suspending fluid was unchanged by this limited aggregation with thrombin. Platelets were recovered by centrifugation and extracted. The lipid extracts were separated by two-dimensional chromatography as before. The percentage in phospholipids in unstimulated platelets was 74.1 ± 10.1%, and in thrombin-stimulated platelets 72.2 ± 7.5%; percentages in ceramide were 1.8 ± 0.5% and 2.2 ± 0.7%; in triacylglycerol 15.4 ± 11.3% and 14.5 ± 9.3%; in diacylglycerol 3.0 ± 2.1% and 3.6 ± 2.4%; and in free fatty acid 1.8 ± 0.6% and 3.5 ± 1.7%. None of these differences was statistically significant by paired difference analysis. In two similar experiments with human platelets, aggregation with thrombin did not change the distribution of the label among these lipid classes.

Enrichment of the cytoskeletons with labeled ceramide. A lipid fraction with the same chromatographic behavior as ceramide (type III, Sigma) comprised 6.7 ± 2.9% of the label in intact rabbit platelets and was greatly enriched in the cytoskeletons (17.4 ± 5.9% for thrombin and 14.8 ± 4.6% for ADP) (Table 2). Even when the data for the intact platelets and cytoskeletons were recalculated, after setting aside the amount of label in cholesterol ester, triacylglycerol, diacylglyc-

erol, monoacylglycerol and free fatty acid, and taking the remaining lipid as 100%, the percentage of label in this ceramide fraction from intact rabbit platelets was only 9.0 ± 4.8%, whereas it was 17.9 ± 6.7% in the thrombin cytoskeletons and 16 ± 4.0% in the ADP cytoskeletons ($P < 0.005$, n=7; $P < 0.025$, n=4 respectively). Intact human platelets had only 1.5 ± 0.8% of the label in this ceramide fraction, but showed enrichment to over 4% in the cytoskeletons ($P < 0.025$, n=4 for thrombin; $P < 0.05$, n=3 for ADP) (Table 2). Recalculation of the values for intact human platelets and cytoskeletons, after setting aside the amount of label in cholesterol ester, triacylglycerol, diacylglycerol, monoacylglycerol and free fatty acid, showed only 1.9% of the label in the ceramide of intact platelets, but 4.5% in the thrombin cytoskeletons and 5% in the ADP cytoskeletons.

When rabbit platelets were labeled with 3.5 μM [^{3}H]palmitic acid or 3.4 μM [^{14}C]palmitic acid, the percentage of label in ceramide in intact platelets was lower (2.9 ± 1.6% and 1.4 ± 0.4%, respectively) than in platelets labeled with 0.04–0.083 μM [^{3}H]palmitic acid (6.7 ± 1.6%), but the enrichment of ceramide in the cytoskeletons was similar (2.6- to 4-fold enrichment).

We did not find appreciable percentages of label in PA, cardiolipin, or cerebroside in the intact platelets or cytoskeletons of either species.

Because of the apparent enrichment of labeled ceramide in the cytoskeletons, confirmation of the identification was sought using Wang and Schick's (17) method of separation of labeled lipids by column chromatography, followed by thin-layer chromatography (Table 3). Although the percentage of the total radioactivity identified as ceramide was not as high as in the earlier experiments, at least a two-fold enrichment of ceramide in the cytoskeletons of rabbit platelets was again apparent. Separation of the lipids in fraction 2 from the chromatography column (ceramides, glycolipids and a small amount of acidic phospholipids) by one-dimensional thin-layer chromatography with chloroform/methanol (150:10, v/v), using ceramide standards, confirmed the ceramide identification and its observed enrichment in the cytoskeletons.

DISCUSSION

As shown previously (1), the uptake by platelets of [^{3}H]palmitic acid, measured when the ambient concentration was 0.04–0.083 μM, reached a maximum within 15 to 30 min under the conditions used in these experiments. After this time the amount of label in the platelets remained essentially constant at 40–50% of the label that had been added to the medium, until the platelets were recovered by centrifugation and resuspended. Other investigators (2,3) observed uptakes of 65% and 40% in 1 to 2 hr. The extent of uptake of labeled palmitic acid, however, was dependent on the concentration of palmitic acid in the incubation medium, since only about 15% of the label that had been incubated with the platelets was taken up when the concentration of labeled palmitic acid was 3.5 μM. The rate of incorporation of [^{3}H]palmitic acid into phospholipids was similar to its rate of uptake into platelets (1) and 70 to 80% of the label was in phospholipids at the time when the platelets were aggregated and the cytoskeletons prepared. All the other reports in the literature (3–6), except that of Burn *et al.* (2), show high percentages (55–79%) of labeled palmitic acid incorporated into platelet phospholipids; Burn *et al.* (2) reported only 3% incorporated into phospholipids after 2 hr.

Table 4 provides a comparison of the results from the present study with those of other investigators who studied the distribution of label from radioactive palmitic acid into various lipids in intact human and rabbit platelets. Although other investigators did not report values for all the lipid classes, the percentages in the various classes are similar to the percentages we found (Table 4). It appears from our observations and those of Andreoli (6) that rabbit platelets incorporate a larger percentage of labeled palmitic acid into PE than do human platelets. It should be noted, however, that Andreoli (6) observed that the radioactivity

TABLE 3

Distribution of Label (%) from [^{3}H]Palmitic Acid Among the Lipids Separated into Three Fractions on a Unisil Column, Followed by Separation of Fraction Two (Ceramide and Acidic Phospholipids) by Two-Dimensional Thin-Layer Chromatography (Solvents II and III)[a]

Fraction	Intact platelets (Unstimulated)	Cytoskeleton (Thrombin)	Cytoskeleton (ADP)
1. Triacylglycerol, diacylglycerol, free fatty acid	11.6	2.9	5.6
2. Ceramide	4.3	10.4	8.2
Acidic phospholipids	1.3	3.8	1.8
3. Phospholipids	82.6	82.3	82.4

[a]Values are percentages of the label applied to the Unisil column and are representative of three experiments with rabbit platelets.

TABLE 4

Comparison of the Distribution of Palmitic Acid Radioactivity Among Lipid Classes of Intact, Unstimulated Platelets

	Percent total palmitic acid radioactivity				
				Present study	
Lipid	Deykin and Desser (5) (Human)	Okuma *et al.* (4) (Human)	Andreoli[a] (6) (Rabbit)	(Human)	(Rabbit)
PC	53.9	63.2	62.6	59.8	49.8
PS/PI	7.0	1.4	2.9	1.8	4.9
PE	6.0	3.6	13.4	4.7	12.3
Ceramide	7.7	nd	nd	1.5	6.7
Triacylglycerol	10.2	11.8	16.3	7.6	10.9
Diacylglycerol	4.1	3.4	nd	1.5	3.5
Free fatty acid	5.7	9.5	nd	5.1	2.8
Cholesterol ester	nd	nd	nd	5.2	2.6
Sphingomyelin	nd	3.9	2.3	6.0	3.5

[a]Calculated from data in Andreoli's Tables 1 and 2 for 60 min incubations.

nd = not determined.

in PC increased over a 60 min incubation period, whereas the radioactivity in PE plateaued by 15 min; this shifted the percentages of label in these phospholipids from being almost the same at 5 min to being predominantly in PC by 60 min. In our experiments, the intact platelets were not extracted until at least 60 min after incubation with [^{3}H]palmitic acid and consequently the percentage of label in PC was four times greater than that in PE. Preliminary experiments were done to insure that the distribution of the label among the lipids of unstimulated platelets did not change over the 1 to 2 hr during which samples were being aggregated for isolation of cytoskeletons.

As in our previous study (1), under the conditions of limited aggregation that were used, 2–7% of the label in intact rabbit or human platelets became incorporated into the cytoskeleton. There were some differences between intact platelets and the isolated cytoskeletons in the distribution of label from [^{3}H]palmitic acid into the various lipid classes. Most of the label in the cytoskeletons was in phospholipids and the distribution among the phospholipid classes was similar in intact platelets and cytoskeletons, except for somewhat more phosphatidylcholine in the cytoskeletons of human platelets than in intact platelets, and slightly less label in phosphatidylserine/phosphatidylinositol in the cytoskeletons of rabbit platelets and thrombin-aggregated human platelets. However, the cytoskeletons contained considerably less label in cholesterol ester, triacylglycerol, diacylglycerol, and free fatty acid compared with the percentage of label in these lipids in intact platelets; the exclusion of labeled triacylglycerol from the cytoskeletons was particularly evident. The cytoskeletons from the platelets of both species were enriched in label in ceramide. These differences were apparent when either thrombin or ADP was used as the aggregating agent, so they are not dependent on reactions involved in the release of granule contents, because only thrombin causes the release of granule contents under the conditions used in these experiments; ADP does not (11).

Contrary to the report by Burn *et al.* (2), we did not observe enrichment of the cytoskeleton from aggregated platelets with labeled free palmitic acid or labeled diacylglycerol. The presence of labeled free palmitic acid in their platelets may be attributable to the lack of albumin in the medium in which they resuspended their platelets; it has been shown by Spector *et al.* (3) that essentially all of the platelet free fatty acid is rapidly transferred to the medium if the medium contains albumin. However, although the free fatty acid in the platelets used by Burn *et al.* (2) may be accounted for in this way, it is difficult to understand why it would be enriched in the cytoskeletons. Burn *et al.* (2) did not provide information about the distribution of radioactivity in intact platelets, but comparison of their chromatograms with those of Deykin and Desser (5) for intact platelets and of our own for both intact platelets and cytoskeletons, indicate that the fraction that they identified as diacylglycerol may have also contained ceramide and cholesterol ester, which Deykin and Desser (5) and our own studies have determined contain appreciable amounts of radioactivity. Thus our results do not support the concept (2) that diacylglycerol and a fatty acid such as palmitic acid, in a 1:1 molar ratio, may take part in a supramolecular complex including α-actinin which connects the cytoskeleton to the membrane.

One of the most evident differences between the intact platelets and the cytoskeletons was the enrichment of the cytoskeletons with labeled ceramide. Although rabbit platelets contained a higher percentage of the label in ceramide than did human platelets, the cytoskeletons from the platelets of both species were enriched in labeled ceramide to the extent of about three times. In control experiments, the labeling of the lipids of unstimulated platelets was compared with that of platelets aggregated to the extent of 25% with thrombin; the results showed that the amount of label in ceramide in intact platelets was not increased by thrombin stimulation, thus ruling out the possibility of formation of ceramide from sphingomyelin during platelet aggregation.

In our earlier report (1) we showed in several differ-

ent ways that the ^{3}H-label associated with the cytoskeletons formed under these conditions of limited aggregation is not labeled material trapped during their preparation and is not due to contamination with membrane fragments. The selective incorporation of labeled lipids into the cytoskeletons that was observed in the present study, particularly the failure to incorporate appreciable labeled triacylglycerol and cholesterol ester into the cytoskeletons, and the enrichment of the cytoskeletons with labeled ceramide, reinforces the conclusion that the labeled lipid with the cytoskeleton does not represent non-specific membrane trapping.

The labeled lipid that we have extracted from intact platelets and platelet cytoskeletons cannot have been covalently bound to protein since we did not carry out the extensive acid hydrolysis that would have been necessary to free it from protein (18). However, this does not rule out the possibility that some covalently bound labeled lipid was present in the cytoskeletons. Thrombin has been shown to increase the extent of palmitoylation of several proteins in platelets (19–21).

Few investigators have analyzed the amounts of the various lipid classes that are associated with the cytoskeleton of human platelets. Schick *et al.* (7) reported a selective enrichment of platelet lipids in the cytoskeletons of human platelets exposed to 1 U/mL of thrombin without the formation of visible aggregates. Of the five major phospholipids, only sphingomyelin and PC were detected in the cytoskeletons. However, more recently, this group reported that small amounts of PE, PS and PI have also been detected in platelet cytoskeletons (9). Cholesterol was the only neutral lipid present in significant quantities (6% of that in the platelets). In the study by Schick *et al.* (7), only one of the four neutral glycolipids, trihexosyl ceramide, was detected and it was about 7% of that in intact platelets. The predominant glycolipid of intact platelets (lactosyl ceramide) was not found. Of the main ganglioside (hematoside) in platelets, 2% was found in the cytoskeletons.

It should be emphasized that our studies differ from those of Schick and his co-workers in that we have examined the amount of label from [^{3}H]palmitic acid in the various lipids, whereas Schick *et al.* (7) and Tuszynski *et al.* (9) assayed the lipid mass that was present in intact platelets and cytoskeletons. The apparent discrepancies between the results with these different methods are probably attributable to major differences in the specific radioactivities of the lipids into which [^{3}H]palmitic acid was incorporated over the time course of these experiments. In addition, under our conditions, much less extensive stimulation of the platelets occurred because we used 0.1 U/mL thrombin for 20–30 seconds, whereas Schick *et al.* (7) used 1 U/mL of thrombin for 3 min. A further difference is that we permitted aggregation to occur, whereas they did not. In our earlier study (1) we showed that aggregation was required for the incorporation of labeled lipid into the cytoskeletons and this incorporation did not occur when platelets were stimulated with ADP without stirring. Alternatively, the enrichment of the cytoskeletons with labeled ceramide may represent the incorporation of a small, readily labeled pool of ceramide. Our finding of enrichment of the cytoskeleton lipid with labeled ceramide may relate to the findings of others (22) that incubation of platelets with thrombin results in a three-fold decrease in lactosyl ceramide, and a corresponding two-fold increase in hematoside. However, Wang and Schick (17) were able to confirm only the net increase in the amount of hematoside and Tao *et al.* (23) reported that treatment of platelets with thrombin did not change the level of ceramide. Our results with intact platelets labeled with palmitic acid agree with those of Tao *et al.* (23). In addition, Wang and Schick (17) have reported that incubation of platelets with thrombin causes a 50% reduction in the exposure of trihexosyl ceramide and globoside, but a 100% increase in the availability of hematoside for reaction with external labeling agents. As pointed out by Schick *et al.* (7,24,25) the presence of glycolipids in cytoskeletons is of considerable interest because glycolipids have been proposed as mediators of cellular adhesion and can act as receptors or as cofactors, modulators, or auxiliary receptors. Schick (24) has also suggested that the plasma membrane and selective lipid moieties may serve as an anchor for cytoskeletons and that a portion of the plasma membrane becomes an integral component of cytoskeletons. A number of investigators [reviewed by Burn (26)] have shown non-covalent interactions of lipids with cytoskeletal proteins. Several proteins have been shown to be incorporated into the cytoskeleton of aggregating platelets (27). For example, vinculin recently has been reported to become associated with the organizing cytoskeleton during the early stages of platelet aggregation (28). It may be that these events involve the incorporation into the cytoskeleton of phospholipids and ceramide that are readily labeled with palmitic acid and that these non-covalently bound lipids have a role in the contribution of the cytoskeleton to the initial stages of platelet aggregation.

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Effect of Excess Dietary Calcium on Colon Mucosal Membranes and Fecal Lipids

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The objective of the present studies was to examine the biochemical alterations in colon apical membranes upon feeding excess calcium to animals on a saturated fatty acid-rich diet. It has been suggested recently that excess dietary calcium may offer a protection to colon membranes as judged by histologic examination. Sprague-Dawley weanling male rats were fed a semisynthetic diet containing 14% beef fat plus 2% corn oil and either the calcium requirement or excess calcium in the form of calcium carbonate. Animals were fed the diets for 4 weeks. Feces were collected in the last 3 days. The results indicate that excess dietary calcium resulted in alteration in the density of 4 protein bands of colon apical membranes upon examination on SDS-gel electrophoresis. These bands contain 20% of membrane proteins. The diet had no effect on either the lipid content or fatty acid composition of the membranes. Excess dietary calcium resulted in a 54% reduction in fecal water bile acids and a 44% reduction in fecal water free fatty acids. The reduction in fecal water lipids was due to alterations in the solubility of these lipids. This was not mediated through alterations in the pH of fecal water. The observed alterations in protein patterns of these membranes may be due to either the reduction of fecal water bile acids and free fatty acids or may be a direct effect of dietary calcium on membrane proteins.
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Epidemiological studies suggest a positive correlation between high fat intake (1-3), especially of fat high in saturated fatty acids (2,4), and the incidence of colon cancer. It has been proposed that high fat intake may increase the concentration of the end products of fat degradation in the colon such as bile acids and free fatty acids and that these may have a detergent-like action on the colon mucosa (5). Exposure of colon mucosa to these lipids results in damage to the mucosa as judged by histologic examination (6,7). The studies were performed by either infusing a fluid containing the lipids intrarectally (8,9) or by administering the lipids as bolus (10), conditions which physiologically deviate from those associated with normal feeding.

It has been proposed that supplementing the diet with excess calcium may provide a protection for the colon mucosa as calcium may bind to free bile acids and free fatty acids and thus render these acids insoluble (5). The insoluble complexes would be less absorbable and thus less harmful to the mucosa. Recent histological studies support this hypothesis (9). However, the biochemical changes that occur in apical membranes of colonocytes as a consequence of high calcium intake have not been examined previously. Therefore, the present studies were designed to examine the effect of calcium supplementation on the composition of colon apical membranes in rats fed a high beef diet. Membrane protein composition was assessed by polyacrylamide gel electrophoresis. Biochemical alterations in membrane lipids and fecal water lipids were also examined.

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Abbreviation: GLC, gas-liquid chromatography.

MATERIALS AND METHODS

Animals and diets. Two groups (12 each) of weanling male Sprague Dawley rats, weighing 90-100 g were fed a diet containing 14% (by weight) beef fat plus 2% corn oil in a semisynthetic basic diet for 4 weeks (Table 1). The diet contained 0.5% calcium which was supplied in the form of a mineral mix which provides the calcium requirement for rats (11). This diet served as control. The experimental diet contained 1% calcium carbonate in addition to the calcium supplied in the mineral mix. The diets were fed *ad libitum.* Individual body weights were recorded weekly. In the last 3 days, animals were transferred to metabolic cages. The feces were collected daily and saved at −20°C. The frozen feces of the three-day collection were pooled and saved in the freezer for future analysis. At the end of the experiment, the animals were killed by decapitation. Blood plasma was collected and analyzed for calcium.

Preparation of colon mucosal apical membranes. Crude colon mucosal membranes were prepared according to Yakymyshyn *et al.* (12). The method was described in detail in previous work from this laboratory (13). In brief, the mucosae were scraped from the colon after having been flushed with saline and longitudinally opened by use of two microscope slides attached to each other. Tissue was homogenized in 50mM mannitol in 2mM Tris-HCl buffer, pH 7.1. The suspension was brought up to 10 mM $CaCl_2$ to precipitate the basal membranes and other cellular organelles which were removed by centrifugation at 3000 × *g* for 10 min. The supernatant was centrifuged

TABLE 1

Composition of the Semisynthetic Diets

Ingredients	Diets	
	Control	Experimental
	(%) by weight	
Cornstarch	30.0	30.0
Casein	26.0	26.0
Sucrose	16.5	15.5
Beef fat	14.0	14.0
Celufil (cellulose)	6.0	6.0
Mineral mix[a]	4.0	4.0
Corn oil	2.0	2.0
Vitamin mix[a]	1.0	1.0
DL-methionine	0.4	0.4
Choline chloride (70%)	0.1	0.1
Calcium carbonate	—	1.0

[a]AIN (11).

at 27,000 × *g* for 30 min to obtain the apical membranes as a pellet. The pellet was suspended in Tris-HCl buffer, pH 7.1, and used as such without further purification on Percoll as suggested by the authors. Adapting this method to the rat colon resulted in a 2-fold enrichment as judged by alkaline phosphatase assay and 4-fold enrichment as judged by sucrase assay. The membrane preparations showed low levels of contamination from lysosomes as judged by the activity of β-glucuronidase, which was 20% of that of the homogenate. There also was no detectable activity for (Na^+-K^+)-ATPase as marker for basolateral membranes.

Membrane lipid composition. Lipids were extracted from membranes by the method of Folch *et al.* (14). Cholesterol and phospholipids were measured in aliquots of lipid extract by the method of Pollet *et al.* (15). Fatty acid composition was determined after methylation (16) using gas-liquid chromatography (GLC) as described below.

Polyacrylamide gel electrophoresis. The method of Laemmli (17) was used to examine the protein pattern of colon membranes prepared from animals fed the experimental diets. Colons from two rats were pooled to obtain enough sample for gel electrophoresis. The slab gels contained 7.5% polyacrylamide. Samples from the control and experimental animals containing 50 μg protein were applied on the same gel along with molecular weight standards. Electrophoresis was run at 37 milliamps for 50 min in the Mighty Small II slab gel unit (Hoefer Scientific, San Francisco, CA) containing 2 gels and SDS buffer. The gels were fixed and stained with Coomassie blue followed by destaining. Gels were dried and the bands were scanned on a transmission densitometer (EC Apparatus Co., St. Petersburg, FL). The areas under the peaks were integrated with a Shimadzu C-RIA (Columbia, MD) integrator. The value of each protein band was determined by averaging the scannings and integrations of each gel three times and was expressed as percentage of total membrane protein.

Fecal water. Frozen samples of feces were ground using a coffee grinder. Fecal water was prepared by shaking the ground feces in distilled deionized water (0.25g/5 mL) for 1 hr followed by centrifugation at 28,000 × *g* for 1 hr according to Rafter *et al.* (9). The supernatant was used as fecal water. The pH of fecal water was measured using a combination electrode.

Fecal water bile acids. Total bile acids were measured in an aliquot of fecal water by the enzymatic method of DeWael *et al.* (18).

Fecal water free fatty acids. Free fatty acids were measured by the colorimetric method of Itaya (19). To examine the composition of free fatty acids, the method of Lepage and Roy (20) was used to methylate fecal water fatty acids. In brief, an aliquot of 100 μL fecal water was added to a screw-capped tube containing 1 mL of methanol/benzene (3:2, v/v) and 1 mL of 5% acetyl chloride in methanol. Tubes were heated at 100°C for 1 hr followed by adding 1 mL H_2O, 1 mL hexane and shaking the tubes. An aliquot of the hexane layer was used for GLC. The composition of methylated fatty acids was examined by GLC on a 6-foot glass column packed with 10% SP-2330 on 100/120 Chromosorb WAW (Supelco, Bellefonte, PA). The temperatures of the injection port and the oven were maintained at 260°C and 190°C, respectively. N_2 was used as the carrier gas. The peaks were identified by comparison with authentic standards (Sigma, St. Louis, MO); peak areas were measured using an integrator.

Serum calcium. Calcium was assayed by the method of Kessler and Wolfman (21) using a kit supplied by Sigma (St. Louis, MO).

Other methods. Protein was assayed by the method of Lees and Paxman (22). The student t-test was used to establish significances of differences between the means (23).

RESULTS

Animals fed the high calcium diet weighed significantly ($p \leq 0.05$) more than the controls after 4 weeks of feeding (Table 2). This increase was 4.1% after four weeks on the high calcium diet. Weekly weights in the first 3 weeks on the diet, however, were not significantly influenced by dietary calcium. By the end of the fourth week on the diet, significant increases in serum calcium were observed. The values for the control (11.27 ± 0.40 mg/dl, n = 12) and the experimental groups (11.64 ± 0.34 mg/dl, n = 12) were within the ranges of the values reported for rats (24). The increase in body weight and the absence of any abnormal symptoms may indicate that the calcium level used in the high calcium diet is subtoxic.

Excess dietary calcium was found to be without effect on the concentrations of membrane cholesterol and phospholipids (Table 3). In addition, membrane fatty acid composition was not influenced by dietary calcium (Table 4). A representative gel showing the protein patterns of membranes of animals on the two diets is depicted in Figure 1. Scanning the gels reveals differences in protein concentrations in four of the bands (Table 5). High

TABLE 2

Effect of Dietary Calcium on Body Weight

Days	Weight, g[a]	
	Control diet	Experimental diet
1	97.3 ± 6.8	97.0 ± 8.0
7	142.2 ± 7.4	139.5 ± 12.7
14	195.8 ± 10.7	193.9 ± 17.1
21	245.6 ± 10.4	250.5 ± 17.1
28	297.5 ± 10.5	309.8 ± 16.1[b]

[a]Values are mean ± S.D. of 12 animals.

[b]Significantly ($P \leq 0.05$) different from control.

TABLE 3

Concentration of Major Lipids in Colon Apical Membranes of Animals Fed Either the Control or the Experimental Diet

Lipids	Lipid content[a]	
	Control	Experimental
Cholesterol	144.8 ± 26.1	155.8 ± 42.4
Phospholipids	184.4 ± 53.8	193.2 ± 54.8

[a]Values (μg/mg protein) are mean ± S.D. of 6 samples.

TABLE 4

Fatty Acid Composition of Colon Apical Membranes of Animals Fed the Experimental Diets[a]

Fatty acid	Control	Experimental
	(%)	
14:0	2.2 ± 1.3	1.1 ± 0.4
14:1	tr[b]	1.4 ± 1.2
16:0	23.9 ± 2.9	23.8 ± 1.4
16:1	1.8 ± 0.8	2.1 ± 0.4
18:0	20.6 ± 1.8	20.6 ± 1.4
18:1	31.5 ± 2.7	31.4 ± 2.4
18:2	5.6 ± 0.9	5.4 ± 0.5
18:3	1.2 ± 0.2	1.2 ± 0.2
20:4	7.6 ± 1.7	7.7 ± 1.3

[a]Values are mean ± S.D. of 6 samples, the balance of 100% represents unidentified fatty acids.

[b]≤0.5% of total fatty acids.

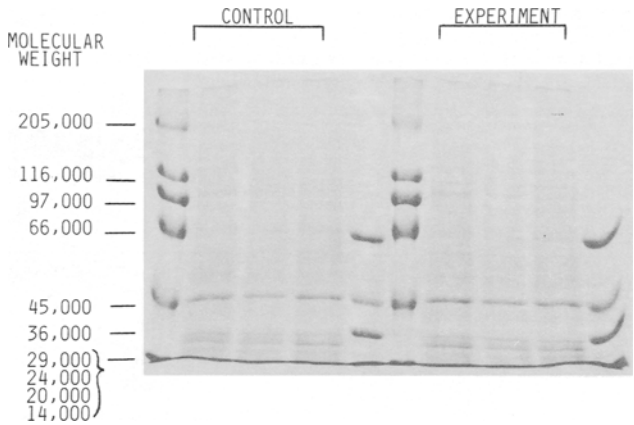

FIG. 1. SDS-gel disk electrophoresis for apical membranes prepared from colons of control and experimental animals (Colons of two animals were pooled). Molecular weight standards were applied in four lanes on the same gel. Each well contained 50 μg membrane protein. The gels were stained, destained, and then scanned.

TABLE 5

Relative Protein Concentrations in Colon Apical Membranes of Animals Fed Either the Control or Experimental Diet[a]

Protein		Relative protein concentration (%)[a]		
MW[b]	Band no.	Control group	Experimental group	Significance[c]
	1	3.83 ± 1.55[2]	4.16 ± 1.30	N.S.
	2	4.77 ± 1.74	3.86 ± 1.58	N.S.
	3	1.75 ± 1.74	1.91 ± 0.81	N.S.
205,000	4	1.73 ± 0.90	2.62 ± 1.08	N.S.
	5	1.95 ± 0.50	1.09 ± 0.62	$p \leq 0.01$
	6	4.79 ± 1.42	4.22 ± 1.06	N.S.
	7	8.08 ± 2.00	4.09 ± 2.25	$p \leq 0.01$
116,000	8	5.84 ± 3.24	10.05 ± 2.58	$p \leq 0.05$
97,400	9	4.28 ± 1.12	3.38 ± 1.50	N.S.
66,000	10	7.24 ± 1.30	8.36 ± 1.12	N.S.
	11	10.12 ± 1.98	11.93 ± 2.12	N.S.
	12	6.54 ± 0.55	4.55 ± 1.98	$p \leq 0.05$
45,000	13	9.90 ± 1.37	10.33 ± 1.30	N.S.
	14	4.14 ± 1.36	4.79 ± 1.42	N.S.
36,000	15	12.56 ± 1.44	14.17 ± 1.82	N.S.
29,000 24,000 20,000 14,000	16	11.56 ± 1.44	10.50 ± 0.97	N.S.

[a]Values are the mean ± standard deviation of 6 samples.

[b]Molecular weight in daltons.

[c]N.S., Nonsignificant.

calcium feeding increased the concentration of band No. 8, corresponding to a molecular weight of 116,000 daltons, as compared to the control. On the other hand, there was a decrease in the protein concentrations of 3 bands, i.e. No. 5, 7 and 12.

There was no significant effect of dietary calcium on fecal water pH. The pH for the experimental diet group was 8.1 ± 0.3 as compared to 8.0 ± 0.4 for the control group. These values represent the mean ± S.D. of 12 animals in each group. Excess calcium intake resulted in a 54% reduction in total fecal water bile acids and 46% in fecal water free fatty acids (Table 6). The main fatty acids in fecal water were found to be 16:0, 18:0 and 18:1 where they comprise 75-78% of total free fatty acids (Table 7). Feeding high calcium diet resulted in a significant reduction in the concentrations of 14:1, 16:0, 18:0, 18:2 and 18:3 fatty acids as compared to controls.

DISCUSSION

The most important finding of the present studies is that alterations in membrane proteins were observed in SDS-

TABLE 6

Fecal Water Bile Acids and Free Fatty Acids of Rats Fed Either the Control or Experimental Diet[a]

	Bile acids	Free fatty acids
Control group	6.23 ± 1.7	4.28 ± 0.94
Experimental group	2.75 ± 1.02	2.29 ± 0.83
Significance	$p \leq 0.01$	$p \leq 0.01$

[a]Values (μmole/g dry weight of feces) are mean ± S.D. of 12 rats.

TABLE 7

Free Fatty Acid Composition of Fecal Water of Animals Fed Either the Control or the Experimental Diet

	Fecal water free fatty acids[a]	
Fatty acids	Control group	Experimental group
14:0	175 ± 61	89 ± 36
14:1	228 ± 153	46 ± 26[b]
16:0	1392 ± 390	491 ± 289[b]
16:1	102 ± 80	106 ± 133
17:0	93 ± 91	60 ± 82
18:0	1047 ± 320	579 ± 298[b]
18:1	576 ± 148	541 ± 307
18:2	185 ± 125	55 ± 34[b]
18:3	132 ± 93	52 ± 42
20:4	67 ± 54	40 ± 22

[a]Values (nmole/g dry weight of feces) are mean ± S.D. of 6 samples.
[b]Significantly (≤0.05) different from controls.

polyacrylamide gels upon feeding a high calcium diet for 4 weeks. The four bands that exhibit these changes comprise about 20% of the total membrane proteins. There was no change in the lipid concentration and fatty acid composition of these membranes suggesting that excess dietary calcium specifically influenced certain membrane proteins.

Our studies are the first to examine the biochemical alteration in these membranes as influenced by dietary calcium. Previous work by others (6,9) examined the effect of excess calcium after fat was given as an oral bolus (10) or after administering bile acids and free fatty acids intrarectally (8,9) to animals, and examined the gross changes in the colon mucosa histologically. The alterations observed in the protein patterns of these membranes suggest significant changes in the function of these membranes. The changes in membrane proteins were associated with reduction in fecal-soluble soaps of both bile acids and free fatty acids. Rafter *et al.* (9) found that addition of 2mM calcium to a perfusion fluid containing 5mM deoxycholic acid reduced the concentration of bile acids in solution by 57%. This value is very close to the value of 54% obtained in the present studies. The presence of these soluble soaps in the colon lumen may be responsible for the altered morphology in colon mucosa (9). The decrease in these fecal water lipids by excess dietary calcium in the present studies was not mediated by a change in the fecal water pH. Previous work (25) demonstrated that calcium soap solubility is influenced by pH. As the pH decreases calcium soap solubility increases (26).

The observed increase in growth rate of animals fed the high calcium diet in the presence of high saturated fat diet as compared to the controls may be due to a decrease in the availability of dietary calcium in the control diet in the presence of high fat content. Nauss *et al.* (27) found that rats fed high mineral mix (containing calcium) in the presence of high beef fat in their diet grew faster than rats fed diet containing smaller amount of mineral mix. In support of our hypothesis, serum calcium of animals fed the high calcium diet for 4 weeks was higher than that of controls.

In summary, we have demonstrated that significant protein alteration in apical membranes has been induced by feeding excess calcium. The magnitude of these alterations suggests functional alteration which deserves further investigation.

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Cell Separation of *Tethya Aurantia*, an Analytical Study of Embryonic and Differentiated Sponge Cells[1]

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The cells of the sponge *Tethya aurantia* var. *californiana* were separated on a Ficoll density gradient and the fractions analyzed for cell types and their lipids. Major cell types were choanocyte, archeocyte, and symbiont. Major differences in archeocyte and choanocyte fatty acid composition were noted for 20:4, 26:1 and 26:2. The fatty acids 26:1, 26:2, and 28:3 were dominant in the phosphatidylcholine fraction. Archeocytes had highest concentrations of 4,7,10,13-20:4 and 5,8,11,14-20:4 (arachidonic) acids which could be derived from symbionts, as odd-chain and methyl-branched fatty acid were also present. Sterol analyses showed cholesterol as a major sterol of the sponge cell fractions and clionasterol (or its 24-isomer) as a major sterol in symbiont cells.
***Lipids 25,* 383-390 (1990).**

Dissociated sponge cells are known to retain the potential for mitogenesis, proliferation and differentiation (1-3). Müller *et al.* (4) recently demonstrated that when a sponge's aggregation factor inserts into its membrane receptor, a series of biochemical events are initiated, which include an increase in extracellular calcium and increased turnover of phosphoinositides. Weissman *et al.* (5) further established the synergistic formation of diacylglycerol and presented experimental evidence for its role as a second messenger and an activator of protein kinase C, an event that may ultimately lead to DNA replication. Both studies draw analogy to intercellular signaling events in higher animals [as reviewed by Berridge, (6)].

A sponge is a collection of cells of two fundamental types. First, the mobile ameboid cells of the mesohyl, the archeocytes and cells of this type with apparent special functions (i.e., spongocytes), are characterized by an "embryonic" nature that allows them either to differentiate into other cell types or to temporarily take on specialized functions (7). These relatively large cells are characterized by a large nucleolus in a rounded, finely granulated nucleus, a well developed Golgi apparatus, and rough endoplasmic reticulum, and large intercellular spaces (8) resembling gap junctions (between 300-1000 Å) which are believed to be important for cells to be electrically coupled and for the passage of small molecules (9,10). Second, the fixed cells of the surfaces, the choanocytes and pinacytes, are terminally differentiated and are collectively characterized by a smaller nucleus (a choanocyte nucleus, which may be haploid, is about 60% that of an archeocyte) with no nucleolus, a greatly reduced number or no mitochondria or Golgi membranes, close intercellular association, and a relatively small size (a choanocyte is usually half the size of an archeocyte) (8). Pinocytes are not adapted for active transport since the deficiency in mitochondria and their thinness make them unsuited for actively maintaining a gradient between the external medium and the mesenchyme. The scarcity of tight junctions makes them ineffective in providing a barrier to ions and water (11). The apparent lack of a nucleolus and a poorly organized rough endoplasmic reticulum suggest that there is less protein synthesis. Although Golgi bodies can be found in choanocytes, glycogen production is greatly reduced (8). In a first approximation, more than 90% of the cells of the sponge can be seen as being of two types (12)—mobile cells of the mesohyl and fixed cells of the surface.

Our first report (13) on lipid differences between cell type was concerned with a Great Barrier Reef sponge *Pseudaxinyssa sp.* This sponge contains long-chain fatty acids and a very unique sterol composition (99%) with two triple alkylated 24-isopropyl sterols. In spite of heavy infestation with cyanobacterial symbionts, these sterols were strongly suspected to be from sponge cell membranes. An asymmetrical distribution of fatty acids and sterols was observed: small, surface-fixed cells (ca. 4×2 μm) contained larger quantities of very long-chain fatty acids (>24 carbons) and smaller quantities of sterols than were present in the large mobile sponge cells. We considered it important to examine in detail a second sponge with "conventional" sterols, but unusual fatty acids. For this purpose, we selected a local sponge, common to the California coast, *Tethya aurantia*, which has an unusually high content of small cells (choanocytes) and long-chain fatty acids, but a sterol content that is common among sponges of the Monterey Bay (USA) and whose symbionts appear to be mainly bacterial and fungal (or red algal).

EXPERIMENTAL PROCEDURES

Sponge sample. Tethya aurantia var. *californiana* ranges in color from orange to yellow and is commonly found on rocky outcrops, pinnacles, and under ledges of the Monterey Bay. Some sponges are known to display seasonal variations in lipid content, as has been shown for *Microciona prolifera* (14). The present cell fractionation was done on sponges collected in the fall of 1988.

Chemicals and materials. The protease enzyme (no. P-2143) and Ficoll (type 400) were purchased from Sigma Chemical Co. (St. Louis, MO). The calcium-magnesium-free artificial sea water (CMF-ASW) was prepared from doubly distilled water and contained 27 g/L NaCl, 1 g/L Na_2SO_4, 0.8 g/L KCl and 0.18 g/L $NaHCO_3$.

[1]This manuscript is no. 24 in the series "Phospholipid Studies of Marine Organisms". For the preceding paper, see Ayanoglu, E., Chiche, B.H., Beattly, M., Djerassi, C., and Duzgunes, N., *Biochemistry*, in press.

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[2]This collection of cells differs from our earlier study [*Lipids 24*, 210-216 (1989)]. In the present study, the sponge cell accumulating in the densest gradient is a storage (or excreting) spherulous cell (29 × 10 μm), whereas earlier, an oversized archeotype (28 × 10 μm) was isolated in the densest gradient.

Abbreviations: BHT, butylated hydroxytoluene; ECL, equivalent chain length; GC, gas chromatography; GC/MS, gas chromatography/mass spectrometry; HPLC, high performance liquid chromatography; NMR, nuclear magnetic resonance; PI, phosphatidylinositol; TCL, thin-layer chromatography; and TMTD, 4,8,12-trimethyltridecanoic acid.

Electron microscopy. Aliquots of sponge cell fractions fixed in 3% glutaraldehyde and Millonig's phosphate buffer (15) were washed (three times) with 0.1 M phosphate buffer for 10 min. Samples were then postfixed in 1% OsO_4 and 0.1 M phosphate buffer and stained after washing (distilled water) with 1% uranyl acetate. After ethanol dehydration, the samples were embedded in VCD-HXSA resin (16). Sections were stained with Reynolds (17) lead citrate and examined with a Philips 410 electron microscope (Philips, Cincinnati, OH) at 60 or 80 KV.

Ficoll gradients. Two types of Ficoll gradients (Fig. 1) were prepared for fractionations by layering the designated (w/w, listed below) concentrations using CMF-ASW. Type I was prepared by layering 5 mL aliquots of 23, 20, 17, 13, 10, and 5%. Type II contained 35, 30, 26, and 23% Ficoll solutions, respectively. The Ficoll gradients were prepared prior to use and stored at 0°C.

Dissociation of sponge cells. This was done according to Thompson *et al.* (18) with the following modifications: The wet sponge (50 g) was washed four times in CMF-ASW, cut into cubes 0.5 cm on a side, soaked in a bath of 500 mL of CMF-ASW, to which 126 mg of protease had been added, and was aerated at room temperature for 2 hr. The resulting cloudy mixture was then filtered through nylon mesh (30 μ) and centrifuged at 650 × *g* (International Equipment Co., Boston, MA, model HR-1) for 10 min to produce an orange-red sponge pellet. The pellet was suspended in 10 mL CMF-ASW and applied to the Ficoll gradients as described.

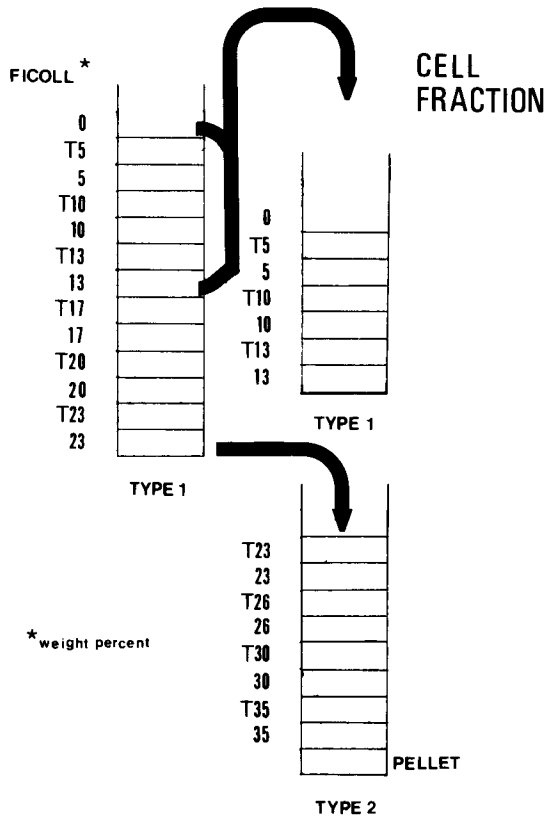


FIG. 1. Schematic of Ficoll fractionation. Labels to the left of each fraction used in text.

Separation of cells. Ten mL of the resuspended pellet was added to the top of a type I Ficoll gradient (Fig. 1) and the system was spun 5 min at 23°C and 650 × *g* in a swing bucket centrifuge. The cells were collected separately in each Ficoll layer and at each interface. The material from the lightest four fractions was combined, washed free of Ficoll in CMF-ASW (50 mL×2) and centrifuged (650 × *g*/10 min). The supernatant was discarded and the cells were added to the top of another Ficoll type I gradient. The cellular material between the different Ficoll concentrations was collected, with the interface of each layer collected as a separate fraction and labeled as the T fraction of the more concentrated layer. All other fraction numbers are the Ficoll concentration from which the cells were collected, as summarized in Figure 1. Fractions derived from Ficoll concentrations 17-23% were obtained from the original gradient. The pellet from the original gradient was reapplied to the top of another Ficoll gradient of heavier concentrations (Type II) and the system was centrifuged as before. After this procedure was run twice, the following combined fraction weights were realized: Zero (0) fraction (31.5 mg), T5 (8.7 mg), 5 (8.6 mg), T10 (6.1 mg), 10 (16.4 mg), T13 (21.7 mg), 13 (33.2 mg), T17 (20.8 mg), 17 (16.1 mg), T20 (36.0 mg), 20 (31.5 mg), T23 (10.8 mg), 23 (32.3 mg), T26 (8.3 mg), 26 (16.5 mg), T30 (12.4 mg), 30 (35.4 mg), T35 (36.0 mg), 35 (36.0 mg), pellet (0.9 mg).

Lipid analysis of cell fractions. The cell fractions were separately extracted with $CHCl_3/CH_3OH$ (1:1, v/v). The soluble material was separated from other cellular debris by filtration through a small plug of glass wool and sand. The extract was dried, 10 mL of 1.5 N HCl (CH_3OH) added, and the mixture heated under reflux for 30 min. The solvents and reagents were removed azeotropically with 10 mL toluene. The resulting esterified material was dissolved in 5 mL hexane/diethyl ether (2:1, v/v), and passed through a florisil column (0.5 g) using (5×1 mL) hexane/diethyl ether (2:1, v/v) as solvent. After evaporation of the solvent (N_2), the resulting fatty acid methyl esters and sterols were collectively analyzed by capillary gas chromatography (GC) using a Hewlett Packard 5790A series gas chromatograph equipped with a 25 m (0.3 mm i.d.) SE-54 coated fused silica column programmed between 170-320°C at 5°C/min (injector, 250°C; detector, 300°C; automatic injector system, Hewlett Packard sampler model 7672A; injector, model 3392A, sampler/event control module, Hewlett Packard model 19405A).

Fatty acid methyl esters (C_9-C_{20}) were identified based on the Hewlett Packard Peak Library, AEROBE, which can assign 119 fatty acid methyl esters common to microbes and sponges. Partially resolvable peaks are described as mixtures in Table 1. For the localization of double bonds or branching, *N*-acyl pyrrolidides were prepared and analyzed by GC/MS (19). For this purpose, fatty acid methyl esters were reacted with pyrrolidine/acetic acid (10:1, v/v) in a capped vial (1 hr, 100°C), followed by extraction with diethyl ether from the acidified solution and purification by preparative TLC (Merck, Ltd., West Germany, aluminum backed sheets precoated with silica gel 60 F_{254}) using hexane/diethyl ether (1:4, v/v) as developing solvent. The samples were analyzed using a Ribermag GC-MS-DS system which combines a Ribermag R 10-10 quadrupole mass spectrometer with a Carlo-Erba series 4160 Fractovap

TABLE 1

Fatty Acids Present in *Tethya aurantia* var. *Californiana* According to Equivalent Chain Length (ECL), as Determined by GC, and Shown in Figure 3

Peak no.[a] (Fig.)	ECL	Identity	Peak no.[a] (Fig.)	ECL	Identity
1	14.000	14:0	25	17.822	unknown
2	14.495	4,8,12 triMe-13:0	26	18.000	18:0
3	14.623	15:0 iso	27	18.634	19:0 iso
4	15.000	15:0	28	18.730	19:0 anteis
5	15.435	16:1 iso 6	29	18.816	unknown
6	15.626	16:0 iso	30	19.001	19:0
7	15.816	16:1 cis 9	31	19.385	5,8,11,14-20:4
8	15.857	16:1 trans 9/15i2OH[b]	32	19.471	6,9,12,15-20:4
9	15.999	16:0	33	19.635	20:0 iso
10	16.412	17:1 iso	34	19.733	unknown
11	16.503	15:0 3OH	35	19.832	unknown
12	16.527	17:1 anteiso	36	20.001	20:0
13	16.573	unknown	37	21.002	21:0
14	16.629	17:0 iso	38	22.001	22:0
15	16.722	17:0 anteiso	39	23.000	23:0
16	16.773	17:1	40	24.002	24:0
17	16.922	unknown	41	24.633	25:0 iso
18	17.000	17:0	42	25.002	25:0
19	17.052	16:1 2OH	43	25.649	5,9-26:2
20	17.236	16:0 2OH	44	25.873	9-26:1
21	17.592	6,12,14-18:3	45	25.997	26:0
22	17.646	18:0 iso	46	26.990	27.0
23	17.720	18:2	47	27.491	28:3
24	17.769	9-18:1			

[a] Peak no. corresponds to abscissa increments of Figure 3. The percent of each compound is found in the ordinate values of Figure 3.
[b] Unseparable fatty acids under our conditions, i.e., 16:1 *t*-9 cannot be separated from 15i2OH.

chromatograph (Carlo Erba, Milano, Italy, co/Haake Buchler Instruments, Inc., Saddle Brook, NJ) containing a fused silica column (28 m × 0.32 mm) with SE-54 (J & W Scientific, Inc., Folsom, CA). The initial oven temperature was 200°C with temperature programming set at 3°C/min. Final temperature was 290°C.

Analysis of phospholipid classes in whole sponge tissue. A sponge sample (4 g) was cut into cubes (1 cm^3) and extracted with $CHCl_3$ containing a trace of butylated hydroxytoluene (BHT) as antioxidant. The extract was concentrated, dried azeotropically (toluene), and diluted to 10 mL with chloroform. The phospholipids were precipitated by addition of acetone (20 times) and collected after centrifugation. An analytical amount of phospholipid was separated on a silica gel plate under conditions in which standard samples of phosphatidylethanolamine, -choline, and -serine gave R_f values of 0.78, 0.45, 0.16, respectively, using the solvent system $CHCl_3$/MeOH/H_2O (65:35:4, v/v/v) for development and molybdenum blue spray for detection. Each phospholipid class and the adsorbent fractions between each class were collected and then transmethylated with BF_3/CH_3OH (10 min, 100°C) in the presence of the silica gel. Phospholipids were also identified by comparison of their migration rates in TLC with those of authentic standards. The results are shown in Table 2. Two fatty acids, 20:4 and 4,8,12-trimethyltridecanoic acid (TMTD), were associated with a fraction that migrated between the PE and PC fractions. This fraction was shown to comigrate with phosphatidylinositol (Sigma, bovine liver). Two-dimensional TLC ($CHCl_3$/CH_3OH/7N NH_4OH, 65:25:4, by vol; and $CHCl_3$/CH_3OH/acetic acid/H_2O, 170:15:15:2, by vol) confirmed the presence of phosphatidylinositol (R_f values, 0.2 and 0.1).

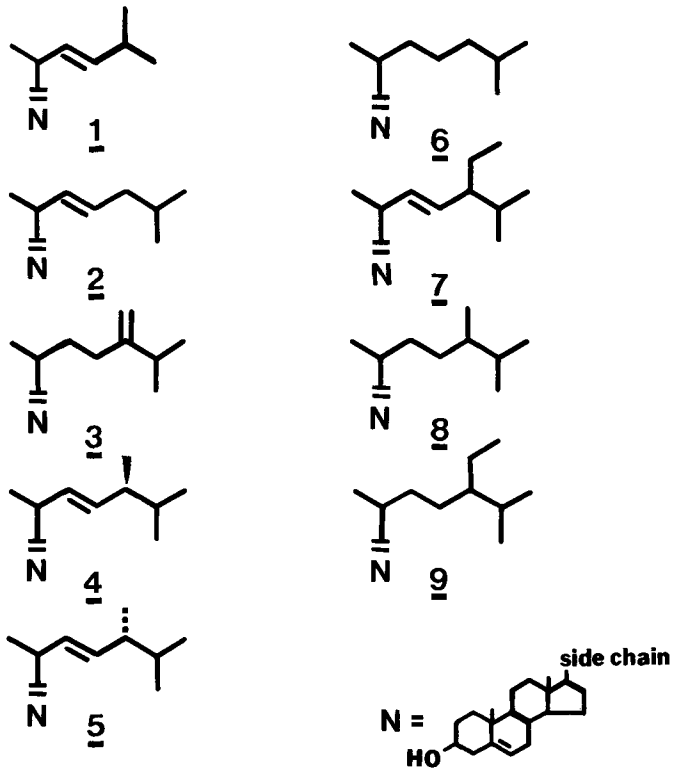
TABLE 2

Percent Fatty Acids in Major Phospholipid Fractions of Whole Sponge

	Phospholipid class			
Fatty acid	PS (0.16)[a]	PC (0.45)[a]	PI	PE (0.78)[a]
4,8,12-triMe-13:0	no	no	4	t
16:0			6	
18:0				
5,8,11,14-20:4	no	t	5	no
6,9,12,15-20:4	no	t	6	no
22:0	9			5
24:0	15			15
26:0				17
26:1	5	40		5
5,9-26:2	3	20	8	15
28:3	9	40	16	32
minors	32[b]		33	11

[a] R_f value for phospholipid class.
[b] There is a 20% unknown at ECL 22.632 not included in this number.

Analysis of sterols independent of other lipids. The sponge (5 g) was extracted with chloroform/methanol (1:1, v/v). The extract was filtered, concentrated, added to 50 mL methanol containing 50 mg KOH, heated under reflux (1 hr), and concentrated again. Methylene chloride was



SCHEME 1. The sterols of *Tethya aurantia* (11).

added to the resulting mixture, which was washed with brine (3×5 mL), dried ($MgSO_4$), filtered, concentrated, and purified by column chromatography (florisil, hexane/ethyl acetate, 8:2, v/v). GC analysis of the combined sterol fraction showed it to be of longer retention time than the fatty acid methyl esters with no significant overlap. The individual sterols (Scheme 1) were isolated by reverse phase high performance liquid chromatography (HPLC) and identified GC retention times and by proton (300 MHz) nuclear magnetic resonance (NMR).

RESULTS

The primary criterion for an efficient separation of cells is a wide range of cell densities. In the present study we chose *Tethya aurantia* var. *californiana* because an initial screening of the electron micrographs (Fig. 2A) showed an abundant number of small cells (12×5 μm), mostly choanocytes (*a* in Fig. 2A) with a nucleus diameter of approximately 2.7 μm and no obvious nucleolus. The intermediate cell archeocyte (*b* in Fig. 2A) was 18 × 10 μm in size with a nucleus diameter of ca. 7 μm and an obvious nucleolus. This photograph agrees completely with one previously published for this sponge (20). The largest cell type (29×16 μm) with a nucleus diameter of 5 μm is spherulous, containing many vacuoles of probable excretory components (Fig. 2B). Finally there are bacterial symbionts (ca. 1.5 μm) between the sponge cells. The extent to which these symbionts occur within the cells cannot be ascertained in these micrographs.[2] The crude ratio of cell areas from these electron micrographs amounts to 1:3:8 for the choanocytes/archeocytes/spherulous cells of this sponge.

The nature of the phospholipid fatty acids of the whole sponge tissue is shown graphically at the top of Figure 3. Phospholipids were further separated and analyzed according to class (Table 2). The major long-chain (C_{24}-C_{30}) fatty acids constitute 70% of the total mixture of fatty acids of the phospholipids of *Tethya aurantia* var. *californiana.* The fatty acids 26:1, 26:2, and 28:3 can be assumed to be the major membrane fatty acids because of their prevalence (99%) in the phosphatidylcholine fraction (Table 2).

Dissociation of the whole sponge *Tethya aurantia* var. *californiana* yielded intact cells independent of the mesophyl matrix. A small number of cells were broken in the dissociation process, resulting in the presence of cell fragments found at the top (fraction 0) of the Ficoll gradient. Electron microscopy showed the dissociated sponge cells to be of a more rounded form. Cell types were assigned by the relative sizes of the cells, as well as nuclear and cytoplasmic characteristics. The dissociated cell mixture appears to be a reasonable representation of the cell population of the whole sponge. This appears substantiated because the fatty acid analysis of the whole sponge agrees qualitatively, and even quantitatively, with the sum of the fatty acid analyses of fractions 0–20 (Fig. 3).

Upon examination of the cell mixture by electron microscopy, symbiont cells could be seen in fraction 0 and in the bottom fraction 35. Chlorophyll was neither observed in the extracts of this sponge nor in cell fractionation, as judged by the absence of the characteristic green color.

The absence of chlorophyll may be due to the fact that the sponge was collected at a depth of forty feet. Orange, the only observed color, was found in fraction 13, T13 (Fig. 3). This color was originally attributed (21) to a porphyrin in sponge cells, but the assignment was later disputed (22) in favor of the more generally found carotene pigments. Associated with these colored fractions is a major acid, 4,8,12-trimethyltridecanoic acid (TMTD). This acid previously has been tied to cells located internally in other sponges (13,23), and has been suggested to be a phytol degradation product. In our analysis, TMTD was found enriched in the phosphatidylinositol fraction (Fig. 3) and in the sponge archeocyte cell type (Fig. 3). Also found in this cell type, and in our analysis specific to archeocytes (Fig. 3), were C_{17} fatty acids as they are common to bacteria. This could imply that a cell-specific symbiont may be present that would also explain TMTD because methyl-branched fatty acids are also common to bacteria (24).

While the above points toward the non-photosynthetic origin of the symbionts, examination of the electron micrographs appears to suggest the presence of eucaryotic symbionts (e.g., b in Fig. 3D). Not only are there many procaryotic symbionts (ca. 0.5–2 μm) living intercellularly, but the archeocytes also appear to have engulfed symbionts of larger size (0.75 to 2.5 μm) with cytoplasmic development beyond that of a bacterium.

Each cell fraction was analyzed for lipid content, which not only provided taxonomic markers for many of the microbial symbionts (25,26), but also indicated the lipid distributions for the different cell types of the sponge. The total symbiont contribution to the sponge fatty acid composition was provided by the analysis of whole sponge tissue (top of Fig. 3), which shows very low percentages

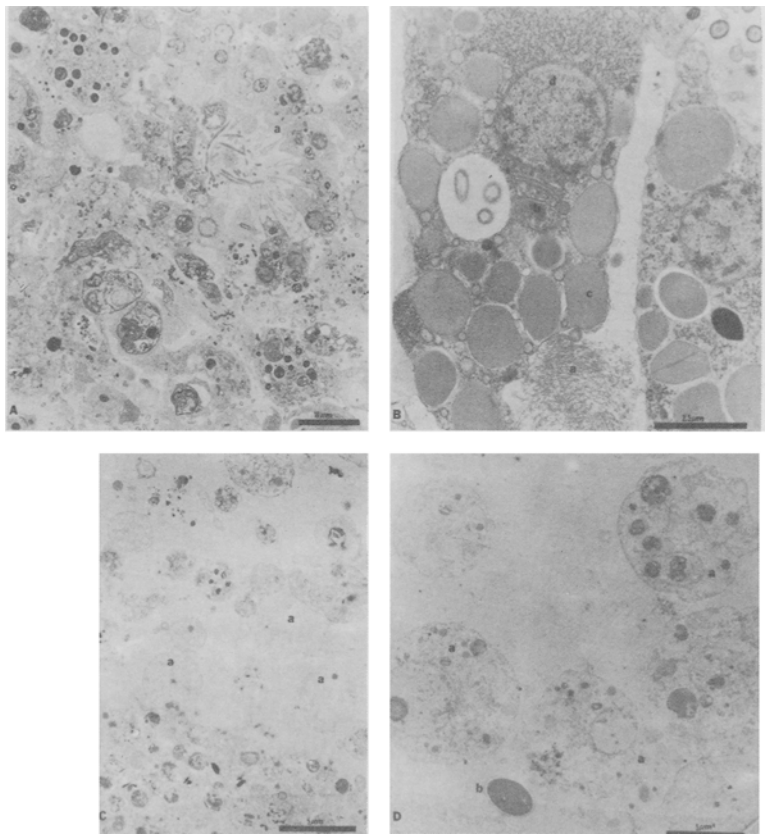


FIG. 2. Electron micrographs of the sponge cells of *Tethya aurantia*. A, whole sponge tissue showing many choanocytes (a) and archaeocytes (b); bar length 10 μm (7500×). B, spherulous nucleated archaeocyte in tissue: (a) collagen; (b) Golgi; (c) vaculated storage precollagen material; (d) nucleus; bar length 2.5 μm. C, dissociated choanocytes (a); bar length 5 μm. D, dissociated archeocytes (a) with endosymbiont (b); bar length 5 μm.

of the most common fatty acids of unicellular symbionts, 16:0 and 18:0. The previously mentioned 17:0 also makes a small contribution. Over 80% of the fatty acid content from whole tissue is from acids of carbon length greater than stearic (18:0), which suggests that symbionts make a small percentage contribution.

Enrichment of fatty acids of bacterial origin is observed in fraction 0 at the top of the Ficoll column (Fig. 3). An electron micrograph shows procaryotes having the size of bacteria (0.5 μm diameter), along with small sponge cells (choanocytes, pinacocytes) with the largest cell diameters being 4 μm. Fatty acid analysis of this fraction shows enrichment of 16:0, 16:1, 17:0, 17:0 iso, 18:1, 18:1 *cis*-11, and 18:0 acids, which are all typical for bacterial populations. These fatty acids are greatly diminished in the next Ficoll fraction.

Fractions T5, T13, and T20 (Fig. 3) appear to be devoid of odd numbered and hydroxy substituted fatty acids. Fraction T5 (Fig. 2C) is shown by electron microscopy to be composed largely of small sponge cells with the largest cells approximately being 4 μm in diameter. Most of these cells are choanocytes which have previously been reported to be quite numerous in cell suspensions, probably because they easily pass through the filter cloth (9). These cells possess higher quantities of very long chain fatty acids (C_{26}, C_{28}) and are lower in phytol (TMTD), 4,7,10,13-20:4 and 5,8,11,14-20:4 acids.

The light Ficoll fractions (0, T5) contained up to six times the 26:2 acid concentration of whole tissue. This trend is also seen in the longer chain acids. As a general rule, the lighter cells seem to have relatively more of the longer chain fatty acids.

Further down the Ficoll column appeared the fractions that have the highest densty of sponge cells, fractions T10-T20. Fraction T13 (Fig. 3) is the most typical of this population, and appears to be mainly composed of archeocytes, derived from the large ameboid nucleated cells of the mesohyl, which were separated by 10–30 μm gaps in tissue. These are large cells with dissociated diameters as large as 9.3 μm, containing high quantities of phytol (TMTD) and 20:4 acids, and a major amount of C_{26} fatty acid.

A significant observation is the extent to which the arachidonic acid resides in the archeocytes. If this is a consequence of the high phagocytic character of an archeocyte, we would expect to find some of this acid in the fractions containing a large number of symbionts (fraction 0 or fraction 35), but in fact these fractions contain only traces of 20:4 acids. The next supposition would be that the engulfed symbionts synthesize arachidonic acid and that this acid is stored by the sponge cell. It seems reasonable that arachidonic acid could have a functional role in the archeocytes since they are mobile and loosely held to the extracellular matrix. Hence, these cells are

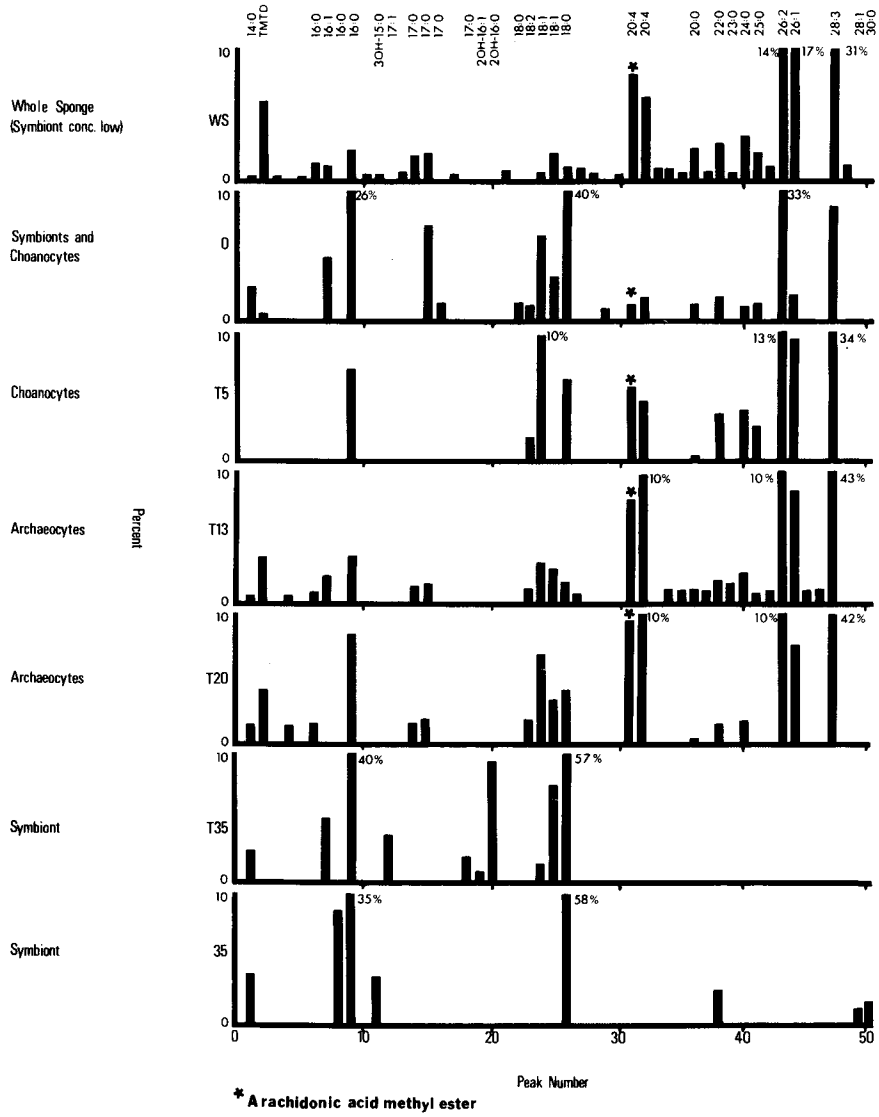


FIG. 3. Fatty acid methyl ester (FAME) analysis of selected Ficoll fractions.

more reliant on the extracellular communication than the more membrane-bound choanocyte cells. Inositolphosphatides containing arachidonic acid in the *sn*-2 position are recognized as playing a special role in cell-signaling in which the signal is received on the cell surface (from hormones, growth factors, neurotransmitters or light) and is then transduced to other second messengers which control many cellular processes such as metabolism, secretion, contraction, phototransduction and cell growth (27–29).

The largest cells (15–20 μm) in *Tethya* tissue (Fig. 2B) are what Fishelson (20) has described as "nucleolated archeocytes" which are characterized by their large nucleolus, numerous globular vesicles and granular cytoplasm. These cells did not survive the Ficoll fractionation in large enough numbers for chemical analysis and were therefore deleted from our electron microscopic analysis. The cells occur in the Ficoll fractions 23–30. Many of the cells appear to be disintegrated and surrounded by polymerized collagen. This is consistent with the role that the cells play in the sponge where, upon ripening, they disintegrate to release fibrous bundles of intracellular matrix (20).

Sterol content of sponge cells. The sterols of *Tethya* are shown in Scheme 1. There are nine major sterols (**1–9**) including the predominant animal sterol cholesterol (**6**, 52%) and the most abundant plant and algal sterol, clionasterol (**9**, 6.1%). In our cell separation procedure, these two sterols were separated as shown in Figure 4. Fraction T5, composed solely of sponge cells, contains only cholesterol. On the other hand, fraction 35, consisting only of symbiont cells, has clionasterol but no cholesterol. Cholesterol (**6**), the major sterol among the sponge cells, does not seem to vary much in quantity from one cell type to another. Unfortunately, the other minor sterols (**1–5, 7–8**) could not be resolved under our chromatographic conditions. Electron micrographs of the symbionts show cells consistent for both unicellular fungi and (red) algae, both of which are known (30) to contain such sterols.

In summary, the cells of *Tethya aurantia* were separated on a Ficoll gradient. A major population of choanocytes (T5) was separated from a major population

of archeocytes (T20), but both fractions contained more symbiont population than the original tissue (WS). The major fatty acid in both sponge cell types was 28:3 (approximately 30%). In the choanocytes, an equal concentration of C_{26} fatty acids was found which was only about half of that found in the archeocytes (T13) (C_{26}/C_{28} = 1:2). The archeocyte cells (T13) also had twice the concentration of 20:4, about half of which was arachidonic acid. Both these fatty acids were associated with the phosphoinositol fraction. Therefore, we suppose that this is an authentic sponge phospholipid which may be involved in cellular communication.

Both choanocytes and archeocytes had cholesterol as the major sterol (Fig. 4), which was equal in concentration to the major fatty acid (28:3). Clionasterol (or its C_{24} epimer) was shown to be the major sterol of the symbionts by its singular presence among the symbiont fractions T35, 35. Therefore, the sterol composition of the whole sponge is a pool whose major components, cholesterol (6) and clionasterol, come from animal (sponge) and plant (symbiont) cells, respectively.

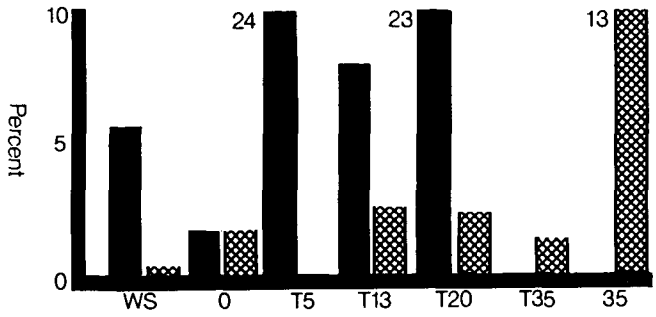


FIG. 4. Relative amounts of the major animal sterol, cholesterol, shown as a solid bar; and the major plant sterol, clionasterol, shown as open mesh bars; in selected Ficoll fractions.

DISCUSSION

Sponges, which can contain a tremendous diversity of sterols as well as fatty acids that are unique to the marine environment, are usually analyzed for lipids as whole organisms. Over 200 marine sterols and more than 50 unusually long-chain (C_{26}-C_{30}) fatty acids have been isolated from sponges since the early 1970's (14,24,31-41). We were perplexed to find fatty acid chain lengths of 24-30 carbons present in substantial quantities in the phospholipids of membrane fractions of the marine demosponges *Reniera* and *Pseudaxinyssa sp.* (13,41,42) and also present in the spherulous cells of *Aplysina fistularis* (33). Furthermore, sponges can contain doubly, triply and quadruply alkylated (relative to cholesterol) side chain sterols, of which *Aplysina fistularis* showed some enrichment in the large mesohyl spherulous cells (42). That these enrichments of unusual fatty acids occurred in the phospholipid fractions suggested that long-chain fatty acids were real membrane components. What remained to be determined was the role of these fatty acids and sterols in the course of primitive cell differentiation that has occurred in sponges. We therefore asked the question: How does fatty acid and sterol content differ from one cell type to another within a specific sponge?

Lipids based on branched-chain fatty acids are characteristic of procaryotes; eucaryotes do not have such lipid systems, with the exception of a few fungi (25,43). The prevalence of certain phospholipid classes can be used for futher differentiation—the major phospholipids of gram positive bacteria are phosphatidylglycerol, in addition to phosphatidylethanolamine; gram negative bacteria possess only the latter. Cyanobacteria are exceptional among bacteria in having only one phospholipid, phosphatidylglycerol, which is ubiquitous (with the exception of actinomycetes) in all bacteria (30). Phosphatidylglycerol is a major lipid of photosynthetic membranes and is localized in the inner mitochondrial membrane of eukaryotes. However, phosphatidylcholine, widespread and major in eukaryotes, is comparatively rare in bacteria. Phosphatidylinositol is not a common constitutent of bacteria, but it is found, along with its derivatives containing mannose, in mycobacteria (actinomycetes), myxobacteriales (gliding bacteria), and corynebacteria (30). It may be significant that some of these more advanced forms of procaryotes (myxobacteria) not only have the phospholipid PI and fatty acid (arachidonic) implicated in cell signalling, these primitive procaryotes also exhibit social behavior in forming loose multicellular aggregates (10). Phosphatidylinositol is also a major lipid in yeasts, another microbe of association (30).

Total saponification of the lipid fraction into fatty acid constituents is often in itself used in the identification of microbes. Generally, the most common bacterial fatty acids are C_{12}-C_{20} saturated and monounsaturated, but not polyunsaturated, fatty acids (a notable exception being the gliding bacteria). Bacteria characteristically contain odd-chain, branched, 2- or 3-hydroxy, or cyclopropyl fatty acids, all of which are rare or absent in higher organisms (30). However, marine algae often contain long-chain polyunsaturated acids (e.g., arachidonic, 20:4). In general, cyanobacteria are very diverse; some are "animal-like" [containing γ-linolenic acid, 18:3(n-6)] while others are "plant-like" [containing α-linolenic acid 18:3(n-3)]. Both n-3 and n-6 18:3 fatty acids may be present in marine fungi which, along with palmitic and stearic acid, may also contain arachidonic acid as a major component (30).

The sterol or nonsaponifiable lipid content of sponges is interesting because it represents an extensive investment in carbon intermediates and energy yet it is nonessential for life since sterols are not required in procaryotes (44). It has been generally accepted that only eucaryotic cells have sterols. However, recent isolations of sterols from certain procaryotes (45), as well as the dependence of others on sterols as growth factors (45), has led to some upgrading of previous thinking that only eucaryotic cells store sterols. Animal sterols are typically represented by cholesterol (derived from lanosterol) while plant sterols have extended alkylation at C-24, as in clionasterol (derived from cycloartenol). Crude sponge extracts usually yield many sterols, e.g., 74 (46) which, at this stage of examination, are impossible to sort into sponge and symbiont sterols.

Sponges are organisms with few cell types without real tissue development. They are unique because of the ease

with which viable cells can be separated. Before our work (13), the differences between the cell types of any particular sponge had been mostly based on electron microscopic evidence. Our lipid analyses of the cell types of the sponge *Tethya aurantia* show that the differentiated choanocytes do differ in their lipid composition from the more "embryonic" archeocyte. If we assume that the long-chain ($>C_{24}$) fatty acids are due only to the sponge cells (not symbiont cells), then choanocytes contain more C_{26}, while archeocytes contain relatively more 20:4 fatty acids. The specialized endoplasmic reticulum, the site of lipid synthesis in higher cells, is greatly diminished in a choanocyte, which raises the question as to the origin of its lipids. Are the lipids manufactured by the choanocyte or are they inherited in a stem cell fashion from the archeocyte? Did the choanocyte manufacture more 26:2 for a specific function or did it lose, through differentiation, the ability to synthesize 28:3? The answers to these questions require further experiments.

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Structure Activity Relationships of Imido *N*-Alkyl Semicarbazones, Thiosemicarbazones and Acethydrazones as Hypolipidemic Agents in Rodents

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A series of nitrogen substituted *N*-butan-3-one derivatives of cyclic imides (phthalimide, substituted phthalimide, *o*-benzosulfimide, 1,8-naphthalimide, 2,3-dihydrophthalazine-1,4-dione and diphenimide) and their semicarbazone, thiosemicarbazone and acethydrazone derivatives were investigated for hypolipidemic activity in rodents. These compounds were generally potent hypolipidemic agents, lowering serum cholesterol levels on an average of 37% and serum triglyceride levels on an average of 29% after 16 days dosing at 20 mg/kg day intraperitoneally (I.P.) in mice. Several analogs, most notably the semicarbazone and acethydrazone derivatives of 1-*N*-(1,8-naphthalimido)-butan-3-one, demonstrated improved hypocholesterolemic activity relative to their ketone percursors. Similarly, the acethydrazone derivatives generally resulted in improved hypotriglyceridemic activity in each series of 2-(3-oxobutyl)-2,3-dihydrophthalazone-1,4-dione analogs tested. The thiosemicarbazones in mice generally resulted in a loss in hypolipidemic activity. Select compounds, 1-*N*-3-methylphthalimido butan-3-semicarbazone (Ig) and 1-(4-methoxyphthalazine-1(2H)-one)yl butan-3(*N*-acetyl)hydrazone (IVg), at 10 mg/kg/day orally administered to rats demonstrated potent hypolipidemic activity after 14 days. These compounds lowered liver, small intestine mucosa and aorta wall tissue lipids, e.g. cholesterol and triglycerides, and raised fecal excretion of cholesterol moderately and of triglyceride significantly. Rat serum lipoprotein fractions after treatment for 14 days showed that the two agents lowered VLDL cholesterol and raised HDL cholesterol content.

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Cyclic imides have been shown to be potent hypolipidemic agents reducing both serum cholesterol and triglyceride levels in rodents more than 35% at 20 mg/kg/day (1–8) even though these compounds demonstrated no acute toxicity at high doses. One of the limiting factors was that whereas VLDL and LDL cholesterol levels were reduced significantly, the HDL cholesterol levels were not increased after two weeks of drug administration. Further evaluation has demonstrated that dosing for eight weeks rather than two weeks with *o*-(*N*-phthalimido)acetophenone led to a 41% increase in HDL cholesterol content (9). These studies suggested that *N*-substitutions are critical to elevating HDL cholesterol and lowering LDL cholesterol in rats. Preliminary studies have shown that 2-(2,4-dimethylphenyl)indan-1,3-dione at 20 mg/kg/day elevated HDL cholesterol of rats by 75% within 14 days (10). Modification of 1-*N*-phthalimidobutan-3-one to afford a semicarbazone derivative has led to a compound which lowered rat VLDL and LDL cholesterol and elevated HDL cholesterol 55% at 10 mg/kg/day after two weeks of drug administration (11). Thus, a series of phthalimide semicarbazone, thiosemicarbazone and acethydrazone derivatives was synthesized for structure activity relationship (SAR) studies and experiments were conducted to determine their effects on lipid metabolism in rodents.

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Abbreviations: HDL, high density lipoprotein; I.P., intraperitoneally; LDL, low density lipoprotein; SAR, structure activity relationship; VLDL, very low density lipoprotein.

MATERIALS AND METHODS

Chemistry. The synthesis of 1-*N*-phthalimidobutan-3-one, 1-*N*-phthalimidopentan-4-one (1), 1-*N*-(*o*-benzosulfimido)-butan-3-one, 1-*N*-(1,8-naphthalimido)butan-3-one (12), 2-(3-oxobutyl)-2,3-dihydrophthalazine-1,4-dione (5) and 1-*N*-diphenimidobutan-3-one (6), utilized as intermediates, has been previously reported. The butan-3-one analogs **Id, If, Ih** and **Ij** were synthesized by Michael addition of methyl vinyl ketone to the appropriately substituted phthalimide adapting the procedure of Irai *et al.* (13). 1-*N*-Phthalimidopentan-3-one, **VIa,** was also prepared by Michael addition of ethyl vinyl ketone generated *in situ* from 1-chloropentan-3-one (14). The 4-alkoxyphthalazin-1(2H)one derivatives **IVd, IVh** and **IV1** were prepared by selective *O*-alkylation of 2-(3-oxobutyl)-2,3-dihydrophthalazine-1,4-dione with the appropriate alkyl halide. The corresponding semicarbazones, thiosemicarbazones and acethydrazones were prepared by reaction of the appropriate ketone with semicarbazide, thiosemicarbazide and acethydrazide, respectively.

Experimental. Melting points were determined on a Thomas-Hoover apparatus (capillary method) and are uncorrected. NMR spectra were determined on a Varian EM 360A spectrometer using tetramethylsilane as an internal standard and deuterochloroform or DMSO-d_6 as the solvent. Infrared spectra were determined on a Beckman Acculab spectrophotometer using the potassium bromide technique. Mass spectra were measured on a Finnigan 4021 spectrometer. All spectral data were consistent with the structures designated herein. Elemental analyses were performed by Atlantic Microlabs, Inc., Atlanta, GA.

Method A. Adopting the procedure of Irai *et al.* (13), 4-chlorophthalimide, 3-methylphthalimide, 4-methylphthalimide or 3-nitrophthalimide (0.025–0.03 mol) were suspended in ethyl acetate, and a catalytic amount of sodium ethoxide was added. The reaction was heated to 70°C. A 20% excess of an equimolar amount of methyl vinyl ketone was added, the solution was refluxed for an additional 3 hr, neutralized with acetic acid, the solvent evaporated *in vacuo,* the residue dissolved in CH_2Cl_2 and washed with water. The CH_2Cl_2 layer was dried over $MgSO_4$, evaporated *in vacuo,* and the residue purified by recrystallization as specified in Table 1 to afford the appropriate butan-3-one.

TABLE 1

Chemical Data for Hypolipidemic Compound

Cpd. No.	m.p.	Solvent (chrom. solvent)	Yield (crude)	Method	Empirical formula	Elemental analyses
Ia	207-210	MeOH/H_2O	79	C	$C_{13}H_{14}N_4O_3$	C,H,N
Ib	215-217	EtOH/H_2O	(90)	D	$C_{13}H_{14}N_4O_2S$	C,H,N,S
Ic	179-181	(EtOAc)	77	E	$C_{14}H_{15}N_3O_3$	C,H,N
Id	108-110	C_6H_6	48	A	$C_{12}H_{10}NO_3Cl$	C,H,N,Cl
Ie	209-211	$CHCl_3$	28	C	$C_{13}H_{13}N_4O_3Cl \cdot 1/3H_2O$	C,H,N,Cl
If	80-82	CH_2Cl_2	30	A	$C_{13}H_{13}NO_3$	C,H,N
Ig	215-217	$CHCl_3$	70	C	$C_{14}H_{16}N_4O_3$	C,H,N
Ih	95-97	$CHCl_3$	72	A	$C_{13}H_{13}NO_3$	C,H,N
Ii	187-189	$CHCl_3$	55	C	$C_{14}H_{16}N_4O_3$	C,H,N
Ij	97-100	—	33	A	$C_{12}H_{10}N_2O_5$	C,H,N
IIa	180-181	MeOH/H_2O	39	C	$C_{12}H_{14}N_4O_4S$	C,H,N
IIb	180-183	MeOH/H_2O	85	D	$C_{12}H_{14}N_4O_3S_2$	C,H,N,S
IIc	157-159	—	83	E	$C_{13}H_{15}N_3O_4S$	C,H,N,S
IIIa	222-223	CH_2Cl_2	(85)	C	$C_{17}H_{16}N_4O_3 \cdot 1/4H_2O$	C,H,N
IIIb	230-231 (dec)	—	81	D	$C_{17}H_{16}N_4O_2S$	C,H,N,S
IIIc	214-215	(EtOAc)/EtOH	14	E	$C_{18}H_{17}N_3O_3 \cdot 1/4H_2O$	C,H,N
IVa	214-216	—	56	C	$C_{13}H_{17}N_5O_4 \cdot H_2O$	C,H,N
IVb	212-214	—	83	D	$C_{13}H_{15}N_5O_2S$	C,H,N,S
IVc	179-180	—	79	E	$C_{17}H_{16}N_4O_3$	C,H,N
IVd	82-83	EtOAc/hexane	(58)	B	$C_{13}H_{14}N_2O_3$	C,H,N
IVe	185-187	isopropanol	72	C	$C_{14}H_{17}N_5O_3$	C,H,N
IVf	193-194	isopropanol	80	D	$C_{14}H_{17}N_5O_2S$	C,H,N,S
IVg	175-177	EtOAc/hexane	78	E	$C_{15}H_{18}N_4O_3$	C,H,N
IVh	48-49	EtOAc/hexane	53	B	$C_{14}H_{16}N_2O_3$	C,H,N
IVi	208-210	isopropanol	(96)	C	$C_{15}H_{19}N_5O_3$	C,H,N
IVj	196-198 (dec)	—	91	D	$C_{15}H_{19}N_5O_2S$	C,H,N,S
IVk	149-151	EtOAc	44	E	$C_{16}H_{20}N_4O_3$	C,H,N
IVl	106-107	MeOH	(77)	B	$C_{19}H_{18}N_2O_3$	C,H,N
IVm	192-193	isopropanol	(92)	C	$C_{20}H_{21}N_5O_3$	C,H,N
IVn	209-211 (dec)	—	92	D	$C_{20}H_{21}N_5O_2S$	C,H,N,S
IVo	119-120	EtOAc/hexane	76	E	$C_{21}H_{22}N_4O_3$	C,H,N
V	148-150	—	65	C	$C_{15}H_{18}N_4O_3$	C,H,N
VIa	91-93.5	EtOH/H_2O	47	—	$C_{13}H_{13}NO_3$	C,H
VIb	189-191	—	82	C	$C_{14}H_{16}N_4O_3$	C,H,N
VII	185-187	—	83	C	$C_{19}H_{18}N_4O_2$	C,H,N

Method B. 2-(3-Oxobutyl)-2,3-dihydrophthalazine-1,4-dione (0.04-0.083 mol) was dissolved in ethanol (200-400 mL) and an equimolar amount of potassium hydroxide added. Upon dissolution an equimolar amount of methyl iodide, bromoethane or benzyl bromide was slowly added and refluxing continued for 3-12 hr. The solvent was removed *in vacuo,* the residue suspended in CH_2Cl_2, washed with 1N NaOH and water, and dried over $MgSO_4$. The solvent was evaporated *in vacuo* and the crude crystalline product recrystallized from the appropriate solvent.

Method C. The appropriate ketone (0.005-0.025 mol) was added to a hydroalcoholic sodium acetate solution containing a 10% excess of an equimolar amount of semicarbazide hydrochloride. The reaction mixture was refluxed until it appeared cloudy, allowed to stand for 2 hr and 40 mL of water added. The precipitate was filtered and recrystallized from the appropriate solvent when necessary.

Method D. Adopting the procedure of Sah and Daniels (15), the appropriate ketone (0.005-0.020 mol) was dissolved in hot ethanol and added to an equimolar solution of thiosemicarbazide in water and acetic acid. The reaction mixture was refluxed until a precipitate formed, then allowed to cool to room temperature. The precipitate was collected by filtration and purified by recrystallization when necessary.

Method E. An ethanolic solution of the appropriate ketone (0.002-0.005 mol) was added to an equimolar solution of acethydrazide in ethanol. The reaction mixture was refluxed for 5-20 min. Compounds **IIc** and **IVc** precipitated from solution and no further purification was necessary. The remaining acethydrazones were obtained by removing the solvent *in vacuo* followed by chromatography and/or recrystallization from the appropriate solvent.

1-N-Phthalimidopentan-3-one (VIa). Phthalimide, 10.0 g (0.068 mol), was suspended in 160 mL of ethyl acetate. Sodium ethoxide (0.130 mol) in 50 mL of ethanol was added and the resultant mixture heated to reflux. 1-Chloropentan-3-one, 8.18 g (0.075 mol), was slowly added and refluxing continued overnight. Upon cooling, the volatile material was removed *in vacuo* to yield a yellow oil which upon recrystallization from ethanol and water afforded 7.3 g (47%) of **VIa**; mp 91-93.5°C. NMR ($CDCl_3$): δ 7.68-8.05 (*m*, 4H, aromatic) 4.00 (*t*, 2H, $-N-CH_2$), 2.78 (*t*, 2H, CH_2CO), 2.45 (*q*, 2H, CH_3CH_2CO), 1.10 (*t*, 3H, CH_3). *Anal.* ($C_{13}H_{13}NO_3$)C,H.

Hypolipidemic screens in normal rodents. The test compounds **Ia-VII** were suspended in an aqueous 1% carboxymethylcellulose solution, homogenized, and administered to CF_1 male mice (~25 g) at 20 mg/kg/day intraperitoneally for 16 days. SAR studies were performed at this dose since this was the optimum dose in the phthalimide, saccharin, diphenimide and 2,3-dihydrophthalzine-1,4-dione studies in rodents (1-8). On days 9 and 16, blood was obtained by tail vein bleeding and the serum separated by centrifugation for 3 min. The serum cholesterol levels were determined by a modification of the Liebermann-Burchard reaction (16). Serum was also analyzed for triglyceride content as determined by a commercial kit (BioDynamices/bmc single vial, triglycerides colorimetric method 348201). Food and water were available *ad libitum* for animals in SAR experiments.

Sprague Dawley rats. Select compounds Ig and IVg were tested in Sprague Dawley male rats (~280 g) by oral administration at 10 mg/kg/day for 14 days. Serum cholesterol and triglyceride levels were measured as previously described. Food and water were available *ad libitum.*

Animals weights and food intake. Food consumption was determined daily (g food/rat/day) for control rats and rats treated orally with compounds **Ig** and **IVg** at 10 mg/kg/day. Body weights were obtained during the experiments and expressed as percentage of the animal's weight on day 0. After dosing for 14 days with compounds, selected organs were excised, trimmed of fat, and weighed.

Liver, small intestine, aorta and fecal lipid extraction. In Sprague Dawley male rats that had been administered the test compounds **Ig** or **IVg** at 10 mg/kg/day for 14 days, the liver, small intestine mucosa from duodenum to ileum, aorta and fecal materials (24 hr collection) were removed and weighed. Homogenates (10%) (w/v) were prepared, extracted (17,18) and analyzed for cholesterol levels (16), triglyceride levels, neutral lipid content (19) and phospholipid content (20). Protein content of the whole homogenate was determined (21).

Serum lipoprotein fractionation. Sprague Dawley male rats (~300 g) were administered compounds **Ig** or **IVg** at 10 mg/kg/day orally. Blood was collected from the abdominal vein and lipoprotein fractions were obtained by the methods of Hatch and Lees (22) and Havel *et al.* (23) as modified for the rat (24). Each of the fractions was analyzed for cholesterol (16), triglyceride, neutral lipids (19), phopholipids (20), and for protein levels (21).

TABLE 2

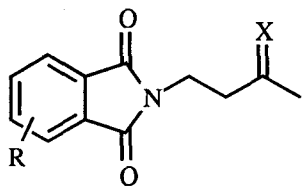
Structure Activity Relationships of Imido, *N*-Alkyl Semicarbazone, Thiosemicarbazones and Acethydrazone in CF_1 Male Mice at 20 Mg/Kg/Day I.P.

Compound (N=6)	(X ± SD)[a] Serum cholesterol mg% Day 9	Day 16	Serum triglyceride mg% Day 14
Ia	31 ± 5*	43 ± 5*	82 ± 7*
Ib	97 ± 4*	79 ± 5	79 ± 5*
Ic	58 ± 5*	41 ± 3*	72 ± 5*
Id	56 ± 4*	56 ± 4*	96 ± 7
Ie	57 ± 3*	54 ± 5*	95 ± 8
If	50 ± 4*	50 ± 5*	63 ± 7*
Ig	52 ± 4*	50 ± 6	63 ± 5*
Ih	66 ± 6	48 ± 4*	78 ± 4*
Ii	44 ± 4*	44 ± 3*	69 ± 5*
Ij	46 ± 4	44 ± 4	76 ± 8*
IIa	44 ± 4*	38 ± 3*	69 ± 5*
IIb	104 ± 5*	98 ± 6*	78 ± 4*
IIc	56 ± 2*	38 ± 3*	88 ± 8
IIIa	49 ± 6*	33 ± 2	79 ± 5*
IIIb	60 ± 4	50 ± 3*	89 ± 5
IIIc	58 ± 3*	37 ± 3*	81 ± 5*
IVa	58 ± 4	52 ± 4*	73 ± 7*
IVb	53 ± 5*	49 ± 4*	67 ± 6*
IVc	47 ± 5*	41 ± 3*	63 ± 5*
IVd	40 ± 4*	40 ± 4*	88 ± 6
IVe	46 ± 4*	41 ± 5*	104 ± 8
IVf	65 ± 3	62 ± 5*	102 ± 7
IVg	55 ± 6*	50 ± 4*	58 ± 4*
IVh	67 ± 4	57 ± 5*	85 ± 6
IVi	42 ± 4*	41 ± 4*	68 ± 5*
IVj	76 ± 5	51 ± 4*	83 ± 4*
IVk	55 ± 4*	55 ± 5*	55 ± 4*
IVl	61 ± 4	39 ± 3*	87 ± 6*
IVm	34 ± 3*	32 ± 3*	85 ± 5*
IVn	57 ± 4*	45 ± 4*	90 ± 6
IVo	70 ± 4	37 ± 4*	83 ± 5*
V	58 ± 4*	60 ± 5*	63 ± 4*
VI	59 ± 4	52 ± 6*	70 ± 5*
VII	44 ± 5*	40 ± 4*	83 ± 6*
Clofibrate 150 mg/kg	64 ± 5	64 ± 4*	82 ± 8*
1% CMC	73 ± 4	75 ± 4	110 ± 8

[a]Student's t-test; *P < 0.001.

RESULTS

The structure activity relationship for hypolipidemic activity (Table 2) in the phthalimide series showed that the *N*-alkyl semicarbazone **Ia** and acethydrazone **Ic** both afforded approximately a 45% reduction of serum cholesterol levels after 16 days dosing at 20 mg/kg/day I.P. in mice (Fig. 1). Serum triglyceride levels were reduced 25% by **Ia** and 35% by **Ic**. Substitution on the aromatic ring of phthalimide showed that only the 3-nitro substitution resulted in a 42% reduction of serum cholesterol levels. None of the other derivatives in the phthalimide series improved the hypocholesterolemic activity relative to the unsubstituted analogs. Substitution of a 3-position methyl group resulting in the butanone **If** and the semicarbazone **Ig** afforded 43% reduction of serum triglyceride levels in mice. In the *o*-benzosulfimide series, the *N*-alkyl semicarbazone **IIa** afforded the best activity with 49% reduction of serum cholesterol and 37% reduction of serum triglyceride levels. The acethydrazone **IIc** also

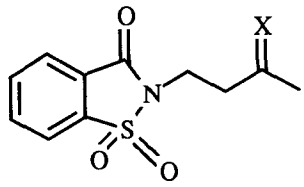


(Ia - j)

	R	X
Ia	H	$NNHCNH_2$ (O)
Ib	H	$NNHCNH_2$ (S)
Ic	H	$NNHC\text{-}CH_3$ (O)
Id	4-Cl	O
Ie	4-Cl	$NNHCNH_2$ (O)
If	$3\text{-}CH_3$	O
Ig	$3\text{-}CH_3$	$NNHCNH_2$ (O)
Ih	$4\text{-}CH_3$	O
Ii	$4\text{-}CH_3$	$NNHCNH_2$ (O)
Ij	$3\text{-}NO_2$	O

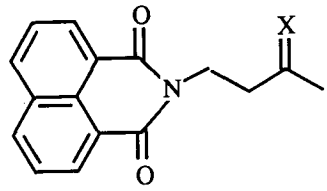
FIGURE 1

resulted in 49% reduction of serum cholesterol levels but the agent was not very effective in lowering serum triglyceride levels. Generally, the thiosemicarbazone derivatives showed minimal hypolipidemic activity in all series tested and compound **IIb** actually demonstrated a significant increase in serum cholesterol levels after 16-day administration (Fig.2). This phenomenon has not been previously observed in the cyclic imide analogs tested. The naphthalimide series followed the same pattern with the semicarbazone **IIIa** and the acethydrazone **IIIc** resulting in reductions of serum cholesterol levels of 56% and 51%, respectively. Among the compounds studied, these derivatives also demonstrated the greatest improvement in hypocholesterolemic activity relative to their respective butanone precursor. However, this series was generally not as potent in lowering serum triglyceride levels as the phthalimide derivatives. In the 2,3-dihydrophthalazine-1,4-dione series, the acethydrazone derivatives **IVc, IVg, IVk** and **IVo** resulted in the best hypotriglyceridemic activity in each group (Fig. 3).



(IIa - c)

	X
IIa	$NNHCNH_2$ (O)
IIb	$NNHCNH_2$ (S)
IIc	$NNHCCH_3$ (O)

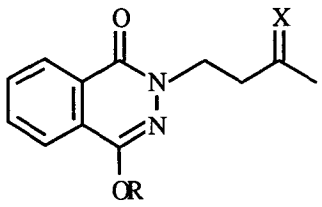


(IIIa - c)

	X
IIIa	$NNHCNH_2$ (O)
IIIb	$NNHCNH_2$ (S)
IIIc	$NNHCCH_3$ (O)

FIGURE 2

Compound **IVc** was one of the more potent hypolipidemic agents with 46% reduction of serum cholesterol levels and 43% reduction of serum triglyceride levels. Replacement of the enolic hydroxyl by a methoxy group affording the butanone **IVd** and semicarbazone **IVe** derivatives resulted in 47% and 46% reductions of serum cholesterol levels respectively, but the reduction of serum triglyceride levels was poor. The acethydrazone **IVg** demonstrated a 34% reduction of serum cholesterol levels and a 47% reduction of serum triglyceride levels. In the 2-(3-oxobutyl)-4-ethoxyphthalazin-1(2H)one series, the semicarbazone **IVi** afforded 46% reduction of serum cholesterol and 38% reduction of serum triglyceride levels. In the



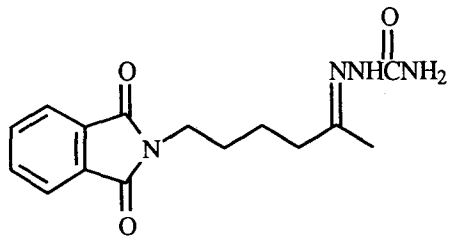
(IVa - o)

	R	X
IVa	H	O ‖ NNHCNH$_2$
IVb	H	S ‖ NNHCNH$_2$
IVc	H	O ‖ NNHCCH$_3$
IVd	CH_3	O
IVe	CH_3	O ‖ NNHCNH$_2$
IVf	CH_3	S ‖ NNHCNH$_2$
IVg	CH_3	O ‖ NNHCCH$_3$
IVh	CH_2CH_3	O
IVi	CH_2CH_3	O ‖ NNHCNH$_2$
IVj	CH_2CH_3	S ‖ NNHCNH$_2$
IVk	CH_2CH_3	O ‖ NNHCCH$_3$
IVl	$CH_2(C_6H_5)$	O
IVm	$CH_2(C_6H_5)$	O ‖ NNHCNH$_2$
IVn	$CH_2(C_6H_5)$	S ‖ NNHCNH$_2$
IVo	$CH_2(C_6H_5)$	O ‖ NNHCCH$_3$

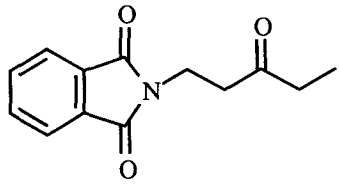
FIGURE 3

benzyl series, the semicarbazone **IVm** and the acethydrazone **IVo** resulted in 58% and 51% reductions of serum cholesterol levels, respectively. Variation of the alkyl chain of 1-*N*-phthalimidobutan-3-one semicarbazone led to no improvement in hypocholesterolemic activity, but the reduction of serum triglyceride levels was improved, i.e. **Va** afforded a 43% reduction and **VIb** afforded a 36% reduction of serum triglyceride levels (Fig. 4). The diphenimide semicarbazone **VIIa** afforded 47% reduction in serum cholesterol content and 24% reduction of serum triglyceride levels.

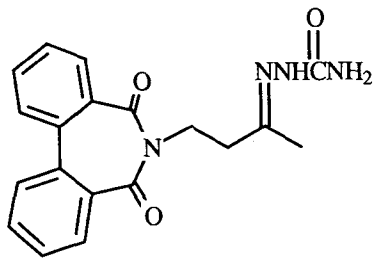
When **Ig** and **IVg** were tested in Sprague Dawley male rats, at 10 mg/kg/day (Table 3) it was observed that after



Va



VIa



VIIa

FIGURE 4

14 days serum cholesterol content was reduced 37% and 42%, respectively and triglyceride content was lowered 28% and 45%, respectively. Compounds **Ig** and **IVg** decreased the total body weight gain by 12 and 14%, respectively, after 14 days administration but did not decrease daily food consumption. The total lipid levels of the rat liver, small intestine and aorta were reduced by **Ig** and **IVg** treatment for 14 days, i.e. cholesterol and triglycerides were reduced in all three tissues (Table 4). After 14 days, the fecal cholesterol content was elevated after drug treatment 14% by **Ig** and 8% by **IVg**. Fecal triglycerides were elevated 81% by **Ig** and 40% by **IVg**. Fecal phospholipid content was elevated 250% by **IVg**. Drug treatment did not affect the weight of the liver or the amount of feces excreted over a 24-hr period.

The serum lipoprotein levels after 14 days (Table 4) showed that **Ig** treatment lowered cholesterol content in the VLDL (56%) and LDL (42%) with a 55% increase in HDL cholesterol content. Compound **IVg** afforded lower cholesterol in the VLDL fraction (25%), but elevated HDL cholesterol 42% after 14 days of treatment. Although VLDL triglyceride content was reduced 39% by **Ig** treatment, there was a corresponding increase in the triglyceride content of the chylomicron, LDL, and HDL fractions. Compound **IVg** lowered chylomicron triglyceride 9%, LDL triglyceride 20% and HDL triglyceride 70%. Phospholipid content was similarly increased in the chylomicron and LDL fractions by **Ig** but was decreased in the VLDL and HDL fractions. Compound **IVg** elevated

TABLE 3

The Effects of 1-*N*-3-Methylphthalimido-butan-3-semicarbazone (Ig) and 1-(4-Methoxyphthalazine-1(2H)-one)yl butan-3-(*N*-acetyl)-hydrazone (IVg) on the Serum Lipids of Sprague Dawley Rats After 14-Day Administration Orally at 10 mg/kg/day

	Serum cholesterol (mg%)		Serum triglyceride (mg%)	
N=6	Day 7	Day 14	Day 7	Day 14
Control 1% CMC	75 ± 5	78 ± 6	109 ± 6	110 ± 6
Treated Ig	53 ± 4*	49 ± 3*	77 ± 6*	79 ± 8*
IVg	54 ± 3*	45 ± 4*	58 ± 5*	61 ± 4*

	Percentage of day zero's body weight (g)		Food consumption gm/day/rat
	Day 7	Day 14	
Control 1% CMC	116	128	20.8
Treated Ig	104	116	22.0
IVg	106	114	25.2

VLDL phospholipid content 61% and HDL phospholipid 114%, but lowered LDL phospholipid 44%.

DISCUSSION

A number of cyclic imides have previously demonstrated potent hypolipidemic activity in rodents effectively lowering both serum cholesterol and triglycerides greater than 35% after 16 days of administration (1-8). Derivatization of the butanone analogs as the semicarbazones, thiosemicarbazones and acethydrazones described herein generally resulted in the retention of significant hypolipidemic activity in rodent screens. Only the 1.8-naphthalimide derivatives, **IIIa** and **IIIc**, demonstrated significant improvements in hypocholesterolemic activity above the parent ring structures. However, if an agent is expected to be effective in hyperlipidemic patients then it should reduce high LDL cholesterol levels and elevate low HDL cholesterol levels. Unfortunately, the commercially available agents used in the clinic today to treat arteriosclerosis and hypolipidemic states are not significantly

TABLE 4

The Effects of 1-*N*-3-Methylphthalimido butan-3-semicarbazone (Ig) and 1-(4-Methoxyphthalazine 1(2H)-one)yl-butan-3-(*N*-acetyl)hydrazone (IVg) on the Tissue Lipids and Serum Lipoprotein Lipids of Sprague Dawley Rats After 14 days at 10 mg/kg/day Orally (N = 6)[a]

	mg Lipid extracted	Cholesterol	Triglyceride	Neutral lipids	Phospholipids	Proteins[b]
				mg/gm wet tissue		
Liver						
Control	58.5 ± 3.5	24.03 ± 1.44	6.37 ± 0.43	44.11 ± 3.10	7.19 ± 0.36	4.50 ± 0.26
Treated Ig	49.0 ± 3.5	20.66 ± 0.97*	5.44 ± 0.29	51.16 ± 3.44	4.29 ± 0.42	4.88 ± 0.25
IVg	57.3 ± 4.7	18.26 ± 1.20*	5.60 ± 0.32	45.87 ± 2.63	6.25 ± 0.24	3.65 ± 0.41
Small Intestine						
Control	45.5 ± 2.78	7.82 ± 0.45	1.12 ± 0.05	6.98 ± 0.41	2.06 ± 0.16	4.20 ± 0.25
Treated Ig	42.7 ± 2.69	6.83 ± 0.35*	0.91 ± 0.06	6.32 ± 0.47	3.49 ± 0.13	3.99 ± 0.22
IVg	41.8 ± 2.53	7.35 ± 0.38	0.95 ± 0.04	6.77 ± 0.33	1.85 ± 0.12	4.75 ± 0.19
Aorta						
Control	11.58 ± 0.57	2.84 ± 0.17	1.86 ± 0.09	3.39 ± 0.20	5.70 ± 0.34	6.99 ± 0.40
Treated Ig	9.84 ± 0.46	2.55 ± 0.16	1.35 ± 0.07*	3.46 ± 0.16	8.26 ± 0.40*	5.38 ± 0.42*
IVg	11.42 ± 0.36	2.07 ± 0.10*	1.36 ± 0.07	2.91 ± 0.17	7.51 ± 0.31*	6.04 ± 0.56
Fecal						
Control	8.01 ± 0.48	2.85 ± 0.14	1.86 ± 0.07	3.39 ± 0.27	1.39 ± 0.10	6.99 ± 0.35
Treated Ig	9.95 ± 0.61	3.24 ± 0.12	3.37 ± 0.09*	3.21 ± 0.23	0.38 ± 0.04*	5.79 ± 0.39
IVg	28.84 ± 0.80*	3.08 ± 0.19	2.61 ± 0.07*	3.02 ± 0.35	4.87 ± 0.15*	9.51 ± 0.65*
				μg/mL of serum		
Chylomicrons						
Control		337 ± 23	420 ± 25	67 ± 4	149 ± 10	184 ± 16
Treated Ig		341 ± 25	613 ± 34	49 ± 3	242 ± 0	
IVg		630 ± 24	382 ± 17	47 ± 3	141 ± 9	188 ± 9
VLDL						
Control		190 ± 11	22 ± 2	98 ± 6	26 ± 2	50 ± 3
Treated Ig		84 ± 10	13 ± 1*	95 ± 5	18 ± 2	47 ± 2
IVg		142 ± 9	22 ± 3	89 ± 6	42 ± 6*	47 ± 8
LDL						
Control		210 ± 13	45 ± 3	10 ± 1	41 ± 2	122 ± 7
Treated Ig		122 ± 8*	56 ± 3*	6 ± 1	55 ± 2	102 ± 8
IVg		202 ± 11	36 ± 2	118 ± 2	23 ± 2*	135 ± 8
HDL						
Control		544 ± 38	27 ± 2	620 ± 50	153 ± 14	65 ± 5
Treated Ig		843 ± 37	74 ± 5*	470 ± 42*	136 ± 9	62 ± 3
IVG		772 ± 27*	8 ± 2*	744 ± 31	327 ± 10*	83 ± 3*

[a]Student's t-test; *p ≤ 0.001.

[b]Protein content of the original homogenates.

effective in elevating HDL cholesterol and lowering LDL cholesterol levels. For example, clofibrate elevated HDL cholesterol 4-16%; decreased serum cholesterol 15% and serum triglyceride 25%; and probucol actually decreased HDL cholesterol 24% (25). Nicotinic acid in man at 3 g/day increases HDL cholesterol 23% and apo AI 7% (25). This agent has been used in the past to treat hypertriglycemia in man. Bezafibrate increases HDL cholesterol 15% and decreases serum cholesterol levels 23% and serum triglyceride levels 17% (26). Fenofibrate elevates HDL cholesterol 9-11% and decreases serum cholesterol levels 20% and serum triglyceride levels 38% (27). CS-514 at 20 mg/kg/day increases HDL cholesterol 14% and decreases serum cholesterol 25% and serum triglyceride 15% in man (28). Lovastatin afforded only a moderate elevation in rat HDL cholesterol 27% (unpublished results). In man, elevating HDL cholesterol and reducing LDL cholesterol supposedly protects against myocardial infarction (29). The semicarbazone derivatives **Ig** and **IVg** apparently can elevate HDL cholesterol significantly at the low dose of 10 mg/kg/day in rats by magnitudes greater than that observed for clinically used hypolipidemic agents.

The modulation of the lipoprotein cholesterol ratio in this direction is important since apo B containing lipoproteins, i.e. LDL, VLDL and IDL, are responsible for carrying cholesterol to the peripheral tissues including the plaques (25) whereas HDL is responsible for removing cholesterol from the peripheral tissue to the liver for the purpose of clearance from the body.

The previously reported cyclic imides were not very effective in elevating rat HDL cholesterol levels although they generally did lower LDL and VLDL cholesterol content with reductions of VLDL triglycerides (1-8). Cyclic imides which have bulkier aromatic substitutions on the imide *N*, e.g. *o*-(N-phthalimido)acetophenone after 8 weeks at 20 mg/kg/day, *N*-(4-methylphenyl)diphenimide (30) and 2-(2,4-dimethylphenyl)indan-1,3-dione (31) at 20 mg/kg/day for 14 days led to elevated levels of HDL cholesterol in rats. The receptor regulating this phenomena may require compounds containing relatively bulky *N*-substituents for modulation of the receptor activity. Phthalimide, sacchairin and cyclic imides without such bulky imido nitrogen substitution may bind the receptor less efficaciously. The semicarbazone derivatives, **Ig** and **IVg**, appear to afford a large increase in rat HDL cholesterol content at 10 mg/kg/day after 14 days of administration.

The agents, **Ig** and **IVg**, did not remove lipids from the rat serum compartment and deposit them in the body tissue; rather some of the lipid content was removed by the fecal route from the body. Previous *in situ* rat intestinal loop studies with **Ig** have demonstrated that the compound reduced cholesterol reabsorption 100% and cholic reabsorption 90% at the therapeutic dose (11). The aorta, small intestine and liver demonstrated reduced lipid levels after **Ig** and **IVg** treatment at 10 mg/kg/day, orally. No increase in any organ weight, e.g. liver, was noted after drug treatment. This data suggests that modulation of the HDL and LDL ratio by these agents was effective in lipid removal from the tissues after two weeks of drug administration. Additional studies with **Ig** have demonstrated inhibition of mouse hepatic acyl-CoA cholesterol acyltransferase, heparin-induced lipoprotein lipase, phosphorylase hydrolase and *sn*-glycerol-3-phosphate acyltransferase activities (11). Inhibition of regulatory enzyme of *de novo* synthesis of triglyceride and cholesterol esters would contribute to the overall lowering of serum lipids by these agents (11). Thus, the semicarbazone and acethydrazone cyclic imides may offer potential clinical hypolipidemic agents for the reduction of lipids in arterial plaques.

ACKNOWLEDGMENT

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METHODS

Enantiomer Separations of Mixtures of Monoacylglycerol Derivatives by HPLC on a Chiral Column

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High-performance liquid chromatographic separations of monoacylglycerol enantiomeric mixtures as their di-3,5-dinitrophenylurethane derivatives were carried out on a chiral stationary phase, N-(*R*)-1-(α-naphthyl)ethylaminocarbonyl-(*S*)-valine bonded to silica gel. The conditions for the quantitative analysis of *sn*-1 and *sn*-3 enantiomeric mixtures of six saturated 1-monoacylglycerols having consecutive even carbon numbers and of *sn*-1 and *sn*-3 enantiomeric mixtures of 1-stearoyl-, 1-oleoyl-, 1-linoleoyl-, and 1-linolenoylglycerols were obtained. The linear relationships of logarithmic retention volumes against the number of carbons and olefinic bonds for the enantiomer homologous series are discussed, and a new concept of enantiomer separation is presented.
***Lipids* 25, 398–400 (1990).**

Recently, enantiomer separations of monoacyl- (1,2), monoalkyl- (3), diacyl- (4,5), and dialkyl- (5) glycerols as their 3,5-dinitrophenylurethane (3,5-DNPU) derivatives have been achieved by high-performance liquid chromatography (HPLC) on the chiral stationary phases, Sumipax OA-2100 N-(*S*)-2-(4-chlorophenyl)isovaleroyl-D-phenylglycine chemically bonded to γ-aminopropyl silanized silica and OA-4100 N-(*R*)-1-(α-naphthyl)ethylaminocarbonyl-(*S*)-valine chemically bonded to silanized silica (Sumitomo Chemical Co., Osaka, Japan). In HPLC of monoacylglycerols (MGs), each racemate of 1-lauroyl-glycerol (1-12:0), 1-14:0, 1-16:0, 1-18:0, 1-18:1, 1-18:2 and 1-18:3 was clearly separated into the respective enantiomers using di-3,5-DNPU derivatives on OA-2100 (1,2).

The chiral phases used in our study have shown an attendant function to separate enantiomeric homologues based on the number of carbons and olefinic bonds, as with normal phase chromatography (1–5). In HPLC of 1-MGs on OA-2100, separations of homologues differing by two carbons and one olefinic bond were poor in comparison to the enantiomer separations. Improvement of the resolutions of 1-MG mixtures has been reported in a preceding paper (2). The present paper describes further improved HPLC conditions using another chiral column OA-4100.

MATERIALS AND METHODS

Samples. Optically active 3-palmitoyl-*sn*-glycerol (*sn*-3-16:0) and *sn*-3-18:0, and racemic 1-palmitoyl-glycerol (*rac*-16:0) and *rac*-18:0 were obtained from Fluka AG (Buchs, Switzerland). *Rac*-12:0 and *rac*-14:0 were obtained from Sigma Chemical Co. (St. Louis, MO). Other MG samples were synthesized in our laboratory based on procedures described in a previous paper (6). It involves interesterification of fatty acid methyl esters with an appropriate amount of glycerol in dimethylformamide at 90°C. The 1-MG products were separated by thin-layer chromatography (TLC) on boric acid-impregnated silicic acid plates. Preparations of di-3,5-DNPU derivatives from 1-MGs were carried out by the procedures of Oi and Kitahara (7,8). The urethane products were purified by TLC on silica gel GF plates using hexane/ethylene dichloride/ethanol (40:15:5, v/v/v) as the developing solvent.

HPLC. HPLC separation was carried out with a Hitachi L-6200 instrument (Hitachi, Ltd., Tokyo, Japan) equipped with a chiral column (stainless steel, 50 cm × 4 mm i.d.) packed with 5 μ particles of Sumipax OA-4100. The analysis was done isocratically using a mixture of HPLC grade hexane/ethylene dichloride/ethanol (40:10:1, v/v/v) as mobile phase at a constant flow rate of 0.5 mL/min at ambient temperature. The separations were monitored with a Shimadzu SPD-6A UV detector (Shimadzu Co., Kyoto, Japan) at 254 nm. Peak area percentages and retention times were measured with a Shimadzu integrator, Chromatopac C-R6A.

RESULTS AND DISCUSSION

Figure 1 shows separations of racemic MGs as di-3,5-DNPU derivatives on a chiral column, OA-4100. Complete enantiomer separations of racemate and almost complete separations of enantiomer homologues are shown in the chromatograms. All the racemic MGs used were separated into enantiomers with separation factors of 1.31–1.35 and peak resolutions of 5.19–7.10. These separation factors and peak resolutions indicate complete separations of racemates into enantiomers on OA-4100. Table 1A and 1B show the chromatographic data for the separations of the enantiomer homologues. The separation factors of the homologues differing by two acyl carbons and by one olefinic bond are 1.04–1.05 and 1.06, respectively. The peak resolutions of the homologues are 1.02–1.48. The OA-4100 column showed 10,600 theoretical plates for the *sn*-3-16:0 peak. The peaks of MG enantiomers generally tailed markedly under the conditions employed, which is attributable to high capacity ratios used (9).

Figure 2 shows the plots of logarithmic retention volumes (Vr) *versus* acyl carbon numbers and olefinic bond numbers for each homologous series of MG enantiomers. The straight lines for *sn*-1 and *sn*-3 enantiom-

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Abbreviations: 3,5-DNPU, 3,5-dinitrophenylurethane; HPLC, high-performance liquid chromatography; MG, monoacylglycerol; TLC, thin-layer chromatography; Vr, corrected retention volume.

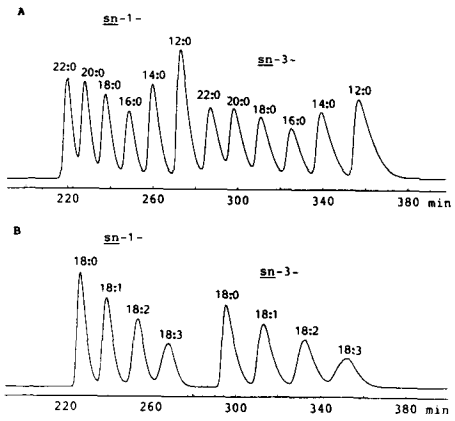

FIG. 1. Separation of MG enantiomers as di-3,5-DNPU derivatives on a chiral column, OA-4100 (50 cm × 4 mm i.d.). A. Carbon number separation of saturated MG enantiomers; B, Olefinic bond-number separation of C_{18} unsaturated MG enantiomers. Mobile phase, hexane/ethylene dichloride/ethanol (40:10:1, v/v/v). Flow rate, 0.5 mL/min. Detection, 254 nm UV.

ers are approximately parallel as reported previously (1,2). The relationship between Vr of *sn*-1 and *sn*-3 enantiomers can be expressed by the following equations:

$$\log \text{Vr}\ (sn\text{-1-N}) = \text{HN} + \text{I} \quad [1]$$

$$\log \text{Vr}\ (sn\text{-3-N}) = \text{HN} + \text{I} + \text{E} \quad [2]$$

$$\text{Log Vr}\ (sn\text{-3-N}) = \log \text{Vr}\ (sn\text{-1-N}) + \text{E} \quad [3]$$

where Vr (*sn*-1-N) and Vr(*sn*-3-N) indicate the retention volumes of *sn*-1 and *sn*-3 enantiomers of the saturated MG having a carbon number N, or those of C_{18} unsaturated MG having an olefinic bond number N. The constants H and E represent the slopes of the lines and the distance between two lines along the vertical axis, respectively. The value of I is a constant for the initial condition of the equation [1].

Compared with separations on OA-2100 in the previous paper (2), it is possible to separate a larger number of *sn*-1 enantiomeric homologues before the appearance of the first peak of *sn*-3 enantiomers on OA-4100. This favorable separation on OA-4100 is mainly explainable on the basis of higher values for E. In the separation based on acyl carbon numbers, lower values of H also contribute to the favorable separation on OA-4100.

The values of H, I, and E on OA-4100 calculated from the retention data of carbon number separation (Table 1A) by the method of least squares were −0.0099, 2.236 and 0.121, respectively. According to the linear relationship, the value of Vr(*sn*-3-(N+12.2)) will be approximately equal to that of Vr(*sn*-1-N). Thus, it is possible to separate six saturated 1-MGs of successive even carbon numbers. The calculated values of H, I, and E on OA-2100 under the same conditions as in this study except for a flow rate of 0.3 mL/min (2) are −0.0127, 2.210, and 0.067, respectively. The limits for sufficient separations of saturated 1-MG mixtures have been three members with successive even carbon numbers.

TABLE 1

Chromatographic Separation of MG Enantiomers on a Chiral Column, OA-4100

	Acyl Position	Acyl Group	Vr[a]	k'[b]	α[c]	Rs[d]	PA[e]
(A)	*sn*-1	22:0	104.8	19.69	1.04	1.08	7.4
		20:0	109.0	20.48	1.04	1.14	7.9
		18:0	113.8	21.38	1.05	1.26	7.3
		16:0	119.3	22.41	1.05	1.25	6.0
		14:0	124.9	23.47	1.05	1.40	8.8
		12:0	131.6	24.73	1.05	1.36	12.8
	sn-3	22:0	138.5	26.04	1.04	1.02	7.7
		20:0	144.1	27.09	1.04	1.10	8.0
		18:0	150.5	28.28	1.05	1.22	7.2
		16:0	157.6	29.62	1.04	1.15	5.9
		14:0	164.7	30.95	1.05	1.26	8.6
		12:0	173.6	32.63			12.4
(B)	*sn*-1	18:0	108.3	20.35	1.06	1.40	15.2
		18:1	114.4	21.51	1.06	1.48	14.0
		18:2	121.7	22.88	1.06	1.24	11.8
		18:3	128.9	25.84	1.11	2.25	9.3
	sn-3	18:0	142.5	26.79	1.06	1.43	15.2
		18:1	151.3	28.43	1.06	1.38	13.8
		18:2	161.0	30.27	1.06	1.15	11.7
		18:3	170.8	32.11			9.0
(C)	*sn*-1	20:4	133.1	25.01	1.35	6.70	52.4
	sn-3	20:4	180.0	33.83			47.6
	sn-1	20:5	139.9	26.30	1.35	6.34	51.0
	sn-3	20:5	189.2	35.56			49.0
	sn-1	22:6	136.9	25.72	1.34	5.88	50.9
	sn-3	22:6	183.7	34.53			49.1

[a]Vr, retention volume (mL) corrected by subtracting the column void volume (5.32 mL).
[b]k', capacity ratio.
[c]α, separation factor (the ratio of the capacity ratio).
[d]Rs, peak resolution. $\text{Rs} = 2(t_2 - t_1)/(w_1 + w_2)$, where t is retention time and w is peak width.
[e]PA, peak area ratio (%).

The values of H, I, and E calculated from the data for olefinic bond-number separations (Table 1B) were 0.025, 2.034, and 0.121, respectively. The values of H, I, and E on OA-2100 (2) are 0.023, 1.893, and 0.057, respectively. The results of the HPLC analyses on OA-4100 suggest a possibility for the separation of mixtures of unsaturated 1-MGs of the same carbon numbers having 0–4 olefinic bonds. On the other hand, the *sn*-1-18:3 and *sn*-3-18:0 peaks overlap on OA-2100.

Thus, the OA-4100 chiral column is suitable for the quantitative analysis of *sn*-1 and *sn*-3 enantiomers of 1-MG mixtures. However, addition of one olefinic bond and two carbons to an enantiomer homologue caused approximately equivalent shifts of logarithmic Vr in the reverse direction. Therefore, it is difficult to obtain fair separations of pairs such as 1-16:0 and 1-18:1. A partial separation was observed for the pair 1-18:3 and 1-20:4, but the pair 1-20:5 and 1-22:6 was poorly resolved on this column (Table 1B and 1C). The separa-

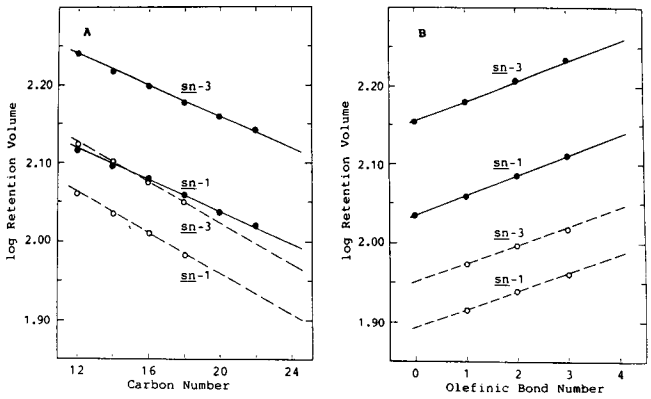

FIG. 2. Plots of log retention volume vs acyl carbon number (A) and olefinic bond-number (B) for MG enantiomers as di-3,5-DNPU derivatives separated by HPLC. ●, HPLC on an OA-4100 chiral column; ○, HPLC on an OA-2100 chiral column. Data of Table 1 and previous paper (2) are used for plotting.

tion of these pairs can be accomplished by prefractionation of the 1-MG samples. TLC on silver nitrate and boric acid-impregnated silicic acid plates, as reported by Morris (10), is useful for prefractionation. The plates were used to separate 1-MG samples on the basis of their degree of unsaturation without isomerization due to acyl migration (2).

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Measurement of the Metabolic Interconversion of Deuterium-Labeled Fatty Acids by Gas Chromatography/Mass Spectrometry[1]

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An analytical method that was developed to analyze deuterium-labeled fatty acids in human blood has been extended to identify labeled fatty acids from C_{14} to C_{24} chain length which are formed by metabolic processes such as desaturation, elongation, or shortening of the labeled fatty acids fed. A new computer and a hardware adder have been utilized to assure reliable data acquisition. Relative standard deviations for the analysis of labeled fatty acids were measured at 0.02, 0.03, and 0.04 at the 5%, 1%, and 0.2% levels of the labeled fatty acid methyl esters, respectively. The method makes extensive use of standards and computer processing for accuracy and high productivity. Data from a chylomicron triacylglycerol fraction are included to demonstrate the sensitivity of detection of metabolites formed by desaturation and elongation.
***Lipids 25,* 401-405 (1990).**

A method to analyze blood lipids from human subjects fed deuterium-labeled fatty acids has been developed over a period of more than a decade (1-6). The method employs analysis of the intact deuterium-labeled fatty acids by a gas chromatographic multiple-ion-monitoring (MID) mass spectrometry technique and isobutane chemical ionization (CI). Analysis of intact fatty acids allows fatty acids labeled with different numbers of deuterium atoms to be fed simultaneously. Feeding multiple labeled fatty acids allows for one fatty acid, e.g., deuterium-labeled 18:1, to be fed to all subjects as an internal control, thus greatly reducing the effect of subject-to-subject variations. The data are calculated as percent labeled fatty acid and in absolute weights, rather than as percent deuterium or deuterium enrichment. Data in this format correlate well with the total analysis of the fatty acids, and the concentration of labeled fatty acids (μg/mL of blood) can be readily compared with the fatty acids that are naturally present in blood.

This paper describes the extension of the method to permit analysis of the C_{14} through C_{24} fatty acids in feeding experiments which have included up to five deuterium-labeled fatty acids. A new computer with a hardware adder has been installed to improve the quality of the acquired data. The lipid metabolism experiments associated with this project make extensive use of selectivity plots and tables, that is, ratios of the compounds found in blood vs. the compounds fed. Inaccuracy in the measurement of deuterium-labeled fatty acids is amplified by the use of these ratios: therefore, the emphasis of this work has been on standard deviation rather than sensitivity.

EXPERIMENTAL

Materials. A 22-component mixture of methyl esters of long chain fatty acids, prepared from commercial methyl esters (Nuchek mixture 68A, 17:0, Nuchek-Prep, Elysian, MN; Sigma 20:5ω3, Sigma Chemical Company, St. Louis, MO; and Nuchek 22:4-ω6), was run several times every day to provide gas chromatography (GC) elution times, pseudo molecular ion isotope cluster patterns, and relative sensitivity data. Heptadecanoic acid was used as an internal standard for quantitations.

Three test mixtures were prepared, consisting of a 20-component standard (Nuchek mixture 68A plus 17:0) with five labeled compounds added at the 5%, 1%, and 0.2% level. In each case the percent value refers to the level of deuterium-labeled compound as a percentage of the amount of the unlabeled compound, not as percentage of the total sample. The compounds added were methyl hexadecanoate-9,10-d_2, methyl octadecanoate-9,9,10,10-d_4, methyl 9-*cis*-octadecenoate-14,14,15,15,17,18-d_6, methyl 12-*cis*,15-*trans*-octadecadienoate-9,10-d_2, and methyl 9-*cis*,12-*cis*-octadecadienoate-15,15,16,16-d_4.

Methyl esters were obtained from an 8-hr chylomicron triacylglycerol blood fraction from a human subject fed a mixture of four deuterium-labeled fatty acids. The fatty acids in the fed triacylglycerol mixture were octadecanoic-9,10,13,13,14,14-d_6 acid, 9-*cis*-octadecenoic-14,14,15,15,17,18-d_6 acid, 9-*cis*,12*cis*-octadecadienoic-15,15,16,16-d_4 acid, and 9-*cis*-12,-*cis*, 15-*cis*-octadecatrienoic-15,16-d_2 acid. A known weight of heptadecanoic acid was added to the chylomicron fraction just before conversion to methyl esters as an internal standard. The subjects, the preparation of labeled compounds, and the separation techniques have been described in publications reporting the results of the metabolic experiments (7,8).

Equipment. The gas chromatograph-mass spectrometer system was a Finnigan model 4000 (Finnigan Corporation, Sunnyvale, CA) upgraded to a model 4500 and equipped with a 30 meter, 0.32mm bore, 0.5 micrometer film thickness, Supelcowax-10, bonded phase, polar, fused silica column (Supelco, Inc., Bellefonte, PA). The exit end of the column was positioned within 20 mm of the mass spectrometer electron beam. The

[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: ADC, analog-to-digital converter; CI, chemical ionization; DAC, digital-to-analog converter; GC, gas chromatography; MID, multiple-ion-monitoring; RSD, relative standard deviation; and TI, total ionization.

capillary GC was operated with a 10:1 input split ratio and was temperature programmed from 150°C to 265°C at 5°C per min. A Finnigan conversion dynode retrofit kit was installed to improve high mass sensitivity.

A Modular Computer Company (Fort Lauderdale, FL) model II Classic computer with 128 k bytes of RAM memory and 13 megabytes of disk memory was used as a data acquisition computer. A 16-bit hardware adder was built using transistor-transistor-logic chips and installed between the Finnigan 12-bit analog-to-digital converter (ADC) and the Modcomp II computer. The adder allows up to 16 ADC values to be added together before it must be read by the computer thus reducing computer timing problems. The hardware adder was equipped with data late and data missed registers to assure that no part of any mass intensity is lost by the computer not reading it on time. The data acquisition computer was connected to a larger time-shared Modcomp computer 500 feet away via a 1 MHz serial link, so that the larger computer could do the mass storage and the data processing.

Data acquisition. Intensity values of the molecular ion regions were measured with a seven-stage MID descriptor, which measured odd-numbered mass intensities only. In the MID procedure the mass spectrometer is stepped through the five to twelve selected mass numbers; held at each mass number for about 30 milliseconds; the ion intensity is recorded; and then stepped to the next mass number. Seven different tables of masses were scanned over the whole of the gas chromatogram. The mass numbers were chosen to measure each saturated and unsaturated fatty acid likely to occur in human blood, allowing for the possibility of the compound to contain up to six deuterium atoms. The program computes the digital-to-analog converter (DAC) values needed to drive the mass scan circuit to follow the mass numbers and time values in the MID descriptor, and provides about 1 millisecond between mass peaks to allow the mass scanning voltage and the electrometer amplifiers to settle to their proper voltages. Mass intensity data collection is timed using the ADC clock to assure that analog-to-digital conversions for every mass peak are summed and included in the calculations. During data acquisition, the generated mass chromatograms are displayed on the data acquisition computer terminal. The data are stored on the acquisition computer disk and transmitted to a time-shared computer, for long-term storage, processing, plotting, and printing.

For diagnostic and mass calibration purposes, a program was written to collect an ADC value at every increment of the mass scanning DAC, through a specified mass range, to generate a profile spectrum. The center of two known peaks were identified with the computer terminal cross hairs controlled by a "mouse", and the exact masses were entered by keyboard.

Data processing. The start and finish of the GC peaks are determined using a first derivative calculation to determine deflection points while the base line is determined by the dead band at the beginning of the MID frame where no peaks are eluting. The program sums the data under the GC curves, keeping the intensity for each mass component separate.

At the time of data acquisition, the operator enters the information necessary to identify the sample and the data necessary for internal standard calculations and mg per mL of blood calculations. The identifiers include the human subject name, the blood fraction, and the hour the sample was taken, which are used to classify the sample in the data management program. The amount in milligrams and the chemical form of the internal standard are used in the quantitation calculations, and the thin-layer chromatography recovery factor and the volume of blood taken from the subject are used to calculate the micrograms of fat per mL of blood. The operator identifies the standard sample which the program uses to automatically update the GC elution times, the molecular ion cluster patterns, and the relative sensitivities. The program sets up simultaneous equations for each GC peak using the molecular ion cluster patterns and the measured sample data, and solves the equations for the relative amount of each component present (5). The data are converted to relative weights using the relative sensitivity values and to moles by proportioning the relative weights to the moles of C_{17} internal standard added. The results of the analysis of some 30 or more fatty acids (up to 100 are possible) are printed in tables and plotted automatically.

The processed data are stored in a relational data base management file which allows the investigator to call up any combination of subject, blood fraction, or hour for tabulation or plotting. Results can be plotted as percent, as weight, or as mg of fatty acid per mL of blood. The data can also be plotted and tabulated in several types of selectivity ratios.

Procedure. One microliter of an isooctane solution containing about one microgram of each ester was injected into the gas chromatography column. Three test mixtures were analyzed three times a day for three days for a total of nine replicates for each sample. The 20-component standard without the labeled compounds was analyzed as the first and second sample each day, and between each set of three samples containing the label, and also as the last sample of the day. In a similar manner, the chylomicron fatty acid methyl esters were analyzed six times and the standard deviation computed.

Chemical ionization spectra of all samples were obtained with isobutane as the reagent gas in order to maximize the molecular ion region of the mass spectrum and to reduce the amount of fragmentation both of the samples and of any background materials present (9). The isobutane pressure of 0.60 Torr was measured by the Finnigan source thermocouple gauge, and the source temperature was held at 150°C.

RESULTS

Table 1 shows the relative standard deviation of nine replicates of three dilutions of five labeled compounds added to the 20-component standard. The data were collected over a period of three days with a newly cleaned ion volume installed each day. The ion volume is an easily changed inside liner for the Finnigan 4500 mass spectrometer source. Standard deviations were calculated for each day, and then for all nine samples. Since there are 20-compounds present, the 5%, 1%,

TABLE 1

Relative Standard Deviation for Deuterium-Labeled Mixtures

Day	Number of samples	16:0 d_2	18:0 d_4	18:1 d_6	18:2CT d_2	18:2 d_4
		5% Label				
First	3	0.024	0.018	0.012	0.009	0.008
Second	3	0.026	0.012	0.003	0.016	0.019
Third	3	0.020	0.036	0.023	0.019	0.016
All three	9	0.021	0.024	0.018	0.015	0.017
		1% Label				
First	3	0.021	0.035	0.027	0.050	0.016
Second	3	0.049	0.008	0.006	0.029	0.012
Third	3	0.031	0.042	0.036	0.010	0.020
All three	9	0.032	0.033	0.030	0.029	0.019
		0.2% Label				
First	3	0.103	0.027	0.037	0.071	0.027
Second	3	0.056	0.035	0.005	0.050	0.024
Third	3	0.040	0.035	0.006	0.035	0.039
All three	9	0.063	0.030	0.023	0.048	0.033

0.2% level of deuterium-labeling represent 0.25%, 0.05%, and 0.01% of the total sample.

The mass chromatogram of the 20-component mixture with five deuterium-labeled methyl esters added is shown in Figure 1. At the 5% level the peaks for the deuterium labeled compounds can be easily seen. The 16:0-d_2 sample is seen as an increase in height and a broadening of the second isotope peak of the pseudo molecular ion of 16:0, which occurs at mass 273. The other labeled compounds are more distinctive because they contain four or six deuterium atoms and are separated from the unlabeled compound.

Figure 2 is the mass chromatogram of the methyl esters of the 8-hr chylomicron triacylglycerol blood fraction of a human subject fed a mixture of four deuterium-labeled fatty acids. The fourth frame contains the C_{18} fatty acids, including the four fed deuterium-labeled C_{18} fatty acids, 18:0-d_6, 18:1-d_6, 18:2-d_4, and 18:3-d_2. The fifth frame contains the C_{20} fatty acids and the peaks for the compounds 20:0-d_6, 20:1-d_6, 20:2-d_4, and 20:3-d_2, due to the elongation of the fed deuterium-labeled fatty acids. The compounds 20:4-d_2, 20:5-d_2, and 22:5-d_2 are due to the elongation and de-

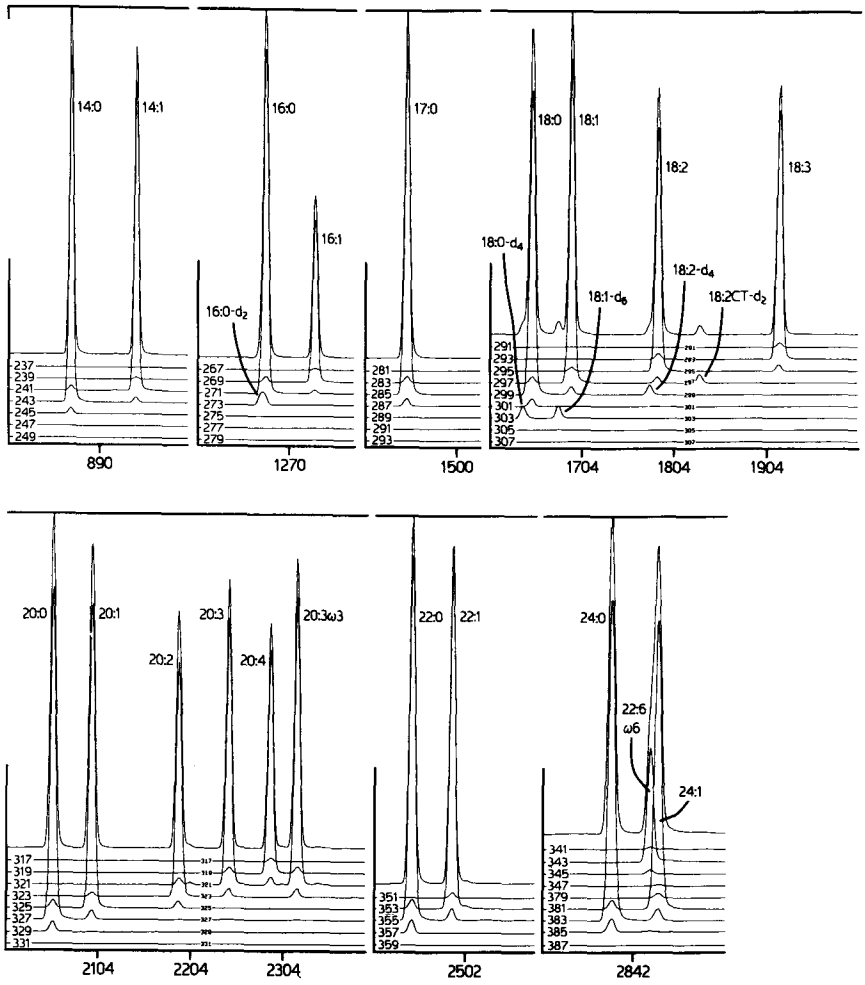

FIG. 1. Mass chromatogram of a 20-component standard with five deuterium-labeled compounds added at the 5% level. Data were recorded with a seven-stage multiple-ion-detection system using chemical ionization. The topmost curve is the total ionization (TI), which is the sum of the curves underneath.

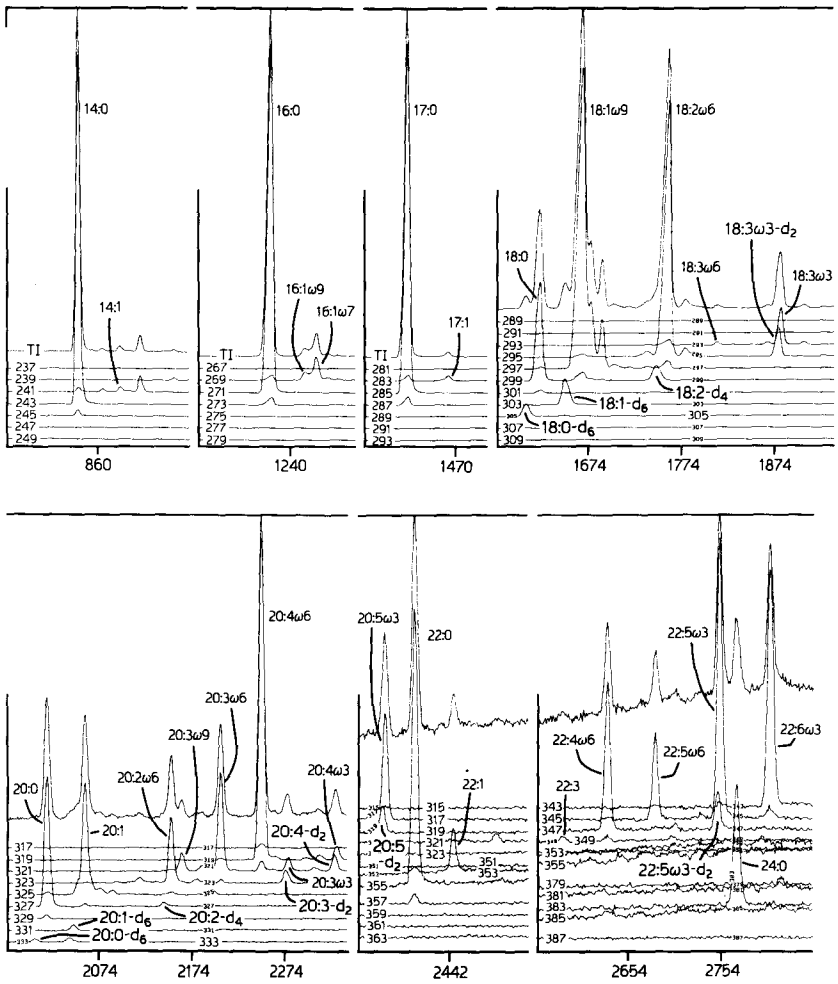

FIG. 2. Mass chromatogram of the fatty acids in the 8-hr chylomicron triacylglycerol fraction of a subject fed four deuterium-labeled C_{18} fatty acids. Data were recorded with a seven-stage multiple-ion-detection system using chemical ionization. The topmost curve is the total ionization (TI), which is the sum of the curves underneath.

saturation of the fed 18:3-d_2. Interpretation of the significance of this data will be reserved for forthcoming papers.

Table 2 gives the mean, standard deviation, and relative standard deviation (RSD) of the percentage data of six replicates of the methyl esters of the chylomicron triacylglycerol fraction. The Table also contains a column which presents the data in micrograms of triacylglycerol per mL of blood, a form very useful for metabolic studies.

DISCUSSION

The Finnigan INCOS 2200 computer originally used with this mass spectrometer was replaced with a Modular Computer Company model II Classic computer. The Modcomp II, with its homemade mass spectrometer interface, has complete control of both the mass scan DAC and the mass intensity ADC, and was programmed to acquire mass spectrometer data without ever missing an ADC reading. Since the computer has control of both the DAC and the ADC adder, mass intensities can be assigned correctly. Newer mass spectrometer data systems using personal computers are limited in their data acquisition capabilities, in that they are not fast enough to accept data, store the data on disk and maintain a computer display without occasionally losing data.

The Modcomp II computer is not fast enough to acquire ADC values at the 40 kHz rate (an ADC value every 25 microseconds) and do minimal user interface control or display. Therefore, 16-bit hardware adder was installed between the ADC and the Modcomp II computer to reduce the time burden. A number between 1 and 16 could be loaded into the adder board by the computer to control the number of ADC values added together before it had to be read by the computer. A count of 16 gave the computer 400 microseconds between data reads, time to perform interface control and data display.

TABLE 2

Analysis of Methyl Esters Derived From Human Blood Chylomicron Triacylglycerol 8-hr Fraction

Compound	μg/mL	Mean	Std. Dev.	RSD
14:0-d_0	11.01	1.631	0.199	0.122
14:1-d_0	0.57	0.085	0.015	0.172
16:0-d_0	104.24	15.432	0.917	0.059
16:0-d_6	0.09	0.013	0.001	0.090
16:1-d_0	8.99	1.330	0.061	0.046
16:1-d_6	0.07	0.011	0.001	0.075
17:1-d_0	0.95	0.141	0.037	0.263
17:1-d_6	0.07	0.010	0.001	0.118
18:0-d_0	34.36	5.079	0.146	0.029
18:0-d_6	4.22	0.624	0.023	0.036
18:1-d_0[a]	210.46	31.110	0.435	0.014
18:1ω9-d_6	11.96	1.768	0.041	0.023
18:2ω6-d_0	200.40	29.597	1.305	0.044
18:2ω6-d_4	11.75	1.735	0.069	0.040
18:3ω3-d_0	21.90	3.235	0.110	0.034
18:3ω3-d_2	18.07	2.662	0.119	0.045
20:0-d_0	1.12	0.165	0.018	0.110
20:0-d_6	0.01	0.001	0.000	0.168
20:1-d_0	1.47	0.217	0.022	0.101
20:1-d_6	0.04	0.005	0.001	0.096
20:2ω6-d_0	1.23	0.182	0.019	0.102
20:2ω6-d_4	0.20	0.030	0.003	0.089
20:3ω9-d_0	0.32	0.047	0.003	0.060
20:3ω?-d_0	0.10	0.015	0.001	0.071
20:3ω6-d_0	1.96	0.289	0.022	0.076
20:3ω6-d_4	0.07	0.011	0.001	0.080
20:4ω6-d_0	9.16	1.354	0.061	0.045
20:3ω3-d_0	0.30	0.045	0.009	0.200
20:3ω3-d_2	0.22	0.033	0.002	0.055
20:4ω3-d_0	0.32	0.048	0.003	0.072
20:4ω3-d_2	0.41	0.060	0.003	0.054
20:5ω3-d_0	3.04	0.450	0.044	0.098
20:5ω3-d_2	0.52	0.076	0.008	0.108
22:0-d_0	0.92	0.137	0.019	0.137
22:1-d_0	0.24	0.036	0.011	0.315
22:4ω6-d_0	2.45	0.363	0.029	0.079
22:5ω6-d_0	1.27	0.188	0.018	0.093
24:0-d_0	0.33	0.049	0.012	0.247
22:5ω3-d_0	5.56	0.822	0.070	0.085
22:5ω3-d_2	0.37	0.055	0.004	0.076
22:6ω3-d_0	5.50	0.813	0.054	0.066
22:6ω?-d_0	0.31	0.046	0.004	0.086

[a]Summed data from mixture of geometric and positional isomers the subject acquired by eating processed food fats. The value for the internal standard, 17:0, was not included in the results.

In order to reduce the likelihood of isotopic effects, the labeled fatty acids were limited to 2, 4, and 6 deuterium atoms. At the molecular weights of these methyl esters, the second isotope peak is about 2.5% of the pseudo molecular ion peak. This means that an unresolved GC peak could consist of four components, d_0, d_2, d_4, and d_6, which are going to interfere with each other during mass analysis. This interference, particularly the d_2-labeled component with the much more abundant naturally occurring d_0 component, ultimately limits the accuracy of the method. These overlapping peaks are resolved by the use of simultaneous equations.

The use of a freshly cleaned ion volume each morning reduced the standard deviation by a factor of two. During the preliminary experiments for the standard deviation studies, it was found that the first sample of the day was also the worst data of the day, and was thus discarded. The use of a calibration standard half way through the day improved the data sufficiently to justify the extra work.

Fatty acids from blood of a human subject who has eaten a normal diet including margarines and hydrogenated salad oils that contain *trans* fatty acids and fatty acids with the double bond in other than the 9 position. On the GC column, such compounds produce unresolved peaks that can extend out two times the width of the 9-*cis*-18:1 peak and cause problems with the automatic data processing. These extra peaks can be seen in Figure 2. The center of this extra wide peak has a longer elution time than the standard, so that it was necessary for the operator to manually enter its elution time in the compound identification table.

The principal limitation on the accuracy of the method is not with the program or the technique, but with drift in mass sensitivity which changes during the day, with the principal effects occurring at the highest and lowest masses. To overcome the effects of this drift, a 22-component standard was run twice a day to update the mass sensitivity table.

The use of a new computer, a hardware adder, freshly cleaned ion source volumes and new programming have improved our ability to measure deuterium-labeled blood fatty acids produced by elongation and desaturation of fed deuterium-labeled fatty acids. These increases in analytical capabilities have significantly expanded the scope and complexity of the human metabolic studies that are possible with deuterium-labeled fatty acids.

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Quantitation of Vitamin K in Human Milk

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A quantitative method was developed for the assay of vitamin K in human colostrum and milk. The procedure combines preparative and analytical chromatography on silica gel in a nitrogen atmosphere followed by reversed phase high performance liquid chromatography (HPLC). Two HPLC steps were used: gradient separation with ultraviolet (UV) detection followed by isocratic separation detected electrochemically. Due to co-migrating impurities, UV detection alone is insufficient for identification of vitamin K. Exogenous vitamin K was shown to equilibrate with endogenous vitamin K in the samples. A statistical method was incorporated to control for experimental variability. Vitamin K_1 was analyzed in 16 pooled milk samples from 7 donors and in individual samples from 15 donors at 1 month post-partum. Vitamin K_1 was present at 2.94 ± 1.94 and 3.15 ± 2.87 ng/mL in pools and in individuals, respectively. Menaquinones, the bacterial form of the vitamin, were not detected. The significance of experimental variation to studies of vitamin K in individuals is discussed.

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Vitamin K is required for the synthesis of proteins of the blood coagulation cascade and a variety of other calcium-binding proteins (1). As breast-fed infants are at risk for hemorrhagic disease of the newborn (HDN) due to vitamin K deficiency, there is considerable interest in documenting the quantity of vitamin K in human milk. Our current knowledge of vitamin K in human milk (2) and the relationship of vitamin K to HDN (3,4) have recently been reviewed.

As vitamin K is unstable in the presence of light and oxygen and its concentration is extremely low in most biological fluids (ng/mL), accurate and reproducible analysis is difficult. With the advent of high resolution HPLC, several studies of vitamin K content in human milk have been reported (5–13), but there is little agreement in the literature. Reported values range about ten-fold for both mature milk and colostrum; i.e., 1.2 to 9.3 and 1.8 to 7.6 ng/mL, respectively. Prior to this work, no systematic study had been reported. In most cases, sampling procedures and recoveries were not reported and statistical methods were not identified. Thus the various reports could not be directly compared. In addition, typically, only a few subjects were studied, and as statistical analyses were limited, experimental variability in the assays could not be determined. The present studies were designed to develop a procedure for reproducible and quantitative assay of vitamin K in human milk which incorporates a method for determining reliability of the assay.

MATERIALS AND METHODS

Materials. Radiolabelled vitamin K_1 (2-[^{3}H]methyl-3-phytyl-1,3-naphthoquinone, 53 mCi/mmol) and MK7 (2-methyl-3-*all-trans*-farnesylgeranylgeranyl-1,4-naphthoquinone) were generous gifts of Hoffman LaRoche (Nutley, NJ). Dihydrovitamin K_1 was synthesized (14) and identified by NMR analysis. All solvents used for extraction and chromatography were HPLC grade or equivalent from J.T. Baker Chemical Co. (Phillipsburg, NJ). Silica (Silicar CC-4) was purchased from Mallinckrodt Inc., Paris, KY, or BioRad Laboratories, Richmond, CA (BioSil HA-325). Tetrabutyl ammonium perchlorate (TBAP) was from Eastman Kodak, Rochester, NY. All other chemicals were of analytical or HPLC grade from Sigma Chemical Co., St. Louis, MO. Nitrogen (99.5%) from a local supplier was further purified by flowing through 6 ft of ice-cooled copper tubing (i.d. = 5 mm) immediately prior to use. Helium was obtained from Liquid Air Corp., San Francisco, CA.

Subject selection. Subjects were healthy women 20–35 years of age, of parity not greater than two, not on any routine medication or steroid contraceptives, and whose infants were growing within normal limits and free of illness.

Collection of milk samples. Milk samples were obtained with Egnell pumps using modifications of published procedures (15). Samples were collected throughout a 24-hr period. Mothers maintained their normal feeding schedules. At each feeding the infant nursed one breast and milk was pumped from the other breast. Mothers were instructed to empty the breast thoroughly by pumping for 15 min or until milk flow was markedly reduced. Milk was collected in pre-weighed, light-protected glass bottles. Immediately after pumping, the mother weighed the milk, mixed it to resuspend lipids, and stored a 15–20 mL sample for analysis. The remainder of the milk was fed to the infant. Breasts were alternated for feeding and pumping at successive feeds.

Samples were light-protected and refrigerated until transported to the laboratory 1 to 2 hr after the last collection. A weighted 24-hr pool was composed in the laboratory for each donor by mixing equal volumes of milk collected at each time point. Weighted pools were frozen at −20°C until transported on dry ice (≤ 6 weeks) to the analytical laboratory.

Sampling. On receipt in the analytical laboratory, frozen samples were thawed at room temperature and divided into appropriate sample sizes (5–10 mL) for

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Abbreviations: HDN, hemorrhagic disease of the newborn; HPLC, high performance liquid chromatography; $k' = (V_e - V_0)/V_0$ (V_e = elution volume; V_0 = void volume); OD, optical density; TBAP, tetrabutyl ammonium perchlorate; UV, ultraviolet.

analysis. Milk was tempered at 37°C for 15 min with gentle swirling (16). Appropriate volumes for assay were transferred into glass vials and stored at −70°C until use. Control pools were constructed by mixing an equal volume of 24-hr pools from each of 7 mothers. Random samples were monitored for bacterial contamination. Samples (50–100 μL) were seeded onto agar plates, incubated 24 hr at 37°C and checked for bacterial colonies (17). Samples which gave ≥ 1,000 colonies in 24 hr were judged contaminated and were not further assayed. The major bacterial species were *S. epidermidis* and *S. aureus.*

Analysis of vitamin K. The procedures we used to extract and quantitate vitamin K from human milk are described below. The Silicar column was used only for the mature milk samples. All procedures involving vitamin K were performed in subdued lighting.

Extraction of vitamin K. Vitamin K was extracted with 3 vol of isopropanol/hexane (3:2, v/v). Additional extractions recovered insignificant amounts of vitamin K. To remove H_2O and precipitate protein, extracts were centrifuged for 15 min at 600 × *g* at room temperature and evaporated three times from 10 vol of chloroform/methanol (2:1, v/v) using a Buchi Model REIII Rotavapor (Brinkmann Instruments, Inc., Westbury, NY). The residue was resuspended in hexane, and the supernatant decanted from the flocculent precipitate, dried under nitrogen and redissolved with 5 mL isooctane.

Open column chromatography. The extract was applied to a 30 mL fritted glass column (25 × 100 mm) containing 5 g Silicar CC-4 overlayed with 2 g anhydrous sodium sulfate which had been equilibrated with 100 mL isooctane. Vitamin K was eluted from the column with 100 mL isooctane/methylene chloride/isopropanol (75:25:0.02, v/v/v). This fraction was evaporated almost to dryness, redissolved in 1 mL hexane, rechromatographed on a second 30-mL fritted glass column (25 × 100 mm) containing 6 g Bio-Sil and eluted with 100 mL hexane/chloroform (75:25, v/v). To control for changes in humidity which significantly affect recovery from silica, open chromatography was performed under positive nitrogen atmosphere in a gas-tight chamber made to our specifications in the Department of Chemistry at the University of Arizona.

HPLC analysis. To purified fractions, 90 pmol menaquinone (MK7) was added and the mixture injected onto a 5 μ C_{18} HPLC column (Radial Pak, Waters, Milford, MA) and eluted with a convex gradient (Waters #3) of ethanol/water (90:10, v/v) to ethanol/hexane (90:10, v/v) as previously described (18). Vitamin K was detected at 254 nm using a Waters Model 450 UV detector. A 2–3 mL portion of the gradient in which vitamin K_1 and MK7 elute was collected, concentrated, re-injected onto a 10 μ C_{18} HPLC column (Radial Pak, Waters) and eluted isocratically with ethanol/hexane/water (90:6.5:3.5, v/v/v) containing 25 mM TBAP. Vitamin K was detected with an amperometric dual glassy carbon electrode (LC-4B/17A, BioAnalytical Systems, Inc., W. Lafayette, IN) using an applied potential of − 0.6 V at the reductive electrode and + 0.2 V at the oxidative electrode. By cyclic voltammetry, in our solvent system, the maximum oxidative and reductive potentials were determined to be + 0.15 and − 0.75 V, respectively. The voltammogram was produced using a BAS model 100 Electrochemical Analyzer at a scan rate of 100 mV/sec with a sample interval of 1 mV/point. The working electrode was a glassy carbon, model No. GCE and the reference electrode was Ag/AgCl. Utilizing these known maximum potentials, conditions for detection were determined using anodic hydrodynamic voltammetry. Constant volumes of a standard solution of vitamin K were injected keeping the cathodic potential constant at +0.2 V and varying the anodic potential. Optimal conditions for chromatography providing the best compromise between sensitivity, selectivity and baseline stability were determined to be oxidation at the downstream electrode at +0.2 V following reduction at −0.6 V. The lower limit of detection under these conditions with a signal to noise ratio of 5:1 (*Federal Register,* Vol. 49, No. 209, October 26, 1984, Rules and Regulations) is 1.4 pmol (0.63 ng). Dual electrode detection was about 3× more sensitive than single electrode detection in the reductive mode. To minimize oxygen in the system, the incoming solvent reservoirs were continually flushed with helium gas.

Scintillation spectrometry. Extracted samples were evaporated to dryness, resuspended in Safety-Solve (Research Products International Corp., Mt. Prospect, IL) and assayed for radioactivity (^3H) using a Beckman LS 3800 Scintillation Spectrometer.

Treatment of glassware. As vitamin K adheres to most plastic surfaces and to glassware, disposable glassware was used where possible. If glassware was to be reused, it was immediately rinsed and soaked in detergent overnight prior to sulfuric acid wash. Glassware was periodically monitored by HPLC for adhering vitamin by exhaustive extraction with solvent using procedures described above.

Quality control procedures. Replicates were assayed individually on separate days to control for day-to-day variation. Control and radiolabelled pools were constructed as follows: A 1-liter pool was prepared from equal quantities of milk from 7 donors and divided in half. One-half received 1 ng/mL [^3H]vitamin K; the other half was unaltered. The tritiated pool validated the recovery procedure up to the HPLC step; the control pool validated the methodology. Each procedure—e.g., extraction, chromatography, etc.—was performed on a single batch on the same day. Authentic vitamin K standards were injected daily interspersed with the samples to verify the chromatography. To consider the assay valid, we required that the integrals and k′ values differ from each other no more than 10%. Standards chosen at random for a single day gave coefficients of variation of 0.005 and 0.022 for integrals and k′ values, respectively (n=3). Due to lack of reproducible recovery of tritium from HPLC columns, these samples were not assayed on HPLC. Instead, MK7 (90 pmol) was added to each sample as internal standard prior to injection onto the first HPLC column.

RESULTS AND DISCUSSION

The mean values, standard deviations and distribution of vitamin K levels in control pools are shown in Figure 1. Assuming the amount of vitamin K in pooled sam-

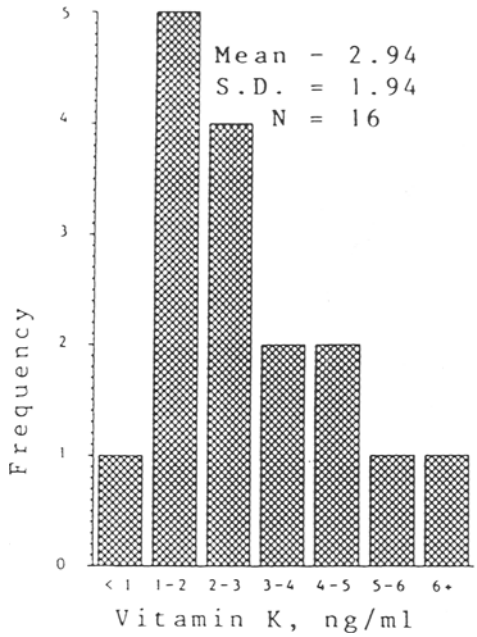

FIG. 1. Distribution of vitamin K, ng/mL, in pooled milk samples.

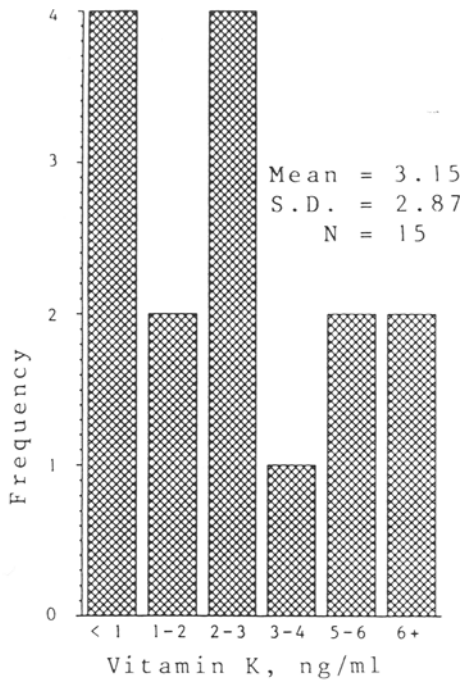

FIG. 2. Distribution of vitamin K, ng/mL, in milk samples from 15 individuals at one month post-partum.

ples to be constant, the variation in the results must be attributed to variation in the experimental method. The most likely source of this variation is non-uniform distribution of the vitamin in the sample due to aggregation of the sample during the freezing and thawing process. In four assays of a single sample of fresh milk, the relative standard deviation was reduced to 32%. Freezing and thawing causes rupture of the fat globule membrane (19), and the resultant aggregated membranes or butter particles which can be seen as small clumps may trap the vitamin, precluding uniform dispersion. In our hands, tempering of the milk sample to 37°C for 10 min was more effective than sonication or vigorous mixing although no optimal method was found for uniform dispersion of the vitamin. Detergents interfered with subsequent chromatographic steps. As is typical for biological samples, the data follow a skewed distribution curve.

Similar results were obtained for the 15 individual samples assayed at 1 month post-partum (Fig. 2). Our means are comparable to those reported earlier by Haroon *et al.* (5). The lower vitamin K concentrations reported by others (7) may be due to lower vitamin K in the diet.

The importance of proper sampling techniques cannot be overemphasized. Vitamin K can be increased as much as 4-fold in hindmilk compared to foremilk (7). This is consistent with higher values reported in hindmilk samples (6). Diurnal variation in milk lipids is significant (20), thus it is important to collect 24-hr pools if possible, or at a minimum, at a set time of day.

Bacterial contamination can confound HPLC assays. Menaquinones, the bacterial forms of vitamin K (2), particularly MK4-6, elute similarly to vitamin K_1. When measures are taken to avoid contamination with skin bacteria, we agree with others (7)—that significant levels of menaquinones are not present in human milk (see also Fig. 5). Meticulous care of glassware is required. Significant quantities of vitamin K_1 can be recovered from glassware washed using routine laboratory procedures (21).

A major difficulty in reproducibly quantitating vitamin K in milk is separating it from the large quantities of triglycerides present (40-60 mg/mL) (2,16). We investigated the modified Folch (22), dry column extraction (23), hexane extraction, and ethanol precipitation methods (6) for extraction of the vitamin. The modified Folch method gave good recovery (81.7, n = 3), but as it extracts lipids more completely, it also produces more contaminating lipid. In addition, it is time-consuming and requires the use of large quantities of biohazardous solvents. Recovery using the dry column procedure was lower, variable (67.05% ± 11.9, n = 4) and labor-intensive. The ethanol precipitation method gave acceptable recovery, (79.0 ± 0.9, n = 4), however, solvent evaporation is fairly time-consuming. The reductive extraction procedure described by Haroon *et al.* (24) for serum gave variable recovery. In contrast, extraction with hexane is rapid, reproducible, and removes less extraneous lipid while giving comparable recoveries to those for other solvent systems (Table 1).

Dihydrovitamin K_1, chloro K, vitamin K epoxide MK4, MK5 and MK7 were evaluated as internal standards. Dihydrovitamin K_1 was poorly recovered from the HPLC column and chloro K was unstable to our

TABLE 1

Recovery of Vitamin K from Human Milk

Step	% Recovered	RSD	n
Extraction and water removal[a]	85.5	10.8	16
Silicar[a]	65.9	26.9	16
Biosil[a]	85.6	21.3	16
HPLC (UV + ECD)[b]	65.2	32.3	18

[a]Recoveries were calculated using [^{3}H]vitamin K, which had been equilibrated with the milk sample as described in the text.
[b]Recovery was calculated using MK7 as an internal standard.

HPLC conditions. The solubility of vitamin K epoxide in isopropanol is substantially different from vitamin K_1 (18). None of the menaquinones we investigated were recovered with the same efficiency as vitamin K_1 from the open columns. Thus we chose a combination of [^{3}H]vitamin K_1 and MK7 as recovery standards. As tritium is not quantitatively recovered from HPLC columns, the recovery from HPLC was monitored by MK7.

In quantitation of recovery, it is essential that concentrations of internal standards comparable to endogenous vitamin K_1 are used and that sufficient time is allowed for equilibration. We found that a minimum of 10 min at room temperature with gentle shaking is required for equilibration of the vitamin prior to extracting. Under these conditions, our extraction recoveries were 85.5% (Table 1). When [^{3}H]vitamin K is added in 100-fold excess, recoveries *appeared* improved (91.4%, n = 43). However, this recovery is not representative of the endogenous vitamin which is present in much smaller concentrations. In addition, endogenous vitamin may be bound or entrapped in the lipid matrix and therefore less readily extracted. Such overestimates of recovery will result in under-reporting of the vitamin. Contrary to reports by others (6), in our hands sonication followed by ethanol extraction *decreased* recovery and increased variability of the extraction (69.8% ± 18.4, n = 12).

When quantitating trace compounds, it must be verified that the endogenous compound is extracted with the same efficiency as the exogenous (25), i.e., that in fact *all* of the vitamin is being recovered. Standard denaturing methods, e.g., mild acid hydrolysis (26) and detergent clarification (27), were unsuccessful due to the instability of the vitamin and interference with chromatographic procedures, respectively. Thus we measured the kinetics of the extraction of the [^{3}H]vitamin which had been equilibrated with the sample. If endogenous vitamin is bound to protein, membrane, etc., one would predict a time-dependent equilibration of ^{3}H-labelled exogenous vitamin and unlabelled endogenous-bound vitamin over time; i.e., if added in similar concentration, the concentration of [^{3}H]vitamin bound (thus not extracted) should decrease over time as it displaces the bound endogenous vitamin. However, as shown in Figure 3, we measured no difference in the amount of vitamin extracted over 24 hr. Thus we conclude that endogenous vitamin K rapidly equilibrates with exogenous vitamin under our conditions.

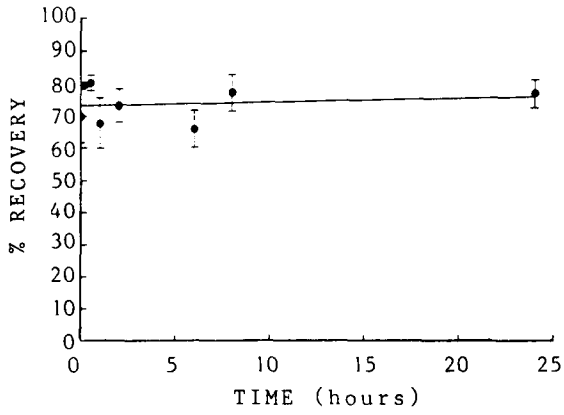

FIG. 3. Equilibration of endogenous with exogenous vitamin K. [^{3}H]vitamin K was equilibrated with milk for 20 min at 25°C, extracted at the indicated times and analyzed by scintillation spectrometry as described in Methods.

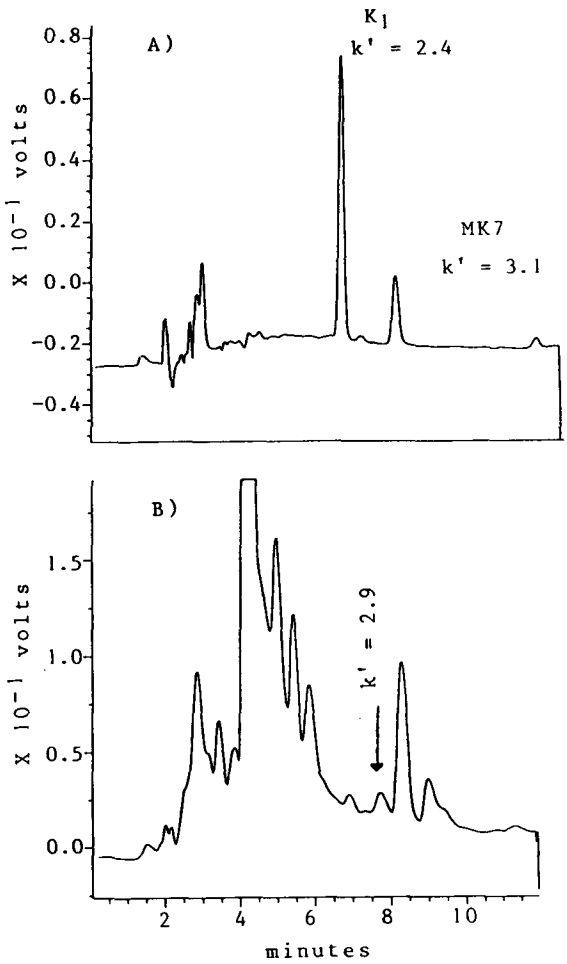

FIG. 4. HPLC chromatography of milk samples following purification by silica chromatography; 4A) Standards; 4B) Purified milk sample. Samples were fractionated on a convex gradient of ethanol/H_2O (90:10, v/v) to ethanol/hexane (90:10, v/v) and detected at 254 nm as described in Methods.

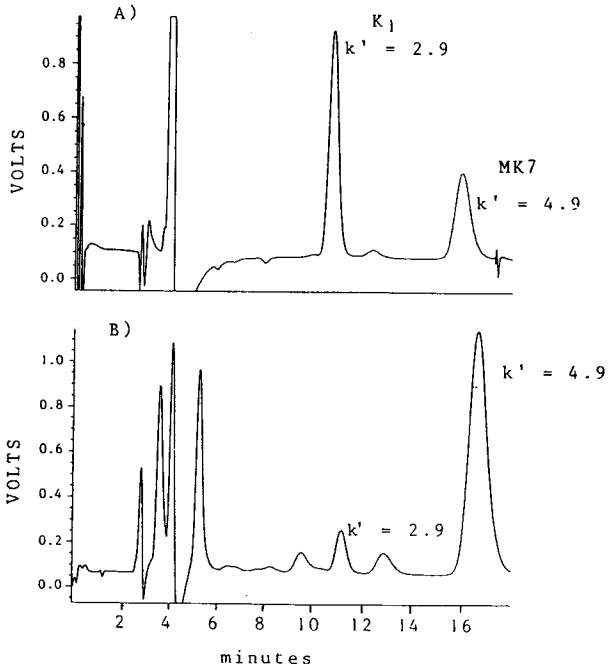

FIG. 5. HPLC chromatography of fraction detected electrochemically; 5A) standards; 5B) Fraction containing vitamin K_1 recovered from HPLC gradient fractionation. Samples were eluted isocratically with ethanol/hexane H_2O (90:6.5:3.5, v/v/v) containing 25 mM TBAP as described in Methods.

Typical HPLC chromatograms are shown in Figures 4 and 5. Authentic standards and the milk fraction from the Bio-sil column following gradient elution and detection at 254 nm are shown in Figures 4A and 4B, respectively. As can be seen in Figure 4B, vitamin K in milk is not resolved by this procedure. The peak with $k' = 2.9$ migrates closely to vitamin K_1 ($k' = 2.4$, Fig. 4A) and is present in milk samples at about 5× the concentrations of vitamin K. The two compounds might easily be confused if HPLC migration were the sole identification. However, optical density (OD) 280/254 ratios of this compound were not consistent with those of authentic vitamin K_1. Further, when this fraction was collected, rechromatographed and detected electrochemically, no compound which co-migrated with authentic vitamin K_1 could be detected. These results demonstrate the importance of using two detection systems to verify the identity of vitamin K. We found UV followed by electrochemical detection to be the most practical and reliable methodology. Other workers have successfully combined UV and fluorometric detection systems (24, 28). Figures 5A and B show authentic standards and a 2-3 mL fraction purified from gradient HPLC as described in Figure 4B, detected electrochemically. Vitamin K_1 was clearly resolved from the internal standard MK7, and no other menaquinones were detected.

In agreement with others (5), we found that a combination of normal and reverse-phase chromatography most effectively resolves vitamin K_1 from contaminating lipids. A number of pre-packed columns including Bond-E-Lut (Analytichem), and a variety of Sep-paks (Waters) including C_{18}, silica and CN for removal of excess triglycerides were investigated. We observed significant lot variability in all of these columns. In addition, as these columns are typically $\leqslant$ 2 cm long, their resolving power was insufficient for our purposes. The water-removal step was essential for proper performance of the silica columns.

Recoveries at each step of the procedure are shown in Table 1. Variability in recovery from the silica columns chiefly reflects differences between batches of silica from the manufacturer. Variation in recovery *within* a batch was typically $\leqslant$ 10%. Open silica chromatography is extremely sensitive to changes in humidity. Thus to control for these changes, and to insure consistent conditions, chromatography was performed under nitrogen in an air-tight chamber.

There are large differences in precision of assay measurements in the literature. This may be due to differences in definition of the term. In most studies, it has not been defined. In one study, precision of assay was defined as variability in measurement of replicates done on the same day (7), in another, variation between means of duplicate assays done on separate days (5). We define the reliability of the assay to be the correlation between successive determinations ($n \geqslant 3$) done on different days on the same sample. In the absence of experimental variation, (100% reliability), repeat determinations would yield the same values, and the correlation between successive determinations would be unity. The low reliability of the assay indicates that external sources of variation are introduced by the experimental method that must be recognized when using the assay to determine values for individuals.

If all the variability in sample determinations was due to variability between individuals, then true individual values could be known precisely. However, in addition to experimental variability, the effects of intra-individual variability must be considered. Effects of intra-individual variability result in loss of power to detect differences in groups (29), attenuation of correlations (30) and a large probability of misclassification of individuals based on a single determination (29). As can be seen by a comparison of Figures 1 and 2, the large experimental variation in the assay precludes precise determination of the effects of inter- and intra-individual variability with this sample size. However, these considerations do not affect the actual mean levels found for populations (29-31).

To illustrate the effect of assay variability on our confidence in the values found in individuals, we constructed confidence intervals for the true value of an individual mother's vitamin K depending on the number of repeat determinations done (Fig. 6). The figure presents 95% confidence intervals based on 1, 2, or 3 determinations. The 45 degree line through the origin corresponds to the average of all determinations done with the lower and upper line representing lower and upper 95% confidence limits, respectively. For example, if a single determination of 5 ng/mL was done, the lower 95% limit is <2 ng/mL and the upper 95% limit is more than 9 ng/mL. Thus with a single determina-

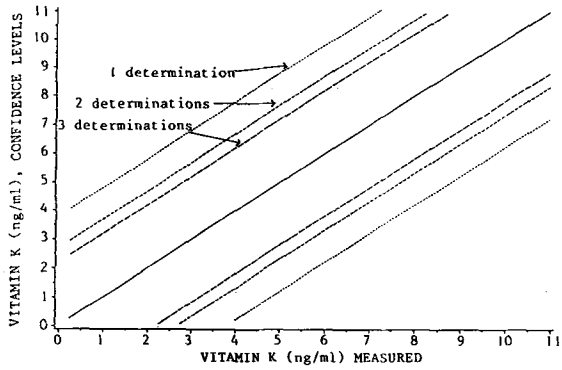

FIG. 6. The 95% confidence intervals of vitamin K in samples according to number of replicates. The lines represent upper and lower confidence levels for 1, 2, and 3 replicates.

tion, we cannot make a precise statement about the true level for an individual. Indeed, in order to know the value of vitamin K_1 in milk in a single sample within 20% with a 95% confidence level would require 7 measurements. This is because of the trace quantities of vitamin K in the presence of large concentrations of lipid. In contrast, in plasma, where we measured vitamin K at 2.0 ± 0.8 ng/mL (32), only 4 measurements would be required to achieve the same level of precision. Similarly, for β-carotene, which is present at ≈100 × higher quantities, in 22 determinations of a single plasma pool, we measured 0.28 ± 0.03 μg/mL. In this case only a single determination would provide the value within 20% with a 95% confidence level.

Currently, available methodology does not allow for a rapid assay of trace quantities of vitamin K in human milk. The major difficulty is the quantitative separation of vitamin K from the overwhelming concentrations of triglyceride in milk. As no biospecific ligand currently exists, considerable research may be required to provide a more rapid assay. The present methodology provides a reliable and practical method for providing estimates of vitamin K in milk using appropriate statistical analyses.

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A Simple Device for the Optimized Isolation of Fat Cells

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We describe a simple cell isolation device that allows the separation and sequential collection of fat cells as they are released from adipose tissue fragments exposed to a collagenase preparation. This is done without interrupting the digestion process. Use of the device allows for appreciable lowering of the degree of cell damage and the extent to which larger cells are ruptured during the isolation process. Fat cells isolated by this technique elicit a higher level of metabolic activity as reflected by their improved response to the lipolytic action of epinephrine.
***Lipids* 25, 412-414 (1990).**

Isolation of fat cells by digestion of adipose tissue fragments with bacterial collagenase is a well-established method (1). Usually, the instrumental recommendations for the digestion step involve no equipment other than a polyethylene bottle (2) in which tissue fragments and fat cells are mixed with collagenase during the overall digestion time. Such a procedure implies that cells are exposed to collagenase for different periods of time, depending on their appearance in the isolated form. Therefore, the cells which are rapidly released in the medium remain unnecessarily in contact with the enzyme(s). This may cause loss of cells, particularly those of larger sizes, as well as alterations in membrane properties. Although the features of the digestion process may vary with types and commercial sources of the collagenase mixture, it can be assumed that the enzymatic treatment should be kept as mild and time-limited as possible. To this end, we have developed a simple cell isolation device (CID) which allows the sequential separation and collection of fat cells as they are released, without interrupting the digestion process. As soon as collected, cells are washed, and properly stored until use. This simple procedure is described in detail in this paper.

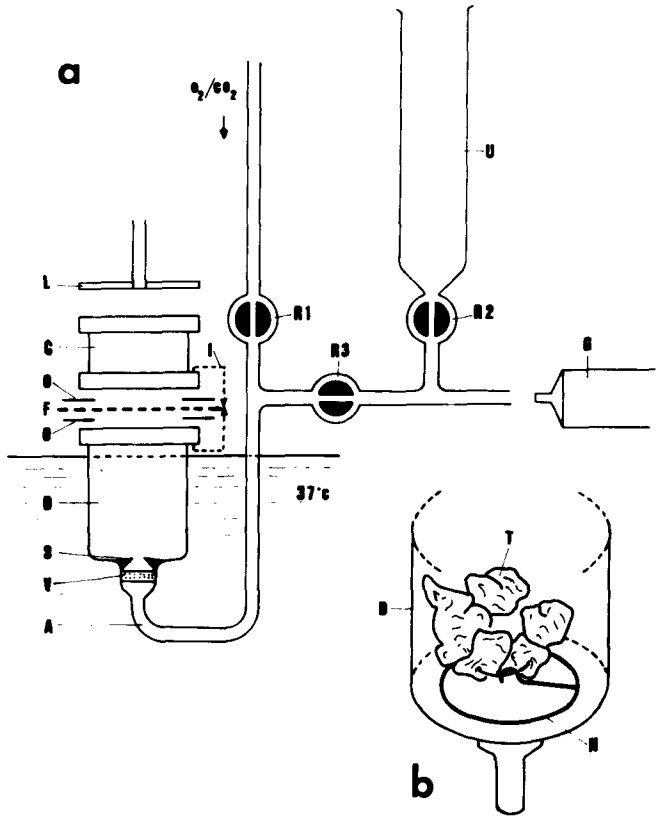

FIG. 1. A, diagrammatic cross-section of the cell isolation device; B, schematic presentation of the digestion chamber D. For text, see the Materials and Methods section.

MATERIALS AND METHODS

Description and functioning of the device. Unless otherwise stated, the CID is made of glass siliconized before use (Fig. 1). A cylindric digestion chamber (D, about 30 mL) is supplied at its base with a fritted glass (V) and above it, a set of convergent glass spikelets (S) which prevents V from being obstructed by tissue fragments. A collection chamber (C, about 15 mL) is separated from D by a polypropylene mesh with a pore size of 250 μm (F). The junction between C and D is made water-tight by two thicknesses of nylon sheet (O) on each side of the sandwiched part of F; the whole is clamped by a system of screws and circular metal springs (I). Chamber C is covered with an open glass cap (L). A two-delivery feeding pipe (A) is operated by two cocks (R_1 and R_2) as follows: R_1 on-R_3 off, delivery of 95% O_2/5% CO_2 mixture; R_1 off—R_3 on, communication with a syringe (G) containing the digestion medium, stored in the reservoir U. The size of the device can be adapted to the amount of tissue to be digested—a 30-mL digestion chamber is suitable for processing 4-6 g of adipose tissue.

Tissue fragments (T, about 250 mg each) to be digested are prevented from floating by mounting with a needle on a thread that is secured to a metal ring (N) located at the bottom of D. Chamber D is immersed in a water-bath at 37°C and filled through A up to a few mm below F, with medium A as defined by Fain (3), to which 3% (w/v) bovine serum albumin (Sigma Chemical Co., St. Louis, MO; Fraction V), 5 mM glucose and the desired concentration of collagenase (Sigma, type II) at pH 7.4 are added. Digestion is started under a gentle flow of 95% O_2/5% CO_2 (R_1 on-R_3 off). Isolated cells are collected every 5-10 min as follows: With R_1 off-R_3 on, the digestion liquid in G is pushed into D until the

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Abbreviations: CID, cell isolation device; and LDH, lactic dehydrogenase.

levels reaches C, above F. Isolated cells pass through F and emerge in C, where they are collected with an Eppendorf pipette. They are immediately washed four times with collagenase-free buffer A at 37°C. Meanwhile, the level of digestion medium is brought back below F by aspiration from G and digestion is continued (R_1 on-R_3 off). The aspirated medium is stocked in U and may be re-used at the next collection cycle. With R_3 off-R_2 on, the medium in U is aspirated in G and reinjected in D (R_2 off-R_3 on). Cycles are repeated as many times as needed until the digestion of fragments T has been completed. The batches of collected cells are pooled and kept under 95% O_2/5% CO_2 in fresh collagenase-free medium A at 37°C before being used for the experiments.

Samples of adipose tissue were taken from the parametrial fat pads of Sprague-Dawley rats after exsanguination under light anesthesia (pentobarbital, i.p., 25 mg/kg body wt). Cells were comparatively isolated using either the commonly recommended method, i.e., batchwise in a polyethylene vial with gentle stirring (20 cycles/min) (Method I) or the CID (Method II).

Other methods. Cell counting was performed on 200-μL aliquots of isolated cell suspensions to which glutaraldehyde (8%, v/v) was added slowly with gentle mixing to a final concentration of 4%. Two drops of saline and ten drops of an aqueous crystal violet stain (25 mg/dl) were added to one drop of the fixed-cell suspension while mixing. After 5 min, aliquots of the stained cell suspension were transferred to a blood cell counting chamber with 0.5 mm depth and 0.0625 mm^2 area. The diameter of at least 1200 cells was measured by means of a photo-microscope eyepiece micrometer. Cell diameters were plotted in class intervals of 5 μm to construct a histogram showing the distribution of fat cells as a function of cell size.

Cell damage was quantitatively estimated by measuring the amount of lactic dehydrogenase (LDH) activity (4) released in the medium by the cells incubated for 20 min at 37°C. Soluble LDH activity was expressed relative to the total LDH activity extracted upon homogenization of identical amounts of the same cell suspension before incubation. One unit of LDH activity corresponds to a change in optical density of 1.0 in 1 min under the assay conditions.

The sensitivity of isolated fat cells to the lipolytic action of epinephrine was assayed as previously described (5), via quantitation of the fatty acids released from cells incubated for 10 min at 37°C in a modified Fain's medium (3) with 5 mM HEPES (Sigma) instead of sodium phosphate and 3% (w/v) defatted albumin. The incubation medium contained in a final volume of 0.55 mL: 0.5 mL buffer A, 0.1 mL fat cells (about 4×10^5 cells) and 0.05 mL 10^{-3} N HCl (containing or not the appropriate amount of epinephrine). Assays were performed in a shaking water bath (30 cycles/min).

RESULTS AND DISCUSSION

As the quantitative aspects of the digestion process varied considerably with individual animals, regional origin of adipose tissue samples and collagenase preparations, representative experiments are presented. All observations reported have been replicated several times with different preparations of adipose tissue samples.

Figure 2 shows the time course of the release of fat cells isolated from tissue fragments (4 g) in a typical experiment using the CID (method II). The yield in isolated cells was maximum at 30–40 min of digestion, and declined after this time. The 60-min overall digestion process supplied 3.4 mL of packed cells. Cell viability was tested sequentially after four washings in collagenase-free buffer A at 37°C. As shown, the LDH release culminated at a value of 8% for the cells collected at 20 and 30 min and declined thereafter; the mean value of LDH release for the total population of isolated cells was 5.7%. Comparatively, 4 g of the same tissue fragments digested by method I for 60 min using the same amount of collagenase (70 mU/mL) as in method II supplied 2.9 mL of packed cells with a mean LDH release of 16%. We routinely observed that: (i) For a given collagenase preparation supplied by the manufacturer under the same reference number, different lots showed variable potencies to isolate fat cells. Therefore, each new lot has to be tested prior to use and stored in sufficient amounts when its efficiency has been established; and (ii) For a given lot of collagenase, the amounts of enzymes suitable for optimum digestion vary depending on the mean size of the adipose cells to be isolated. Tissue fragments containing fat cells with relatively low mean diameters required higher concentration of collagenase to be digested in good yield with essentially no additional cell damage; in turn, the use of lower concentrations of collagenase was required to isolate larger cells with an acceptable degree of cell integrity. Routinely, we adapted the concentration of collagenase used for digestion to the mean

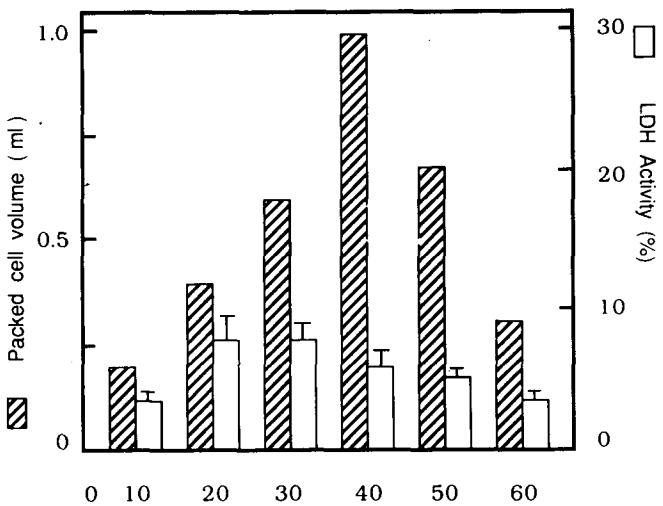

FIG. 2. Adipose tissue fragments were prepared from the parametrial fat of a female rat (290 g). The mean cellular diameter was 100 μm. Adipose tissue fragments (4 g) were immersed in the digestion chamber containing 70 U/mL collagenase at 37°C. Digestion was carried out for 60 min with sequential collections of isolated cells at 10-min time intervals. For each collected sample, the volume of isolated cells was recorded. Percents (mean ± SD, n = 3) of LDH activity released in the medium refer to the amount of LDH activity contained in the total pool of cells collected at this given time, taken as 100%.

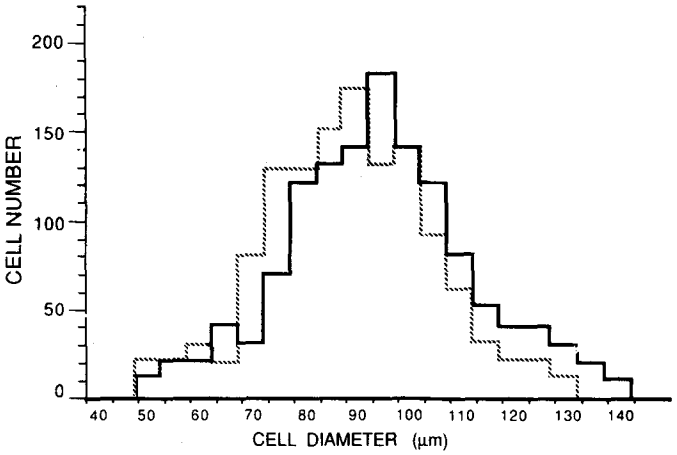

FIG. 3. Histograms of the distribution in intervals of 5 μm of fat cell diameters from parametrial fat tissue obtained from a 315-g rat. The histograms were constructed by comparatively sizing five replicated 260 fat cell samples from each cell population. The CVs for the sizing operation were 0.6 and 0.5% for the diameters of cells obtained by method I (without CID, ---) and method II (with CID, ——), respectively. The two curves are significantly different ($\chi^2 = 40.9$, $p<0.002$).

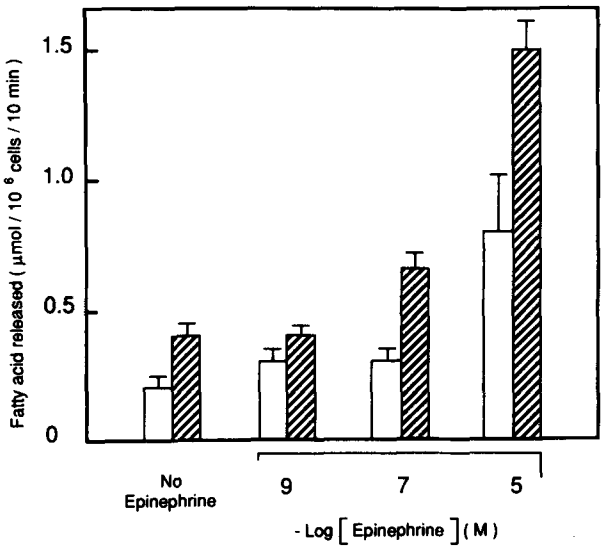

FIG. 4. Comparative evaluation of the responses to the lipolytic action of epinephrine of adipose cells isolated by method I (without CID, open columns) or method II (with CID, striped columns) from parametrial fat pads of female Sprague-Dawley rats weighing 240-260 g (mean cell diameter, 90 μm; collagenase, 100 U/mL). Values represent the mean (± SD) of two experiments carried out in duplicate.

cell diameter of a given adipose tissue by selecting ranges of collagenase concentrations of 50-100 U/mL or 100-200 U/mL for adipose tissue samples whose mean cell diameters were higher or lower than 80-90 μm, respectively. These latter values approximately correspond to cells from parametrial fat pads of female Sprague-Dawley rats weighing 280 g.

Figure 3 shows the histograms of the distribution of fat cell diameters resulting from the digestion of aliquots of the same adipose tissue sample by methods I and II. The comparison of the five means of 260 fat cell diameters revealed a mean of 93 ± 11.4 μm with a range of 50-140 μm for the cells isolated with the CID (method II), as compared to a mean of 88 ± 12.0 μm with a range of 50-130 μm for the cells isolated by method I. When the population of larger cells was arbitrarily defined as that including cells with a diameter larger than 90 μm, we found that the number of such cells was decreased by 45% with method I, as compared to 24% with method II. As noted by other researchers (6-9), the collagenase digestion affects the distribution of cell diameters mainly at the expense of the larger cells. By using the CID, one can therefore appreciably lower both the degree of cell damage and the extent to which larger cells are ruptured during the digestion process.

It is known that exposure of fat cells to crude preparations of proteolytic enzymes may impair their metabolic activities (10). Keeping the collagenase action at a minimum, as achieved by method II, results in a better lipolytic response to the receptor-mediated action of epinephrine (Fig. 4). As expressed relative to basal (no epinephrine) levels, epinephrine at 10^{-9} M and 10^{-7} M had a small (<50%) stimulating effect on the release of fatty acids with method I, whereas at 10^{-7} M the release was stimulated by 113% with method II. At a saturating concentration (10^{-5} M), epinephrine stimulated cells isolated by both methods, but the mean level of fatty acid release was still 88% higher with method II than with method I. In summary, populations of fat cells isolated using the CID are more likely to reflect the cell size distribution and metabolic activity of fat cells in adipose tissue.

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Separation and Detection of Phospholipid Hydroperoxides in the Low Nanomolar Range by a High Performance Liquid Chromatography/Ironthiocyanate Assay

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A new method for the detection of phospholipid hydroperoxides in the low nanomolar range has been developed by using a high performance liquid chromatography system combined with a post-column reaction. The detection is based on the oxidation of Fe(II) to Fe(III) by hydroperoxides and the subsequent formation of an ironthiocyanate complex, which can be determined spectrophotometrically at 505 nm. By this method it is possible to separate and quantitate phosphatidylethanolamine hydroperoxide and phosphatidylcholine hydroperoxide formed in erythrocyte ghost membranes during photooxidation.
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Lipid peroxidation in biological membranes is thought to affect membrane fluidity and ion-permeability as well as the function of membrane bound enzymes, which, in turn, can result in cellular damage (1,2). The primary products of lipid peroxidation are lipid hydroperoxides, which in the presence of transition metals are degraded into secondary oxidation products, such as hydrocarbons and carbonyl compounds (3), some of which have been shown to be cytotoxic (4). To help in understanding the mechanism of lipid peroxidation in membranes, it is important not only to detect the total amount of hydroperoxides, but also to identify and quantitate the various hydroperoxides formed from different lipid classes. However, the most common methods to measure lipid peroxidation in biological samples lack specificity. The thiobarbituric acid (TBA) assay measures breakdown products from all lipid hydroperoxides, primarily malondialdehyde (MDA), but other products can interfere (5). The iodometric assay (6) and the ironthiocyanate assay (7) detect the total amount of hydroperoxide groups, but are unable to reveal their origin.

As a consequence, there has been an increased interest in the separation of lipid hydroperoxides by high performance liquid chromatography (HPLC). The detection of conjugated dienes at 233 nm has been used for quantification (8,9). The individual phospholipid hydroperoxides, e.g., phosphatidylethanolamine hydroperoxide (PE-OOH) and phosphatidylcholine hydroperoxide (PC-OOH), can be separated by normal phase HPLC, but not on reversed phase (10,11). However, separation of hydroperoxides from the corresponding hydroxy derivatives, which also contain conjugated dienes, is difficult in normal phase HPLC (11). Therefore, the idea of using a post-column reagent, which reacts with the hydroperoxide group after HPLC separation, was introduced. Previously published methods were based on a post-column reaction between the hydroperoxide, a peroxidase and isoluminol or luminol, and required a luminometer or a fluorometer (10,12). However, other compounds which can oxidize isoluminol will also give a response (13).

The purpose of the present work was to develop a selective and sensitive HPLC method for phospholipid hydroperoxides with post-column detection based on the principle of the ironthiocyanate assay (7). The peroxides oxidize Fe(II) to Fe(III), which subsequently reacts with SCN^- to form a violet complex with an absorbtion maximum at 505 nm. The reaction has the advantage that it proceeds very rapidly and does not require heating of any kind. The method has been evaluated by studying photooxidation of erythrocyte ghosts, which has proved a convenient model for the oxidation of biological membranes.

MATERIALS AND METHODS

Materials. Hematoporphyrin was obtained from Sigma Chemical Co. (St. Louis, MO). Chloroform, methanol and water used for HPLC were redistilled. All other chemicals were analytical grade (Merck, Darmstadt, Federal Republic of Germany).

Preparation of phospholipid hydroperoxides. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were extracted from rat liver, according to Folch *et al.* (14) and separated on a silica gel column (Baker Chemical Co., Phillipsburg, N.J.) using mixtures of chloroform and methanol with increasing polarity for elution. The purity of the fractions was verified by thin-layer chromatography (TLC).

The phospholipids were photooxidized in methanol containing 0.1 mM methylene blue (12). After photooxidation, the reaction mixture was passed through a silica gel 100 column (1 cm × 5 cm) with methanol. Using this procedure, methylene blue remains on the column. PC-OOH and PE-OOH were separated from unoxidized phospholipids by TLC (Merck, silica gel 60, 0.25 mm) using the developing solvent hexane/chloroform/methanol/acetic acid (30:40:20:10, v/v/v/v) containing 1.8 g boric acid per 100 mL (15). To detect the hydroperoxides, a lane on the TLC plate was sprayed with *N,N*-dimethyl-*p*-phenylenediamine reagent (16) which produces a pink color reaction on the TLC plate when reacting with a hydroperoxide. The areas corresponding to PE-OOH and PC-OOH were scraped off, and the substances extracted with 3 × 5 mL of chloroform/methanol (2:1, v/v). The hydroperoxide concentration in the final products was determined by an iodometric assay (6).

HPLC conditions. Chromatography was carried out at ambient temperature, using a Nucleosil 100-5 silica gel column (4.6 mm × 30 cm; Microlab Aarhus A/S, Denmark). The mobile phase consisted of a gradient between

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Abbreviations: EDTA, ethylenediaminetetraacetic acid; HPLC, high performance liquid chromatography; MDA, malondialdehyde; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PC-OOH, phosphatidylcholine hydroperoxide; PE-OOH, phosphatidylethanolamine hydroperoxide; TBA, thiobarbituric acid; TLC, thin-layer chromatography.

eluent A (chloroform) and eluent B (methanol/5 mM choline chloride, 9:1, v/v), starting 10% B and leading up to 58% B after 12 min. The gradient was then switched back to 10% B. The solvent flow rate was maintained at 1 mL/min.

Certain commercially available HPLC grade eluents were shown to contain considerable amounts of impurities which react with the post-column detection reagent; therefore, freshly redistilled eluents of normal laboratory grade must be used.

The equipment for HPLC (KONTRON) included 2 pumps, model 414, a Rheodyne injector, a UVIKON 720 detector, and an ANACOMP computer for eluent programming and data collection. The post-column reagent was introduced through a T-piece at a flow rate of 1 mL/min using a Waters pump model 510. The reaction time of approximately 25 seconds was obtained by using a 4-m reaction coil (i.d. 0.5 mm). The sample size was 100 μL and the effluent was monitored at 505 nm.

Post-column detection reagent. The reagent was prepared by mixing 10 mL of ferrous sulfate solution ($FeSO_4.7H_2O$, 1 mg/mL) with 10 mL of barium chloride solution ($BaCl_2.2H_2O$, 0.8 mg/mL) and 40 μL of concentrated hydrochloric acid. The mixture was allowed to precipitate and was then centrifuged for 10 min at 1500 × *g*. The supernatant was added to 380 mL of redistilled methanol, containing 600 mg NH_4SCN. The reagent was stable for 1–2 days at room temperature and up to one week at 4°C.

Photooxidation of erythrocyte ghost. Venous blood (ethylenediaminetetraacetic acid, EDTA; 10 mM) was obtained from the cubital vein of healthy male volunteers. Erythrocyte membranes (unsealed white ghosts) were prepared by standard lysing and washing procedures (17). The incubation system which contained ghost membranes (0.5 mg protein/mL), 14 μM hematoporphyrin, 7 mM sodium phosphate, pH 7.5, was irradiated for various lengths of time by light with a maximum emission at 366 nm. At the end of the incubation period, 1 mL of the incubation mixture was withdrawn for the measurement of MDA equivalents, using the TBA method described by Bidlack and Tappel (18), and 4 mL was extracted according to Bligh and Dyer (19). The lipid extract was dried under vacuum in a rotatory evaporator and redissolved in 1 mL of chloroform; 100 μL of this solution was subjected to HPLC analysis.

RESULTS AND DISCUSSION

HPLC analysis. The lipid hydroperoxides were separated by the silica gel HPLC column and then mixed with the post-column eluent as described in Materials and Methods. The separation of the two phospholipid classes was optimized by varying the gradient of chloroform and methanol:choline chloride. Figure 1 shows the separation of PE-OOH and PC-OOH in a typical HPLC chromatogram. The slope of the baseline is caused by the changes in the absorption of the solvent system. The slope is delayed for about 5 min, corresponding to the passage time of the eluent.

Hydroperoxides from other lipid classes, such as cholesterol, cholesterol ester and free fatty acid are eluted within the first 7 min and are not fully separated. Thus, in order to determine these species quantitatively, a suitable solvent system for the separation of neutral lipids must be introduced.

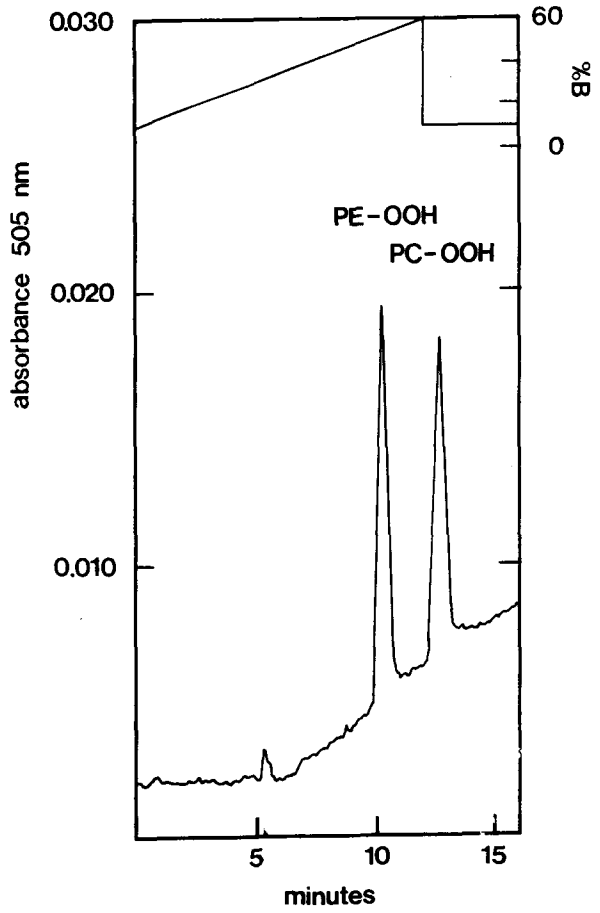

FIG. 1. HPLC separation of 488 pmol PE-OOH and 507 pmol PC-OOH. The gradient is shown on top of figure. Eluent A, chloroform; eluent B, methanol/5 mM choline chloride (9:1, v/v/v); flow, 1 mL/min; and detection, 505 nm.

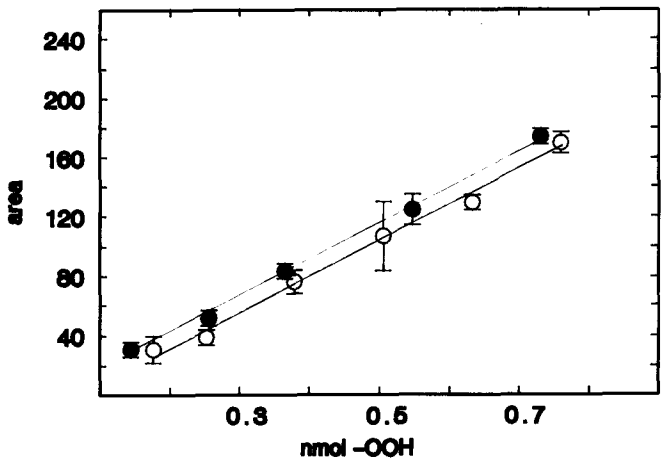

FIG. 2. Relationship between injected amount of hydroperoxide and peak area. ●, PE-OOH; and ○, PC-OOH.

By injection of varying amounts of pure PE-OOH and PC-OOH a linear response was obtained, as shown in Figure 2. The concentration of the standard solution is

determined by the iodometric assay. It is demonstrated that the correlation between the amount of hydroperoxide and the absorbance at 505 nm for both PE-OOH and PC-OOH shows linearity at concentrations between 0.2 and 0.8 nmoles. This is comparable with the detection limit of the HPLC method with electrochemical detection described by Yamada *et al.* (20), but their system does not separate PE-OOH and PC-OOH very well. The detection limit of the methods described by Miyazawa *et al.* (12) and Yamamoto *et al.* (10) is lower than in the present method (0.010 nmoles as compared to 0.2 nmoles), however these methods require a fluorometer or a luminometer. In the HPLC/isoluminol assay for determination of hydroperoxides (10), oxygen radical scavengers, such as antioxidants and chloroform, interfere by quenching the response. This means that chloroform, which is often used in lipid analysis, cannot be used as an eluent, however, in the presently described assay, chloroform does not influence the reaction and can readily be used as an eluent. Antioxidants, which give negative responses in the HPLC/isoluminol assay, give no response in this assay and thus do not interfere.

Unoxidized phospholipids are eluted with the same retention time as the corresponding hydroperoxides, but do not contribute to the absorbance at 505 nm. This has been confirmed by injection of up to 200 nmoles of purified PE or PC. The coefficients of variation (standard deviation/mean) of the peak areas were around 2% for PE-OOH, and around 3% for PC-OOH ($n \geq 3$).

Ghost incubation. Figure 3 presents an HPLC chromatogram of a lipid extract from erythrocyte ghosts photoxidized for 120 min. This chromatogram differs from the standard chromatogram by the presence of a peak at approximately 6 min, and a shoulder at the PC-OOH peak. The peak eluted after 6 min represents neutral lipid peroxides formed from membrane cholesterol or free fatty acids, but since these are eluted with approximately the same retention time, it has not yet been possible to determine the origin of this peak. The reason for the shoulder at the PC-OOH peak is still unknown.

As can be seen in Figure 4, the content of both PE-OOH and PC-OOH increases during the first 2 hr of photooxidation. Thereafter, a small decrease is seen, due to decreased formation and/or increased breakdown of the hydroperoxides.

The time courses of the formation of PE-OOH and PC-OOH are approximately the same, as shown in Figure 4.

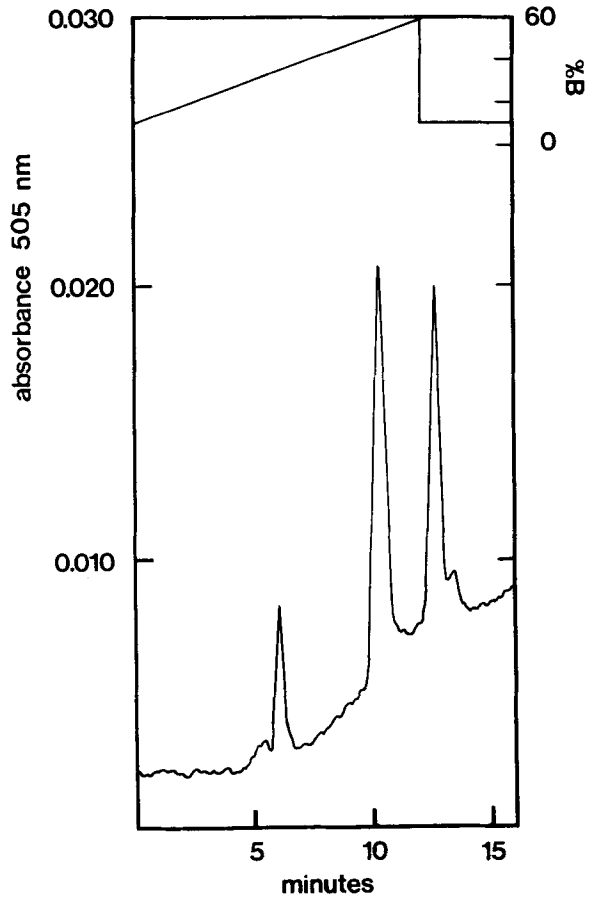

FIG. 3. HPLC separation of a lipid extract from an erythrocyte ghost incubation. Erythrocyte ghosts were photooxidized by irradiation as described under Materials and Methods. The gradient is shown on top of the Figure. Eluent A, chloroform; eluent B, methanol/5 mM choline chloride (9:1, v/v); flow, 1 mL/min; and detection, 505 nm.

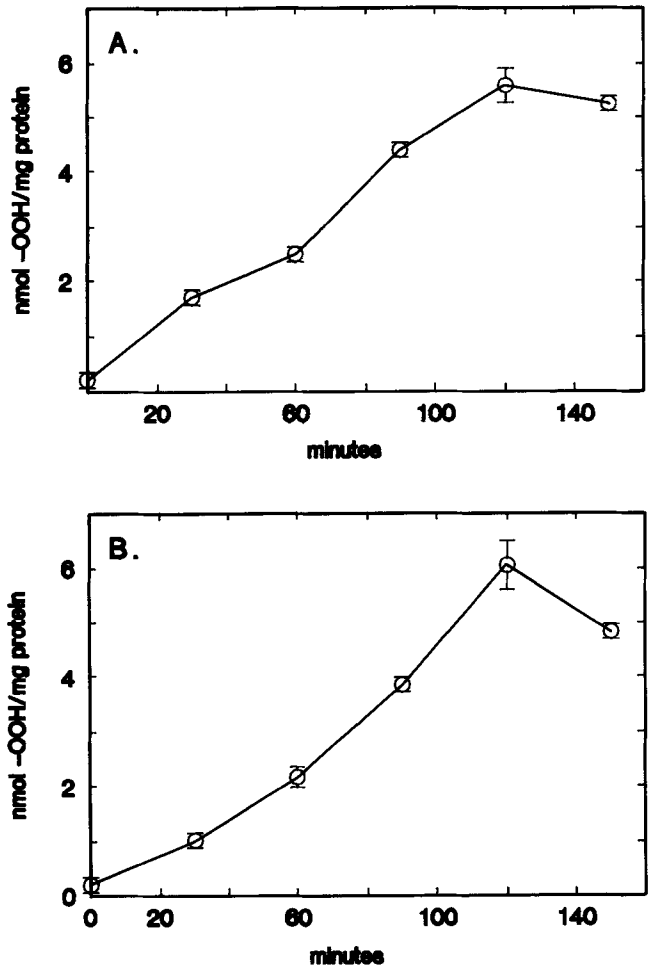

FIG. 4. Time course of the formation of phospholipid hydroperoxide. Erythrocyte ghosts were photoxodized by irradiation as described under Materials and Methods. Mean ± SEM. A, PE-OOH; and B, PC-OOH.

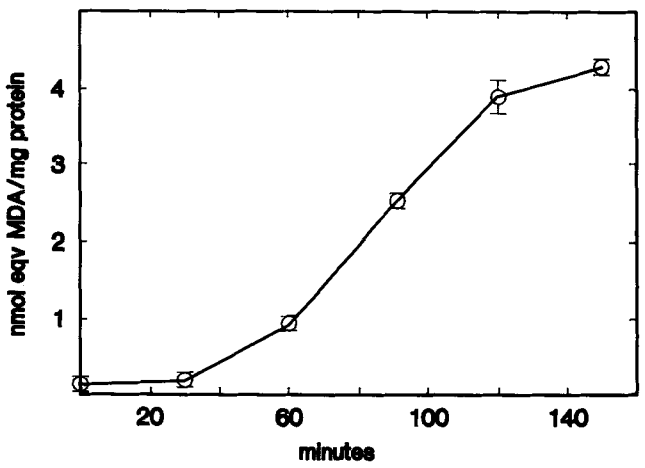

FIG. 5. Time course of the formation of MDA production. Erythrocyte ghosts were photooxidized by irradiation as described under Materials and Methods. Mean ± SEM.

The production of MDA equivalents (Fig. 5) is delayed as compared to the formation of hydroperoxides. This could be expected since MDA equivalents are breakdown products from hydroperoxides. MDA is not produced quantitatively from lipid hydroperoxides, but assuming that a constant percentage of lipid hydroperoxides are decomposed to form MDA, it is possible to compare Figures 4 and 5. The production starts at approximately 30 min and increases during the entire photooxidation period.

It can thus be concluded that the HPLC/ironthiocyanate method for detection of hydroperoxides is more sensitive and makes it possible to detect lipid peroxidation at an earlier stage than the TBA test. The method seems very suitable to measure lipid peroxidation in lipid extracts from biological samples.

Changes in the fatty acid composition of erythrocyte membranes caused by diet, as is found when fish oils are ingested, might influence the formation of phospholipid hydroperoxides. This aspect is currently being investigated.

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REVIEW

Lipids in Gap Junction Assembly and Function

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Gap junctions (GJ) are important regulators of cellular function. They provide channels for the direct movement of small molecules between cells and thus control cell-to-cell transfer of metabolites and the transmission of various stimuli. Gap junctions have been shown to be involved in a multitude of cellular processes ranging from cell synchronization and neuronal function to cell differentiation and carcinogenesis. Much knowledge has been gained in recent years concerning the structure and molecular organization of GJ proteins; yet, the mechanisms that control and modulate gap junction assembly and function are still not well understood. Although it is quite apparent that the GJ proteins assemble in the lipid milieu of the plasma membrane, and that the cluster of proteins assembled in the junction do function in a lipid environment, there is a general paucity of information on the role of lipids in the gap junction assembly process and in the function of gap junctions.

The present review is a comprehensive account of current knowledge on gap junction lipids. We also discuss what is known to date on the involvement of lipids in gap junction formation. Special emphasis is being placed on the potential role of membrane cholesterol in gap junction assembly and function.
***Lipids 25,* 419-427 (1990).**

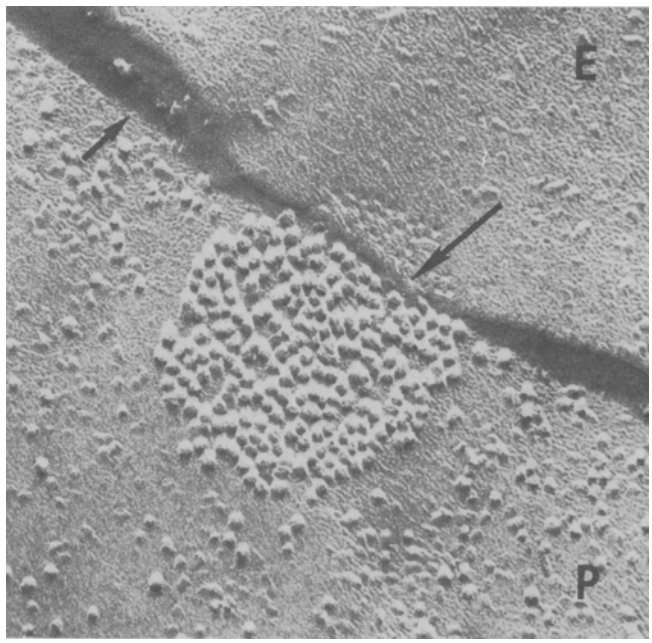

FIG. 1. Freeze-fracture electron micrograph of a mature gap junction between two adjacent Novikoff hepatoma cells (27). Uniformly-sized gap junction particles (9 nm diameter) arranged in a plaque are visualized on the membrane P fracture face of one cell with pits being observed on the E face of the apposed membrane. The large arrow points towards the narrow "gap" between the closely aligned membranes within the junction, while the smaller arrow identifies the extracellular space between non-junctional membranes (Reproduced from the *Journal of Cell Biology* by copyright permission of the Rockefeller University Press).

Gap junctions (GJ) are plasma membrane microdomains of specifically organized and tightly packed protein molecules which span the cell membrane and form conductive channels between adjacent cells (for reviews, see refs. 1-6). The channels of gap junctions permit the exchange of ions and of small hydrophilic molecules of up to about 1000 Daltons. Intercellular communication *via* gap junctions can be followed by monitoring electrical transmission (7-11), or the transfer of radioactive tracers (12) or fluorescent dyes between cells (2,8,13-16). Also, metabolic cooperation between cells has been taken as a physiologically relevant measure of intercellular communication *via* gap junctions (17-20).

Gap junctions can be visualized by freeze-fracture electron microscopy (EM). Using this technique, junctions appear as arrays of membrane particles which measure about 9 nm in diameter (6,21-26). The gap junction particles are arrayed in matched plaques on the closely apposed membranes of two adjacent cells. Figure 1 shows such a gap junction plaque between adjacent Novikoff hepatoma cells as seen by freeze-fracture EM (27). A more detailed picture of the gap junction channel has been derived from X-ray diffraction studies (22,28,29) and Fourier analysis of images of hydrated and frozen gap junction membranes (24). Figure 2 shows a view of a gap junction in which head-to-head alignment of the protein hexamers in the closely apposed bilayer membranes creates a hydrophilic channel between the adjacent cells (28).

Gap junctions were early shown to be involved in the transfer of stimuli between neurons (for reviews, see refs. 3,8,30,31). The transmission of other stimuli (8,26,32-34) and intercellular transport of metabolites through junctions (18,35-38) have been reported for a variety of other types of cells. For example, synchronized heart beat (10,39,40) and smooth muscle function (32,41-43) are coordinated *via* gap junctions. Also, the involvement of gap junctions in cell development and differentiation (44-50) is well recognized. In cell transformation and teratogenesis, alterations in GJ have been correlated with

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Abbreviations: chol, cholesterol; EM, electron microscopy; FP, formation plaque; GJ, gap junction; LDL, low density lipoprotein; lysoPC, lysophosphatidylcholine; lysoPE, lysophosphatidylethanolamine; NHC, Novikoff rat hepatoma cells; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL, phospholipid; PS, phosphatidylserine; SPH, sphingomyelin; TLC, thin-layer chromatography.

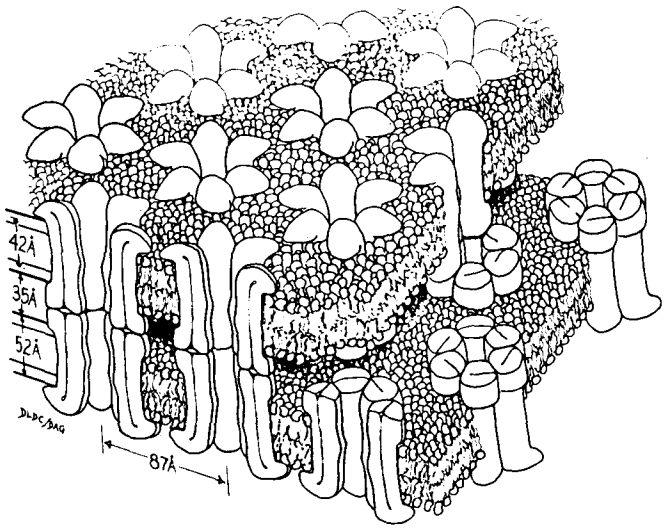

FIG. 2. View of a gap junction structure (open channels) according to Makowski *et al.* (28). Head-to-head aligned protein hexamers ("connexons") traverse the pair of closely apposed bilayer membranes of two adjacent hepatocytes. Dimensions given are based on X-ray diffraction data (courtesy of Dr. Makowski; reproduced from the *Journal of Cell Biology* by copyright permission of the Rockefeller University Press).

changes in cell growth and expression of normal cellular features (51–62), which has led to models for the role of gap junctions in the regulation of cell growth (2,19,45, 60,62,63).

Gap junction structure and function have been studied extensively in normal as well as in transformed cells. Studies have been concerned with the mechanism of GJ initiation and GJ development and with the molecular organization, stability and function of gap junctions. The isolation and characterization of GJ-specific proteins, peptides and antibodies and the cloning of GJ c-DNA's have been the focus of much recent attention (4–6,26,64–72). We know that families of related GJ proteins, often termed "connexins", exist in different tissues. Models have been proposed for the arrangement of these proteins within apposed bilayers consistent with the experimental evidence for the formation of hydrophilic transmembrane cell-to-cell channels. Specific protein sequences are being identified that are thought to be the key to protein assembly in clusters and thus essential to channel formation and function.

Although gap junction proteins reside in the lipid milieu of the plasma membrane and certainly are influenced by their lipid environment, the role of membrane lipids and the effect of lipids on gap junction assembly, stability and function are not yet well understood.

The present review is intended as an up-to-date account of what is known about lipids in gap junctions. We will summarize what has been done and where we stand. We will point out the experimental difficulties that still need to be faced and will attempt to search for avenues that can be followed.

LIPID COMPOSITION OF GAP JUNCTIONS

Analyzing the lipids of gap junctions has remained a difficult task. This is primarily due to difficulties in isolating "pure junctions" without lipid loss or alteration. The preparation of gap junctions usually requires extensive treatment of plasma membrane fractions with detergents or alkali (73–77), conditions which are not favorable for preserving gap junction lipids in their native state. Thus, lipid hydrolysis, lipid oxidation, and selective lipid losses can occur during the isolation of gap junctions (78). Moreover, contamination with non-junctional membrane does affect the lipid content of gap junction preparations and, most likely, also affects lipid composition. Furthermore, simple and convenient methods for assessing the purity of GJ preparations are not available. Although enrichment of GJ proteins in a preparation can now be monitored more readily by use of antibodies, the "lipid domain" associated with a gap junction remains poorly defined and difficult to preserve. Electron microscopy is still the method of choice to identify gap junctions and to assess the "purity" of gap junction preparations.

Because of these imponderabilities, lipid contents and lipid compositions of gap junctions reported in the literature must be considered with caution. They also must be seen in light of the methods used for the preparation of gap junctions and for the isolation of the gap junction lipids.

Currently available methods for the isolation of gap junctions have foremost been developed to preserve the structural integrity of the protein components. Therefore, it is presently not known how and to what extent treatment of membrane preparations with detergents and/or alkali (73–77) affects gap junction lipids. There is an urgent need for such an evaluation. It appears likely that new experimental approaches will need to be sought for the isolation of gap junctions that will also preserve the integrity of gap junction lipids.

Gap junction phospholipids. Phospholipids are predominant constituents of plasma membranes (for a review, see ref. 79). Hence, their presence in gap junction preparations can be expected. The first GJ phospholipid analyses were reported for mouse *liver* (73,80) and rat liver (76). Phosphatidylcholine (PC) was shown to occur in gap junctions as a major phospholipid class; smaller quantitites of phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI) were also found. On the other hand, sphingomyelin (SPH) was reported to occur in gap junction preparations obtained from mouse liver plasma membranes (80), but apparently was not detected in rat liver GJ preparations (76). Because sphingomyelin is an abundant constituent of liver plasma membranes (79–81), it would be of interest to determine whether or not spingomyelin occurs in liver gap junctions.

Comparative studies reported by Henderson *et al.* (75) have clearly shown that the quantity of phospholipid that can be recovered from gap junctions does very much depend on the method by which the gap junctions are isolated. Mouse liver gap junctions prepared by the enzyme-sarcosyl method (73) contained almost four times as much phospholipid as gap junctions prepared with Triton X-100 and urea (75). Because individual phospholipid classes were not quantified in the latter study, it is not quite clear at this point whether phospholipid losses were selective and dependent on the detergent used.

In the present context, studies on *lens fiber cell* membranes are also of interest because these membranes contain extensive structural arrays which closely resemble

TABLE 1

Cholesterol (Chol) and Phospholipid (PL) Contents and Chol/PL Molar Ratios of Gap Junction (GJ) Preparations and Plasma Membranes

Membrane Preparation	Cholesterol[a]	Phospholipids[a]	Chol/PL Molar Ratio	Reference
Mouse liver GJ[b]	591 ± 80 nmol	384 ± 43 nmol	1.5 ± 0.09	Henderson *et al.* (75)
Mouse liver GJ[c]	521 ± 32 nmol	102 ± 36 nmol	5.6 ± 1.6	Henderson *et al.* (75)
Mouse liver plasma membrane	428 ± 133 nmol	473 ± 190 nmol	0.9 ± 0.1	Henderson *et al.* (75)
Chick lens fiber cell GJ	1171 nmol	372 nmol	3.1	Alcala *et al.* (83)
Chick lens fiber cell membrane	1036 nmol	494 nmol	2.1	Alcala *et al.* (83)
Bovine lens GJ-rich membrane	571 nmol	479 nmol	1.2	Malewicz *et al.*[d]
Bovine lens fiber GJ	0.2 mg (518 nmol)	0.6 mg (850 nmol)[e]	0.6[e]	Zampighi *et al.* (85)
Bovine lens membrane from equator[f]	—	—	0.5	Broekhuyse *et al.* (86)
Bovine lens membrane from cortex[f]	—	—	0.8	Broekhuyse *et al.* (86)
Bovine lens membrane from nucleus[f]	—	—	1.6	Broekhuyse *et al.* (86)

[a] Per mg protein.

[b] GJ isolated by the collagenase/sarcosyl procedure.

[c] GJ isolated by the Triton X-100/urea procedure.

[d] Malewicz, B., Lampe, P., Johnson, R.G., and Baumann, W.J., unpublished data.

[e] Calculations are based on a phospholipid average molecular weight of 700.

[f] Membrane preparations were treated with urea.

gap junctions as they are seen in other tissues. An overview on lens gap junctions has been published recently (82).

Studies on lens GJ have indicated that sphingomyelin may be a major phospholipid in these preparations. Gap junction-enriched preparations obtained by the sarcosyl method from chick lens fiber cell membranes were shown to contain about 40% SPH, 31% PC, 22% PE, and 4% PI as compared to 29% SPH, 27% PC, 39% PE, and 4% PI determined for the total lens fiber plasma membrane (83). The data are based on phosphorus assays of phospholipid fractions that were isolated by thin-layer chromatography (TLC). The results are in good agreement with quantitative TLC densitometric measurements that we have carried out on GJ-enriched bovine lens plasma membrane preparations.[1] We showed that phospholipids of bovine lens gap junction preparations contain significant amounts of SPH (37%) and somewhat lesser amounts of PC (27%) and PE (31%). In addition, small percentages of lysophosphatidylcholine (lysoPC) and lysophosphatidylethanolamine (lysoPE) were present.

A special role of sphingomyelin in the assembly of lens gap junctions may be indicated by the observation that major changes in the sphingomyelin composition of lens epithelial cell plasma membranes occur during cell elongation (84). Cell elongation in the lens has been associated with the onset of a massive formation of gap junctions. Not only does the content of sphingomyelin increase spontaneously at the time of epithelial cell elongation, but it is accompanied by significant changes in sphingomyelin acyl chain composition. While the content of oleic acid (18:1) decreases (from 16 to 5%) at the time of cell elongation, the content of fatty acids with 20 or more carbon atoms increases substantially. A particularly pronounced increase was observed in nervonic acid (24:1), from about 17% to 39% of total sphingomyelin fatty acids (84), which could suggest that longer-chain sphingomyelins may be required to sustain the gap junction assembly process.

The *total phospholipid content* of junction-rich preparations (Table 1) from bovine lens is thought to lie between about 479 nmol/mg protein[1] and 850 nmol/mg protein (calculated from 0.6 mg/mg protein; ref. 85). For chick lens fiber cell gap junctions, a phospholipid content of 372 nmol/mg protein was reported (83). Mouse hepatic GJ isolated by the collagenase/sarcosyl method showed a phospholipid content of 384 nmol/mg protein (75). However, only 102 nmol phospholipid/mg protein was found when Triton X-100/urea was used for the isolation of junctions (75). In another study, "vesicular" (light) and "nexus" (heavy) subfractions of mouse liver GJ preparations obtained by the sarcosyl method were shown to differ considerably in phospholipid content (80). While vesicular fractions were reported to contain as much as 6.5 mg phospholipid/mg protein (about 9000 nmol/mg), nexus fractions contained only 0.05 mg/mg (about 70 nmol/mg) protein (80).

Gap junction glycolipids. Little work has been done so far on gap junction glycolipids. Mouse liver GJ obtained by the sarcosyl method were shown to contain two types of glycolipids which were separable by adsorption TLC ($CHCl_3/CH_3OH/4N\ NH_3$, 9:7:2, v/v/v; ref. 80). It was possible to label these glycolipids with [^{14}C]glucosamine. After separation of GJ preparations into "vesicular" and "nexus" subfractions, the two labeled glycolipids were only associated with the "vesicular" fraction (80). Further work is required to determine whether glycolipids are actually integral components of gap junctions.

Gap junction neutral lipids. The neutral lipid fractions isolated from GJ preparations usually contain small amounts of free fatty acids, triglycerides and cholesterol esters, but substantial quantities of cholesterol.

Free fatty acids have been reported to occur in rat liver gap junctions (76), but only in small amounts in mouse liver GJ (75). In mouse liver GJ preparations, free fatty acids were detected by TLC in "vesicular" subfractions, but not in the "nexus" subfractions (80). Analyses done in our laboratory showed that about 170 nmol of free fatty

[1] Malewicz, B., Lampe, P., Johnson, R.G., and Baumann, W.J., unpublished data.

acids per milligram of sample protein were present in GJ-enriched plasma membrane fractions from bovine lens.[1] On the other hand, free fatty acids were found in GJ from chick lens fiber cells only in low abundance (83). It is not clear at this point whether the differences in GJ free fatty acid levels do reflect true species or tissue specificities, or whether they may at least partly be explained by lipid hydrolysis that could occur during gap junction and lipid isolation.

Triglycerides and *cholesterol esters* were reported to be present in small amounts in "vesicular" and "nexus" subfractions of mouse liver GJ preparations (80). Neutral lipids which co-migrated with triglycerides and/or cholesterol esters (petroleum ether/diethyl ether/acetic acid, 95:10:1, v/v/v) were also found in rat liver GJ (76). More detailed quantitative data are clearly needed.

Cholesterol has been shown to be the major neutral lipid in liver gap junctions (22,75,76,78,80) as well as in junction-rich lens membrane preparations (ref. 83; see Table 1). Mouse hepatic gap junctions isolated by the sarcosyl method and by the Triton X-100 method have been shown to contain 591 and 521 nmol cholesterol/mg protein, respectively (75). These values are quite similar to the cholesterol levels of 571 nmol/mg protein which we determined for GJ-rich preparations from bovine lens.[1] Similarly, cholesterol levels of about 518 nmol/mg protein (0.2 mg/mg protein) were reported for bovine lens junctions based on an estimation of TLC fractions after charring as compared to standards (85). More highly purified chick lens fiber cell gap junctions, which were prepared by the urea-deoxycholate method, were shown to contain higher cholesterol levels (1171 nmol chol/mg protein; ref. 83).

It is important to note that there seems to be a reasonably good agreement in the GJ cholesterol/protein ratios reported for most cell types (Table 1). Moreover, the cholesterol contents (per mg protein) of mouse liver gap junctions, for example, seem to be quite independent of the GJ isolation procedure used, whereas the phospholipid contents are not (75). This could suggest that cholesterol is more closely associated with the gap junction protein and is possibly a more integral part of the gap junction plaque than are most phospholipids.

Gap junction cholesterol-to-phospholipid molar ratios. The membrane cholesterol to membrane phospholipid molar ratio is often used as an approximate measure of membrane "fluidity". It is well known that in comparison to other cellular membranes, such as those of the endoplasmic reticulum, plasma membranes are characterized by a higher cholesterol-to-phospholipid (chol/PL) molar ratio.

In Table 1, we have compiled cholesterol and phospholipid contents, as well as chol/PL molar ratios for plasma membranes and gap junction preparations. Although there are some exceptions (85), a general pattern emerges: *the chol/PL molar ratios of gap junction preparations are typically much higher than those of the native plasma membranes.* The chol/PL ratios reported for mouse liver GJ (75), for example, vary between 1.5 and 5.6, dependent upon the type of detergent used for the preparation of the junctions (Table 1). By comparison, the chol/PL molar ratio of mouse liver plasma membrane was reported to be 0.9 (75). The chol/PL ratios reported for junction-rich lens membranes can range from 3.1 (83) to 1.2[1] (Table 1). Observed differences appear to be largely due to the method of junction enrichment used.

Thus, a high chol/PL molar ratio may, indeed, be characteristic of gap junctions. This, in turn, could suggest that the high cholesterol levels associated with gap junctions may be required for the ordering and anchoring of the clusters of GJ proteins within the plaques to preseve their functional integrity.

The filipin evidence. Filipin is a polyene macrolide antibiotic with a high affinity for cholesterol as well as for some other sterols. When inserted into a membrane, filipin forms complexes with membrane sterols. These complexes are thought to be accommodated between the two bilayer leaflets within the core of the bilayer membrane (87). The filipin-cholesterol aggregates can be observed by freeze-fracture electron microscopy. They appear as 15–25 nm protrusions on plasma membrane P-faces or as depressions on the E-faces.

Filipin has frequently been used as a cholesterol probe in GJ studies. Yet, despite the apparent abundance of cholesterol in GJ preparations, it has not been possible to visualize cholesterol associated with gap junctions using filipin. The earliest filipin experiments with gap junction preparations were done with cardiac muscle cells (88). Because the gap junction domains remained void of the characteristic filipin protrusions or depressions, it was concluded that gap junctions contain only little or no cholesterol. Similarly, GJ of cultured hepatocytes and of sebaceous tumor cells did not give positive sterol evidence with filipin, which suggested that "these regions are low in cholesterol" (89,90). Other investigators have since confirmed this observation on other cell types, such as human skin fibroblasts (91), human ovarian and adrenal adenocarcinomas (92), rat hepatoma cells grown in culture (93), hepatocytes (94), insect epidermal samples (95), and frog heart fibers (96). In all these instances, the gap junction plaque areas did not give any indication of filipin-sterol complex formation, whereas the membrane regions surrounding the GJ domains showed the filipin-characteristic membrane protrusions.

There is increasing evidence, however, that factors other than low membrane cholesterol levels may be responsible for negative filipin results. It has been shown, for example, that surface proteins (94) can interfere with filipin labeling. Other more recent studies on gap junctions have pointed towards the high protein concentration and the resulting "membrane rigidity" within the gap junction membrane domain as a possible cause for the "negative" filipin tests (91,95,96). Although the presence of sterol clearly appears to be a prerequisite for observing the filipin aggregates, other factors seem to matter as well. We suggest that in order for the sterol/filipin complexes to be formed and to appear as membrane protrusions, filipin must not only penetrate into the respective membrane domain, but the affinity of the sterol for filipin must exceed the affinity of the sterol for other membrane constitutents, and the respective sites must lend themselves to deformation in order to show the protrusions. Therefore, only a "positive" filipin test should be interpreted as evidence for the presence of sterol in a given membrane domain (97,98). "Negative" filipin tests should be considered inconclusive.

It is of interest in this context, that developing junction domains, in which particle density is still quite low

as compared to mature junctions, also do not give a positive filipin test (92–94). These gap junction formation plaques (FP) are deficient in intramembranous particles and do not display cytoskeletal membrane coats. Nevertheless, they do not produce the sterol-characteristic membrane protrusions with filipin. It is not clear at this point whether the deficiency in intramembranous particles can be attributed to low cholesterol levels in FP or whether "ordering factors" in developing junctions are responsible for the apparent lipid differentiation which may preclude filipin complexation and/or deformation of the plasma membrane.

EFFECTS OF LIPIDS ON GAP JUNCTIONS

The effects of lipids on gap junction assembly, stability and function are of primary interest. Although involvement of lipids in the development and function of gap junctions can be expected, conclusive experimental evidence has been forthcoming slowly.

Effect of fatty acids. While there is some uncertainty in regard to the levels of free fatty acids that are associated with gap junctions (see above), there is substantial evidence that exogenous fatty acids added to cell or organ cultures affect GJ function. Unsaturated fatty acids, in particular, have been shown to modulate gap junctions (99–102). Thus, supplementation of culture media with myristoleic (14:1), palmitoleic (16:1), oleic (18:1), linoleic (18:2), linolenic (18:3) or arachidonic acid (20:4) at micromolar concentrations was shown to *inhibit* GJ-mediated intercellular communication between Chinese hamster cells as judged by monitoring metabolic cooperation (101,102). Oleic acid (18:1) was a more potent inhibitor of GJ-mediated metabolic cooperation than was linoleic (18:2), linolenic (18:3), palmitoleic (16:1) or myristoleic (14:1) acid. The *trans* 16:1 and 18:1 isomers were less effective than the *cis* isomers (101). Saturated fatty acids, as well as short-chain acids, had no significant effect on intercellular communication. Undecanoic (11:0) and undecenoic (11:1) acids both showed moderate inhibition (101). Also, rat myometrium exposed to arachidonic acid for 48 hr was shown to reduce the number of GJ *in vitro* (99).

The mechanism of GJ down-regulation by fatty acids is presently not known. There is also insufficient experimental evidence to determine with any degree of certainty whether the fatty acids exert their effect in the "free" state or after incorporation into other lipids. The greater inhibitory effect of *cis* fatty acids than the *trans* isomers (101) could be taken in support of the idea that increased membrane fluidity may play a role in inhibiting GJ-mediated intercellular communication. However, actual changes in membrane fluidity and resulting GJ inhibition have not been documented so far. On the other hand, it is known that there is only a narrow concentration range for modulating gap junction communication by fatty acids and that modulation occurs just before toxic fatty acid levels are reached (101,102). In this respect, inhibition of GJ communication by fatty acids shows interesting parallels with fatty acid-induced inhibition of cell adhesion (103) and the effect of fatty acids on capping (104).

In contrast to other unsaturated fatty acids, arachidonic acid has been reported to *stimulate* gap junctions in some systems. Thus, canine tracheal smooth muscles treated *in vitro* with arachidonic acid (5×10^{-5} M; 30 min) significantly increased the number of gap junctions and the percentage of GJ membrane relative to total plasma membrane area (100). This effect of arachidonic acid on gap junctions does not seem to be a direct fatty acid effect, but rather appears to be mediated through products of prostaglandin metabolism (100). Such a mode of action would be consistent with the observations that PGE_2 and PGI_2 also increased GJ formation in canine tracheals and that indomethacin, which inhibits cyclooxygenase, abolished the stimulatory effect of arachidonic acid on gap junctions.

Effect of diacylglycerols. Only small quantitites of diacylglycerols are known to be present in plasma membranes; the occurrence of diacylglycerols in GJ preparations has not been reported to date. However, it was shown for Chinese hamster V79 cells that diacylglycerols containing at least one unsaturated fatty acid, when added to the culture medium, inhibited GJ-mediated communication between the cells (101). Similarly, Lucifer yellow transfer between isolated rat liver cells was inhibited by unsaturated diacylglycerols (105), whereas saturated diacylglycerols did not show an inhibitory effect. The position of the unsaturated fatty acids on the glycerol backbone does not appear to matter, because 1,2-diolein and 1,3-diolein inhibited intercellular metabolic cooperation to a similar extent (101). Also, *rac*-1-oleoyl-2-acetyl-glycerol was shown to be an effective inhibitor of junctional permeability in rat liver epithelial cells (105).

The mechanism of down-regulation of GJ-mediated intercellular communication by diacylglycerols is not certain. While a direct effect cannot be excluded, considerable evidence points towards the possibility that down-regulation may be mediated through a mechanism that involves protein kinase C (101,105,106). This hypothesis is consistent with the observation that 8-*N,N*-(diethylamino)octyl-3,4,5-trimethoxybenzoate (TMB-8), an inhibitor of protein kinase C, counteracts diacylglycerol-induced down-regulation of GJ (105). Also, tumor promoters, such as phorbol esters, that enhance protein kinase C activity, were shown to decrease GJ-mediated cell-to-cell communication (12,57,59,107).

Effect of prostaglandins. Several studies suggest that prostaglandins affect GJ development and functions *in vivo* and *in vitro*. Prostaglandins are thought to modulate GJ-mediated transmission of electrical signals (34,42,108) and the transfer of metabolites between cells (109). In addition, prostaglandins are known to affect synchronization of smooth muscle contraction, which is thought to be dependent upon gap junctions (32,34,42,109–112).

Evidence that prostaglandins affect gap junctions has been gathered on cultured cells as well as organ cultures of canine trachea (100). Short-term exposure (30 min) of canine tracheal muscle strips to PGE_2 or PGI_2 increased the number of junctions several-fold as it increased the percentage of GJ relative to total plasma membrane area (100). In the latter study, PGE_2 was shown to be more effective than PGI_2. In rat serosal cells grown in culture, PGE_1 increased the number of gap junctions; however, $PGF_{2\alpha}$ showed an inhibitory effect (113).

When human WI-38 and C-6 glioma cells were incubated with prostaglandin E_1 at micromolar levels (7 hr), the number and size of gap junctions was increased

and junctional permeability was enhanced when measured by following the rate of transfer of fluorescent probes (114). Simultaneously, the levels of intracellular cyclic-AMP were shown to increase in WI-38 and C-6 glioma cells up to 8- and 100-fold, respectively (114).

It is not clear at present whether prostaglandins directly modulate gap junctions or whether the effect is indirect. The possibility has been considered that up-regulation of GJ permeability may involve cyclic-AMP (114). Indeed, cyclic-AMP has been shown to increase GJ permeability in a number of systems (115–119). In view of the complex variety of cellular processes that are affected by prostaglandins and cyclic-AMP, conclusive cause-and-effect relationships have not yet been established.

Effect of cholesterol. The special role of cholesterol in modulating membrane structure and function is well recognized today (for a review, see ref. 120). Cholesterol is known to broaden the gel-to-liquid crystalline phase transition of phospholipid membranes and to affect membrane permeability and membrane fluidity (120–124). It also has been established that the cholesterol content of plasma membranes is much higher than that of other subcellular fractions. The distribution of cholesterol within the plane of the plasma membrane is typically not uniform (97,125).

As mentioned above, gap junctions appear to be characterized by a high level of cholesterol (per mg protein) and by a high cholesterol-to-phospholipid molar ratio. GJ cholesterol levels are consistently higher than the cholesterol levels of the respective plasma membranes (Table 1). This predominance of cholesterol in gap junctions has prompted us to focus specific attention on the role of cholesterol in gap junction assembly, structure and function.

We had observed earlier that Novikoff hepatoma cells (NHC) grown in culture readily take up cholesterol which is provided in the medium (126–128). Cholesterol levels in NHC can thus be modulated over a wide range of concentrations. Most of the cholesterol taken up by the cells remains unesterified and is incorporated into the plasma membranes[2] (128).

The effect of cholesterol on gap junction assembly and function was studied in the NHC system by growing the cells in serum-containing medium which was supplemented with cholesterol. Cholesterol-enriched cells were then dissociated, recovered and reaggregated to analyze the development of junctional structures after freeze-fracture using quantitative electron microscopy (7). Junctional permeability was also monitored by following dye transfer between cells after intracellular injection of Lucifer yellow (129,130).

We found that upon cholesterol supplementation, gap junction assembly and permeability were stimulated or inhibited, depending upon the cholesterol concentration. Maximum stimulation was observed when the cells were grown for 24 hr in 20 μM cholesterol-supplemented medium which resulted in up to a seven-fold increase in the number of aggregated GJ particles, and in up to a three-fold increase in GJ plaque area[2] (128–131). Maximum stimulation was observed when cell cholesterol levels reached approximately 55 nmol chol/mg cell protein, *i.e.*, at an increase of about 50% above control levels. At this point, plasma membrane cholesterol levels reached 250–280 nmol of membrane cholesterol/mg protein, and the plasma membrane cholesterol-to-phospholipid ratio reached 0.45, as compared to 0.25 for control cells[2] (128). In cells grown in 20 μM cholesterol-supplemented medium, the rate of Lucifer yellow transfer between cells also was significantly increased and optimal (129,130). Cholesterol supplementation did not significantly change the percentage of cells forming junctions. Hence, cholesterol does not appear to affect initiation of junction assembly, but rather junction growth.

When cells were grown in medium supplemented with 40 μM or higher cholesterol levels, junction formation became inhibited. At 40 μM cholesterol supplementation, when cell cholesterol levels doubled, junction formation was lower than in control cells[2] (128–131).

Stimulation of gap junction formation was also observed when the cells were treated with low density lipoprotein (LDL) instead of cholesterol[3] (132). Interestingly, optimal GJ stimulation occurred at essentially the same cell cholesterol levels (about 55 nmol chol/mg cell protein) independent of whether cholesterol or LDL was added to the medium.[3] Stimulation by LDL was dosage dependent. At higher LDL levels, stimulation of gap junction formation was lost and eventually control levels were reached. However, there was an interesting difference between the two modes of cholesterol administration. While cholesterol uptake by the more physiological LDL route became down-regulated at cell cholesterol levels higher than the optimal cholesterol concentration, uptake of free cholesterol continued. Yet, the stimulatory effect on gap junctions was lost in both instances and inhibition occurred at high cholesterol concentrations.

The mechanism by which gap junction assembly is up- and down-regulated is not clear at present. One could speculate, for example, that cholesterol may specifically affect the synthesis of gap junction proteins. This, in turn, would be consistent with the observation that cycloheximide blocked the cholesterol-induced increase in GJ assembly without affecting basal levels of assembly (130). On the other hand, it has not been possible so far to demonstrate either cholesterol-induced stimulation or inhibition of NHC gap junction protein synthesis by use of cross-reacting connexin-43 antibodies (133,134). Thus, cycloheximide may be acting on the synthesis of other proteins that are involved in GJ assembly. The complex nature of the GJ assembly process is also indicated by the observation that colchicine abolished the cholesterol-induced stimulation of GJ formation, suggesting an effect on transport processes.[3]

At present, one explanation for the observed stimulation by cholesterol of gap junction formation in Novikoff hepatoma cells would be that cholesterol is required as an essential structural component for the assembly and function of gap junctions. On the other hand, the lack of stimulation at higher cholesterol levels appears to coincide with a state of down-regulation in these cells, at least in respect to LDL uptake. Clearly, additional experimental evidence is required to pin-point the mechanisms by which gap junction assembly is regulated.

[2]Malewicz, B., Meyer, R.A., Johnson, R.G., and Baumann, W.J., manuscript in preparation.

[3]Meyer, R.A., Malewicz, B., Baumann, W.J., and Johnson, R.G., manuscript in preparation.

CONCLUSION

Important strides have been made in our understanding of the role of lipids in gap junctions. It has already become quite clear that various lipid mediators of cell function are also involved in the regulation of gap junctions. Furthermore, evidence is mounting which suggests that lipids are integral structural components of the gap junction plaque. A picture is emerging in which specific lipids appear to be required for the assembly of gap junction particles in functional arrays. These specialized lipid domains located in closely apposed plasma membrane regions would provide the structural rigidity to anchor the clusters of apposed gap junction proteins and thus assure the functional integrity of gap junction channels. It will be a challenging task to bring into balance the vast knowledge that has already been gained on gap junction proteins with what we still need to learn about the role of lipids in gap junction assembly, structure and function.

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ARTICLES

Hydrolysis of Fluorescent Pyrene-Acyl Esters by Human Pancreatic Carboxylic Ester Hydrolase and Bile Salt-Stimulated Lipase

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Fluorescent esters containing pyrenedecanoic acid (P_{10}) or pyrenebutanoic (P_4) acid (P_4cholesterol, P_{10}cholesterol, P_4- and P_{10}-containing triacylglycerols) were synthesized and used as substrates for human pancreatic carboxylic ester hydrolase and bile salt-stimulated lipase from human milk. Both enzymes were purified by immunoaffinity chromatography. All fluorescent pyrene derivatives were hydrolyzed by pancreatic carboxylic ester hydrolase and bile salt-stimulated lipase, but at different rates. The hydrolytic rates of the "short" acyl esters (P_4-containing esters) were higher than those of the "long" ones (P_{10}-containing esters). Conditions were optimized for sensitivity of the assay using fluorescent cholesteryl esters. The pH optimum was 7.5–8.0. Sodium cholate exhibited a stronger activating effect than taurocholate or taurodeoxycholate (maximal activation was achieved with 5 mmol/L cholate and with a molar ratio cholesteryl ester/cholate around 1:10). Both pancreatic carboxylic ester hydrolase and bile salt-stimulated lipase from milk were strongly inhibited by the other amphiphiles tested, namely phosphatidylcholine and Triton X-100, and were inactivated by low concentrations (10 μmol/L) of the serine-reactive diethyl-paranitrophenyl phosphate (E_{600}). Both enzymes were strongly inhibited by relatively low concentrations of plasma low density lipoproteins. These studies indicate that the fluorescent esters containing pyrene fatty acids can be used as substrates for assaying and investigating the properties of pancreatic carboxylic ester hydrolase as well as bile salt-stimulated lipase from milk.

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Human pancreatic carboxylic ester hydrolase (1–7) and cholesteryl ester hydrolase (E.C. 3.1.1.1) exhibit a broad substrate specificity for lipid-soluble substrates (such as esters of cholesterol, glycerol or vitamin A) and for synthetic water-soluble carboxylic esters (3,4,8–10). Pancreatic carboxylic ester hydrolase and bile salt-stimulated lipase from human milk share similar enzymatic properties (subtrate specificity, activation by bile salts) (11,12) and can be purified by the same immunoaffinity column prepared with antibodies directed against pancreatic carboxylic ester hydrolase (12).

The determination of pancreatic carboxylic ester hydrolase activity has been performed using various titrimetric, radiometric or spectrophotometric methods with lipid-soluble substrates (tributyrin, oleoyl cholesterol) (10, 13–15) or with water-soluble carboxylic esters (phenyl- and nitrophenyl acyl esters) (3,4,8,9,14). In spite of their interest, these methods suffer from either poor sensitivity (unlabeled substrates) or lack of specificity (colored synthetic substrates are hydrolyzed by "non specific carboxylesterases") (16,17) or require specific apparatus (18,19).

We have recently synthesized several fluorescent esters of cholesterol and glycerol, containing pyrene-fatty acids with "short" and "long" chains (17,20–22). These pyrene-acyl derivatives have been successfully used as a substitute of the natural substrates in very sensitive and specific enzymatic assays for determining the activities of lipases and cholesteryl ester hydrolases (18–21). However, the presence of the pyrene nucleus, which is more bulky and less hydrophobic than an alkyl chain equivalent in length (i.e., about 6 carbons), could induce changes in the physicochemical properties of these substrates and in their recognition by the hydrolytic enzymes. Thus, we tested the hydrolysis of various fluorescent cholesteryl esters and triacylglycerols (containing pyrene fatty acids with a "short" or "long" acyl chain) by purified carboxylic ester hydrolase and bile salt-stimulated lipase. We report the optimal conditions of a fluorometric assay for both enzymes.

MATERIALS AND METHODS

Chemicals. Oleoyl cholesterol, triolein and tributyrin, pyrenebutanoic acid (P_4), pyrenedecanoic acid (P_{10}), egg yolk phosphatidylcholine, E_{600} (diethyl-paranitrophenyl phosphate), sodium cholate, taurocholate and taurodeoxycholate were purchased from Sigma (St. Louis, MO). Other reagents or chemicals were from Prolabo (Paris, France) or Merck (Darmstadt, FRG).

The fluorescent cholesteryl esters, pyrenebutanoyl cholesterol (or P_4cholesterol) and pyrenedecanoyl cholesterol (or P_{10}cholesterol), the fluorescent triacylglycerols, tri-pyrenebutanoyl glycerol (or triP_4G), tri-pyrenedecanoyl glycerol (or triP_{10}G) and 1-pyrenedecanoyl-2,3-dioleyl glycerol (or P_{10}OOG) and the radiolabeled [9,10-^{3}H]oleoyl cholesterol and [1-^{14}C]butyryl cholesterol were synthesized as previously described (21,23) following the method of Neises and Steglich (24).

Enzyme purification. Human pancreatic carboxylic ester hydrolase from pancreatic juice and bile salt-stimulated lipase from human milk were purified using an immunoaffinity column as previously described (12).

Preparation of lipoproteins. Human plasma low density lipoproteins were prepared by sequential ultracentrifugation (L8-70 Beckman Ultracentrifuge), according to Havel *et al.* (25), using pooled plasma from healthy subjects. Lipoproteins and lipoprotein-depleted plasma were extensively dialyzed against 0.15 mol/L NaCl containing 0.3 mmol/L EDTA, for 3 days, at 4°C, and kept at 4°C until use.

Substrate preparation and enzymatic assays. The substrates (fluorescent and unlabeled) and cholate were dissolved in chloroform, evaporated under nitrogen and dispersed in the assay buffer by sonication in ice (2 cycles

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Abbreviations: P_4, pyrenebutanoic acid; P_{10}, pyrenedecanoic acid; P_4cholesterol, pyrenebutanoyl cholesterol; P_{10}cholesterol, pyrenedecanoyl cholesterol; triP_4G, tri-pyrenebutanoyl glycerol; triP_{10}G, tri-pyrenedecanoyl glycerol; P_{10}OOG, 1-pyrenedecanoyl-2,3-dioleoyl glycerol; Ch, cholate; TCh, taurochoate; TdCh, taurodeoxycholate; Tx, Triton X-100; PC, phosphatidylcholine.

of 1 min at maximal power, using a MSE Sonicator model 7100) and immediately used for the enzymatic assays. The microemulsion was homogenous (the size of the substrate particles was 21±4 nm, as determined using a Coulter NM4 Nanosizer) and stable for at least 12 hr.

Pancreatic carboxylic ester hydrolase and bile salt-stimulated lipase activities were determined using the following standard conditions: the fluorescent ester was diluted with the corresponding unlabeled ester (and, when specified, with a tracer dose of the radiolabeled ester). P_{10}cholesterol was diluted with oleoyl cholesterol, P_4cholesterol with butyryl cholesterol, triP_{10}G and P_{10}OOG with triolein, triP_4G with tributyrin. The reaction mixture contained 20 μmol/L fluorescent ester and 500 μmol/L unlabeled ester (and 50,000 dpm radiolabeled substrate/assay, when indicated in the text), 5 mmol/L sodium cholate, 0.2 mol/L citrate-phosphate pH 7.5 and pure enzyme solution (0.5–1 μg protein/assay) in a final volume of 100 μL. After incubation for 30 min at 37°C, the liberated fatty acids were extracted and quantified by fluorometry (Jobin-Yvon spectrofluorometer model JY3C), using standards of P_4 or P_{10}, as previously described (21–23). Enzyme activities are expressed as nmol of total (fluorescent and unlabeled) fatty acid liberated per min and per mg protein, assuming that the fluorescent and the related unlabeled substrates are hydrolyzed at the same rate (this will be demonstrated in Table 1).

The study of the acyl chain length specificity was done using two types of assays containing a mixture of fluorescent and radiolabeled substrates. The "radiolabeled standard" assay was performed under the standard conditions as indicated above. In order to discriminate between acyl chain length specificity and influence of the physical state of the substrate, we used a second type of assay containing "all the substrates mixed together," i.e. [^{14}C]butyryl cholesterol (50,000 dpm/assay), [^{3}H]oleoyl cholesterol (50,000 dpm/assay), 10 μmol/L P_4cholesterol, 10 μmol/L P_{10}cholesterol, 250 μmol/L butyryl cholesterol, 5 mmol/L sodium cholate, 0.2 mol/L citrate-phosphate pH 7.5 and enzyme solution (1 μg protein/assay) in a final volume of 100 μL. After incubation for 30 min at 37°C, the liberated fatty acids were extracted and determined by radiometry and by fluorometry. The counting and the isotopic ratio of the liberated radiolabeled fatty acid, [^{14}C]butyrate/[^{3}H]oleate, were determined using a Packard scintillation counter (model Tricarb 4530). The fluorescent fatty acids were extracted with 0.5 mL water and 2 mL of chloroform/methanol/N sulfuric acid, (2:1:0.1, v/v/v) and separated by thin-layer chromatography on silica gel G analytical plates (developing solvents petroleum ether/diethyl ether/acetic acid, 80:20:1, v/v/v). The two fractions of fluorescent fatty acids (P_4 and P_{10}) were scraped off and eluted with 10 mL of chloroform/methanol (90:10, v/v). The solvents were evaporated to dryness, the fatty acids dissolved in 1 mL of chloroform/methanol (2:1, v/v) and fluorescence was read as previously described (21,22).

Proteins were determined according to Lowry *et al.* (26).

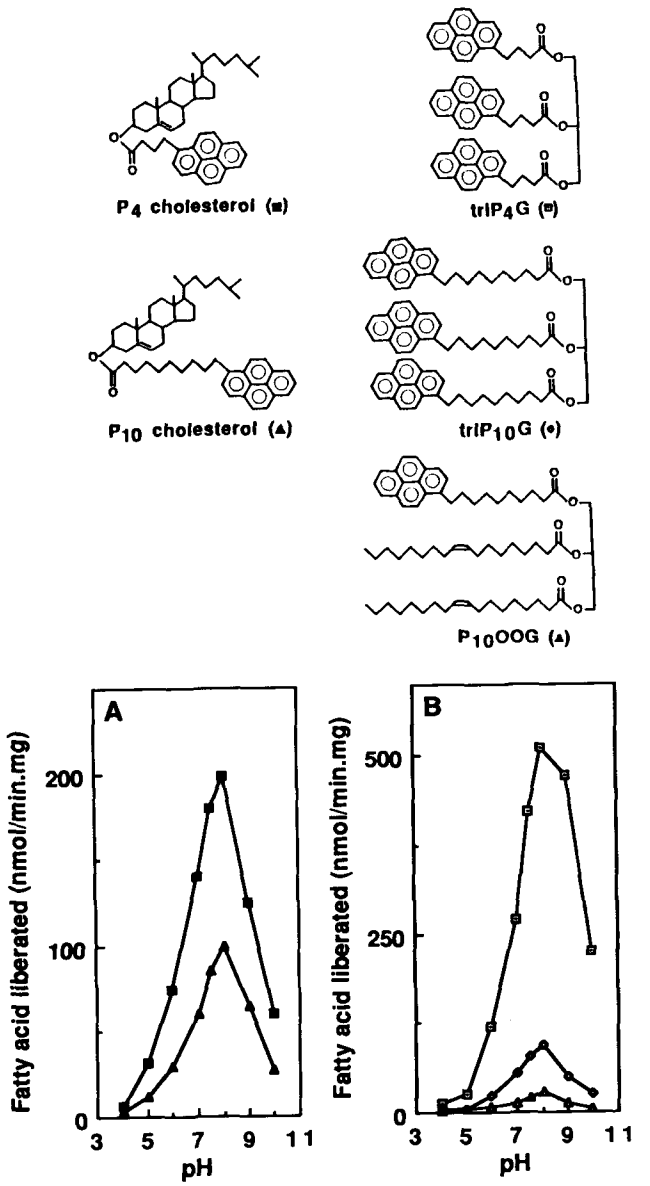

FIG. 1. Effect of pH on the hydrolysis of fluorescent esters of cholesterol (A) and glycerol (B) by pancreatic carboxylic ester hydrolase. The reaction mixture contained 20 μmol/L fluorescent acyl esters diluted with 500 μmol/L unlabeled substrates, (triP_4G (□) with tributyrin; triP_{10}G (○) and P_{10}OOG (△) with triolein, P_4cholesterol (■) with butyryl cholesterol, P_{10}cholesterol (▲) with oleoyl cholesterol), 5 mmol/L sodium cholate, 0.2 mol/L buffer at variable pH (citrate-phosphate from pH 4.0 to 7.0, Tris-HCl from pH 7.5 to 8.5, and glycine/NaOH from pH 9.0 to 10) and enzyme (1 μg/assay) in a final volume of 100 μL. After incubation (30 min at 37°C), the fluorescent fatty acids liberated were extracted and quantified using standards of P_4 and P_{10}. Enzyme activities are expressed as nmol of total fatty acids liberated per min and mg protein. The chemical structures of the pyrene derivatives used are shown above.

RESULTS

pH Optimum profiles and substrate specificity. The hydrolytic activity of the purified pancreatic carboxylic ester hydrolase was assayed using fluorescent cholesteryl esters and triacyl-glycerols with "short" and "long" acyl chains. The enzyme hydrolyzed the 5 fluorescent substrates (pH optimum around 7.5–8.0) at different hydrolytic rates: "short" acyl chain esters (P_4cholesterol and triP_4G) were hydrolyzed more rapidly than the

TABLE 1

Comparison of the Hydrolytic Activity of Pancreatic Carboxylic Ester Hydrolase on "Short" and "Long" Fluorescent and Radiolabeled Cholesteryl Esters

	Enzyme activity (nmol/min/mg)			
	P_4cholesterol	Butyryl cholesterol	P_{10}cholesterol	Oleoyl cholesterol
1 - "Radiolabeled standard" assay[a]				
Fluorescent fatty acid	7.6 ± 0.3	—	3.9 ± 0.1	—
Total fatty acid	197.6 ± 12[b]	201 ± 10	101.4 ± 5[b]	102 ± 6
2 - Assay with "all the substrates mixed together"[c]				
Fluorescent fatty acid	5.3 ± 0.2	—	2.7 ± 0.1	—
Total fatty acid	137.8 ± 6[b]	139 ± 7	71.2 ± 4[b]	70 ± 6

[a]The "radiolabeled standard" assay with "short" chain substrates contained P_4cholesterol + butyryl cholesterol (radiolabeled and unlabeled); that with "long" chain substrates contained P_{10}cholesteryl + oleoyl cholesterol (radiolabeled and unlabeled). For experimental details, see Materials and Methods.

[b]These values were calculated assuming that the fluorescent and the "natural" fatty acids (on one hand, P_4cholesterol and butyryl cholesterol, and, on the other hand, P_{10}cholesterol and oleoyl cholesterol) were hydrolyzed at the same rate.

[c]The assay with "all the substrates mixed together" contained a mixture of "short" chain and "long" chain substrates: P_4cholesterol + P_{10}cholesteryl + butyryl cholesterol + oleoyl cholesterol (butyryl cholesterol and oleoyl cholesterol, both radiolabeled and unlabeled). The liberated fatty acids were extracted and determined (fluorometrically or radiometrically) as reported in the Materials and Methods section. All values of pancreatic carboxylic ester hydrolase activity are expressed as nmol of fatty acid liberated (either fluorescent fatty acid only or total fatty acids)/min/mg protein.

All results are the mean of 3 separate experiments.

"long" chain esters. Quite similar results were obtained with bile salt-stimulated lipase (data not shown).

In order to evaluate the acyl chain length specificity of the pancreatic carboxylic ester hydrolase without or with minimal influence of the physical state of the substrate, the hydrolytic activity was tested using two types of assays (Table 1). Under the conditions of the "radiolabeled standard" assays, fluorescent and radiolabeled (or unlabeled) cholesteryl esters with "equivalent" chain length were hydrolyzed at the same rate, as shown by the identity of the values determined radiometrically and fluorometrically (upper part of Table 1). Pyrene nucleus did not seem to interfere with the substrate specificity of pancreatic carboxylic ester hydrolase, since P_4cholesterol and [^{14}C]butyryl cholesterol on one hand, and P_{10}cholesterol and [^{3}H]oleoyl cholesterol on the other hand, were hydrolyzed at similar rates. "Short" chain esters are the preferred substrates of the enzyme since the hydrolytic rate of P_4cholesterol (or butyryl cholesterol) is about twice that of P_{10}cholesterol (or oleoyl cholesterol). In order to examine whether these differences resulted from acyl chain length specificity of the enzyme or from differences in the physical state of the substrate, an assay was performed with "all the substrates mixed together" (assuming that they are homogenously mixed). As shown in Table 1 (lower part), the ratios of hydrolysis of P_4cholesterol/P_{10}cholesterol and of [^{14}C]butyryl cholesterol/[^{3}H]oleoyl cholesterol were similar (about 2): this indicates that the "short" chain cholesteryl esters were hydrolyzed twice as fast as the "long" chain esters.

Linearity with time and enzyme concentration. Under standard conditions, hydrolysis of both "short" and "long" chain substrates (P_4cholesterol and P_{10}cholesterol) was linear up to 30–40 min (Fig. 2A) and up to 15 μg protein/mL (1.5 μg/assay).

Effect of bile salts and other tensio-active compounds.

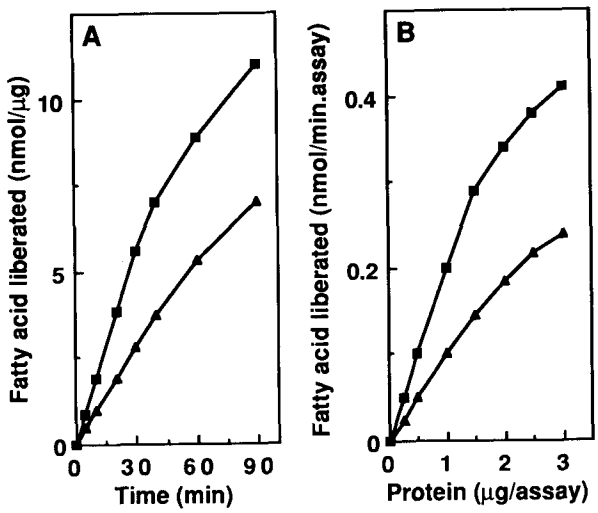

FIG. 2. Linearity with incubation time (A) and enzyme concentration (B) of the hydrolysis of P_4cholesterol (■) and P_{10}cholesterol (▲) by pancreatic carboxylic ester hydrolase. Enzyme activity was determined using the standard assay, but varying the incubation time up to 90 min (A) or varying the enzyme concentration up to 30 μg/mL (i.e. 3 μg/assay) (B).

The study of the effect of increasing concentrations of primary (cholate and taurocholate) and secondary (taurodeoxycholate) bile salts on the hydrolysis of cholesteryl esters by the purified pancreatic carboxylic ester hydrolase demonstrated the requirement of bile salts for optimizing substrate hydrolysis (Fig. 3A). In the absence of bile salt (substrate dispersion stabilized by 0.1% serum albumin), the rate of hydrolysis for the fluorescent cholesteryl ester was very low (around 0.25–0.5% of the maximal activity observed in presence of sodium cholate). Among the 3 bile salts tested,

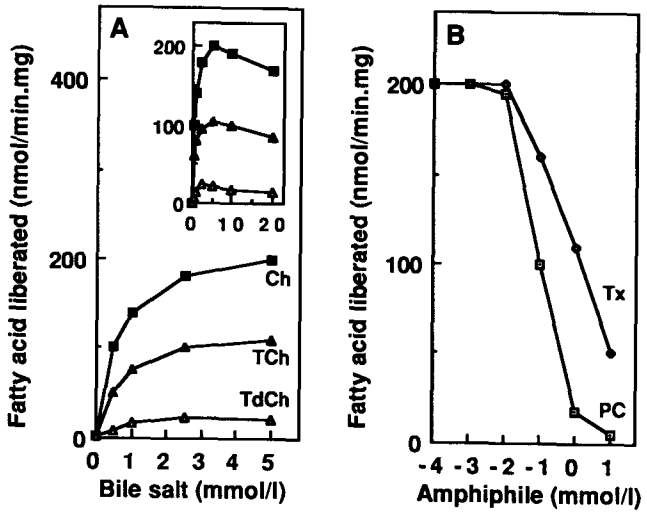

FIG. 3. Effect of bile salts (A), Triton X-100 and phosphatidylcholine (B) on the hydrolysis of P_4cholesterol by pancreatic carboxylic ester hydrolase. In A, enzyme activity was determined under standard conditions, but with addition of 0.1% bovine serum albumin (in order to stabilize the substrate emulsion in the absence of bile salt) and bile salts at varying concentrations (from 0 to 5 mmol/L, and from 0 to 20 mmol/L in insert); sodium cholate (Ch), taurocholate (TCh), and taurodeoxycholate (TdCh). In B, enzyme activity was determined under standard assay conditions (with 5 mmol/L sodium cholate), with increasing concentrations of Triton X-100 (Tx) or phosphatidylcholine (PC) (concentrations expressed as log mmol/L).

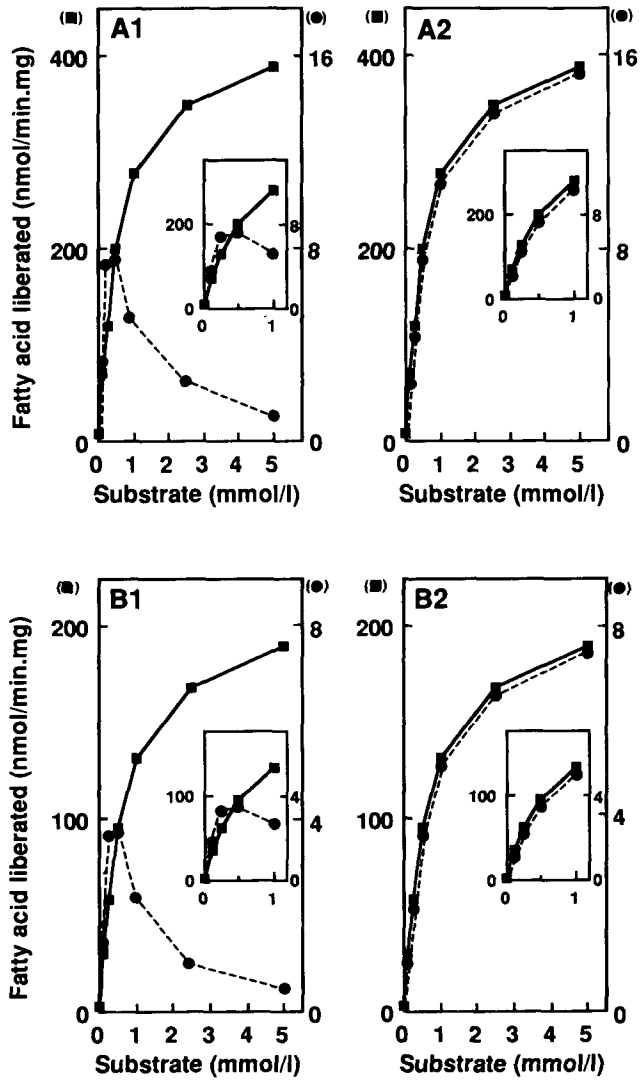

FIG. 4. Influence of the substrate concentration on pancreatic carboxylic ester hydrolase activity. The enzyme activity was followed with increasing substrate concentrations (from 20 μmol/L to 5 mmol/L) and sodium cholate (for keeping constant the molar ratio substrate/cholate at 1:10). Other conditions are similar to those reported in Materials and Methods. In A, P_4cholesterol (diluted with butyryl cholesterol); in B, P_{10}cholesterol (diluted with oleoyl cholesterol); in A1 and B1, the fluorescent substrate was used at a fixed concentration (20 μmol/L) and only the unlabeled substrate increased; in A2 and B2, both fluorescent and unlabeled substrates increased simultaneously (while the ratio of fluorescent/unlabeled substrate was kept constant at 1:25). Results are expressed as nmol of total fatty acids released per min per mg protein, assuming that the fluorescent and the natural fatty acids are hydrolyzed at the same rate (-■-, left scale), and as nmol of fluorescent fatty acids released per min per mg protein (-●-, right scale).

maximal activation was achieved with 5–10 mmol/L cholate, whereas the maximal activating effect of taurocholate and taurodeoxycholate did not exceed 55 and 15% of that obtained with cholate, respectively. Thus, under optimal assay conditions, the molar ratio of cholesteryl esters to cholate was chosen at 1:10. As shown in Fig. 3B, the hydrolysis of the fluorescent cholesteryl esters by pancreatic carboxylic ester hydrolase was inhibited by Triton X-100 and even more strongly so by phosphatidylcholine.

Effect of substrate concentration. In order to optimize assay conditions, the hydrolytic activity of the pancreatic carboxylic ester hydrolase on the fluorescent and unlabeled substrates was simultaneously studied by varying the substrate concentration (with a constant molar ratio cholate/cholesteryl ester of 10:1). In a first set of experiments (Figs. 4A1 and B1), the fluorescent substrate concentration was kept constant and only the unlabeled substrate increased whereas in the second set of experiments (Figs. 4A2 and B2), both fluorescent and unlabeled substrates increased simultaneously (with a constant molar ratio of fluorescent/unlabeled substrate of 1:25). The rate of hydrolysis was calculated assuming that P_4cholesterol and butyryl cholesterol on one hand and that P_{10}cholesterol and oleoyl cholesterol on the other hand were hydrolyzed at the same rate (as demonstrated in Table 1). As shown in Figure 4, the hydrolytic rates increased with increasing substrate concentrations and the shapes of the curves of V (expressed as total fatty acid liberated) versus S were quite similar in both types of experiments. These results confirm that the fluorescent and the unlabeled substrates are hydrolyzed at the same rate. In contrast, as expected, the shape of the curves of fluorescent substrate hydrolysis was different in the two sets of experiments. In the first set of experiments (constant amount of fluorescent substrate), the hydrolysis of the fluorescent substrate increased up to 0.5 mmol/L, then decreased (Figs. 4A1 and B1), whereas in second set of experiments (constant molar ratio of fluorescent to unlabeled substrates) it increased continuously (Figs. 4A2 and B2).

Comparison of the enzymatic properties of carboxylic ester hydrolase from pancreatic juice and bile salt-

TABLE 2

Comparison of the Characteristic Properties of Pancreatic Carboxylic Ester Hydrolase and Bile Salt-Stimulated Lipase Using Fluorescent P_4cholesterol as Substrate

	Carboxylic ester hydrolase	Bile salt-stimulated lipase
Substrate specificity		
Acyl chain length	"short"	"short"
P_4cholesterol/P_{10}cholesterol	1.96 ± 0.10	1.98 ± 0.12
butyryl cholesterol/oleoyl cholesterol	2.01 ± 0.11	1.99 ± 0.10
pH optimum	7.5 ± 8.0	7.5 ± 8.0
Effectors[a]		
Cholate (5 mmol/L)	195 ± 8	185 ± 7
Taurocholate (5 mmol/L)	105 ± 5	97 ± 7
Taurodeoxycholate (5 mmol/L)	23 ± 1	21 ± 3
Triton X-100		
(1 mmol/L)	110 ± 8	105 ± 5
(10 mmol/L)	55 ± 5	54 ± 4
Phosphatidylcholine		
(0.1 mmol/L)	92 ± 6	85 ± 5
(1 mmol/L)	14 ± 3	12 ± 2
E_{600}[b] (10 μmol/L)	10 ± 2	11 ± 1
Effect of human serum components		
Human serum (1%, v/v)	25 ± 3	22 ± 3
Low density lipoprotein (0.3 mg apoB/mL)	17 ± 2	15 ± 3
Lipoprotein-depleted plasma (1%, v/v)	197 ± 5	186 ± 7

[a]Enzyme activities are expressed as nmol of fatty acid liberated/min/mg protein.

[b]The enzyme was preincubated with 10 μmol/L E_{600} for 15 min at room temperature, before assaying the activity.

These results are the mean of 3 separate experiments.

stimulated lipase from milk. The pH optimum of bile salt-stimulated lipase (around 7.5–8.0) and its substrate specificity towards the fluorescent cholesteryl esters (hydrolysis ratio P_4cholesterol/P_{10}cholesterol around 2) were quite similar to those of pancreatic carboxylic ester hydrolase. The hydrolysis of P_4cholesterol by both enzymes was similarly activated by bile salts: maximal hydrolysis was observed in the presence of 5 mmol/L cholate (optimal ratio cholate/cholesteryl esters around 10). Triton X-100 and phosphatidylcholine exhibited the same inhibitory effect on the hydrolysis of P_4cholesterol by bile salt-stimulated lipase and pancreatic carboxylic ester hydrolase.

In order to study the possibility to use this fluorometric assay for determining the activity of pancreatic carboxylic ester hydrolase or bile salt-stimulated lipase in the serum, we have tested the effect of various serum components on the apparent activity of both enzymes. As reported in Table 2, the hydrolysis of the fluorescent substrates was almost completely inhibited by 1% serum. This inhibitory effect was due to the low density lipoprotein fraction, whereas the lipoprotein-depleted plasma used in the same proportion exhibited no (or only a very slight) inhibitory effect.

DISCUSSION

Fluorescent esters of cholesterol or glycerol containing "short" (P_4) or "long" chain (P_{10}) pyrene fatty acids were shown to be substrates for pancreatic carboxylic ester hydrolase. The broad substrate specificity of the enzyme is quite consistent with previous reports (3,8,9). Carboxylic ester hydrolase exhibited also a broad acyl chain length specificity towards fluorescent substrates, with a marked preference for "short" acyl chain esters. This can be explained either by a true substrate specificity or by differences in the physical state of the substrates. Such differences could considerably affect adsorption of the enzyme to lipid surfaces, its interfacial denaturation and its apparent hydrolytic activity (27,28). In order to exclude differences in the substrate physical state, we performed an assay containing "all the substrates mixed together" (mixture of "short" and "long" cholesteryl esters), assuming that all the substrates were homogenously dispersed and that the formation of domains in the supersubstrate is negligible. The results obtained under the latter conditions confirmed (i) that both natural and fluorescent "short" chain esters were hydrolyzed at the same rate and (ii) that the "short" chain esters were hydrolyzed twice as fast as the "long" chain ones. This is in good agreement with the well-known substrate specificity of the enzyme which prefers "short" chain acyl esters (13,14) and suggests that the pyrene nucleus is not a factor of major steric hindrance for the interaction of the fluorescent substrate with the enzymatic site. The data concerning the hydrolysis of P_4cholesterol by the pancreatic carboxylic ester hydrolase are in good agreement with those obtained with other cholesterol esterases of lymphoid cell lines (21). As previously discussed (21), we expected P_4cholesterol to exhibit properties similar to an ester with a 10-carbons chain based on the size of pyrene nucleus which is equivalent in length to an alkyl chain of about 5–6 carbons. P_4cholesterol was hydrolyzed by the pancreatic carboxylic ester hydrolase. However, at a rate quite similar to that of cholesterol butyrate. These results can probably be explained by the polarity of

pyrene nucleus which is greater than that of a 6-carbon alkyl chain. This relatively higher polarity of P_4cholesterol compared to that of decanoyl cholesterol (equivalent in terms of chain length) was also apparent from the difference in the relative retardation factor (R_f) values in thin-layer chromatography (data not shown). As already discussed for the hydrolysis of cholesteryl esters containing colored fatty acids by cellular cholesterol esterases (29), the apparent substrate specificity seems to be dependent on the chain length as well as on the polarity of the acyl chain (i.e., on the dispersibility of the substrate in the aqueous medium). These conclusions are in agreement with the results of Tsujita and Brockman (27), as the higher polarity of the pyrene-containing esters should favor their location at (or near) the lipid-water interface, thus increasing their accessibility to the enzyme. Similar conclusions can be drawn from the study of the hydrolysis of pyreneacyl esters of glycerol by pancreatic carboxylic ester hydrolase: triP_4G was hydrolyzed more rapidly than triP_{10}G, as tributyrin was hydrolyzed faster than triolein, in agreement with the substrate specificity of the enzyme previously reported (13,14).

The hydrolysis of fluorescent esters by pancreatic carboxylic ester hydrolase and bile salt-stimulated lipase requires the presence of bile salts. Primary bile salts are preferred, as previously reported for other lipid-soluble substrates (9,10). Primary bile salts (cholate and taurocholate) interact with a specific binding site of pancreatic carboxylic ester hydrolase inducing a dimerization of pancreatic carboxylic ester hydrolase and leading to its ability to hydrolyze lipid-soluble substrates (24,25). In contrast, sodium taurodeoxycholate, a secondary bile-salt, had a low activating effect on the hydrolysis of the fluorescent cholesteryl esters, in agreement with results previously reported for non-fluorescent lipids (30,31).

The inhibitory effect of other amphiphiles is consistent with the recent results of Tsujita and Brockman (27,28) which showed that phosphatidylcholine molecules located at the lipid-water interface prevent the adsorption of the enzyme to substrate-containing surfaces thus rendering the substrate less accessible to the enzyme.

The inhibition of pancreatic carboxylic ester hydrolase by blood plasma (or serum) can be accurately attributed to the plasma lipoproteins (Table 2). At the molecular level, this inhibitory effect could be the result of either substrate competition (if lipoproteins are a substrate for the carboxylic ester hydrolase), or of mixing of lipoprotein phospholipids with the substrate microemulsion (either by transfer of phospholipids from lipoproteins to cholesteryl ester-cholate particles, by fusion of these particles with lipoproteins, or by transfer of cholesteryl ester molecules from particles to lipoproteins). From a practical point of view, these results could explain the apparent lack of pancreatic carboxylic ester hydrolase activity in sera from normal subjects and from patients affected with pancreatitis (data not shown).

The fluorescent substrates can be used under the same conditions for assaying the bile salt-stimulated lipase from milk and pancreatic carboxylic ester hydrolase, as shown by the similarity of their hydrolytic properties (Table 2). The data are quite consistent with previous results on the functional and structural properties of both enzymes (11,13).

The sensitivity of the fluorescent assay described in this paper is dependent on the limit of detection of the fluorescent product (around 5 pmol of P4/assay). It corresponds to the theoretical limit of detection of carboxylic ester hydrolase activity of 0.3 nmol/min/mg protein, corresponding in turn to around 20 ng protein of purified enzyme per assay under the experimental conditions used. It is noteworthy that the purity of the substrate and of the reagents and solvents used is essential for optimal sensitivity. The sensitivity of the assays with fluorescent substrates is much higher than that with colored substrates as previously discussed (17,20-22) and is in the same range as that of the radiolabeled assay (the limit of which mainly results from the purity of the radiolabeled substrate). The sensitivity can be improved by increasing the concentration of the fluorescent substrate in the assay. However, this is less desirable because the pyrene-containing substrates are relatively expensive (like radiolabeled substrates). Thus, it was for "economic" reasons that in our standard fluorometric assay relatively low concentrations of both substrates were used, and that the ratios of fluorescent to unlabeled substrates were chosen (see Fig. 4A1 and B1; i.e., maximal hydrolysis of the fluorescent substrate). When compared to radiolabeled substrates, the major advantage of fluorescent substrates lies in the fact (i) that fluorescent compounds can be widely used whereas the use of radiolabeled substrates is severely restricted in several countries, (ii) that the properties of the fluorescent group (here pyrene) linked to the acyl chain can be useful for studying the properties of the enzymatic site and of the substrate-enzyme interactions (since little chemical modifications of the fluorescent group can induce some significant change in the physicochemical properties of the acyl chain, e.g. polarity, electric charge, steric hindrance), and (iii) that the fluorescent lipids can be useful for cell biological experiments (32,33).

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Effect of Temperature on the Incorporation into Phospholipid Classes and Metabolism *via* Desaturation and Elongation of n-3 and n-6 Polyunsaturated Fatty Acids in Fish Cells in Culture

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The incorporation of 18:2n-6, 18:3n-3, 20:4n-6 and 20:5n-3 was greater at 10°C than at 22°C in Atlantic salmon (AS), rainbow trout (RTG-2) and turbot (TF) cells. However, there were generally no significant differences between the amount of incorporation of all four polyunsaturated fatty acids (PUFA) into total lipid within a cell type at either 22°C or 10°C. The distributions of the PUFA between individual phospholipid classes at 22°C was essentially the same in AS and TF cells—with the C_{18} PUFA the order of incorporation in these cells was phosphatidylcholine (PC) > phosphatidylethanolamine (PE) > phosphatidic acid/cardiolipin (PA/CL); with 20:4n-6 the order was PE and phosphatidylinositol (PI) > PC; with 20:5n-3, PE > PC. In RTG-2 cells at 22°C the distributions of the C_{18} PUFA were similar to the other cell lines, but with 20:4n-6 the order was PC > PI > PE, and with 20:5n-3 it was PC > PE. At 10°C the incorporation of C_{18} PUFA into PC increased and into PE and PA/CL decreased, in general, in all cell lines. Incorporation of 20:5n-3 into PC and PE was increased and decreased at 10°C, respectively, in AS and TF cells, whereas in RTG-2 cells the changes at 10°C were opposite i.e., increased in PE and decreased in PC. With 20:4n-6, incorporation into PC at 10°C was increased in all cell lines with decreased incorporation into PI in AS and RTG-2 cells and into PE in AS and TF cells, whereas incorporation of 20:4n-6 into PE increased in RTG-2 cells. The metabolism *via* desaturation and elongation of the n-3 PUFA was greater than that of the equivalent n-6 PUFA in all cell lines, irrespective of temperature. There was less conversion of the C_{18} PUFA at 10°C than at 22°C in RTG-2 and TF cells, but the conversion of 18:3n-3 by AS cells was increased at 10°C. Temperature had no effect on the conversion of the C_{20} PUFA.
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The polyunsaturated fatty acids (PUFA) of terrestrial mammals are dominated by the n-6 series, whereas in aquatic and especially marine organisms, n-3 PUFA predominate (1–4). Mammals have an absolute requirement for 18:2n-6 (5), whereas their requirement for 18:3n-3 has been difficult to establish (6). In contrast, fish have a well defined requirement for 18:3n-3 (7) and also long chain n-3 PUFA in the case of some marine fish (7,8), whereas the qualitative and quantitative requirements for 18:2n-6 or other n-6 PUFA have not been conclusively established (7–10). However, the majority of mammalian cell lines investigated appear to grow in the absence of PUFA, both essential and non-essential (11). Routinely, most tissue culture media contain no lipid or essential fatty acids other than those present in the serum supplement. The lipids and fatty acids thus available to tissue culture cells are dependent upon the type of serum utilized. Previously, we have shown that with fish cell culture the use of fetal calf serum results in cells that are deficient in n-3 PUFA, with often increased levels of n-6 PUFA (12). This imbalance can be of major significance when the cells are used in lipid metabolism studies.

Consequently, we investigated the effects of supplementing the media with various n-6 and n-3 PUFA on the fatty acid compositions of fish cells in culture (13). Rainbow trout *(Salmo gairdneri)* (RTG-2) cells expressed Δ6 and Δ5 desaturase activities and so could elongate and desaturate 18:2n-6 and 18:3n-3 to 20:4n-6 and 20:5n-3, respectively, but Δ4 desaturase activity was apparently absent (13). However, turbot *(Scophthalmus maximus)* (TF) cells were deficient in the C_{18} to C_{20} elongase and/or Δ5 desaturase, and could not produce the C_{20} PUFA from C_{18} PUFA, but Δ4 desaturase activity was apparent (13). The desaturase activities of both cell lines showed a preference for n-3 PUFA.

These experiments were performed at 22°C, around the optimum temperature for these cell lines in culture [(14), and Dr. B. Hill, personal communication]. However, the normal ambient temperature in U.K. waters for the fish species the cell lines were derived from would rarely exceed 10°C, generally within a range of 5–15°C (15). Furthermore, temperature has been shown to have major effects on the membrane phospholipid structure and acyl chain composition in fish (16). Therefore, we have now investigated the effects of temperature on the incorporation of various ^{14}C-labelled PUFA into the phospholipids of three fish cell lines in culture. The metabolism of the incorporated PUFA *via* desaturation and chain elongation was also studied to determine the effects of temperature on this pathway in cultured cells. Both n-3 and n-6 PUFA were investigated to determine if culture temperature had differential effects on the metabolism of the two PUFA series.

MATERIALS AND METHODS

Cell lines and media. The cells were dedifferentiated lines with fibroblastic or epithelial morphology. The RTG-2 cell line (14) and the Atlantic salmon *(Salmo salar)* (AS) cell line (17) were obtained from Flow Laboratories (Rickmansworth, U.K.) and were maintained in Eagle's minimal essential medium (EMEM) containing 0.3% sodium bicarbonate and 1% non-essential amino acids. The TF cell line was supplied by Dr. B. Hill, MAFF Fish Diseases Laboratory (Weymouth, U.K.), and was maintained in

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Abbreviations: ANOVA, analysis of variance; AS, Atlantic salmon; BHT, butylated hydroxytoluene; EMEM, Eagle's minimal essential medium; FCS, fetal calf serum; GC, gas chromatography; HPLC, high performance liquid chromatography; HPTLC, high performance thin-layer liquid chromatography; PA, phosphatidic acid; PA/CL, phosphatidic acid/cardiolipin; PBS, phosphate buffered saline; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PUFA, polyunsaturated fatty acid(s); PS, phosphatidylserine; RTG-2, rainbow trout; SM, sphingomylelin; TF, turbot; TLC, thin-layer chromatography.

TABLE 1

Fatty Acid Compositions of FCS and of Total Lipid from AS, RTG-2 and TF Cells Cultured in 10% FCS at 22°C

Fatty Acid	FCS	AS	RTG-2	TF
14:0	2.4 ± 0.9	1.6 ± 0.2	1.5 ± 0.3	1.6 ± 0.2
16:0	21.8 ± 0.5	14.2 ± 0.9	15.0 ± 0.7	13.7 ± 0.9
18:0	10.4 ± 0.8	8.2 ± 2.0	5.9 ± 1.0	10.9 ± 0.8
Total Saturate[a]	37.5 ± 1.6	25.1 ± 4.1	22.9 ± 3.9	26.9 ± 2.2
16:1n-7	5.9 ± 1.2	5.9 ± 0.4	8.8 ± 0.8	8.4 ± 1.0
18:1n-9	18.7 ± 1.1	37.7 ± 9.9	42.0 ± 6.1	21.9 ± 3.9
18:1n-7	6.1 ± 0.6	3.5 ± 0.7	4.4 ± 0.7	4.0 ± 1.0
20:1n-9	1.1 ± 0.2	0.7 ± 0.4	2.3 ± 0.3	0.7 ± 0.2
Total Monoenoic[b]	34.0 ± 2.5	49.7 ± 6.7	59.8 ± 6.0	35.4 ± 5.1
18:2n-6	5.0 ± 0.6	1.8 ± 0.7	1.0 ± 0.2	1.6 ± 0.3
18:3n-6	0.2 ± 0.1	0.2 ± 0.1	*f*	0.1 ± 0.1
20:3n-6	1.7 ± 0.2	1.3 ± 0.3	0.5 ± 0.1	0.9 ± 0.2
20:4n-6	7.7 ± 1.0	5.7 ± 2.8	2.1 ± 0.4	4.3 ± 0.3
22:4n-6	0.6 ± 0.1	0.6 ± 0.6	0.2 ± 0.1	0.8 ± 0.3
Total n-6 PUFA[c]	15.7 ± 1.0	9.8 ± 2.9	4.9 ± 0.6	8.5 ± 0.8
18:3n-3	0.4 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
18:4n-3	0.2 ± 0.2	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
20:5n-3	0.8 ± 0.3	0.8 ± 0.2	0.7 ± 0.1	0.6 ± 0.1
22:5n-3	2.8 ± 0.9	2.0 ± 0.2	0.8 ± 0.2	1.2 ± 0.3
22:6n-3	2.9 ± 0.3	2.7 ± 0.1	1.5 ± 0.3	4.1 ± 1.0
Total n-3 PUFA[d]	7.1 ± 0.7	5.8 ± 0.3	3.1 ± 0.4	6.1 ± 1.1
Total PUFA[e]	23.9 ± 1.1	16.8 ± 1.9	13.2 ± 1.9	27.6 ± 3.4
n-3/n-6	0.5 ± 0.1	0.6 ± 0.3	0.6 ± 0.2	0.7 ± 0.2
Total dimethylacetals	*f*	5.1 ± 0.1	3.1 ± 0.6	5.9 ± 1.8
Total unidentified	*f*	3.3 ± 0.7	1.0 ± 0.3	4.2 ± 1.0

The fatty acid compositions are presented as a % of weight and are means ± SD (n=3). FCS, Fetal calf serum; PUFA, polyunsaturated fatty acids. Totals include small percentages of [a]15:0, 17:0 and 20:0; [b]22:1 and 24:1; [c]20:2n-6; [d]20:3n-3 and 20:4n-3; [e]n-9 PUFA; and [f]none detected.

Leibovitz L-15 medium containing 0.35% sodium chloride. Both media were further supplemented with antibiotics (50 I.U/mL penicillin and 50 μg/mL streptomycin) and 10% fetal calf serum (FCS). The fatty acid composition of the FCS is given in Table 1.

Cell growth and incubation conditions. The cells were routinely cultured at 22°C in sealed 175 cm^2 plastic flasks (Gibco-Nunc Ltd., Paisley, U.K.) in 50 mL media. For provision of experimental material, the cultures were subcultured within 24 hr of reaching confluence, usually about six days after a 1 to 4 split. Experimental cultures were transferred to the appropriate temperature (10°C or 22°C) after splitting and were grown to confluence, which was approximately 10 days at 10°C.

Incubation of cell cultures with ^{14}C-labelled PUFA. Upon reaching confluence, the medium was aspirated and the cells washed with 10 mL per flask of phosphate buffered saline (PBS), or PBS/1.75% NaCl in the case of TF cells, and 30 mL fresh media without the serum supplement, added per flask. The 1-^{14}C-labelled 18:2n-6, 18:3n-3, 20:4n-6 and 20:5n-3 (0.9 μCi, 0.6 μM per flask) were added, carrier free, in ethanol (final ethanol conc., 0.3%). One 175 cm^2 flasks was used per isotope at each temperature per experiment, and each experiment was performed in triplicate or quadruplicate. Incubation continued at the experimental temperature for a further seven days.

Harvesting of cells and lipid extraction. The medium was aspirated and the cultures washed twice with 40 mL ice-cold PBS or PBS/NaCl. The cells were harvested *via* trypsinization, washed a futher two times with 10 mL cold PBS or PBS/NaCl and the cell numbers determined by counting in a haemocytometer. Lipid was extracted essentially according to Folch *et al.* (18) as previously described in detail (12). All solvents contained 0.05% butylated hydroxytoluene (BHT) as antioxidant. Incorporation of radioactivity into total lipid was expressed relative to lipid content as the latter was unaffected by the trace mass of fatty acid added. Protein content was also constant in each cell line during these experiments and so expression of radioactive incorporation into total lipid relative to protein content gave the same statistical variation. Fatty acid analyses were performed as described previously (13).

Incorporation of radioactivity into phospholipid classes. A sample of total lipid (100 μg) was applied in a 1-cm streak onto a high performance thin-layer chromatography (HPTLC) plate and the phospholipid and other polar lipid classes separated using methyl acetate/isopropanol/chloroform/methanol/0.25% aq. KCl (25:25:25:10:9, by vol.) (19). The lipid classes were visualized by brief exposure to iodine vapor, marked, and the iodine removed under vacuum. The individual classes were scraped into scintillation minivials, 2.5 mL optifluor (Canberra

Packard Ltd., Berkshire, U.K.) added, and radioactivity determined using a Packard 2000CA Tri-Carb liquid scintillation analyzer.

Incorporation of radioactivity into PUFA. Total lipid methyl esters were separated according to degree of unsaturation on silver nitrate-impregnated thin-layer chromatography (TLC) plates (20). The plates were developed for 18 cm in toluene/ethyl acetate (90:10, v/v), dried under vacuum, and further developed for 6 cm in diethyl ether/acetic acid (90:10, v/v). The TLC plates were subjected to autoradiography for five days using Konica A2 X-ray film. After development, the labelled bands were scraped into scintillation vials and radioactivity determined as above.

Some bands (n-6 trienes and tetraenes and n-3 tetraenes and pentaenes) required further fractionation by gas chromatography (GC). The bands were eluted from the TLC plate by hexane/diethyl ether (1:1, v/v), washed with 20% NaCl to remove $AgNO_3$, and supplemented with a methyl ester mixture to facilitate the identification and collection of separated methyl esters. The methyl esters were separated in a Pye 104 gas chromatograph using a glass column (2 m × 4 mm i.d.) packed with 10% CP Wax 51 on chromosorb 100-200 mesh (Chrompack U.K., Ltd.). The instrument was equipped with a stream splitter situated before the flame ionization detector, which enabled 90% of the sample to be collected outside the chromatograph. Fractions of the column effluent corresponding to individual methyl esters were trapped on filters coated with optifluor scintillation liquid, and the filters placed in scintillation vials with a further 10 mL optifluor. Radioactivity was determined as described above.

Materials. EMEM, L-15, PBS, glutamine, sodium bicarbonate, antibiotics, FCS and trypsin/EDTA were all obtained from Flow Laboratories (Rickmansworth, U.K.). [1-^{14}C]18:2n-6 (56 mCi/mmol), [1-^{14}C]18:3n-3 (54 mCi/mmol), [1-^{14}C]20:4n-6 (55 mCi/mmol) and [1-^{14}C]20:5n-3 (58 mCi/mmol) were obtained from Amersham International, (Buckinghamshire, U.K.). BHT and silver nitrate were from Sigma Chemical Co. Ltd. (Poole, 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, Federal Republic of Germany). All solvents were HPLC grade and were obtained from Rathburn Chemicals (Walkerburn, U.K.).

Statistical analysis. All results are the means of three or four experiments ± 1 S.D. The data contained in Tables 2-7 were subjected to three-way analysis of variance (ANOVA) using a VAX mainframe with GENSTAT software for unbalanced trials. This allowed comparisons of cell type, PUFA, temperature, cell type × PUFA, cell type × temperature, PUFA × temperature and cell type × PUFA × temperature. F values (probabilities) generated by ANOVA are reported in the text where required and are specifically indicated as ANOVA data. Where appropriate, significance of differences (due to temperature) between individual means were determined by the least significant difference (lsd) test. These differences are reported in the text and Tables as significant if $p < 0.05$.

RESULTS AND DISCUSSION

The PUFA status of the cell lines grown in FCS at 22°C is shown in Table 1. The n-3 PUFA deficiency resulting in low n-3/n-6 ratios is clearly evident. In these short term experiments, reducing the culture temperature to 10°C did not significantly alter the fatty acid composition or lipid class composition of the cells cultured in 10% FCS. Due to the lack of mass effects it was necessary to use radioactively labelled tracers to investigate the effect of short-term cold stress to the cells.

ANOVA showed that there was significant variation in almost all the data due to cell type, PUFA and temperature, separately. Significant interactions were observed with cell type × PUFA and cell type × temperature, but generally not with temperature × PUFA or cell type × temperature × PUFA. The differential metabolism of various PUFA by the three cell lines has been reported earlier (13,21-24) and so generally only the effects of temperature are discussed here in detail.

There were no significant differences between the amounts of ^{14}C-labelled 18:2n-6, 18:3n-3, 20:4n-6 and 20:5n-3 incorporated into total lipid at either 22°C or 10°C, irrespective of cell type (ANOVA, $p=0.746$) (Table 2). There are few similar comparative data on other cell lines, but studies have shown no difference in the amount of incorporation of ^{14}C-labelled 20:4n-6 and

TABLE 2

The Effects of Temperature on the Incorporation of [^{14}C]PUFA into Total Lipid from AS, RTG-2 and TF Cells

	AS		RTG-2		TF	
[^{14}C]PUFA Added	10°C	22°C	10°C	22°C	10°C	22°C
18:2n-6	569 ± 152[a]	419 ± 107	532 ± 20[a]	350 ± 14	644 ± 106[a]	311 ± 53
18:3n-3	509 ± 142	529 ± 74	641 ± 6[a]	254 ± 39	639 ± 170[a]	245 ± 49
20:4n-6	480 ± 198	428 ± 89	604 ± 53[a]	223 ± 34	634 ± 84[a]	322 ± 23
20:5n-3	593 ± 198	499 ± 140	510 ± 16[a]	294 ± 35	617 ± 112[a]	327 ± 44

Confluent cultures were incubated with 0.9 μCi of [^{14}C]PUFA (final conc., 0.6 μM), added carrier free in ethanol (final conc., 0.3%), for seven days at the temperature indicated after culture at that temperature for a week. Cells were harvested and counted, lipid extracted and weighed and radioactivity determined as described in Materials and Methods. Results are dpm/μg lipid and are means ± S.D. (n=3-4).

[a]The values at 10°C were significantly different ($p < 0.05$) from those at 22°C.

20:5n-3 into human fetal skin fibroblasts (25), human keratinocytes (26) and plaice neutrophils in suspension (27). However, the total incorporation of [^{14}C]18:2n-6 into human fibroblasts was significantly less than the incorporation of C_{20} PUFA (25).

The incorporation of PUFA into the cells was greater at 10°C than at 22°C (ANOVA, $p<0.001$) (Table 2). The lsd test showed the increased incorporation was significant in RTG-2 and TF cells, but generally not in AS cells. This was due to significantly greater incorporation of [^{14}C]PUFA into AS cells at 22°C as compared to RTG-2 and TF cells (lsd, $p<0.05$), whereas there were no significant differences between the cell lines in the incorporation of any of the [^{14}C]PUFA at 10°C. The greater incorporation of the PUFA into cultured fish cells at the lower temperature is noteworthy as it reflects whole fish data, where increased PUFA in phospholipids has been reported in response to cold acclimation (28–30). Consistent with this, all four PUFA were incorporated predominantly into phospholipids of the cultured fish cells and the effect of temperature was significant (ANOVA, $p=0.011$) (Tables 3–5).

TABLE 3

The Effects of Temperature on the Incorporation of [^{14}C]PUFA into AS Cell Phospholipid Classes

Lipids	[1-^{14}C]18:2n-6		[1-^{14}C]18:3n-3	
	10°C	22°C	10°C	22°C
Total polar	86.7 ± 1.1	89.1 ± 5.1	83.2 ± 0.6[a]	86.0 ± 4.8
Total neutral	13.3 ± 1.1	10.9 ± 5.1	16.8 ± 0.6	14.0 ± 4.8
PC	49.7 ± 1.8[a]	41.9 ± 2.5	57.7 ± 2.6[a]	49.0 ± 1.8
PE	22.6 ± 1.7	27.4 ± 3.1	15.9 ± 1.7[a]	20.9 ± 1.1
PS	6.2 ± 1.4	5.9 ± 0.7	3.0 ± 0.5	3.3 ± 0.2
PI	3.5 ± 0.4	4.4 ± 0.8	1.8 ± 0.5	2.5 ± 0.4
PA/CL	3.8 ± 0.8[a]	8.1 ± 1.3	3.8 ± 1.0[a]	8.7 ± 2.3
SM	0.2 ± 0.1	0.6 ± 0.2	0.1 ± 0.1	0.3 ± 0.2
Cer/Sul	0.7 ± 0.3	0.9 ± 0.3	1.0 ± 0.8	1.4 ± 1.0
Lipids	[1-^{14}C]20:4n-6		[1-^{14}C]20:5n-3	
	10°C	22°C	10°C	22°C
Total polar	95.7 ± 1.4	96.2 ± 0.9	97.6 ± 0.3	97.5 ± 1.0
Total neutral	4.3 ± 1.4	3.8 ± 0.9	2.4 ± 0.3	2.5 ± 1.0
PC	13.9 ± 0.6	9.7 ± 0.4	29.0 ± 1.4	28.0 ± 3.4
PE	37.8 ± 1.7	39.4 ± 0.8	55.7 ± 0.7	57.8 ± 3.1
PS	4.5 ± 0.4[a]	3.7 ± 0.5	4.8 ± 0.3	4.4 ± 0.3
PI	36.7 ± 2.2	40.0 ± 0.4	5.3 ± 0.9	4.5 ± 0.6
PA/CL	2.3 ± 0.4	3.1 ± 0.9	1.8 ± 0.7	2.1 ± 0.4
SM	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
Cer/Sul	0.5 ± 0.5	0.2 ± 0.2	0.9 ± 0.9	0.6 ± 0.1

Incubation conditions are as described in Table 2. Phospholipid classes were separated *via* HPTLC and radioactive incorporation determined as described in the Materials and Methods section. Results are expressed as a percentage of total incorporation and are means ± S.D. (n=4).

[a]The values at 10°C were significantly different ($p<0.05$) from those at 22°C.

TABLE 4

The Effects of Temperature on the Incorporation of [^{14}C]PUFA into RTG-2 Cell Phospholipid Classes

Lipids	[1-^{14}C]18:2n-6		[1-^{14}C]18:3n-3	
	10°C	22°C	10°C	22°C
Total polar	94.7 ± 0.6	94.5 ± 0.7	93.2 ± 0.5	95.2 ± 1.2
Total neutral	5.3 ± 0.6	5.5 ± 0.7	6.2 ± 0.5	4.8 ± 1.2
PC	63.9 ± 0.4	60.1 ± 6.0	70.4 ± 0.6	66.1 ± 5.2
PE	16.9 ± 0.3	15.6 ± 1.8	13.5 ± 0.9	15.0 ± 2.1
PS	4.4 ± 0.3	5.2 ± 1.9	3.1 ± 0.2	3.3 ± 0.5
PI	5.0 ± 0.5	5.9 ± 0.8	1.8 ± 0.2	2.4 ± 0.8
PA/CL	3.8 ± 0.4[a]	6.1 ± 1.0	4.4 ± 1.2[a]	6.4 ± 1.4
SM	0.1 ± 0.1	0.6 ± 0.4	0.1 ± 0.1	0.5 ± 0.3
Cer/Sul	0.5 ± 0.1	1.1 ± 0.5	0.5 ± 0.2[a]	1.4 ± 0.7
	[1-^{14}C]20:4n-6		[1-^{14}C]20:5n-3	
	10°C	22°C	10°C	22°C
Total polar	97.6 ± 0.3	97.6 ± 0.2	97.9 ± 0.4	98.4 ± 0.7
Total neutral	2.4 ± 0.3	2.4 ± 0.3	2.1 ± 0.4	1.6 ± 0.7
PC	37.7 ± 1.1	36.0 ± 7.1	60.9 ± 1.4	65.3 ± 5.3
PE	29.8 ± 0.3[a]	22.6 ± 2.9	31.3 ± 0.8[a]	25.1 ± 3.7
PS	3.3 ± 0.2	3.9 ± 0.3	2.3 ± 0.1	2.7 ± 0.4
PI	25.5 ± 0.6	32.5 ± 3.8	2.3 ± 0.2	2.5 ± 0.4
PA/CL	1.0 ± 0.1	1.7 ± 0.2	0.9 ± 0.1	1.6 ± 0.4
SM	—	0.5 ± 0.1	—	0.5 ± 0.3
Cer/Sul	0.3 ± 0.2	0.5 ± 0.2	0.3 ± 0.1	0.7 ± 0.1

Incubation conditions, experimental details and expression of results are as described in Table 3, except for n=3. Footnotes as for Table 3.

The incorporation of C_{20} PUFA into phospholipids was greater than the incorporation of C_{18} PUFA into phospholipids in all cell lines irrespective of culture temperature (ANOVA, $p<0.001$) (Tables 3–5). Previously, we found that about 79% of both 20:4n-6 and 20:5n-3 were incorporated into phospholipids in plaice neutrophils (27). Similarly, 83–88% of 18:2n-6, 20:4n-6 and 20:5n-3 were incorporated into phospholipids in human fibroblasts (21), 70–80% of 20:4n-6 and 20:5n-3 were incorporated into phospholipids in human keratinocytes (26) and about 60% of 18:2n-6 and 70% of 20:4n-6 were incorporated into phospholipids in Ehrlich ascites cells (31).

The distributions of the incorporated [^{14}C]PUFA between individual phospholipid classes at 22°C was essentially the same in AS and TF cells (Tables 3 and 5). With the C_{18} PUFA, the incorporation in AS and TF cells was greatest in PC then PE, followed by PA/CL; with 20:4n-6 the incorporation was greater in PE and PI than in PC; and with 20:5n-3 incorporation was primarily into PE followed by PC (Tables 3 and 5). In RTG-2 cells at 22°C the distributions of the C_{18} PUFA were similar to the other cell lines, but with 20:4n-6 the incorporation into PI was less than into PC but greater than into PE, and 20:5n-3 incorporation into PC greatly exceeded that into PE (Table 4). In comparison, a greater incorporation into PC than into PE was reported for 18:2n-6, 20:4n-6 and

TABLE 5

The Effects of Temperature on the Incorporation of [^{14}C]PUFA into TF Cell Phospholipid Classes

Lipids	[1-^{14}C]18:2n-6		[1-^{14}C]18:3n-3	
	10°C	22°C	10°C	22°C
Total polar	93.0 ± 3.7	94.8 ± 0.7	92.7 ± 3.1	93.7 ± 1.5
Total neutral	7.0 ± 3.7	5.2 ± 0.7	7.3 ± 3.1	6.3 ± 1.5
PC	55.4 ± 0.8[a]	43.1 ± 1.5	56.7 ± 1.7[a]	42.2 ± 2.2
PE	21.6 ± 2.2	24.7 ± 1.1	21.9 ± 2.6[a]	31.0 ± 3.7
PS	4.5 ± 1.3[a]	9.4 ± 1.1	4.6 ± 1.0[a]	8.0 ± 1.1
PI	4.4 ± 1.9	5.9 ± 0.8	2.2 ± 0.4	2.7 ± 0.2
PA/CL	5.9 ± 0.4[a]	10.2 ± 0.4	6.6 ± 1.3[a]	8.7 ± 0.3
SM	0.7 ± 0.7	1.1 ± 1.1	0.4 ± 0.2	0.8 ± 0.9
Cer/Sul	0.5 ± 0.1	0.4 ± 0.1	0.4 ± 0.2	0.3 ± 0.1
Lipids	[1-^{14}C]20:4n-6		[1-^{14}C]20:5n-3	
	10°C	22°C	10°C	22°C
Total polar	97.1 ± 0.8	97.7 ± 0.5	94.7 ± 4.6[a]	97.6 ± 0.1
Total neutral	2.9 ± 0.8	2.3 ± 0.5	5.3 ± 4.6	2.4 ± 0.1
PC	20.1 ± 4.7[a]	13.8 ± 3.7	40.3 ± 3.2[a]	27.9 ± 0.9
PE	33.3 ± 0.7[a]	41.7 ± 4.6	41.8 ± 8.7[a]	59.2 ± 0.6
PS	5.3 ± 2.5	4.0 ± 1.5	3.6 ± 0.2	3.4 ± 0.9
PI	36.4 ± 8.7	35.8 ± 10.4	5.7 ± 0.6	4.1 ± 0.4
PA/CL	1.4 ± 0.5	2.1 ± 0.6	2.1 ± 0.1	2.6 ± 0.6
SM	0.2 ± 0.2	0.2 ± 0.2	0.4 ± 0.4	0.1 ± 0.1
Cer/Sul	0.3 ± 0.1	0.2 ± 0.2	0.8 ± 0.3	0.4 ± 0.3

Incubation conditions, experimental details and expression of results are as described in Table 3, except n=3. Footnotes as for Table 3.

20:5n-3 in human fibroblasts (21), 18:2n-6 in Ehrlich ascites cells (31), and 20:4n-6 and 20:5n-3 in plaice neutrophils (27). However, the incorporation of 20:4n-6 and 20:5n-3 into human keratinocytes was primarily into PE (26), as was found with 20:5n-3 and, to a lesser extent, 20:4n-6 incorporation into AS and TF cells (Tables 3 and 5). The selectivity for the incorporation of 20:4n-6 into PI in the cultured fish cells in this study was very prominent and has been previously noted in plaice neutrophils in suspension (27). This selectivity was apparent in some mammalian cells in culture (26,31), but not all (25). Based on the high proportion of 20:4n-6 found in PIs from fish tissues (32–34) and the fact that most eicosanoid synthesis in fish appears to be from 20:4n-6 despite the general preponderance of 20:5n-3 (10,35), we have postulated that PI plays an important role in the provision of eicosanoid fatty acid precursor in fish (10,32,33). The data from this study are consistent with this hypothesis.

Temperature significantly affected the incorporation of PUFA into all individual phospholipid classes in all cell lines (ANOVA, $p<0.05$) except for PI (ANOVA, $p=0.164$). In general, at 10°C the incorporation of C_{18} PUFA into PC increased, and the incorporation into PE and PA/CL decreased in all cell lines (Tables 3–5). Incorporation of 20:5n-3 into PC and PE was also increased and decreased, respectively, at 10°C in AS and TF cells (Tables 3 and 5), whereas in RTG-2 cells the changes at 10°C were opposite, i.e., increased in PE and decreased in PC (Table 4). At 10°C, the incorporation of 20:4n-6 into PC was increased in all cell lines and decreased into PI in AS and RTG-2 cells and into PE in AS and TF cells, whereas in RTG-2 cells the incorporation of 20:4n-6 into PE increased. The precise physiological significance of these changes in the incorporation or distribution of incorporated PUFA in individual phospholipid classes is unclear and there are no directly comparable data in the literature. However, there is considerable evidence for phospholipid restructuring in response to environmental temperature in fish (16), including changes in acyl chain unsaturation (28–30), proportions of PE and PC (28,30) and individual molecular species (36). The changes reported here were relatively consistent and, in particular, the increased incorporation of all PUFA into PC at 10°C (except for 20:5n-3 in RTG-2 cells) is noteworthy. Clearly, these responses to culture temperature could have important consequences for membrane structure and possibly function.

The amounts of ^{14}C-radioactivity recovered in the fatty acid fractions corresponding to the [^{14}C]PUFA added to the cells showed that the n-3 PUFA were metabolized *via* desaturation and elongation to a greater extent than the equivalent n-6PUFA in all cell lines and irrespective of culture temperature (ANOVA, $p<0.001$ for both C_{18} and C_{20} acids) (Tables 6 and 7). Similarly, Maeda *et al.* (37) found that a range of six mammalian cultured cell lines all converted [1-^{14}C]18:3n-3 to longer chain and more unsaturated fatty acids to a far greater extent than [1-^{14}C]18:2n-6. At 22°C, 35.4, 66.6 and 80.6% of [1-^{14}C]18:3n-3 compared with 9.0, 17.8 and 60.1% of [1-^{14}C]18:2n-6 were converted by AS, RTG-2 and TF cells, respectively (Table 6). Furthermore, 16.8, 17.6 and 19.2% of [1-^{14}C]20:5n-3 compared with 4.4, 2.3 and 8.0% of [1-^{14}C]20:4n-6 were converted, primarily *via* elongation, in AS, RTG-2 and TF cells, respectively (Table 7). Similarly, after four days culture, elongation of 20:5n-3 was four-fold greater than the elongation of 20:4n-6 in human skin fibroblasts (38).

The main products of 18:3n-3 metabolism at 22°C were 18:4 in TF cells, 18:4 and 20:4 in AS cells and 20:4 and 20:5 in RTG-2 cells (Table 6). Like RTG-2 cells, AS and TF cells also converted 18:3n-3 through to 20:5, but very little radiolabel was found in 22:6 in any cell line. Upon addition of [^{14}C]18:3n-3 to six mammalian cells lines, radioactivity was recovered in 18:4 and 20:4 in all lines, but in 20:5n-3 in only two lines and radioactivity was not found in 22:6 in any of the six lines (37). The main products of 18:2n-6 metabolism at 22°C were 18:3 and 20:3 in TF cells, 18:3 and 20:2 in AS cells and 18:3, 20:2 and 20:3 in RTG-2 cells (Table 6). Only small amounts of radiolabel were found in tetraene and pentaene n-6 PUFA. In comparison, only 5% and 6% of radioactivity from [^{14}C]18:2n-6 was found in 20:4n-6 when added to cultures of human skin fibroblasts (39) and keratinocytes (40), respectively, whereas with mouse keratinocytes (40) and 3T3 cells (39), the conversion of [^{14}C]18:2 to 20:4 was 13% and 16%, respectively. Maeda *et al.* (37) found that only two out of six mammalian cell lines could metabolize [^{14}C]18:2n-6 through to 20:4n-6, and in both cases the conversion was under 5%.

TABLE 6

The Effects of Temperature on the Metabolism *via* Desaturation and Elongation of Incorporated [1-^{14}C]18:3n-3 and [1-^{14}C]18:2n-6 in AS, RTG-2 and TF Cells

	[1-^{14}C]18:3n-3					
	AS		RTG-2		TF	
PUFA	10°C	22°C	10°C	22°C	10°C	22°C
18:3	58.1 ± 1.8[a]	64.6 ± 4.3	50.6 ± 3.1[a]	33.4 ± 1.1	63.2 ± 7.5[a]	19.4 ± 3.8
18:4	24.7 ± 2.9[a]	12.2 ± 1.5	21.1 ± 1.2[a]	13.1 ± 0.2	26.4 ± 8.5[a]	59.5 ± 7.7
20:3	3.4 ± 1.1[a]	5.7 ± 2.8	2.7 ± 0.2[a]	5.1 ± 0.3	3.1 ± 0.8	2.5 ± 0.8
20:4	8.9 ± 0.6[a]	11.8 ± 1.5	11.3 ± 1.0[a]	24.6 ± 1.1	4.3 ± 1.9[a]	8.1 ± 0.9
20:5	3.5 ± 0.3	4.7 ± 2.7	12.9 ± 1.8[a]	21.5 ± 2.8	1.4 ± 0.5[a]	6.0 ± 1.0
22:5	0.9 ± 0.4[a]	0.4 ± 0.3	0.6 ± 0.1[a]	1.7 ± 0.1	0.5 ± 0.2[a]	1.5 ± 0.1
22:6	0.5 ± 0.2	0.6 ± 0.3	0.9 ± 0.3	0.7 ± 0.1	0.7 ± 0.4[a]	1.3 ± 0.2
	[1-^{14}C]18:2n-6					
	AS		RTG-2		TF	
PUFA	10°C	22°C	10°C	22°C	10°C	22°C
18:2	89.3 ± 0.6	91.0 ± 2.3	85.1 ± 1.2	82.2 ± 1.3	72.5 ± 0.7[a]	39.9 ± 4.0
18:3	4.7 ± 0.5	3.0 ± 1.2	4.7 ± 0.5	4.0 ± 0.4	13.9 ± 0.6[a]	44.4 ± 2.4
20:2	3.4 ± 0.6	3.6 ± 0.9	3.8 ± 0.5	5.2 ± 1.3	4.0 ± 0.9	3.3 ± 0.4
20:3	1.8 ± 0.3	1.4 ± 0.7	4.5 ± 0.5[a]	6.8 ± 1.2	6.4 ± 2.4[a]	10.7 ± 0.7
20:4	0.3 ± 0.1	0.4 ± 0.1	1.2 ± 0.1	0.7 ± 0.2	1.2 ± 0.7	1.1 ± 0.2
22:4	0.1 ± 0.1	–	0.2 ± 0.1	0.3 ± 0.2	0.1 ± 0.1	0.5 ± 0.5
22:5	0.4 ± 0.1	0.6 ± 0.2	0.4 ± 0.1	0.7 ± 0.2	0.5 ± 0.1	0.3 ± 0.1

Incubation conditions were as described in Table 2. Total lipid was transmethylated and the fatty acid methyl esters separated and radioactive incorporation determined by a combination of silver nitrate TLC and radio GC as described in the Materials and Methods section. Results are expressed as a percentage of recovered radioactivity and are means ± S.D. (n=3-4). Footnotes as for Table 3.

TABLE 7

The Effects of Temperature on the Metabolism *via* Desaturation and Elongation of Incorporated [1-^{14}C]20:5n-3 and [1-^{14}C]20:4n-6 in AS, RTG-2 and TF Cells

	[1-^{14}C]20:5n-3					
	AS		RTG-2		TF	
PUFA	10°C	22°C	10°C	22°C	10°C	22°C
20:5	82.4 ± 2.1	83.2 ± 1.2	85.0 ± 1.1	82.4 ± 1.5	79.6 ± 0.3	80.8 ± 3.6
22:5	15.2 ± 3.9	14.1 ± 1.5	12.6 ± 0.6[a]	16.6 ± 1.3	17.2 ± 1.7	17.5 ± 3.8
22:6	2.4 ± 2.1	2.7 ± 1.1	2.4 ± 0.8	1.0 ± 0.2	3.3 ± 1.4	1.7 ± 0.2
	[1-^{14}C]20:4n-6					
	AS		RTG-2		TF	
PUFA	10°C	22°C	10°C	22°C	10°C	22°C
20:4	96.5 ± 1.2	95.6 ± 1.1	97.3 ± 0.3	97.7 ± 0.4	94.3 ± 3.4	92.0 ± 2.5
22:4	2.5 ± 0.7	3.4 ± 0.6	1.7 ± 0.2	1.6 ± 0.3	4.1 ± 1.9	6.2 ± 2.9
22:5	1.0 ± 0.7	1.0 ± 0.4	1.0 ± 0.3	0.6 ± 0.2	1.7 ± 1.5	1.8 ± 0.8

Incubation conditions, experimental details and expression of results are as described in Table 6. Footnotes as for Table 3.

The results of the present study are generally consistent with data obtained previously in unlabelled PUFA supplementation experiments (13,23). In those studies performed at 22°C, AS and RTG-2 cells exhibited significant Δ6 and Δ5 desaturase activities but lacked Δ4 desaturase activity, whereas TF cells were found to have a very active Δ6 desaturase but had a low activity of C_{18} to C_{20} elongase and/or Δ5 desaturase (13,23). However, supplementing TF cells with 20 μM 20:5n-3 at 22°C suggested that they possessed Δ4 desaturase activity, but the data presented here, using only 0.6 μM [1-^{14}C]20:5n-3 and [1-^{14}C]20:4n-6, show that very little radioactivity was associated with the products of Δ4 desaturase in all cell lines, including TF. Nonetheless, elongation to C_{22} PUFA was significant, especially with 20:5n-3 (Table 6). Overall, the results tend to suggest that the specificity for n-3 PUFA is not situated in one activity alone, but is a general characteristic of the whole desaturation/elongation pathways in these fish cell lines.

Temperature significantly affected the metabolism *via* desaturation and elongation of the C_{18} PUFA (ANOVA, $p<0.001$). There were significant decreases in the metabolism *via* desaturation and elongation of the C_{18} PUFA at 10°C as compared with 22°C in RTG-2 and TF cells, but in AS cells at 10°C the metabolism of the C_{18} PUFA was increased, significantly in the case of 18:3n-3 (Table 6). With TF cells, the most significant decreases in radioactivity in elongation/desaturation products of [1-^{14}C]-18:3n-3 and [1-^{14}C]18:2n-6 at 10°C were associated with the products of Δ6 desaturase activity, 18:4n-3 and 18:3n-6, respectively. With RTG-2 cells the most significant decreases in the amount of radioactivity at 10°C were associated with 20:4n-3 and 20:5n-3 in the case of [1-^{14}C]18:3n-3, and 20:3n-6 in the case of [1-^{14}C]18:2n-6. In contrast, the amount of radioactivity associated with the products of Δ6 desaturase activity increased at 10°C in AS and RTG-2 cells (Table 6). However, temperature had no significant effect on the conversion of the C_{20} PUFA (ANOVA, $p=0.187$) (Table 7). Overall, these results suggest that the main effects on the desaturation pathways in these cell lines of reducing the culture temperature were decreased Δ6 desaturase activity in TF cells, decreased Δ5 desaturase and/or C_{18} to C_{20} elongase in RTG-2 cells and increased Δ6 desaturase in AS cells. The Δ6 desaturase activity of RTG-2 cells may also be decreased, but the increased radioactivity in 18:4n-3 may be a consequence of reduced conversion of the 18:4n-3.

It appears that only AS cells are responding to culture temperature in the generally expected manner, i.e., lower temperature stimulating desaturase activity to increase the unsaturation of membranes. With RTG-2 and TF cells it was not clear if this was occurring. However, the mechanisms of temperature acclimation with respect to fatty acid metabolism in whole fish have also been complicated by the fact that the total capacity for the production of unsaturated fatty acids is often higher in warm- than cold-acclimated fish (16). Certainly, it appears that the direct kinetic effect of temperature on enzyme activity is an important factor in these experiments using cultured fish cells, particularly with RTG-2 and TF cells. In this respect, with the fish species these cell lines are derived from, it may be more correct to regard 10°C as the normal temperature and 22°C as the stressed temperature.

In conclusion, these experiments on the metabolism of various ^{14}C-labelled n-3 and n-6 PUFA have given considerable information of the adaptive responses of fish cells to changes in culture temperature. Lowering the culture temperature increased the amount of PUFA incorporated into total lipid, altered the distributions of the PUFA within the phospholipid classes and affected the further metabolism *via* desaturation/elongation of incorporated C_{18} PUFA. Although n-3 PUFA were metabolized in the desaturation/elongation pathways to a greater extent than n-6 PUFA, the effects of temperature were generally the same in both PUFA series.

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Phospholipid and Fatty Acid Composition of Frog (*Rana esculenta*) Liver–a Circannual Study

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Liver lipid composition of the frog *Rana esculenta* was examined on a circannual basis. In particular, phospholipid and cholesterol content, relative phospholipid distribution, and fatty acid patterns have been studied. Seasonal acclimatization is associated with significant modifications of phospholipid content and of the relative proportion of phospholipid classes, while cholesterol level is unchanged throughout the year. In regard to the fatty acid composition of total phospholipids as well as of the four major phospholipid classes—phosphatidylcholine (PC); phosphatidylethanolamine (PE); sphingomyelin (SM); phosphatidylserine (PS)—it appears that the liver of "summer animals" is characterized by a higher unsaturation index due to a decrease of saturated fatty acids and to an increased content of n-3 and n-6 polyunsaturated fatty acids. The results suggest that relevant compositional changes occur mainly in spring and autumn: these changes could be interpreted as being the result of both a nutritionally- and thermally-induced seasonal adaptation directed toward the preservation of membrane-associated physiological activities that are linked to the transition from the active to the inactive state of the animal.

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Poikilothermic animals are characterized by their ability to adapt their metabolic activities to variations of environmental conditions that occur during the normal seasonal cycle. It has been shown in amphibia that lipid, glycogen and protein contents of the liver (1–3), liver enzyme activities (4–6), blood hormone levels (7–9), and hormone sensitivity (10–13) undergo marked variations throughout the year.

On the other hand, few reports have been concerned with adaptive changes of membrane lipid composition due to changes that occur during early development, metamorphosis, or thermal acclimation (14–17). As it is well established that physicochemical characteristics of biomembranes are strongly affected both by physical (e.g. temperature, pressure, pH) and chemical parameters such as relative distribution of phospholipid classes, chain length and degree of unsaturation of the constitutent fatty acids, cholesterol content, and cholesterol/phospholipid molar ratio (18), it is of interest to investigate whether seasonal changes affect membrane composition of frog liver. This kind of investigation may lead to a better understanding of the mechanisms involved in the process of acclimatization, thus providing a basis to explain how liver modulates function in lower vertebrates.

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Abbreviations: PL, phospholipid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SM, sphingomyelin; PS, phosphatidylserine; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; PG, phosphatidylglycerol; PA, phosphatidic acid; CL, cardiolipin.

MATERIALS AND METHODS

Animals. Male and female frogs *Rana esculenta* 23.2 ± 5.5 g, average body weight ±SD) were kept in an outdoor terrarium throughout the year. Animals were fed naturally; neither forced feeding nor special diets were employed. Every month, 3–6 animals of each sex were caught at random and, after given anaesthesia with 0.2% (w/v) MS 222 (tricaine methanesulfonate), were sacrificed by decapitation. Livers were removed, washed in cold 0.15M saline, blotted, weighed and immediately used. Alternatively, excised livers were rapidly frozen and stored at −60°C.

Lipid extraction and determination. Liver lipids were extracted according to the method of Bligh and Dyer (19) in the presence of 50 μg/mL BHT (2,6-di-*tert*-butyl-*p*-cresol) to prevent oxidation. The chloroform phase was concentrated under a stream of nitrogen, and aliquots were used for phospholipid and cholesterol estimation. A preliminary purification of total lipids was carried out by thin layer-chromatography (TLC) on silica gel H plates (Merck, Darmstadt, FRG) using petroleum ether (b.p. 40–60°C)/diethyl ether/formic acid (80:20:2, v/v/v) as developing solvent. The total phospholipid fractions were then scraped off the plate and re-extracted as described above.

Phospholipid classes were separated by two-dimensional TLC using 20 × 20 cm silica gel 60 F254 coated plates (0.25 mm thick, Merck) and the solvent system chloroform/methanol/25% ammonia/water (90:54:5.5:5.5, by vol) in the first dimension and chloroform/methanol/acetic acid/water (90:40:12:2, by vol) in the second. Phospholipid fractions were visualized by ultraviolet (UV) light (at 254 nm) or by directly exposing the plates to iodine vapors, and were identified, whenever possible, by comparing their retardation factor (Rf) with those of authentic standards (Sigma, St. Louis, MO) cochromatographed under the same conditions. The silica gel areas corresponding to specific phospholipid fractions were scraped off the plate and extracted as mentioned above. Phospholipid phosphorus was determined by the method of Bartlett (20) after digestion with 70% perchloric acid according to Marinetti (21).

Cholesterol was determined by the cholesterol oxidase method using a high performance Monotest kit supplied by Boehringer (Mannheim, FRG).

Fatty acid composition. Fatty acids were methylated according to Morrison and Smith (22) and analyzed in a Perkin-Elmer 8310 gas chromatograph equipped with a flame ionization detector and interfaced with a Perkin-Elmer LC1-100 integrator. Analyses of fatty acid methyl esters were carried out on GP 3% SP 2310/2% SP-2300 on 100-120 Chromosorb W AV (column, 6′×1/8″; Supelco, Bellefonte, PA). The column temperature was programmed as follows: 5 min at 170°C, then a stepwise (3°C/min) increase to 250°C, and finally 10 min at 250°C; the nitrogen flow rate was 25 mL/min. Fatty acids were identified by comparing their retention times with those of

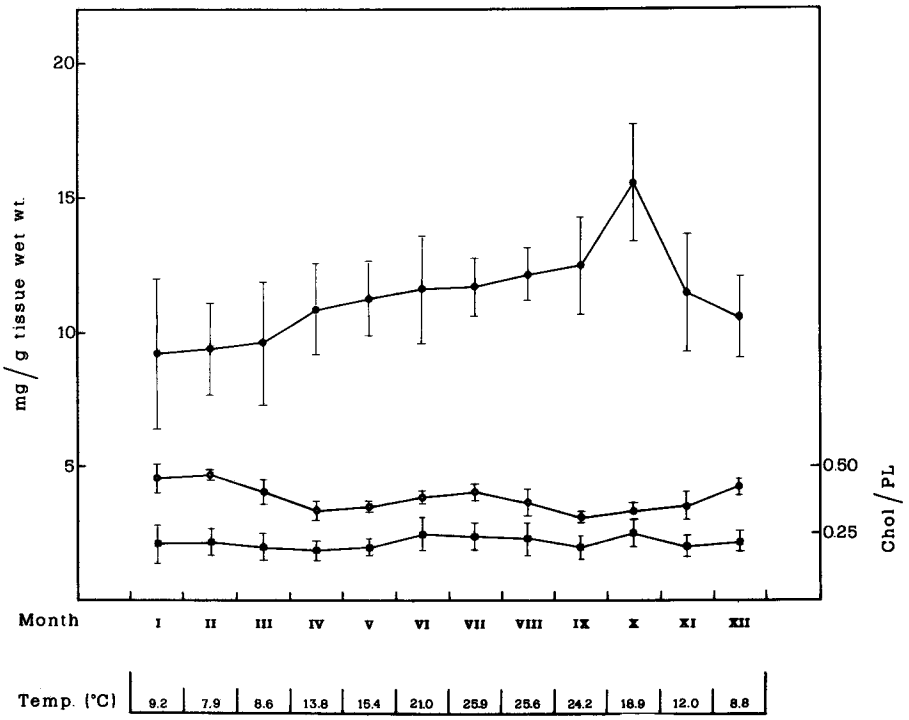

FIG. 1. Circannual variations of total phospholipids (●—●), cholesterol (■—■) and cholesterol/phospholipid (Chol/PL) molar ratio (O—O) in frog liver. Each point represents the average value obtained from 6–12 independent liver preparations carried out on an equal number of animals of both sexes; vertical bars indicate SD. For statistical analysis, see text. The average temperatures observed in Rome throughout the year (1987) are also reported.

authentic standards (Sigma). For each examined phospholipid class, fatty acids covered more than 93% of total integrated area, as assessed by the recovery of 15:0 and 17:0 internal standards.

Statistical analysis. Data were analyzed by one-way analysis of variance and by the Newman-Keuls' multiple-range test using the SPSS/PC$^+$ statistical package (SPSS Inc., 1984–1985) on an IBM-PS/2 computer.

RESULTS

Preliminary examination of the data revealed that, generally, liver phospholipid and cholesterol contents were slightly higher in female than in male frogs. Nevertheless the observed differences, because of the large individual variability, never reached statistical significance; for this reason, our results refer to mean values of pooled observations made on 6–12 animals of both sexes.

The circannual variations of total phospholipids and cholesterol contents together with cholesterol/phospholipid molar ratios are reported in Figure 1; the average temperature observed for each month in Rome in 1987 is also reported. Phospholipid content of frog liver is strongly affected by the seasonal cycle (January through December, where I corresponds to January), the differences observed throughout the year being highly significant ($p<0.005$, from analysis of variance). In particular, phospholipid content is low during winter. It gradually increases to reach its maximum in October (about 70% increase with respect to January, and significantly different —$p<0.05$— with respect to all other months as assessed by the Newman-Keuls' test), then sharply declines to winter levels. The total lipid/protein ratio did not change significantly throughout the year (not shown) consistent with previous reports (5,6). Cholesterol content does not appear to be affected by season, undergoing only limited and insignificant oscillations relative to the average level (± SD) of 2.16 ± 0.21 mg/g tissue wet weight. Conversely, a significant variation ($p<0.005$, from analysis of variance) is observed as far as the cholesterol/phospholipid molar ratio is concerned.

The relative content of different phospholipid classes of frog liver is reported in Figure 2—A,B,C. The major phospholipid classes (PC,PE,SM and PS) represent an average 83.51–94.25% of total phospholipids recovered from TLC plates, the contribution of all other phospholipid classes being between 8.12–14.48% depending on season. We were unable to identify, using a wide set of standards, a spot with R_f values 0.57 (1st dimension) and 0.41 (2nd dimension), which accounted for 1.30 ± 0.60% (average ± SD) of lipid phosphorus (not reported in the figure). Our data indicate that the large variation of total phospholipid content observed in frog liver throughout the year results mainly from the gradual increase of PC which reaches its maximum in September–October (about 50% of total phospholipids) coupled with an increase of PE with its maximum in November (about 32%). An opposite pattern of variation is observed for SM (October minimum about 10%, April maximum about 17%) and for PS (about 4% and 6% in July and April, respectively). In any case, the seasonal pattern reported for the different phospholipid classes is statistically significant ($p<0.05$ at least; by analysis of variance). The

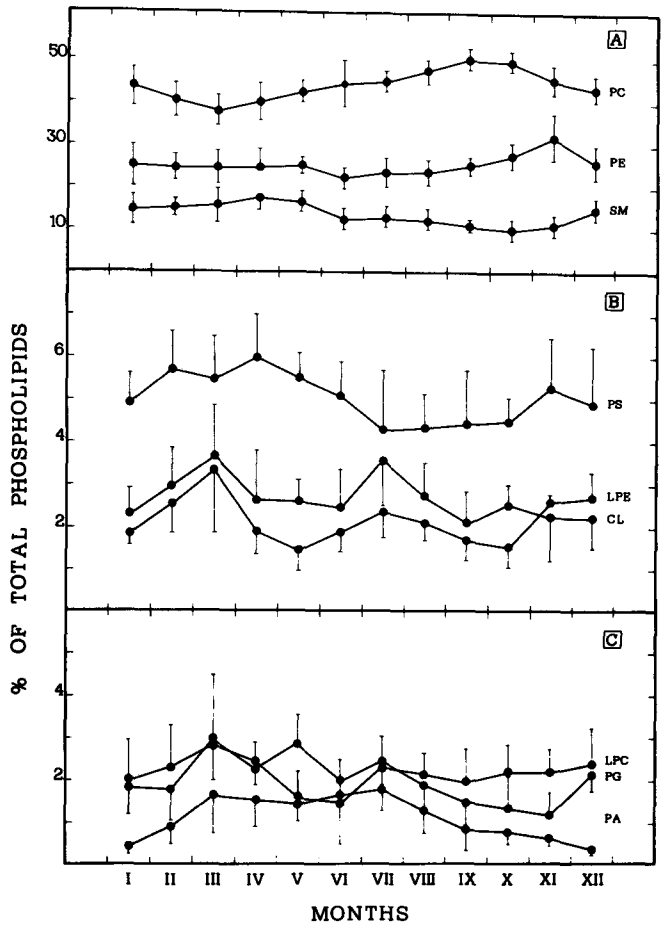

FIG. 2. Circannual variation of relative distribution of phospholipid classes in frog liver. Each point is the average of 6–12 independent preparations as in Figure 1; vertical bars indicate SD. For statistical analysis, see text.

Newman-Keuls' test indicates ($p<0.05$) the following: PC percentage observed in September and October differs from other reported values; PE percentage observed in November differs from the rest of the year; SM values observed in March-April-May differ from values observed in the June-October period.

The fatty acid distribution in frog liver phospholipids is presented in Tables 1-5. Analysis of the reported data shows that both in total phospholipid extract (Table 1) and in TLC purified phospholipid classes, namely PC,PE,SM and PS (Table 2-5), the major fatty acids are: 16:0 and 18:0 among saturated fatty acids; 16:1, 18:1 and 24:1 among monounsaturated fatty acids; and 18:2, 20:4 and 22:6 among polyunsaturated fatty acids. Furthermore, fatty acid composition appears to be characteristic for each phospholipid class with noticeable seasonal differences. When applying the Newman-Keuls' test to compounded data reported in Tables 1-5, in order to estimate significant differences ($p<0.05$, at least) shown by percentages observed for each month with respect to the rest of the year, we obtain the following results: i) In total phospholipids (Table 1) saturated fatty acids increase in November and December with respect to the rest of the year; the polyunsaturated fatty acid increase observed in the April-June period is significant with respect to November and December; the total unsaturated fatty acids increase significantly in the March-July period with respect to the November-February period; the saturated/unsaturated ratio in the November-February period is higher with respect to the April-July period; the unsaturation index in the November-February period is significantly lower with respect to the rest of the year. ii) In PC (Table 2), the saturated fatty acid percentage in February differs from that of the rest of the year; the saturated/unsaturated ratio is also higher in February with respect to the rest of the year. iii) In SM (Table 4), the saturated fatty acid percentage observed in May is significantly different from that reported for the November-February period; the saturated/unsaturated ratio in May is different form the rest of the year. iv) In PS (Table 5), the saturated fatty acid percentage in June-August-September-December is different from the rest of the year; the monounsaturated fatty acid percentage in June is different with respect to February-March and September-October; the total unsaturated fatty acid percentage in June-July-August and December is different from that observed for the rest of the year.

DISCUSSION

Phospholipids constitute the fluid matrix of biological membranes, and their quantitative and qualitative composition largely affects the functions of these structures (18,23). In poikilothermic animals, adaptive changes occur following acclimation to different environmental temperatures with the apparent purpose to maintain the fluidity of lipid bilayer, thus maintaining membrane functions as suggested by the hypothesis of "homeoviscous adaptation" (24-26). It has been reported that in amphibia, liver fatty acid unsaturation is increased in response to cold adaptation (14), but the effects of seasonal acclimatization on phospholipid composition had not been investigated previously. The results reported in this study give a first account of liver phospholipid composition in frogs under natural conditions of seasonal variability and suggest a compositional rearrangement of membranes throughout the year. Our data, although they suggest compositional changes of the average membranous structures of the cell, point to the existence of a pronounced seasonal rythmicity of: i) total liver phospholipid content; ii) cholesterol/phospholipid molar ratio; iii) relative distribution of phospholipid classes; and iv) fatty acid composition of major phospholipids. In addition, our results indicate that major modifications occur in two well-defined periods of the year, namely spring and autumn.

It is of interest in this context to remember that frog hepatocytes undergo marked morphological changes with season—the onset of the breeding period (spring) is associated with a gradual increase of membranous structures inside the cell as well as increased protein synthesis which reach their maxima when the process of gametogenesis is terminated (autumn). On the other hand, winter hepatocytes are characterized by a reduction of endoplasmic reticulum; thus the peak of total phospholipids observed in October could be related to the reported marked increase of cellular membranes (27). At the same time, the relative consistency of cholesterol levels is not surprising in view of the contradictory reports concerning the behavior of this sterol following

TABLE 1

Fatty Acid Composition of Total Phospholipids in Frog Liver

Fatty acid	Months (January-December)											
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII
14:0	0.92	1.06	0.86	1.01	0.75	0.75	0.44	0.60	0.75	0.79	1.82	1.25
16:0	28.79	28.84	25.78	25.18	24.50	19.39	26.62	27.57	28.51	31.83	25.22	27.34
16:1	5.51	4.26	6.12	6.29	6.83	7.85	6.98	7.86	7.54	5.16	5.32	4.67
18:0	11.10	10.68	4.99	4.79	5.23	3.52	6.68	5.81	4.95	5.98	26.01	22.39
18:1	17.64	17.96	14.52	15.21	19.69	18.05	23.31	20.33	17.35	13.58	12.48	16.84
18:2	17.52	16.63	20.40	19.39	20.46	20.15	17.81	16.98	16.16	14.33	2.07	4.67
20:3	tr.	0.23	0.49	0.54	0.85	0.98	0.94	0.66	0.38	0.44	0.83	1.08
20:4	9.93	7.96	13.18	12.71	12.10	13.33	9.93	10.67	11.37	9.09	6.25	8.63
20:5	1.57	2.24	1.00	1.84	0.45	1.70	3.19	2.87	2.55	5.31	6.12	2.64
24:0	0.60	0.19	0.15	0.25	0.27	0.35	tr.	tr.	tr.	0.85	tr.	0.17
22:4	0.30	1.25	1.17	1.41	0.32	0.53	tr.	tr.	tr.	0.53	0.62	0.20
22:5	1.22	2.17	2.06	2.43	2.63	2.45	tr.	tr.	tr.	tr.	2.48	0.59
22:6	5.03	6.62	9.26	9.06	8.97	8.89	9.77	10.09	10.41	12.78	12.81	9.50
Sat.[a]	41.41	40.77	31.78	31.23	30.75	24.01	33.74	33.98	34.21	39.45	53.05	51.15
Monounsat.[b]	23.15	22.22	20.64	21.50	26.52	25.90	30.29	27.59	24.89	18.74	17.80	21.51
Polyunsat.[a]	35.57	37.10	47.56	47.38	45.78	48.03	41.64	41.27	40.87	42.48	31.18	27.31
Total unsat.[a]	58.72	59.32	68.20	68.88	72.30	73.93	71.93	68.86	65.76	61.22	48.98	48.82
Sat./unsat.[a]	0.70	0.69	0.46	0.45	0.42	0.32	0.47	0.49	0.52	0.64	1.08	1.05
U.I.[a]	142.69	154.52	191.22	194.15	188.98	207.60	183.04	177.43	179.08	189.37	137.00	149.39

Average values (pooled from 3 male and 3 female frogs) are reported as relative percentages by weight; Standard Deviation did never exceed 20%. Unsaturation Index (U.I.) was calculated as the sum of the percentage by weight of each fatty acid times the number of olefinic bonds. The significance of circannual variability was assessed by one-way analysis of variance of compounded data ([a]$p<0.005$; [b]$p<0.05$) as well as by a Newman-Keuls' test (see text). Fatty acid percentages below 0.1% are indicated by tr. (traces). Sat. = saturated; unsat. = unsaturated.

TABLE 2

Fatty Acid Composition of Phosphatidylcholine in Frog Liver

Fatty acid	Months (January-December)											
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII
14:0	3.85	1.26	1.56	1.00	1.90	2.53	3.27	3.08	2.89	2.28	2.47	3.80
16:0	44.53	53.85	40.87	35.34	36.78	39.68	34.93	35.75	36.26	36.24	39.00	41.18
16:1	9.09	10.15	10.29	10.38	10.04	9.34	10.25	10.05	10.18	11.39	8.53	8.80
18:0	4.58	6.10	6.21	4.68	7.79	5.37	8.81	5.67	4.10	4.11	4.91	5.38
18:1	25.25	17.67	18.68	19.95	20.16	19.23	17.79	17.82	17.84	17.24	25.33	26.44
18:2	9.08	9.05	16.54	22.15	15.79	15.50	15.15	15.84	16.16	16.55	13.75	9.10
20:3	0.25	0.43	0.27	0.45	0.46	0.58	0.37	0.93	1.22	0.21	0.37	0.43
20:4	1.33	0.83	2.47	1.64	2.58	2.66	2.50	2.73	3.15	4.82	1.10	1.78
22:6	1.52	0.61	3.08	4.38	4.47	5.07	6.90	8.16	8.17	7.24	4.50	3.07
Sat.[a]	52.96	61.21	48.64	41.02	46.47	47.58	47.01	44.50	43.25	42.63	46.38	50.36
Monounsat.[b]	34.34	27.82	28.97	30.33	30.20	28.57	28.04	27.87	28.02	28.63	33.86	35.24
Polyunsat[a]	12.18	10.92	22.36	28.62	23.30	23.81	24.92	27.66	28.70	28.82	19.72	14.38
Total unsat.[a]	46.52	38.74	51.33	58.95	53.50	52.38	52.96	55.53	56.72	57.45	53.58	46.62
Sat./unsat.[a]	1.14	1.58	0.95	0.69	0.87	0.91	0.89	0.80	0.76	0.74	0.86	1.01
U.I.[c]	67.73	54.25	91.25	108.85	100.34	102.40	110.87	124.17	125.68	124.79	93.90	80.32

Average values (pooled from 3 male and 3 female frogs) are reported as relative percentages by weight; Standard Deviation did never exceed 20%. Unsaturation Index (U.I.) was calculated as in Table 1. The significance of circannual variability was assessed by one-way analysis of variance of compounded data ([a]$p<0.005$; [b]$p<0.05$; [c]$p<0.01$) as well as by a Newman-Keuls' test (see text). Sat. = saturated; unsat. = unsaturated.

TABLE 3

Fatty Acid Composition of Phosphatidylethanolamine in Frog Liver

	Months (January-December)											
Fatty acid	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII
14:0	4.43	2.74	1.50	2.30	2.09	2.54	1.89	2.00	2.20	3.62	3.84	4.57
16:0	28.44	29.66	21.96	27.37	29.16	25.15	24.64	23.22	22.18	24.87	27.60	28.14
16:1	7.52	6.26	6.93	7.65	5.27	5.66	7.28	8.17	8.50	9.96	8.54	8.80
18:0	12.41	12.75	12.51	15.04	14.42	15.73	13.25	10.73	9.88	9.80	10.60	11.21
18:1	25.19	25.07	21.39	28.03	20.75	23.95	21.95	25.32	26.19	25.79	25.93	26.73
18:2	8.17	8.64	11.18	11.43	11.07	8.82	9.59	9.94	9.34	7.11	8.10	6.88
20:3	0.55	0.77	0.24	0.19	0.18	0.23	0.20	0.28	0.31	0.15	0.28	0.55
20:4	4.36	4.75	13.13	5.90	8.52	8.82	9.89	10.29	10.70	6.04	5.22	5.08
20:5	3.27	3.34	2.57	1.43	2.23	1.65	2.62	2.34	2.24	4.69	4.06	4.06
22:4	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.
22:5	tr.	tr.	2.27	tr.	tr.	1.53	0.50	tr.	1.72	0.71	tr.	tr.
22:6	5.58	5.99	7.50	3.52	5.94	5.88	6.42	7.03	7.70	7.56	5.77	5.15
Sat.	45.28	45.15	35.97	44.71	45.67	43.42	39.78	35.95	34.26	38.29	42.04	43.92
Monounsat.[a]	32.71	31.33	28.32	35.68	26.02	26.91	29.23	33.49	34.69	35.75	34.47	35.53
Polyunsat.	21.93	23.49	36.79	22.47	27.94	26.93	29.22	29.88	32.01	26.26	23.43	21.72
Total unsat.	54.64	54.82	65.11	58.15	53.96	56.54	58.45	63.37	66.70	62.01	57.90	57.25
Sat./unsat.[b]	0.83	0.82	0.55	0.77	0.85	0.77	0.68	0.57	0.51	0.62	0.73	0.77
U.I.[b]	118.00	122.60	167.32	110.75	129.30	134.43	142.72	148.08	158.42	145.18	127.38	122.47

Average values (pooled from 3 male and 3 female frogs) are reported as relative percentages by weight; Standard Deviation did never exceed 20%. Unsaturation Index (U.I.) was calculated as in Table 1. The significance of circannual variability was assessed by one-way analysis of variance of compounded data ([a]$p<0.005$; [b]$p<0.05$) as well as by a Newman-Keuls' test (see text). Sat. = saturated; unsat. = unsaturated. Fatty acid percentages below 0.1% are indicated by tr. (traces).

TABLE 4

Fatty Acid Composition of Sphingomyelin in Frog Liver

	Months (January-December)											
Fatty acid	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII
14:0	4.63	1.90	2.38	2.03	2.41	3.77	3.69	3.63	4.17	3.66	4.54	6.19
16:0	38.75	42.34	39.58	36.84	32.05	32.39	36.91	36.70	38.45	33.70	30.04	28.37
16:1	11.29	7.49	7.83	8.26	9.28	9.57	9.71	9.34	7.87	10.46	12.85	14.00
18:0	6.91	4.85	13.67	13.97	23.19	9.11	9.05	9.11	9.49	6.53	6.45	8.49
18:1	19.15	13.33	16.45	16.37	15.16	25.44	22.99	24.23	23.45	27.70	28.95	26.23
18:2	4.60	4.14	3.97	4.29	4.26	7.97	6.79	6.70	7.69	4.76	5.93	7.38
20:0	0.72	0.73	0.85	0.98	1.08	0.79	0.99	0.91	0.82	0.98	0.82	0.75
22:0	0.95	0.53	0.95	1.36	1.31	2.00	1.33	1.32	1.29	1.80	0.97	1.19
24:0	2.85	1.96	2.71	3.04	3.52	2.92	2.07	2.30	2.08	3.29	2.71	2.61
24:1	10.15	22.51	12.49	12.63	7.74	6.02	6.24	5.59	4.68	7.09	6.67	4.75
Sat.[a]	54.81	52.31	60.15	58.22	63.56	50.98	54.04	53.97	56.30	49.96	45.53	47.60
Monounsat.[a]	40.59	43.33	36.77	37.46	32.18	41.03	38.94	39.16	36.00	45.25	48.47	44.98
Polyunsat[b]	4.60	4.14	3.97	4.29	4.26	7.97	6.79	6.70	7.69	4.76	5.93	7.38
Total unsat.[a]	45.19	47.47	40.74	41.75	36.44	49.00	45.73	45.86	43.69	50.01	54.40	52.36
Sat./unsat.[b]	1.21	1.10	1.48	1.39	1.74	1.04	1.18	1.18	1.29	0.99	0.84	0.91
U.I.[a]	47.52	51.62	44.73	46.05	40.71	56.98	52.74	53.79	51.38	54.78	60.34	59.76

Average values (pooled from 3 male and 3 female frogs) are reported as relative percentages by weight; Standard Deviation did never exceed 20%. Unsaturation Index (U.I.) was calculated as in Table 1. The significance of circannual variability was assessed by one-way analysis of variance of compounded data ([a]$p<0.005$; [b]$p<0.05$) as well as by a Newman-Keuls' test (see text). Sat. = saturated; unsat. = unsaturated.

TABLE 5

Fatty Acid Composition of Phosphatidylserine in Frog Liver

Fatty acid	Months (January–December)											
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII
14:0	4.07	3.35	3.95	5.93	3.15	5.10	4.40	3.91	3.91	6.46	5.31	5.65
16:0	30.16	30.58	26.84	27.30	26.44	27.14	25.68	28.54	33.56	28.45	29.07	27.70
16:1	10.49	11.31	5.93	6.35	5.13	13.13	10.27	10.09	9.83	9.21	10.68	12.45
18:0	18.08	23.11	23.45	21.70	22.03	13.03	18.22	15.77	10.81	20.07	17.64	12.26
18:1	27.76	22.66	29.36	32.14	34.53	32.33	27.23	26.24	24.71	24.33	25.00	31.24
18:2	5.97	5.64	4.37	3.88	3.45	6.83	7.86	7.21	6.76	3.77	5.47	6.36
20:3	tr.	tr.	tr.	tr.	0.44	tr.	tr.	tr.	tr.	tr.	tr.	tr.
20:4	1.77	2.25	2.45	0.77	2.29	0.66	2.85	3.17	3.83	3.09	3.18	0.95
22:4	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr	tr.	tr.	tr.
22:5	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.
22:6	1.67	2.76	3.57	1.91	2.81	1.75	3.47	4.47	6.56	4.29	3.63	3.38
24:0	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.
Sat.[a]	52.31	57.04	54.24	54.93	51.62	45.27	48.30	48.22	48.28	54.98	52.02	45.61
Monounsat.[b]	38.25	33.97	35.29	38.49	39.66	45.46	37.50	36.33	34.54	33.54	35.68	43.69
Polyunsat.[c]	9.41	10.65	10.39	6.56	8.99	9.24	14.18	14.85	17.15	11.15	12.28	10.69
Total unsat.[a]	47.66	44.62	45.68	45.05	48.65	54.70	51.68	51.18	51.69	44.69	47.96	54.38
Sat./unsat.[b]	1.10	1.28	1.19	1.22	1.06	0.83	0.93	0.94	0.93	1.23	1.08	0.84
U.I.[c]	67.34	70.83	75.28	60.82	72.86	72.31	85.45	89.78	102.78	79.54	81.05	80.52

Average values (pooled from 3 male and 3 female frogs) are reported as relative percentages by weight; Standard Deviation did never exceed 20%. Unsaturation Index (U.I.) was calculated as in Table 1. The significance of circannual variability was assessed by one-way analysis of variance of compounded data ([a]$p<0.005$; [b]$p<0.01$; [c]$p<0.05$) as well as by a Newman-Keuls' test (see text). Sat. = saturated; unsat. = unsaturated. Fatty acid percentages below 0.1% are indicated by tr. (traces.

temperature adaptation (28). The changes reported could be interpreted in terms of an adaptatory mechanism: chemical modifications of membrane lipid composition could provide a suitable microenvironment for metabolic activities associated with the transition from the active (summer frogs) to the inactive (winter frogs) state.

The observed variation of saturated/unsaturated fatty acid ratios which takes place in the transition from the active to the inactive state could be related to the feeding habits of frogs. Animals do not feed actively before the end of the breeding period (March–April) and stop eating in autumn when entering hibernation. This could also explain the increased presence of polyunsaturated n-3 and n-6 fatty acids of dietary origin. The exposure of poikilothermic vertebrates to low environmental temperatures results in an increased content of shorter chain as well as of unsaturated fatty acids in membrane phospholipids (24,29). This phenomenon was first observed by Sinensky (30) and referred to as "homeoviscous adaptation"—an adaptatory mechanism able to preserve the membrane lipid environment under optimal fluidity conditions. Such a mechanism was found to be in effect in frog liver (14) when animals caught in autumn were exposed to low temperature (7°C). Our data suggest an increased overall fluidity of liver membrane phospholipids in spring and summer, at variance with both the report of Baranska and Wlodawer (14) and the "homeoviscous adapatation" hypothesis. This discrepancy might be due to the fundamental differences existing between "acclimatization," i.e. the adaptatory mechanism to a natural ecosystem (depending on photoperiod, food availability, social factors as well as on usual environmental parameters), and "acclimation," i.e. the experimental adaptation to a single, well-controlled environmental parameter (24).

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The Association of Lysophosphatidylcholine with Isolated Cardiac Myocytes

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The ability of exogenous lysophosphatidylcholine to produce electrophysiological derangements and cardiac arrhythmias in the heart has been documented. The action of lysophosphatidylcholine is thought to be mediated via its association with the membrane. The present study examined the nature of the association of lysophosphatidylcholine with isolated rat myocyte membrane. The association was studied by incubating myocytes in a lysophosphatidylcholine-containing medium. The association of lysophosphatidylcholine with the myocyte sarcolemma was not affected by palmitic acid and glycerophosphocholine but was reduced by platelet-activating factor (PAF). The addition of albumin (5 mg/mL) at the end of the incubation period effectively removed the lysophosphatidylcholine from the myocytes. Our results suggest that most of the lysophosphatidylcholine in isolated myocytes was associated preferentially with the outer leaflet of the myocyte sarcolemma. This type of association might be responsible for the lysophosphatidylcholine-induced electrophysiological alterations in the heart.
Lipids 25, 450–454 (1990).

Lysophosphatidylcholine is the major lysophospholipid in mammalian tissues. Due to its cytolytic nature, the level of lysophosphatidylcholine in tissue is under rigid control. The accumulation of lysophosphatidylcholine in the ischemic heart has been demonstrated (1–4), and this accumulation has been postulated as one of the biochemical factors for the production of cardiac arrhythmias (1,5,6). Although the exact role of lysophosphatidylcholine in the development of cardiac arrhythmias during ischemia has yet to be established, its presence in the perfusate produces reductions of action potential amplitude, resting membrane potential and membrane excitability, occurrence of slow conduction and increases in automaticity in isolated Purkinje fibers (7–9). These electrophysiological alterations produced by exogenous lysophosphatidylcholine appear to mimic those observed in the ischemic tissue. Perfusion of the isolated heart with lysophosphatidylcholine has been shown to produce cardiac arrhythmias in a time- and concentration-dependent manner (10,11). Recently, a temporal relationship between the elevation of lysophosphatidylcholine levels and the production of arrhythmias in the ischemic heart has been established (12). These studies provide support to the notion that lysophosphatidylcholine is involved in the generation of arrhythmias during cardiac ischemia (5,6).

Although the ability to produce cardiac arrhythmias by exogenous lysophosphatidylcholine has been well documented, the underlying mechanism for the lysophospholipid to produce the membrane dysfunction is largely unknown. Using autoradiographic techniques, the majority of radiolabelled lysophosphatidylcholine associated with the cardiac tissue was found to be localized in the sarcolemma (13). The association of lysophosphatidylcholine with the sarcolemma is thought to be critical for its action to produce electrophysiological abnormalities and cardiac arrhythmias (5). However, the nature of association of lysophosphatidylcholine with the cardiac membrane is undefined.

The complexity of the whole heart is not suitable for studies on the association of lysophosphatidylcholine with cell membranes. Such association can, however, be easily examined on isolated cells which can be placed in direct contact with a buffer containing a defined concentration of lysophosphatidylcholine. In the present study, the nature of the association of lysophosphatidylcholine with the myocyte membrane was investigated.

MATERIALS AND METHODS

Materials. 1-[1-^{14}C]Palmitoyl lysophosphatidylcholine (specific radioactivity, 58.5 mCi/mmol) was purchased from Amersham (Oakville, Canada). Joklik minimum essential medium was obtained from Gibco (Burlington, Canada). Collagenase (from *Clostridium histolyticum*) was purchased from Boehringer Mannheim (Dorval, Canada). Hyaluronidase (type I-S), lysophosphatidylcholine (from egg yolk), glycerophosphocholine, platelet-activating factor (PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine from bovine heart), palmitic acid and bovine serum albumin were obtained from Sigma (St. Louis, MO). Lidocaine was a product of Astra Pharmaceuticals (Mississauga, Canada). Other chemicals and reagents were obtained from Fisher Chemical of Canada (Edmonton).

Myocyte isolation. Cells were isolated as described by Bihler *et al.* (14). Briefly, male Sprague-Dawley rats, 250–300 g, were sacrificed by cervical dislocation and the hearts were rapidly removed. The hearts were perfused in the Langendorff mode for 5 min with a modified Ca^{2+}-free Joklik minimum essential medium maintained at 37°C and aerated with 95% O_2/5% CO_2. Subsequently, the hearts were perfused with the same medium containing 1 mg/mL collagenase and 1 mg/mL hyaluronidase for 20 min. After perfusion, the ventricles were cut into small pieces and incubated in a shaking water bath with a Ca^{2+}-free medium containing 30 mM K^+. The disaggregated cells were filtered through nylon mesh and washed twice in Ca^{2+}-free medium containing 2% and 0.1% bovine serum albumin, respectively. Batches of highly purified rod-shaped myocytes were obtained by this procedure. The cells were quiescent and displayed functional integrity. In addition, the myocytes were not hypersensitive to Ca^{2+}

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Abbreviation: PAF, platelet-activating factor.

and would survive in Ca^{2+}-containing media for long periods of time (14).

Labelling of myocytes with [^{14}C]lysophosphatidylcholine. The myocytes were washed twice in albumin-free Joklik medium containing 1.25 mM Ca^{2+} and 5.4 mM K^+ (pH 7.4). The cells were suspended in the same buffer and aliquots of the suspension (final concentration 30–60 μg protein/mL) were incubated in Joklik medium containing 50 μM labelled lysophosphatidylcholine (2.5 nCi/mL). Incubations without myocytes were used to determine the background counts. After incubation, an aliquot (1 mL) of the incubating mixture was layered on top of 400 μL of dibutylphthalate/dioctylphthalate (3:1, v/v) placed in a microcentrifuge vial. The preparation was centrifuged at 12,000 × *g* for 15 s and 200 μL of the medium (top layer) was saved for further analysis. Subsequent to the removal of the remaining medium and oil, the cell pellet in the microcentrifuge vial was digested overnight by incubation with 500 μL of tissue solubilizer. Both the radioactivity in the medium and in the digested cell pellet were determined by liquid scintillation counting. Background was determined by conducting experiments in the absence of myocytes and accounted for 1.7 ± 0.7% of total radioactivity (n=9).

The protein concentrations of the myocyte suspensions were determined by the method of Lowry *et al.* (15). The amount of lysophosphatidylcholine associated with the myocytes was expressed as nmol lysophosphatidylcholine/mg protein. Analysis of variance and Student's t-tests for paired or unpaired data were used for statistical analyses where appropriate. Regression analysis was used to determine the slope and intercept of the double reciprocal plot of the amount of lysophosphatidylcholine associated with the myocytes *versus* the concentrations of the lysophospholipid in the medium.

RESULTS

The amount of lysophosphatidylcholine associated with the myocytes was determined by incubating the myocyte suspension with medium containing labelled lysophosphatidylcholine. Subsequent to incubation, the myocytes were separated from the incubating medium by sedimentation through an oil layer (dibutylphthalate/dioctylphthalate). The effect of temperature on the association of lysophosphatidylcholine with the myocytes was determined. There was no significant difference in the amount of lysophosphatidylcholine associated with the myocytes at 23, 30 and 37°C over various time periods of incubation. Hence, all subsequent studies were conducted at 23°C. The time course for the association of lysophosphatidylcholine with the myocytes is depicted in Figure 1. The amount of lipid associated with the myocytes was linear up to 10 min of incubation. The amount of lysophosphatidylcholine associated with the myocytes as a function of the lipid concentrations in the medium is illustrated in Figure 2. Analysis of the data by double reciprocal plot showed that the association constant (K_m) of lipid with myocytes is 9.0 μM of lysophosphatidylcholine.

The effects of lidocaine and Ca^{2+} concentration on the association of lysophosphatidylcholine with the myocytes are summarized in Table 1. Although both lidocaine and reduced Ca^{2+} concentrations were shown to decrease lysophospholipid-induced arrhythmias in perfused rat hearts (16,17), the presence of lidocaine or alteration of the Ca^{2+} concentration in the medium did not affect the association of lysophosphatidylcholine with the myocytes. The effects of palmitic acid, glycer-

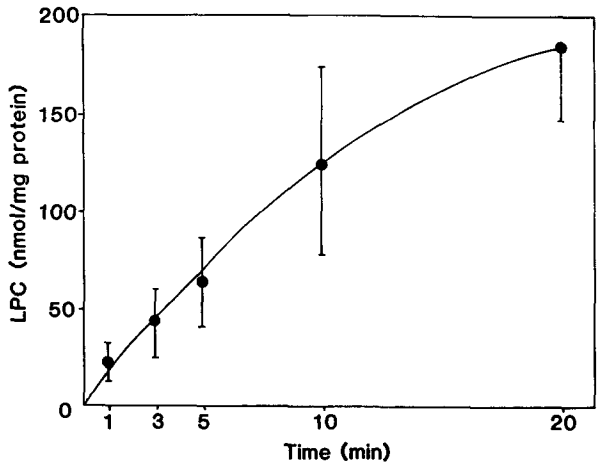

FIG. 1. The effect of time on the association of lysophosphatidylcholine (LPC) with isolated myocytes. Experiments were performed as described in the Method section. Myocytes were incubated at 23°C in a medium containing 50 μM lysophosphatidylcholine for the various time periods. Values represent mean ± standard deviation of 4 to 6 separate experiments.

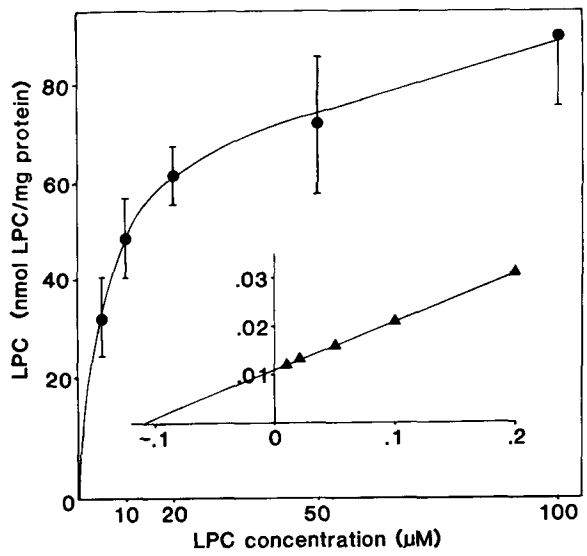

FIG. 2. The effect of lysophosphatidylcholine (LPC) concentration on the association of lysophosphatidylcholine with isolated myocytes. Myocytes were incubated at 23°C in a medium containing various concentrations of lysophosphatidylcholine. The amount of lysophosphatidylcholine associated with the myocytes was determined after 5 min of incubation. Inset represents the double reciprocal plot of the same data. The slope and intercepts were determined by linear regression. Values represent mean ± standard deviation of 8 to 10 separate experiments.

TABLE 1

The Effects of Lidocaine and Ca^{2+} Concentration on the Association of Lysophosphatidylcholine with Isolated Myocytes[a]

	Lysophosphatidylcholine (LPC) associated with myocytes (nmol LPC/mg protein)	n
Control	81.7 ± 17.2	10
Lidocaine 10 μg/mL	81.5 ± 12.0	10
Lidocaine 20 μg/mL	81.5 ± 9.7	10
Control	72.0 ± 12.2	10
Low Ca^{2+} (0.625 mM)	77.0 ± 9.7	10
High Ca^{2+} (2.5 mM)	81.0 ± 10.3	10

[a]The amount of lysophosphatidylcholine associated with the isolated myocytes was determined after 5 min of incubation with 50 μM lysophosphatidylcholine at 23°C. Values represent mean ± S.D., n = number of experiments each determined in triplicate.

TABLE 2

The Effects of Platelet-Activating Factor, Glycerophosphocholine and Palmitic Acid on the Association of Lysophosphatidylcholine with Isolated Myocytes[a]

	Lysophosphatidylcholine (LPC) associated with myocytes (nmol LPC/mg protein)	n
Control	70.4 ± 12.8	9
Platelet-activating factor	36.8 ± 5.9**	9
Control	84.0 ± 26.2	9
Glycerophosphocholine	76.8 ± 24.3	9
Control	70.9 ± 14.6	8
Palmitic Acid	73.5 ± 9.0	9

[a]The amounts of lysophosphatidylcholine associated with isolated myocytes in each group were determined after 5 min of incubation with 50 μM lysophosphatidylcholine at 23°C in the absence (control) and the presence of 50 μM of the test compounds. Values represent mean ± S.D., n = number of experiments each determined in triplicate.

**P<0.01 with the respective control.

ophosphocholine and platelet-activating factor (PAF) on this association were also investigated and the results are depicted in Table 2. Palmitic acid and glycerophosphocholine, the metabolic products of lysophosphatidylcholine, did not affect the association of lysophosphatidylcholine with the myocytes. However, the presence of PAF significantly reduced such association. The mechanism for the attenuation of lysophosphatidylcholine association with the myocytes by PAF (50 μM) was investigated by a kinetic approach and the results are shown in Figure 3. Analysis of the results by a double reciprocal plot revealed that PAF inhibited the association of lysophosphatidylcholine with the myocytes in a competitive manner.

The association of lysophosphatidylcholine with the myocytes might be limited to the outer leaflet of the sarcolemma or might include the incorporation of the lysophospholipid to the inner leaflet of the sarcolemma and other cellular components. In order to identify the amount of lysophosphatidylcholine associated with the outer leaflet of the sarcolemma, the lysophospholipid in the outer leaflet was removed by albumin (18,19). The ability of albumin to remove lysophosphatidylcholine from the outer leaflet of the plasma membrane has been well documented (18). Myocytes were incubated with lysophosphatidylcholine-containing medium, and fatty-acid free albumin (5 mg/mL) was added to the mixture after 5 min of incubation. The myocytes were separated from the medium by centrifugation through the oil layer, and the radioactivity in the digested cell pellet was determined. Lysophosphatidylcholine associated with the outer leaflet of the sarcolemma was estimated from the difference in radioactivity in the sedimented myocytes with or without albumin treatment after incubation. As depicted in Table 3, only 1.6% of total radioactivity associated with the myocytes remained in the pellet after albumin treatment. The result suggests that the vast majority of the lysophosphatidylcholine associated with the myocytes was located in the outer leaflet of the sarcolemma. The lysophosphatidylcholine in the outer leaflet could be removed by repeated washing of the myocytes with buffer (Table 3).

DISCUSSION

In previous studies, the association of lysophosphatidylcholine with erythrocytes was examined by incubating the cells with a lysophospholipid-containing medium. Subsequent to incubation, the cells were separated from the medium by centrifugation, and washed extensively with either normal or albumin-containing solutions (18). In order to calculate the total amount of lysophosphatidylcholine associated with the cells, the radioactivity in the erythrocytes after each wash was employed to extrapolate the value for zero wash (18). In receptor binding studies, the millipore filtration technique was used to separate the cells or cell fragments from the radioactivity in the medium, thus eliminating the need for centrifugation and extrapolation of data. However, preliminary experiments with the millipore filtration technique in the absence of myocytes showed that the background remained high even after extensive washing. The high affinity of lysophospholipid to the filter material made this relatively simple procedure unsuitable for our study. The present technique (by sedimentation of the myocytes through an oil layer) provided us with a rapid and clean procedure to separate the myocytes from the medium. Less than 1% of total radioactivity associated with the myocytes was lost during transit through the oil layer. In addition, there was no substantial trapping of the medium by the myocytes.

One limitation of the present study is the range of incubation time and the concentrations of lysophosphatidylcholine used to examine its association with the myocytes. Maximum association was not observed even after 20 min of incubation and with the highest concentration of lysophosphatidylcholine tested (100 μM). Due to the cytolytic nature of lysophosphatidylcholine (20), studies at higher concentrations and longer incubation time were not feasible. It should be noted that arrhythmias and electrophysiological alterations were observed in the cardiac tissues and the isolated heart within

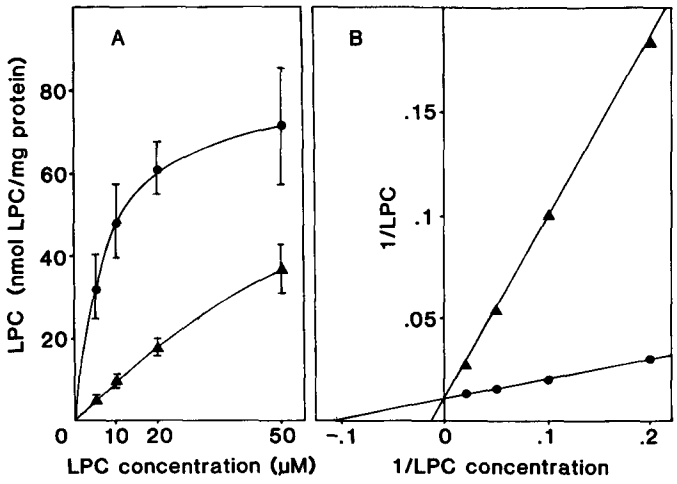

FIG. 3. The effect of PAF on the association of lysophosphatidylcholine (LPC) with isolated myocytes. The amount of lysophosphatidylcholine associated with myocytes was determined as described in Figure 2. The symbol ● represents controls and ▲ represents the presence of 50 μM PAF in the incubation medium. Panel B shows the double reciprocal plot of the data in panel A. The slopes and intercepts were determined by linear regression. Values represent mean ± standard deviation of 4 to 6 separate experiments.

TABLE 3

The Effects of Albumin and Washing on the Association of Lysophosphatidylcholine with Isolated Myocytes[a]

	Radioactivity remaining with myocytes (%)	n
Myocyte pellet through oil layer		
Control	100	10
Addition of albumin	1.6 ± 0.4	4
Myocyte pellet after washing with medium		
1 wash	58	2
5 washes	4	2
10 washes	1	2

[a]Isolated myocytes were incubated with a medium containing lysophosphatidylcholine (50 μM) for 5 min at 23°C. Fatty-acid free albumin (5 mg/mL) was added directly after incubation. After 5 min, the myocytes were separated from the medium by centrifugation through an oil layer as described in the method section. Myocyte pellets obtained before the addition of albumin were used as controls (100%). In another study, the myocytes after incubation were washed in Joklik medium. After centrifugation, the supernatant was removed, and the myocytes were resuspended with an equal volume of medium. The radioactivity associated with the myocytes after each wash was determined. The loss of myocytes (16, 51 and 70% after 1, 5 and 10 washes, respectively) was corrected in the calculation of radioactivities. Values represent mean ± S.D., n = number of experiments each determined in triplicate.

minutes after exposure to 5-100 μM lysophosphatidylcholine.

The ability to separate rapidly the myocytes from the incubation medium enabled us to study the association of lysophosphatidylcholine with the myocytes under these conditions. From the time and concentration studies, it is clear that such association followed Michaelis-Menten kinetics and that most of the lysophospholipid was associated with the outer leaflet of the sarcolemma. This is not surprising since most of exogenous lysophosphatidylcholine in erythrocytes after a short incubation period was also found to be located in the outer leaflets of the plasmalemma. Our results revealed that after 5 min of incubation, a large amount of lysophosphatidylcholine (70-85 nmol/mg protein) was associated with the myocytes. One facile explanation is that most of the lysophosphatidylcholine associated with the outer leaflet of the myocyte sarcolemma was in the micellar form since the concentrations of lysophosphatidylcholine used in the present study (5-100 μM) were above the critical micelle concentration (20). In view that lidocaine and Ca^{+2} have profound effects on lysophosphatidylcholine-induced cardiac arrhythmias, it was not clear if their actions were mediated via the modulation of the lysophospholipid associated to the sarcolemma. Our results indicate that their effects appear to be independent of lysophosphatidylcholine binding to the sarcolemma.

An important role for the association of lysophosphatidylcholine with the sarcolemmal membrane in order for the lysophospholipid to exert its arrhythmogenic effect has been suggested (5). Indeed, the data of Akita *et al.* (21) showed that intracellular microinjections of lysophosphatidylcholine to raise the intracellular concentration of lysophosphatidylcholine did not produce any significant electrophysiological effects. Their data is compatible with the concept that only lysophosphatidylcholine associated with the sarcolemmal membrane is capable of exerting its arrhythmogenic action. At present, the exact mechanism for the association of the lysophosphatidylcholine with the outer sarcolemma leaflet is still unknown. Studies on the association of lysophosphatidylcholine with the sarcolemma in the presence of its metabolites and analog revealed that the association was inhibited by PAF but not glycerophosphocholine or fatty acid. We postulate that the amphipathic nature of lysophospholipid is an important factor for its association with the sarcolemma of the myocytes.

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Influence of Dietary Linoleic Acid and *trans* Fatty Acids on the Fatty Acid Profile of Cardiolipins in Rats

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Cardiolipins (CL) have unique fatty acid profiles with generally high levels of polyunsaturated fatty acids, primarily 18:2n-6, and low levels of saturated fatty acids. In order to study the effect of dietary fatty acid isomers on the fatty acid composition of cardiolipins, rats were fed partially hydrogenated marine oils (HMO), rich in 16:1, 18:1, 20:1, and 22:1 isomeric fatty acids, supplemented with linoleic acid at levels ranging from 1.9% to 14.5% of total fat. Although the dietary fats contained 33% *trans* fatty acids, the levels of *trans* fatty acids in CL were below 2.5% in all organs. The fatty acid profiles of cardiolipins of liver, heart, kidney and testes showed different responses to dietary linoleic acid level. In liver, the contents of 18:2 reflected the dietary levels. In heart and kidney, the levels of 18:2 also parallelled increasing dietary levels, but in all groups fed HMO, levels of 18:2 were considerably higher than in the reference group fed palm oil. In testes, the 18:2 levels were unaffected by the dietary level of 18:2 and HMO.
***Lipids 25*, 455–459 (1990).**

Partially hydrogenated fats of vegetable or marine origin are used in the production of spreads and margarines (1). Dietary *trans* and other isomeric fatty acids, which are constituents of partially hydrogenated fats, are readily incorporated into the major phospholipids, such as phosphatidylcholines (PC) and phosphatidylethanolamines (PE), as well as into triglycerides and cholesterol esters (2–4). They also have been reported to pass the placental barrier in the rat (5). Furthermore, the occurrence of *trans* fatty acids has been demonstrated in human tissues, such as heart and adipose, at levels of 0.1–5% (6,7). Effects of *trans* fatty acids on the activities of desaturases, which convert linoleic acid to long-chain polyunsaturated fatty acids, have also been reported (8). Few studies on the effects of isomeric fatty acids, however, have included the cardiolipins which typically are located in the inner mitochondrial membrane. Only data on the cardiolipins of liver and heart have previously been published which showed that the level of *trans* fatty acids in cardiolipins (CL) is in the range of 1–2% when *trans* fatty acids in PC or PE reach about 15% (9,10).

It has been demonstrated that cytochrome oxidase, which is the terminal enzyme of the mitochondrial respiratory chain, has a specific requirement for cardiolipins (CL) (11). Thus, the fatty acid composition of the dietary lipids may affect the activity of cytochrome oxidase (12).

The objective of this paper is to show how the dietary level of linoleic acid affects the fatty acid composition of cardiolipins in various organs of rats fed diets containing partially hydrogenated fats with a high content of *trans* fatty acids, either 16:1, 18:1, 20:1, and 22:1 from marine oil, or 18:1 from vegetable oil.

MATERIALS AND METHODS

Seven groups of male weanling Wistar rats, each group consisting of 8 animals, were fed diets containing 20 wt% of fat (Table 1). A detailed analysis of the dietary fats and a description of the analytical procedures have been published previously (13). In groups 1–5, the dietary fats consisted of 16 wt% of hydrogenated marine oils (HMO) plus 4 wt% of different combinations of olive oil (OO) and sunflower seed oil (SO). The total *trans* fatty acid content of these fats was 33%, and the linoleic acid content ranged from 1.9% to 14.9%. Group 6 was fed 10 wt% of partially hydrogenated soybean oil (HSOY) plus 9 wt% OO and 1 wt% SO, providing 32% *trans* fatty acids and 8% linoleic acid. Group 7 was fed palm oil containing 10.1% linoleic acid and no *trans* fatty acids. After 10 weeks, the rats were killed by decapitation. Liver, heart, kidneys, and testes were extracted according to Folch *et al.* (14). The lipids were separated by thin-layer chromatography (TLC) using a two-step procedure to avoid contamination of CL with free fatty acids (13). The cardiolipins were saponified and the fatty acids were esterified (13). The fatty acid methyl esters were analyzed by gas-liquid chromatography (GLC) (1) using 10% SP-2330 as stationary phase for the separation of total fatty acids and 10% OV-275 for measuring the distribution of *cis* and *trans* isomers. The identity of specific polyunsaturated fatty acids, such as 20:3n-6, 20:3n-9 and 20:4n-6, was verified by capillary GLC on a 0.32mm × 50m Sil-88 column (Chrompack, Middelburg, The Netherlands).

RESULTS AND DISCUSSION

The fatty acid profiles presented (Tables 2 and 3) demonstrate that dietary fats affect CL in various organs to a different extent. A common feature, however, is that very little *trans* fatty acid is found in CL. Only *trans* 16:1, *trans* 18:1, and *cis,trans* 18:2 are found, whereas no *trans* 20:1 or *trans* 22:1 fatty acids were incorporated into CL. In liver and heart, primarily *trans* 18:1 is deposited. Previous studies by Sgoutas *et al.* (9) also demonstrated that rats fed 20 wt% of partially hydrogenated soybean oil plus 2% corn oil had approximately 3% *trans* 18:1 in liver CL. Hsu and Kummerow (15) found high levels (18–20%) of *trans* 18:1 in heart CL from rats fed partially hydrogenated fats with or without supplementation with corn oil. In

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Abbreviations: CL, cardiolipin; HMO, partially hydrogenated marine oil; PC, phosphatidylcholine; PE, phosphatidylethanolamine; OO, olive oil; SO, sunflower seed oil; HSOY, partially hydrogenated soybean oil; PALM, palm oil; TLC, thin-layer chromatography; GLC, gas-liquid chromatography.

TABLE 1

Fatty Acid Compositions of Dietary Fats[a]

Dietary fat	16% HMO + 4% OO	16% HMO +3% OO +1% SO	16% HMO +2% OO +2% SO	16% HMO +1% OO +3% SO	16% HMO +4% SO	10% HSOY +9% OO +1% SO	20% PALM
Fatty acid	(%)	(%)	(%)	(%)	(%)	(%)	(%)
Saturated acids	30.1	29.8	29.6	29.5	29.3	16.1	51.8
14:1	0.4	0.4	0.4	0.4	0.4	—	—
16:1 *t*	5.2	5.2	5.2	5.2	5.2	0.1	—
16:1 *c*	3.1	3.0	2.9	2.9	2.8	0.6	—
18:1 *t*	7.4	7.4	7.4	7.4	7.4	26.6	—
18:1 *c*	19.7	16.8	13.9	11.4	8.2	42.7	36.8
20:1 *t*	6.6	6.6	6.6	6.6	6.6	—	—
20:1 *c*	5.9	5.9	5.9	5.9	5.9	—	—
22:1 *t*	6.6	6.6	6.6	6.6	6.6	—	—
22:1 *c*	5.1	5.1	5.1	5.1	5.1	—	—
c,t/t,c dienes	7.4	7.4	7.4	7.4	7.4	4.7	—
18:2 *c,c*	1.9	5.1	8.2	11.3	14.5	8.0	10.1
Σ *trans* fatty acids	33	33	33	33	33	32	—
Ratio % 18:2/% *trans*	0.06	0.15	0.25	0.34	0.44	0.25	—

[a]OO, olive oil; SO, sunflower seed oil; HSOY, partially hydrogenated soybean oil; PALM, palm oil.

agreement with our data, Blomstrand and Svensson (10) reported low contents of *trans* fatty acids in heart CL from rats fed 20% partially hydrogenated peanut oil, containing *trans* 18:1, or 20% partially hydrogenated capelin or herring oil, containing *trans* 16:1, *trans* 18:1, *trans* 20:1, and *trans* 22:1, all supplemented with sufficient linoleic acid. Our data for liver and heart also demonstrate that incorporation of *trans* fatty acids in CL is independent of the dietary levels of 18:2 within a wide range.

Apparently, no data on the influence of partially hydrogenated fats on the fatty acid profiles of CL from kidney and testes have previously been published. In kidney, the major *trans* fatty acid is *trans* 16:1 indicating that β-oxidation of longer chain fatty acids, i.e. 20:1 and 22:1, beyond 18:1 has taken place, contrary to what appears to take place in liver and heart. In testes CL, no *trans* fatty acids were found. This agrees with the observations by Jensen (16) who fed to rats purified 20:1 or 22:1 fractions from partially hydrogenated fish oil and found little incorporation of monoenes into testicular total lipids. Apparently, *trans* fatty acids, irrespective of their chain length, are not deposited in testicular CL.

The dietary levels of linoleic acid influence the organ polyunsaturated fatty acids (PUFA) profiles in various ways. In the liver, an increase in the dietary level of 18:2 is reflected in CL. Furthermore, the presence of partially hydrogenated marine oils (HMO) or HSOY in the diet does not affect the level of 18:2 in CL, as seen for groups 3,6, and 7, which were fed similar levels of 18:2. At low dietary levels of 18:2 (groups 1 and 2), significant incorporation of 20:3n-6 into the CL took place which probably reflects inhibition of the Δ5-desaturase by HMO (8,17).

In heart CL of rats fed HMO, the level of 18:2 is substantially higher than the levels found in HSOY- and palm oil (PALM)-fed rats, even at low dietary concentrations. Furthermore, substantial levels of 20:3n-6 are found in CL of the rats fed HMO. This may reflect reduced conversion of 18:2n-6 into 20:4n-6 and 22:5n-6 (13) in the heart increasing the 18:2n-6 and 20:3n-6 pools in cytidine diphosphate-diglycerides (CDP-DG), which apparently provide substrates for formation of CL. Both HMO and HSOY seem to interfere with the formation of 22:5n-6, which is a prominent fatty acid in the heart, which is also associated with phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (13).

In the kidney, increased incorporation of 18:2n-6 into CL is also observed upon feeding HMO even at low dietary levels of 18:2, reflecting the larger metabolic pool of 18:2 that is available for incorporation into CL. This could be a result of decreased conversion of n-6 fatty acids due to inhibition of Δ6- and Δ5-desaturation, which is also evidenced by the low levels of 20:4n-6 in the HMO-fed groups. Contrary to the heart, kidney CL does not contain any C_{22} fatty acids, in agreement with the low levels of these fatty acids found in other kidney phospholipids (18).

The fatty acid profiles of testicular CL demonstrate little dependence on dietary fatty acids, but are remarkably different from CL of other organs in containing high levels of palmitic acid, low levels of linoleic acid, and in being rich in 22:5n-6. The data indicate that HMO even at low levels of dietary 18:2 does not interfere with the fatty acids in testicular CL. The 22:5n-6 is a fatty acid which is typically found in lipids of rat testes (18). The high levels of 22:5n-6 observed could be indicative of a very efficient conversion of 18:2n-6 favored by the absence of *trans* fatty acids in this organ or by conversion of arachidonic acid formed in other organs, e.g. the liver, as demonstrated by Blank *et al.* (19).

TABLE 2

Fatty Acid Compositions of Cardiolipins from Rat Liver and Heart

Dietary fat	16% HMO+ 4% OO	16% HMO+ 3% OO+ 1% SO	16% HMO+ 2% OO+ 2% SO	16% HMO+ 1% OO+ 3% SO	16% HMO+ 4% SO	10% HSOY+ 9% OO+ 1% SO	20% PALM
Fatty acid	(%)	(%)	(%)	(%)	(%)	(%)	(%)
Liver							
12:0	0.6 ± 0.1	0.5 ± 0.2	0.6 ± 0.1	0.6 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.5 ± 0.2
14:0	0.6 ± 0.0	0.6 ± 0.1	0.6 ± 0.1	0.4 ± 0.2	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
16:0	2.5 ± 0.3	2.0 ± 0.3	2.9 ± 0.3	1.7 ± 0.2	1.3 ± 0.1	2.2 ± 0.2	2.0 ± 0.2
16:1 *t*	0.5 ± 0.0	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.5 ± 0.1	0.6 ± 0.0	—
16:1 *c*	6.4 ± 0.3[a,b]	4.8 ± 0.2[a,b]	4.1 ± 0.3[b]	3.5 ± 0.4[a,b]	3.2 ± 0.2	2.6 ± 0.6[a]	4.0 ± 0.5
18:0	1.2 ± 0.5	0.7 ± 0.3	2.5 ± 0.7	0.4 ± 0.1	0.3 ± 0.0	0.5 ± 0.1	0.4 ± 0.1
18:1 *t*	0.2 ± 0.0	0.4 ± 0.2	0.8 ± 0.2	0.2 ± 0.2	0.2 ± 0.1	0.5 ± 0.1	—
18:1 *c*	26.7 ± 0.1	20.4 ± 0.2	17.9 ± 1.9	15.4 ± 0.7[a,b]	13.9 ± 0.9[a,b]	25.4 ± 1.1	26.3 ± 1.0
18:2 *c,t*	0.7 ± 0.2	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.2	0.5 ± 0.0	0.9 ± 0.2	—
18:2 *c,c*	48.4 ± 0.7[a,b]	58.3 ± 1.9	58.0 ± 0.9	69.1 ± 2.0[a,b]	72.0 ± 1.3[a,b]	57.3 ± 2.8	55.5 ± 2.8
18:3/20:1	0.9 ± 0.1	0.8 ± 0.1	0.8 ± 0.0	0.8 ± 0.0	0.6 ± 0.1	0.5 ± 0.2	0.7 ± 0.1
20:3n-9	1.0 ± 0.1	0.5 ± 0.1	0.4 ± 0.0	0.5 ± 0.1	0.8 ± 0.1	0.5 ± 0.1	—
20:3n-6	7.8 ± 0.5[a,b]	6.5 ± 0.5[a]	4.8 ± 0.4	3.6 ± 0.1	3.3 ± 0.2	4.2 ± 0.4	3.7 ± 0.2
20:4n-6	1.2 ± 0.1[a]	1.4 ± 0.3[a]	3.8 ± 0.8	1.1 ± 0.2[a]	1.2 ± 0.2[a]	1.7 ± 0.2[a]	3.1 ± 0.3
22:5n-6	0.3 ± 0.1[a]	0.2 ± 0.1[a]	0.3 ± 0.1[a]	0.2 ± 0.1[a]	0.2 ± 0.2[a]	0.2 ± 0.1[a]	1.5 ± 0.7
22:6n-3	1.0 ± 0.1	0.6 ± 0.2	0.7 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.9 ± 0.2	1.0 ± 0.3
Heart							
12:0	0.2 ± 0.1	0.3 ± 0.0	0.3 ± 0.1	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.1	0.4 ± 0.0
14:0	0.4 ± 0.1	0.4 ± 0.0	0.3 ± 0.1	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.1	0.2 ± 0.0
16:0	0.7 ± 0.1	0.6 ± 0.2	0.5 ± 0.2	0.5 ± 0.2	0.5 ± 0.0	0.7 ± 0.1	0.8 ± 0.1
16:1 *t*	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.1	—
16:1 *c*	2.6 ± 0.1[a,b]	1.7 ± 0.1[a,b]	1.6 ± 0.3[a,b]	1.2 ± 0.1[a,b]	1.1 ± 0.2[a,b]	0.5 ± 0.1	0.5 ± 0.1
18:0	0.5 ± 0.0	0.5 ± 0.3	0.4 ± 0.3	0.3 ± 0.1	0.3 ± 0.0	0.4 ± 0.1	0.4 ± 0.1
18:1 *t*	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.3 ± 0.1	—
18:1 *c*	9.7 ± 0.2[a,b]	6.5 ± 0.3[a,b]	4.9 ± 0.4[a,b]	3.4 ± 0.2[a,b]	3.2 ± 0.3[a,b]	14.0 ± 0.7[a]	12.8 ± 0.2
18:2 *c,t*	0.3 ± 0.1	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.1	0.2 ± 0.1	0.4 ± 0.2	—
18:2 *c,c*	71.5 ± 0.4	79.4 ± 0.5[a,b]	83.5 ± 1.1[a,b]	87.0 ± 0.4 [a,b]	87.6 ± 0.7[a,b]	73.8 ± 1.5[a]	69.3 ± 0.7
18:3n-3	0.3 ± 0.0	0.2 ± 0.1	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.1	0.2 ± 0.2	0.1 ± 0.0
20:1	1.5 ± 0.1	1.3 ± 0.3	1.3 ± 0.3	1.3 ± 0.1	1.3 ± 0.2	0.3 ± 0.1	0.2 ± 0.0
20:3n-6	7.6 ± 0.2[a,b]	4.7 ± 0.3[a,b]	2.9 ± 0.2[a,b]	2.3 ± 0.1[a,b]	1.7 ± 0.1	2.0 ± 0.1[a]	1.5 ± 0.1
20:4n-6	2.6 ± 0.2[a,b]	2.8 ± 0.2[a,b]	2.8 ± 0.2[a,b]	2.6 ± 0.2[a,b]	2.6 ± 0.2[a,b]	3.9 ± 0.3	5.4 ± 0.1
22:5n-6	0.2 ± 0.0[a,b]	0.3 ± 0.1[a,b]	0.3 ± 0.1[a,b]	0.1 ± 0.1[a,b]	0.3 ± 0.1[a,b]	0.8 ± 0.3[a]	5.5 ± 0.7
22:6n-3	0.7 ± 0.1	0.6 ± 0.2	0.4 ± 0.1	0.2 ± 0.0	0.2 ± 0.1	1.8 ± 0.3	2.1 ± 0.1

[a]Significantly different from PALM $P<0.05$.
[b]Significantly different from HSOY $P<0.05$.

At present, little is known about the effect of fatty acid composition of phospholipids on membrane-bound enzyme systems. Of particular interest is the cytochrome oxidase, as it has been demonstrated that CL is associated with the active site of this enzyme (11). The present paper demonstrates that CL from various tissues may differ considerably in fatty acid profiles and in the degree of unsaturation. Few papers have previously been published on the effect of dietary fatty acids on the metabolic capacity of mitochondria. Results obtained with partially hydrogenated vegetable oils (20,21) have indicated that isomeric fatty acids have no influence on the activity of mitochondria. Blomstrand and Svensson (10) reported that the *cis/trans* ratio of partially hydrogenated marine oils affected the fatty acid profiles of heart CL as well as the metabolic activity. Heart mitochondria from rats fed partially hydrogenated capelin oil (36% *trans*, 11% 18:2) had increased 18:2 levels in CL and showed a level of adenosine triphosphate (ATP) synthesis similar to that of a peanut oil group, whereas a partially hydrogenated herring oil group (24% *trans*, 11% 18:2) had 18:2 levels in CL similar to the reference group but significantly decreased ATP synthesis. However, the diets used differed in total 22:1 contents. The very high levels of linoleic acid in heart mitochondrial CL may reflect an attempt to accommodate the respiratory capacity of the mitochondria to the oxidation of acetyl-CoA and acyl-CoA formed by increased peroxisomal and mitochondrial degradation of dietary long-chain fatty acids (22,23) present in HMO. On the other hand, it has also been demonstrated that rats fed HMO and low levels of linoleic acid have different populations of mitochondria compared with rats fed vegetable oil (24,25). This may represent an alternative explanation of the high 18:2 levels found in heart CL from HMO-fed rats.

TABLE 3

Fatty Acid Compositions of Cardiolipins from Rat Kidney and Testes

Dietary fat	16% HMO+ 4% OO	16% HMO+ 3% OO+ 1% SO	16% HMO+ 2% OO+ 2% SO	16% HMO+ 1% OO+ 3% SO	16% HMO+ 4% SO	10% HSOY+ 9% OO+ 1% SO	20% PALM
Fatty acid	(%)	(%)	(%)	(%)	(%)	(%)	(%)
Kidney							
12:0	0.3 ± 0.1	—	0.2 ± 0.1	0.5 ± 0.1	—	0.4 ± 0.0	0.2 ± 0.1
14:0	0.9 ± 0.1	1.0 ± 0.1	1.2 ± 0.1	1.0 ± 0.1	0.7 ± 0.1	0.6 ± 0.0	0.5 ± 0.0
16:0	3.8 ± 0.4	3.7 ± 0.1	3.9 ± 0.2	3.9 ± 0.1	3.9 ± 0.2	4.8 ± 0.4	7.2 ± 0.1
16:1 *t*	1.1 ± 0.1	1.3 ± 0.1	1.4 ± 0.1	1.5 ± 0.1	1.4 ± 0.0	1.9 ± 0.2	—
16:1 *c*	3.9 ± 0.1	3.1 ± 0.0	3.1 ± 0.2	2.9 ± 0.2	2.9 ± 0.2	2.4 ± 0.2	1.8 ± 0.1
18:0	0.8 ± 0.3	0.6 ± 0.0	0.8 ± 0.1	0.9 ± 0.2	0.7 ± 0.1	0.8 ± 0.0	1.0 ± 0.0
18:1 *t*	0.4 ± 0.0	0.4 ± 0.1	0.5 ± 0.0	0.4 ± 0.1	0.2 ± 0.0	1.2 ± 0.2	—
18:1 *c*	21.5 ± 0.7[a]	18.1 ± 0.2[a,b]	16.2 ± 0.4[a,b]	14.6 ± 0.4[a,b]	14.0 ± 0.7[a,b]	22.9 ± 0.9[a]	26.8 ± 0.8
18:2 *c,t*	0.9 ± 0.2	0.5 ± 0.2	0.6 ± 0.1	0.7 ± 0.0	0.4 ± 0.1	1.0 ± 0.2	—
18:2 *c,c*	62.1 ± 0.6[a]	66.0 ± 0.3[a,b]	66.6 ± 1.2[a,b]	68.6 ± 1.4[a,b]	70.6 ± 1.1[a,b]	58.3 ± 1.7[a]	52.8 ± 1.5
18:3/20:1	0.6 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.3 ± 0.0	—	—
20:3n-6	2.4 ± 0.2[b]	2.5 ± 0.1[b]	2.3 ± 0.1	1.9 ± 0.2	1.8 ± 0.1[a]	2.0 ± 0.0	2.2 ± 0.0
20:4n-6	1.6 ± 0.1[a,b]	2.5 ± 0.2[a]	2.9 ± 0.2[a]	2.9 ± 0.4[a]	3.1 ± 0.3[a]	3.5 ± 0.3[a]	5.9 ± 0.5
Testes							
12:0	1.0 ± 0.2	0.9 ± 0.1	0.8 ± 0.1	1.0 ± 0.2	1.0 ± 0.1	0.9 ± 0.0	0.6 ± 0.1
14:0	0.5 ± 0.0	0.4 ± 0.0	0.5 ± 0.0	0.6 ± 0.1	0.6 ± 0.0	0.5 ± 0.0	0.6 ± 0.1
16:0	37.7 ± 2.3	35.1 ± 0.7	39.3 ± 1.4	37.5 ± 2.9	42.4 ± 2.2	37.3 ± 2.0	37.2 ± 1.4
16:1 *c*	1.5 ± 0.1	1.5 ± 0.1	1.4 ± 0.1	1.5 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	1.5 ± 0.1
18:0	2.9 ± 0.3	2.4 ± 0.1	2.7 ± 0.2	2.7 ± 0.4	3.0 ± 0.2	2.6 ± 0.2	2.5 ± 0.2
18:1 *c*	22.8 ± 1.0	22.1 ± 0.2	21.1 ± 0.3	19.9 ± 1.2[a]	18.6 ± 0.8[a]	21.5 ± 0.6[a]	23.8 ± 0.8
18:2 *c,c*	19.0 ± 1.4	23.0 ± 0.6	21.1 ± 1.3	22.0 ± 1.7	18.4 ± 1.6	20.0 ± 1.3	18.4 ± 1.4
18:3	0.3 ± 0.2	0.4 ± 0.1	0.2 ± 0.1	0.5 ± 0.1	0.3 ± 0.1	0.7 ± 0.2	0.4 ± 0.2
20:3n-9	0.6 ± 0.1	0.6 ± 0.1	0.7 ± 0.0	0.7 ± 0.1	0.4 ± 0.0	0.7 ± 0.2	0.5 ± 0.1
20:3n-6	3.2 ± 0.2	3.3 ± 0.1[a]	2.9 ± 0.1	2.9 ± 0.2	2.4 ± 0.1	2.8 ± 0.1	2.6 ± 0.2
20:4n-6	2.6 ± 0.2	2.7 ± 0.0	2.6 ± 0.1	2.7 ± 0.1	2.5 ± 0.1	3.0 ± 0.1	3.2 ± 0.1
22:4n-6	0.5 ± 0.0	0.5 ± 0.0	0.6 ± 0.0	0.7 ± 0.1	0.6 ± 0.1	0.6 ± 0.0	0.5 ± 0.0
22:5n-6	5.1 ± 0.3	5.1 ± 0.4	4.5 ± 0.2	4.9 ± 0.0	6.4 ± 0.4	5.3 ± 0.3	5.5 ± 0.8
22:6n-3	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.2	0.1 ± 0.0	0.1 ± 0.0	0.3 ± 0.0	0.2 ± 0.0

[a]Significantly different from PALM $P<0.05$.
[b]Significantly different from HSOY $P<0.05$.

The fatty acid profiles of CL are different from those of other phospholipids. Since it has not been possible (26) to demonstrate selectivity for species of CDP-diglyceride intermediates in the formation of CL, it remains most likely that re-acylation is the route by which the very special fatty acid profiles of CL are established and maintained.

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Gas Chromatographic Analysis of Reactive Carbonyl Compounds Formed from Lipids upon UV-Irradiation

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Peroxidation of lipids produces carbonyl compounds; some of these, e.g., malonaldehyde and 4-hydroxynonenal, are genotoxic because of their reactivity with biological nucleophiles. Analysis of the reactive carbonyl compounds is often difficult. The methylhydrazine method developed for malonaldehyde analysis was applied to simultaneously measure the products formed from linoleic acid, linolenic acid, arachidonic acid, and squalene upon ultraviolet-irradiation (UV-irradiation). The photoreaction products, saturated monocarbonyl, α,β-unsaturated carbonyls, and β-dicarbonyls, were derivatized with methylhydrazine to give hydrazones, pyrazolines, and pyrazoles, respectively. The derivatives were analyzed by gas chromatography and gas chromatography-mass spectrometry. Lipid peroxidation products identified included formaldehyde, acetaldehyde, acrolein, malonaldehyde, n-hexanal, and 4-hydroxy-2-nonenal. Malonaldehyde levels formed upon 4 hr of irradiation were 0.06 μg/mg from squalene, 2.4 μg/mg from linolenic acid, and 5.7 μg/mg from arachidonic acid. Significant levels of acrolein (2.5 μg/mg) and 4-hydroxy-2-nonenal (0.17 μg/mg) were also produced from arachidonic acid upon 4 hr irradiation.
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Polyunsaturated fatty acids and their esters are known to produce lipid peroxidation products, upon ultraviolet-irradiation (UV-irradiation) (1). Among the lipid peroxidation products, β-dicarbonyls, α,β-unsaturated aldehydes, 2,4-alkadienals and 4-hydroxy-2-alkenals have received much attention as biologically active agents (2). Some of these, e.g., malonaldehyde, exhibit toxic activities by reacting with biological nucleophiles (3). Several α,β-unsaturated compounds, including acrolein, crotonaldehyde, and methyl vinyl ketone, were reported to be mutagenic toward *Salmonella typhimurium* strains (4,5). Reactivity of these compounds may be associated with their carcinogenic activity as a result of adduct formation with deoxyribonucleic acid (DNA) (6). For example, malonaldehyde, which is formed in significant levels from squalene, linoleic acid and linolenic acid upon UV-irradiation (7,8), crosslinks to proteins and binds covalently to nucleic acids (9-11). Formation of biologically active compounds, such as malonaldehyde, at the skin surface may be related to the toxic effects of solar irradiation.

Analysis of these reactive carbonyls is often difficult (7). Recently, malonaldehyde was reacted with methylhydrazine to form 1-methylpyrazole, which was subsequently analyzed by gas chromatography (GC) (7,12). This method was originally developed to measure malonaldehyde, but it should be possible to simultaneously measure other lipid peroxidation products because methylhydrazine reacts with saturated monocarbonyls, α,β-unsaturated carbonyls, and β-dicarbonyls to form hydrazones, pyrazolines, and pyrazoles, respectively. In fact, several reactive carbonyl compounds have been identified as methylhydrazine derivatives in photoirradiated corn oil and beef fat (7).

In the present study, the reactions of methylhydrazine with several α,β-unsaturated aldehydes and β-dicarbonyls were examined in order to develop a method for the simultaneous quantitation of lipid peroxidation products by GC. The method was then used to investigate the reactive carbonyls produced from squalene and fatty acids upon UV-irradiation.

MATERIALS AND METHODS

Reagents. Acrolein, methyl vinyl ketone, and methacrolein (Aldrich Chemical Co., Milwaukee, WI) were dried over anhydrous sodium sulfate and purified by distillation immediately prior to use. All other aldehydes and fatty acids were purchased from Aldrich and used without further purification. *trans*-4-Hydroxynonenal was a gift from Drs. Carl Winter and Hank Segall (University of California, Davis, CA). All other chemicals were obtained from reliable commercial sources at the highest grade available and used without further treatment.

Preparation of standard derivatives. 1-Methyl-2-pyrazole was prepared from methylhydrazine and malonaldehyde *bis*(dimethyl acetal) by the method described by Umano *et al.* (7). For this purpose, 9 carbonyl compound (acrolein, methyl vinyl ketone, methacrolein, or crotonaldehyde) in diethyl ether was added dropwise into an etheric solution of methylhydrazine over the specified time period. The reaction mixture was dried over anhydrous sodium sulfate. After removal of sodium sulfate, the reaction product was isolated by distillation under reduced pressure. Experimental conditions for each derivative are shown in Table 1.

In the case of 1,3,5-trimethyl-2-pyrazole, 2.4 g of 2,4-pentanedione was dissolved in 25 mL deionized water, and the pH was adjusted to 4 with 1N HCl solution. The solution was added dropwise over 30 min into 25 mL deionized water containing 1.6 g methylhydrazine. The solution was stirred with a magnetic stirrer at 0°C. After the reaction, the solution was adjusted to pH 12 with 2N NaOH and then extracted two times with 10 mL of diethyl ether. The extract was dried over anhydrous sodium sulfate for 12 hr. After removal of diethyl ether by distillation, 1,3,5-trimethyl-2-pyrazole was isolated by fractional distillation under reduced pressure (5 mm Hg). Pale, yellow needlelike crystals (2.4 g) were obtained. Spectral data of the derivatives are listed in Table 2.

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Abbreviations: GC, gas chromatography; HPLC, high performance liquid chromatography; NPD, nitrogen-phosphorus detector; MS, mass spectrometry; UV, ultraviolet; FT-IR, Fourier transform infrared spectroscopy; FT-NMR, Fourier transform nuclear magnetic resonance.

TABLE 1

Reaction Conditions Used for Preparation of Standard Derivatives

Derivative	Carbonyl	Amount of carbonyl (g)/ ether (mL)	Amount of methyl hydrazine (g)/ ether (mL)	Reaction time (min)	Amount of product (g)
1-Methyl-2-pyrazoline	acrolein	5.6/50	5.4/75	60	2.5
1,3-Dimethyl-2-pyrazoline	methyl vinyl ketone	3.4/20	2.0/20	20	2.5
1,4-Dimethyl-2-pyrazoline	methacrolein	2.0/20	1.4/20	20	0.7
1,5-Dimethyl-2-pyrazoline	crotonaldehyde	3.0/30	2.0/30	45	1.0

TABLE 2

Spectral Data of Methylhydrazine Derivatives of Carbonyls

Derivative	MS	NMR	IR
1-Methyl-2-pyrazoline	M^+ = 84 (79), 83 (100), 56 (38), 43 (31), 42 (67), 41 (17).	δ 2.66 (2H, *t*, *J* = 9.1 Hz), 2.82 (3H, *s*), 2.49 (2H, *t*, *J* = 9.1 Hz), 6.79 (1H, *s*).	cm^{-1} 3072 (w), 2980 (s), 2820 (s), 1574 (m), 1449 (m), 1239 (m).
1,3-Dimethyl-2-pyrazoline	M^+ = 98 (65), 97 (100), 69 (11), 56 (30), 55 (11), 43 (20), 42 (46), 41 (10).	δ 1.98 (3H, *s*), 2.63 (2H, *t*, *J* = 9.0 Hz), 2.74 (3H, *s*) 2.99 (2H, *t*, *J* = 9 Hz).	cm^{-1} 2961 (s), 2815 (s), 1616 (w), 1448 (m), 1332 (m), 983 (m).
1,4-Dimethyl-2-pyrazoline	M^+ = 98 (42), 83 (58), 56 (37), 44 (21), 43 (43), 42 (100), 40 (15), 39 (25).	δ 1.16 (3H, dd, *J* = 7.0 Hz, 2.2 Hz) 2.44 (1H, *t*), 2.79 (3H, *s*), 3.06 (1H, *t*), 3.24 (1H, *t*), 6.65 (1H, *s*).	cm^{-1} 3061 (w), 2965 (s), 2868 (s), 2807 (s), 1574 1456 (m), 1130 (m), 944 (m), 810 (m).
1,5-Dimethyl-2-pyrazoline	M^+ = 98 (38), 83 (58), 56 (36), 44 (20), 43 (45), 42 (100), 40 (14), 39 (27).	δ 1.30 (3H, d, *J* = 6.0 Hz), 2.22 (2H, *t*, *J* = 11.0 Hz), 2.77 (3H, *s*) 2.83 (1H, *m*) 6.74 (1H, *s*).	cm^{-1} 3071 (m), 2970 (s), 2845 (s), 1576 (m), 1453 (m), 1343 (m), 956 (m), 882 (m).
1,3,5-Trimethyl-pyrazole	M^+ = 110 (100), 109 (87), 95 (35), 82 (12), 68 (16), 56 (19), 44 (11), 43 (32), 42 (27), 41 (11).	δ 2.17 (6H, *s*), 3.63 (3H, *s*), 5.70 (1H, *s*).	cm^{-1} 3050 (w), 2942 (s), 1553 (s), 1460 (s), 1382 (s), 1280 (m), 776 (s).

Recovery studies on methylhydrazine derivatives. Recovery efficiencies of methylhydrazine derivatives in the present experimental systems were determined using 10 mL hexane solutions containing 150 μg each of 1-methylpyrazole, 1,3,5-trimethylpyrazole, 1,3-dimethylpyrazoline, 1,4-dimethylpyrazoline, or 1,5-dimethylpyrazoline. The hexane solutions were extracted three times with 0.1N HCl/10% NaCl. The combined aqueous extracts were adjusted to pH 12 with 2N NaOH. The total volume was adjusted to 40 mL and then extracted continuously with 7 mL of dichloromethane for 3 hr. Dichloromethane extracts were dried over anhydrous sodium sulfate for 12 hr. A dichloromethane solution (150 μL) of *N*-methyl acetamide (0.5 mg/mL) was added to the extracts as a GC internal standard. Samples were adjusted to 10 mL in volume and then analyzed by GC.

Determination of reaction yields of methylhydrazine derivatives. Methylhydrazine, in slight excess, was added at room temperature to a hexane solution (25 mL) containing the carbonyl compound (3-30 mg). After the solution was allowed to stand for 1 hr, the volume was adjusted to 50 mL with hexane, and then the product was analyzed by GC.

UV irradiation on linoleic acid, linolenic acid (n-3), arachidonic acid, squalene, and cholesterol in hexane. Hexane solutions (50 mL) containing 1 mg/mL of fatty acid, squalene, or cholesterol were irradiated in a sealed Pyrex tube for 3 hr with UVA (λ = 350 nm) lamps. Control samples containing 50 mL of the above solution were kept in the dark. Immediately after irradiation, 50 μL methylhydrazine was added to derivatize the carbonyl products in the sample. In experiments with cholesterol and arachidonic acid, 10 μL of methylhydrazine was added to the samples. The solutions were allowed to react at room temperature for 1 hr prior to extraction.

The reaction mixtures in hexane were extracted three times with 0.1N HCl/10% NaCl. The pH of the combined aqueous extracts was adjusted to 12 with 2N NaOH. The total volume was adjusted to 40 mL with deionized water and then extracted continuously with 7 mL of dichloromethane for 3 hr. Dichloromethane extracts were dried over anhydrous sodium sulfate for 12 hr. After removal of sodium sulfate, a dichloromethane solution (150 μL) of N-methyl acetamide (0.5 mg/mL) was added to the extracts as a GC internal standard, and the volume was adjusted to 10 mL. A dichloromethane solution (300 μL)

of 2-methylpyrazine (74 μg/mL) was added as a second GC internal standard in the final step prior to GC analysis. Samples were then analyzed by GC.

Instrumental methods. Samples from recovery experiments were analyzed with a Hewlett Packard (HP) 5880 GC equipped with a 30 m × 0.25 mm i.d. DB-WAX fused silica capillary column (J & W Scientific, Folsom, CA) and a nitrogen-phosphorus specific detector (NPD). GC peak areas were calculated with an HP 5880 GC series 3 terminal. The column was programmed from 60°C (1 min hold) to 170°C (15 min hold) at 6°C/min. Helium was used as carrier gas at a linear velocity of 32 cm/sec. The injector was operated at 250°C with a 50:1 split ratio. The detector temperature was 300°C.

Samples from irradiation experiments were analyzed with an HP 5890 GC equipped with a 30 m × 0.25 mm i.d. DB-WAX fused silica capillary column and an NPD. GC peak area was calculated with a Spectra Physics 3290A reporting integrator. Column temperature was programmed from 30°C (0.5 min hold) to 200°C (20 min hold) at 6°C/min. Helium was used as carrier gas at a flow rate of 33 cm/sec. The injector was operated at 210°C with a 35:1 split. The detector temperature was 250°C.

A VG-Trio 2 Mass Spectrometer interfaced to an HP 5890 GC was used to obtain (MS) of GC peaks. Infrared (IR) spectra were obtained on an HP 5965 Infrared Detector interfaced to an HP 59970 IRD Chem Station and an HP 5890 GC. GC conditions for both instruments were the same as those described previously. The ^1H nuclear magnetic resonance (NMR) spectra, except for 1,3,5-trimethylpyrazole, were obtained on a 300 MHz General Electric QE-300 (FT-NMR) spectrometer. The ^1H NMR spectrum for 1,3,5-trimethylpyrazole was recorded on a Varian EM-390 NMR spectrometer.

RESULTS AND DISCUSSION

Recovery efficiencies of some representative pyrazoles and pyrazolines from the present experimental system are shown in Table 3. Recovery of the derivatives was quite satisfactory. Yield percentages of the methylhydrazine derivatives are shown in Table 3. GC of the reaction mixtures taken after 5 min of methylhydrazine addition showed complete disappearance of the starting carbonyl compounds indicating that the reaction was complete within 5 min. Reaction of methylhydrazine with *trans*-4-hydroxynonenal produced two products. The identical MS [M^+, 184; *m/z* 83 (100), 42 (86), and 41 (41)] and IR (cm^{-1} 3647, 3070, 2940, and 1592) spectra of the two compounds suggested that they were isomers. The low yields of 1,3,5-trimethylpyrazole may be due to formation of polymeric material via osazone intermediates. The low yields of 1,5-dimethyl-2-pyrazoline may be due to the low purity of the starting crotonaldehyde used.

Figure 1 shows a typical GC of methylhydrazine derivatives obtained from the products of arachidonic acid after irradiation for 4 hr with UVA light. Products identified in the irradiated arachidonic acid are shown in Table 4. Only empirical formulae were obtained for some of the compounds (Table 4) due to lack of authentic standards. The major photooxidation products from arachidonic acid were acrolein, malonaldehyde, and n-hexanal. Arachidonic acid also formed acetaldehyde during irradiation; however, the peak corresponding to the hydrazone was too broad to be properly integrated.

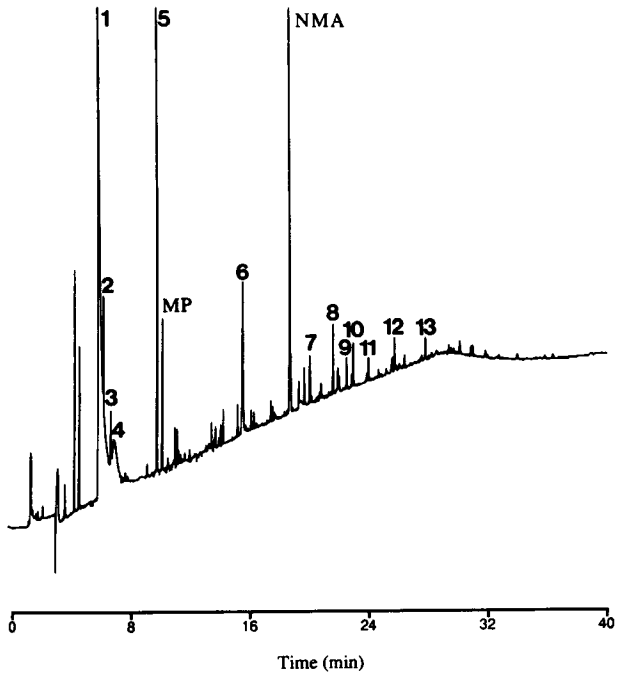

FIG. 1. Gas chromatogram of methylhydrazine derivatives obtained from photoirradiated arachidonic acid (MP, 2-methylpyrazine; NMA, N-methyl acetamide).

TABLE 3

Reaction Yields of Derivatives from Methylhydrazine and Carbonyls and Their Recovery Efficiencies

Carbonyl	Methylhydrazine derivative	Yield (%)[a]	Recovery (%)
Acrolein	1-methyl-2-pyrazoline	94 ± 7.0	80 ± 3.0
Malonaldehyde	1-methylpyrazole	86[b]	87 ± 4.0
Methyl vinyl ketone	1,3-dimethyl-2-pyrazoline	96 ± 6.0	85 ± 5.0
Crotonaldehyde	1,5-dimethyl-2-pyrazoline	57 ± 9.0	86 ± 4.0
Methacrolein	1,4-dimethyl-2-pyrazoline	102 ± 6.0	84 ± 4.0
2,4-Pentanedione	1,3,5-trimethylpyrazole	70 ± 4.0	88 ± 4.0

[a]Relative to starting carbonyl.

[b]Calculated from data of Umano *et al.* (7).

Values are mean ± standard deviation of three replicates.

TABLE 4

Products Identified in UV-Irradiated Arachidonic Acid

GC peak number in Figure 1	Derivatives	Original carbonyl	Amount of product (μg/mg acid)[a]	
			Control	Irradiated
1	Formaldehyde-N-methyl-hydrazone	formaldehyde	1.6	6.5
2	1-Methyl-2-pyrazoline	acrolein	0.16	2.5
3	Unknown	unknown	n.d.[b]	0.14
4	Acetaldehyde-N-methyl-hydrazone	acetaldehyde	peak[c]	peak[c]
5	1-Methylpyrazole	malonaldehyde	0.74	5.7
6	Hexanal-N-methyl-hydrazone	n-hexanal	0.10	0.74
7	Unknown	unknown	0.41	0.20
8	2-Pyrazoline derivative ($C_6H_{12}N_2$)	2-alkenal (C_5H_8O)	0.04	0.20
9	Pyrazole derivative ($C_6H_{10}N_2$)	β-dicarbonyl ($C_5H_8O_2$)	0.04	0.09
10	2-Pyrazoline derivative ($C_7H14_4N_2$)	2-alkenal ($C_6H_{10}O$)	0.06	0.13
11	2-Pyrazoline derivative ($C_8H_{16}N_2$)	2-alkenal ($C_7H_{12}O$)	n.d.	0.10
12	1-Methyl-5-(1-hydroxy)-[d] hexyl-2-pyrazoline	4-hydroxy-2-nonenal	0.05	0.10
13	1-Methyl-5-(1-hydroxy)-[d] hexyl-2-pyrazoline	4-hydroxy-2-nonenal	n.d.	0.07

[a]Amounts estimated from comparison to 1-methylpyrazole standard curve.

[b]Not detected.

[c]Peak was not integrated due to broad shape.

[d]Isomers.

TABLE 5

Amount of Methylhydrazine Derivatives Identified in UV-Irradiated Linoleic Acid, Linolenic Acid, and Squalene

Derivative	Original carbonyl	Linoleic acid (μg/mg acid)[a]		Linolenic acid (μg/mg acid)		Squalene μg/mg squalene)	
		Control	Irradiated	Control	Irradiated	Control	Irradiated
1-Methyl-2-pyrazoline	acrolein	0.15	n.d.[b]	1.0	0.36	n.d.	n.d.
1-Methylpyrazole	malonaldehyde	0.16	0.06	0.46	2.41	n.d.	0.06
Hydrazone derivative	monocarbonyl	2.8	5.7	3.0	11.0	1.2	10.9
Hexanal-N-methylhydrazone	hexanal	0.38	0.39	0.06	0.11	n.d.	n.d.
Hydrazone derivative	monocarbonyl	8.7	11.7	3.7	8.0	6.2	20.5
2-Pyrazoline derivative ($C_7H_{14}N_2$)	2-alkenal ($C_6H_{10}O$)	n.d.	n.d.	n.d.	0.07	n.d.	n.d.
2-Pyrazoline derivative ($C_8H_{16}N_2$)	2-alkenal ($C_7H_{12}O$)	n.d.	n.d.	n.d.	0.30	n.d.	n.d.
1-Methyl-5-(1-hydroxy)-hexyl-2-pyrazoline	4-hydroxy-2-nonenal	0.26	0.09	n.d.	0.07	n.d.	n.d.

[a]Estimated amounts; derived from comparison of peak areas with 1-methylpyrazole standard curve.

[b]Not detected.

Peaks corresponding to the methylhydrazine derivatives of 4-hydroxynonenal were also found in the irradiated arachidonic acid (peaks 12 and 13 in Fig. 1). The compounds were similarly found in the control samples, but were increased significantly in the irradiated samples.

Results from UVA irradiation of linoleic acid, linolenic acid, and squalene are summarized in Table 5. Empirical formulae were only obtained for some of the compounds (Table 4). Products showing significant changes as a result of irradiation are listed. After irradiation, malonaldehyde levels increased significantly in linolenic acid and squalene but not in linoleic acid. Amounts of malonaldehyde produced from fatty acids upon photooxidation increased in the following order: squalene, linolenic acid, arachidonic acid (Tables 4 and 5). This trend was consistent with that predicted from thiobarbituric acid tests on UV-irradiated or autoxidized squalene, linolenic acid, and arachidonic acid (13). Concentrations of malonaldehyde from UVA-irradiated linolenic acid were more than twice those found in UVB-irradiated linolenic acid (7) [UVB (λ = 300 nm) lamps].

In the present study, acrolein, which is known to have certain genotoxic activities (14), increased only in the arachidonic acid sample. Acrolein was also reported to form in photoirradiated corn oil (7). Cholesterol irradiat-

ed under the conditions used in the present study showed no significant changes as compared to the nonirradiated control.

4-Hydroxy-2-alkenals, which are formed by lipid peroxidation (3) and show mutagenic activity (5), are commonly measured by high performance liquid chromatography (HPLC) (2,15). They were also analyzed by gas chromatography-mass spectrometry (GC-MS) as trimethylsily-pentafluorobenzyl oxime derivatives (16). 4-Hydroxy-3-nonenal was found in photoirradiated linoleic acid, and arachidonic acid, but not in photoirradiated squalene (Tables 4 and 5). The 4-hydroxy-2-alkenals are considered to be genotoxic due to their reactivity toward the sulfhydryl groups of DNA (17,18). *trans*-4-Hydroxy-2-nonenal was also found in the products formed by biological lipid peroxidation of linoleic, linolenic, and arachidonic acids (3).

Reactive carbonyl compounds, such as acrolein and malonaldehyde, are difficult to measure accurately in the free form (7) because they undergo self-polymerization as well as condensation with other nucleophilic materials that may be present in the sample. Problems that have been encountered in the analysis of these compounds have been solved in the present study by the use of stable methylhydrazine derivatives. The results we obtained showed that the original method of malonaldehyde analysis can simultaneously analyze other lipid peroxidation products. Because N-methylhydrazine reacted with malonaldehyde also in aqueous rat liver microsomes, the method might be useful for biological samples as well.

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Dietary Linoleic Acid and Polyunsaturated Fatty Acids in Rat Brain and Other Organs. Minimal Requirements of Linoleic Acid

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Starting three weeks before mating, 12 groups of female rats were fed different amounts of linoleic acid (18:2n-6). Their male pups were killed when 21-days-old. Varying the dietary 18:2n-6 content between 150 and 6200 mg/100 g food intake had the following results. Linoleic acid levels remained very low in brain, myelin, synaptosomes, and retina. In contrast, 18:2n-6 levels increased in sciatic nerve. In heart, linoleic acid levels were high, but were not related to dietary linoleic acid intake. Levels of 18:2n-6 were significantly increased in liver, lung, kidney, and testicle and were even higher in muscle and adipose tissue. On the other hand, in heart a constant amount of 18:2n-6 was found at a low level of dietary 18:2n-6. Constant levels of arachidonic acid (20:4n-6) were reached at 150 mg/100 g diet in all nerve structures, and at 300 mg/100 g diet in testicle and muscle, at 800 mg/100 g diet in kidney, and at 1200 mg/100 g diet in liver, lung, and heart. Constant adrenic acid (22:4n-6) levels were obtained at 150, 900, and 1200 mg/100 g diet in myelin, sciatic nerve, and brain, respectively. Minimal levels were difficult to determine. In all fractions examined accumulation of docosapentaenoic acid (22:5n-6) was the most direct and specific consequence of increasing amounts of dietary 18:2n-6. Tissue eicosapentaenoic acid (20:5n-3) and 22:5n-3 levels were relatively independent of dietary 18:2n-6 intake, except in lung, liver, and kidney. In several organs (muscle, lung, kidney, liver, heart) as well as in myelin, very low levels of dietary linoleic acid led to an increase in 20:5n-3. Dietary requirements for 18:2n-6 varied from 150 to 1200 mg/100 g food intake, depending on the organ and the nature of the tissue fatty acid. Therefore, the minimum dietary requirement is estimated to be about 1200 mg/100 g (i.e., the level that ensures stable and constant amounts of arachidonic acid).

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There have been many studies of essential polyunsaturated fatty acids since the first report by Burr and Burr (1). Linoleic acid is now universally recognized to be an essential nutrient. On the other hand, α-linolenic acid was considered non-essential until recently. The effects of polyunsaturated fatty acid deficiency have been extensively studied; prolonged deficiency leads to death in animals. This deficiency is partially corrected in cellular structures by the endogenous synthesis of polyunsaturated fatty acids from oleic acid, which can itself be synthesized *de novo*. However, the resulting fatty acid has only 20 carbon atoms and 3 double bonds (2-4).

Polyunsaturated fatty acid deficiency alters the composition and structure of membranes in all types of cells, including those of the nervous system. The presence of α-linolenic acid, as well as various dietary, hormonal, or toxic factors, can affect linoleic acid metabolism (5-7). Essential fatty acid deficiency affects all nerve functions, including conductivity, and there are changes in the electroretinogram and in behavior (8,9). The effects of deficiency have also been described in man and can result from maternal or perinatal deficiency, undernutrition in the adult, or unsuitable enteral or parenteral feeding (10,11).

The polyunsaturated fatty acids in membranes are not the same as the dietary precursors (linoleic and α-linolenic acids), but have longer and more highly unsaturated chains (mainly arachidonic and cervonic acids). These acids, in particular arachidonic acid, are the precursors of important hormonal substances (prostaglandins and leukotrienes), but their structural role is also important (4) since they play a major role in the structure, enzymatic activities, and function of the membrane (12-15).

Studies carried out in man and in animals have suggested that the minimal dietary requirement of linoleic acid is probably about 3-5% of calories (i.e., 1.5-2.5 g/100 g food intake). The variability in requirements of different organs has not been studied. Our previous results showed that the minimal α-linolenic acid requirement (i.e., to maintain a constant amount of 22:6n-3 in the various organs) in the developing rat is 0.4% of calories (200 mg/100 g food intake) in all organs studied (16). A deficiency in α-linolenic acid is reflected in a decrease in cervonic acid (22:6n-3) in all cerebral cells and organelles, and this is compensated for by an increase in 22:5n-6 (16,17).

The objective of this work was to determine the linoleic acid requirement of the brain and other organs when the requirement in α-linolenic acid was concurrently satisfied. Requirements are defined as the minimal amount of linoleic acid that maintains a constant amount of 20:4n-6 in all tissues, and the minimal amount of dietary α-linolenic acid to obtain a constant amount of 22:6n-3 in all tissues being 0.4% of calories (16).

MATERIAL AND METHODS

A strain of Wistar rats was given a semi-synthetic diet containing 2% sunflower oil for at least two generations to stabilize fatty acid content in all tissues. The diet was prepared in our laboratory. The overall composition of the diet is given in Table 1. It includes a vitamin supplement containing vitamin E as an antioxidant. Three weeks before mating, 12 groups of 8 females were fed one of 12 diets differing in their linoleic and α-linolenic acid content (six groups were fed a diet containing 150 mg/100 g α-linolenic acid, the other six received a diet containing 300 mg/100 g). Three days after parturition, the litters were adjusted to 10 animals.

In each of the 12 diets (containing 5% lipids), the

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Abbreviations: ANOVA, analysis of variance, EPA, eicosapentanoic acid.

linoleic acid content was obtained by mixing different quantities of rapeseed, soybean, linseed, or hydrogenated palm oil (Table 2). The fatty acid contents of the diets are given in Table 3. The n-6/n-3 ratio ranged from 1 to 10, and the maximum dietary linoleic acid content was 3000 mg/100 g. A 13th diet, containing 10% lipids, consisted of a mixture of soybean and sunflower oil so as to supply 6200 mg linoleic acid and 300 mg α-linolenic acid.

Animals were housed in plastic cages, humidity and temperature were kept constant (65% and 21°C, respectively) and a 12-hr day-night cycle was maintained. There were no significant differences in food intake or body weight between groups.

Twenty-one-day-old rats were killed by decapitation followed by exsanguination. Organs were removed and any remaining blood washed out. Myelin and nerve endings (synaptosome) were prepared as previously described and their purity determined by electron microscopy, enzyme marker assay, and characterization of specific proteins by electrophoresis and radioimmunoassay (17).

Total lipids were extracted using a modification of Folch's method and methyl esters were obtained according to Morisson and Smith (18) and analyzed by capillary column gas chromatography as previously reported (17). Statistical analyses were performed using Students t-test or analysis of variance (ANOVA). Data are given as means, and S.D. were always less than 15%.

Analyses on whole organs were performed on at least six individual animals from at least three different litters. Six retinas from three animals were pooled and three pools were analyzed. Myelin and nerve endings were prepared from four pooled animals and five preparations from five different litters were used in the experiments.

TABLE 1

Diet Constituents[a]

		Experimental	
	Sunflower oil (g/kg)	5% Lipid (g/kg)	10% Lipid (g/kg)
Delipidated casein	220	220	220
DL-methionine	1.6	1.6	1.6
Cellulose	20	20	20
Starch	463.4	443.4	405.4
Saccharose	225	215	203
Oil	20	50	100
Vitamin supplement	10	10	10
Mineral supplement	40	40	40

[a]The diet containing sunflower oil was fed to the rats before the animals received the experimental diet. The experimental diet contained a mixture of various oils (see Table 2). Composition of the vitamin supplement (United State Biochemical Corp., Cleveland, OH; mg/kg, triturated in dextrose) was as follows: α-tocopherol (1000 i.u./g), 5.0; L-ascorbic acid, 45.0; choline chloride 75.0; D-calcium pantothenate, 3.0; inositol, 5.0; menadione, 2.25; niacin 4.5; para-aminobenzoic acid, 5.0; pyridoxine HCl, 1.0; riboflavin, 1.0; thiamin HCl, 1.0; retinol acetate , 900,000 i.u.; ergocalciferol (vitamin D_2), 100,000 i.u.; biotin, 20 mg; folic acid, 90 mg; vitamin B_{12} 1.35 mg. The caloric density was 3780 Kcal/kg.

TABLE 2

Oil Content of Diets Varying in Linoleic Acid[a]

n-6/n-3 ratio	n-3 = 300 mg/100 g		n-3 = 150 mg/100g	
1	Rapeseed	25.40	Rapeseed	9.48
	Linseed	7.38	Linseed	4.10
	Hydrogenated palm	67.22	Hydrogenated palm	86.42
2	Rapeseed	66.80	Rapeseed	30.64
	Linseed	2.06	Linseed	1.38
	Hydrogenated palm	31.14	Hydrogenated palm	67.98
4	Rapeseed	56.03	Rapeseed	41.32
	Soybean	27.25	Sunflower	7.30
	Hydrogenated palm	16.67	Hydrogenated palm	51.38
6	Rapeseed	19.02	Rapeseed	41.08
	Soybean	64.78	Sunflower	17.18
	Hydrogenated palm	16.20	Hydrogenated palm	41.74
8	Soybean	83.70	Rapeseed	40.72
	Sunflower	9.62	Sunflower	27.16
	Hydrogenated palm	6.68	Hydrogenated palm	32.12
10	Linseed	9.76	Rapeseed	40.48
	Sunflower	90.24	Sunflower	37.36
			Hydrogenated palm	22.16

[a]Experimental animals were fed a 5% lipid diet.

TABLE 3

Fatty Acid Composition of Diets Varying in Linoleic Acid[a]

Fatty acids	n-3 = 300 mg						n-3 = 150 mg					
14:0	1.1	0.5	0.3	0.3	0.2	0.1	1	1	0.7	0.6	0.5	0.4
16:0	27.5	16.5	13	14.1	11.5	6.1	39.9	33.3	25.6	21.5	17.5	14.4
18:0	8.1	6	4.8	5.2	4.4	3.8	14.9	10.5	9.6	8.3	7.0	5.9
18:1n-9	48.2	57.4	46.2	31.8	23.2	21.3	37.7	39.8	47.4	46.2	44.2	42.7
18:1n-7	1.3	—	3.4	2.6	1.7	1.4	—	—	—	—	—	—
18:2n-6	6.7	12.6	25	38.8	51.7	60.6	3	6.9	12.7	19.3	26.2	32.1
18:3n-3	6.6	5.8	6.1	6.3	6.5	5.9	3	3.6	3.1	3.1	3.2	3.3
20:0	0.3	0.4	0.4	0.4	0.3	0.2	0.4	0.3	0.4	0.4	0.4	0.4
20:1n-7 + n-9	0.2	0.6	0.6	0.3	0.2	0.1	0.1	0.3	0.4	0.4	0.4	0.5
22:0	—	0.2	0.2	0.2	0.3	0.5	—	—	0.1	0.2	0.2	0.3
n-6/n-3 ratio	1.0	2.2	4.1	6.2	7.9	10.3	1	1.9	4.1	6.2	8.2	9.7

[a]Results are expressed in mg/100 mg fatty acids.

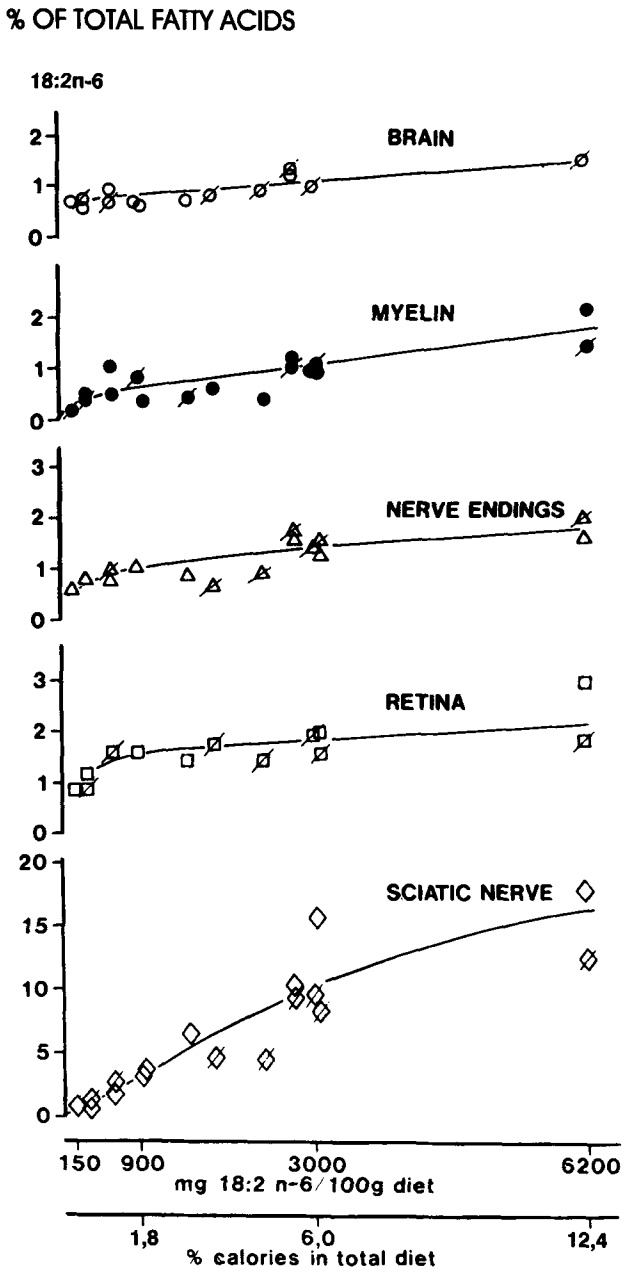

FIG. 1. Relationship between dietary content and concentration of linoleic acid in nervous tissues. Values are % of total fatty acids. All variations were statistically significant between 150 and 6200 mg linoleic acid/100 g diet ($p < 0.05$ for brain, myelin, nerve endings; $p < 0.01$ for sciatic nerve). Open symbols, the dietary content of α-linolenic acid was 150 mg/100 g diet. Symbols with a bar, the dietary content of α-linolenic acid was 300 mg/100 g diet.

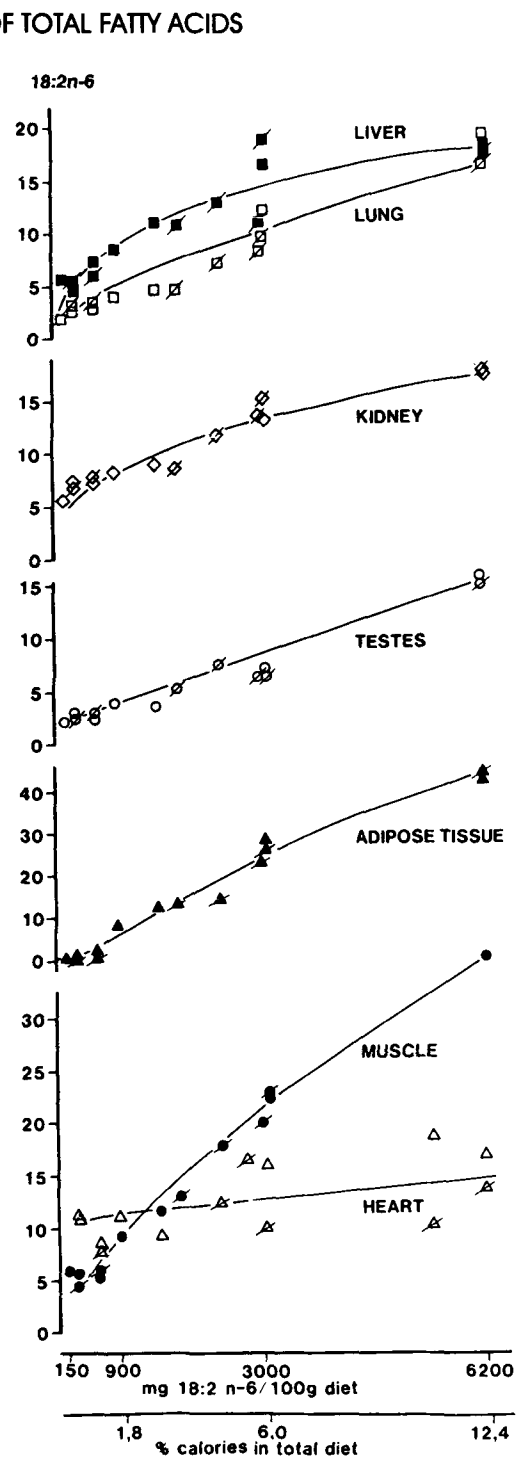

FIG. 2. Relationship between dietary content and concentration of linoleic acid in various organs. Values are % of total fatty acids. Except for heart, all variations were statistically significant ($p < 0.01$). Open symbols, the dietary content of α-linolenic acid was 150 mg/100 g diet. Symbols with a bar, the dietary content of α-linolenic acid was 300 mg/100 g diet.

RESULTS

Linoleic acid. In the nervous system (Fig. 1), the level of 18:2n-6 was very low (about 1% of fatty acids in brain, retina, synaptosomes, and myelin). Increasing dietary 18:2n-6 resulted in only a small increase in levels in brain, retina, synaptosomes and myelin; a 41-fold increase in dietary 18:2n-6 increased tissue levels to a maximum of 2% of total fatty acid. On the other hand, peripheral nerve (sciatic nerve) responded in a linear manner to increasing amounts of dietary 18:2n-6; a 41-fold dietary increase resulted in a nearly 20-fold increase of 18:2n-6 in sciatic nerve. At 6200 mg/100 g food intake, the amount of 18:2n-6 found in sciatic nerve represented 15% of total fatty acids.

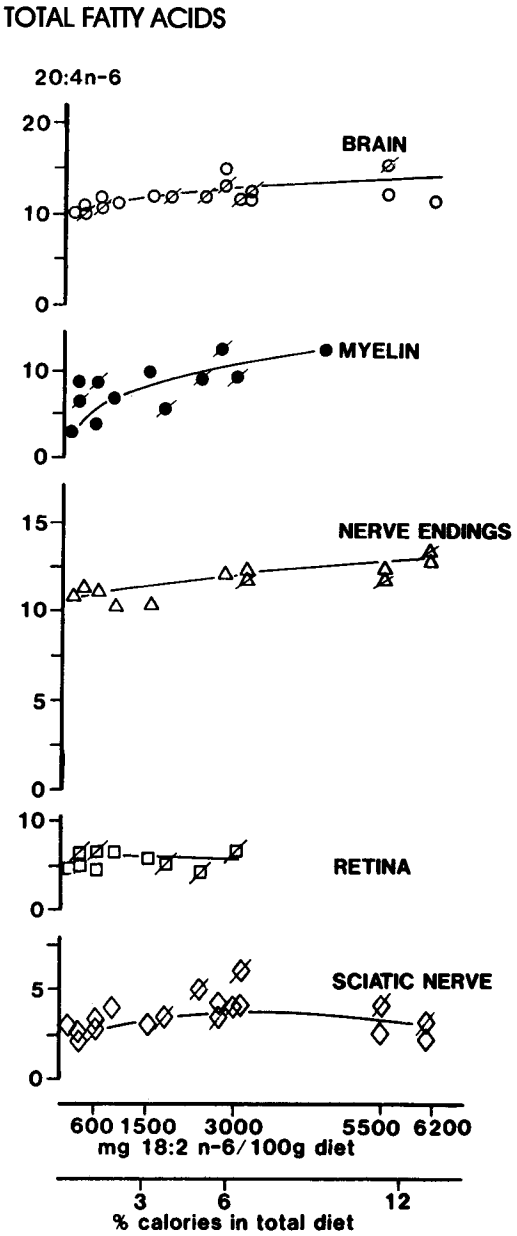

FIG. 3. Arachidonic acid concentration in nervous tissues in relation to dietary linoleic acid content. Values are % of total fatty acids. Except for myelin ($p < 0.05$), no significant variations were found. Open symbols, the dietary content of α-linolenic acid was 150 mg/100 g diet. Symbols with a bar, the dietary content of α-linolenic acid was 300 mg/100 g diet.

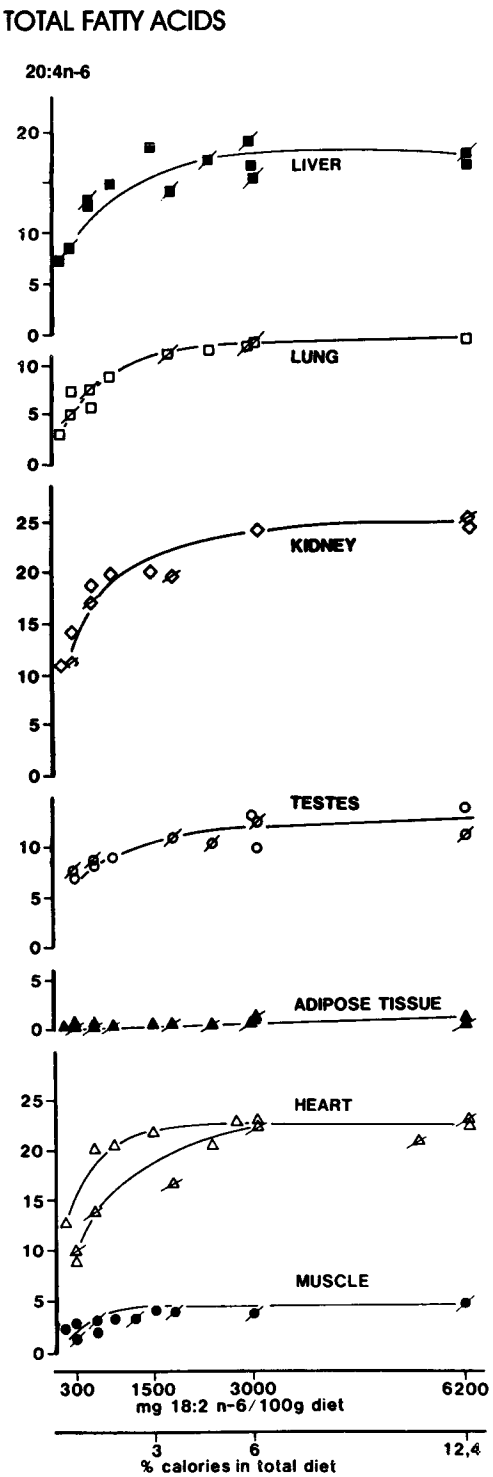

FIG. 4. Arachidonic acid concentration in various organs in relation to dietary linoleic acid content. Values are % of total fatty acids. Except for muscle and adipose tissue, all variations were statistically significant ($p < 0.05$ for testes, $p < 0.01$ for liver, lung, kidney, and heart). Open symbols, the dietary content of α-linolenic acid was 150 mg/100 g diet. Symbols with a bar, the dietary content of α-linolenic acid was 300 mg/100 g diet.

Accumulation of 18:2n-6 in nerve structures was independent of dietary α-linolenic acid (18:3n-3), both when dietary 18:3n-3 remained close to the optimal value (150 or 300 mg/100 g food intake, this study), or when dietary intake was very low (down to 20 mg/100 mg food, unpublished results).

In contrast to nervous tissue, all the other organs examined (Fig. 2) showed an increase in 18:2n-6 that was parallel to dietary 18:2n-6. This increase was marked in muscle (from 5% to 32% of fatty acids) and adipose tissue (from trace level to nearly 40%). In liver, lung, kidney, and testicle the increase was smaller; in heart a plateau was reached at 150 mg/100 g food intake.

Arachidonic acid (20:4n-6). The amount of 20:4n-6 was tightly controlled by the organism and was independent of dietary 18:3n-3. For nervous tissue, the constant level was reached at 150 mg 18:2n-6/100 g food intake

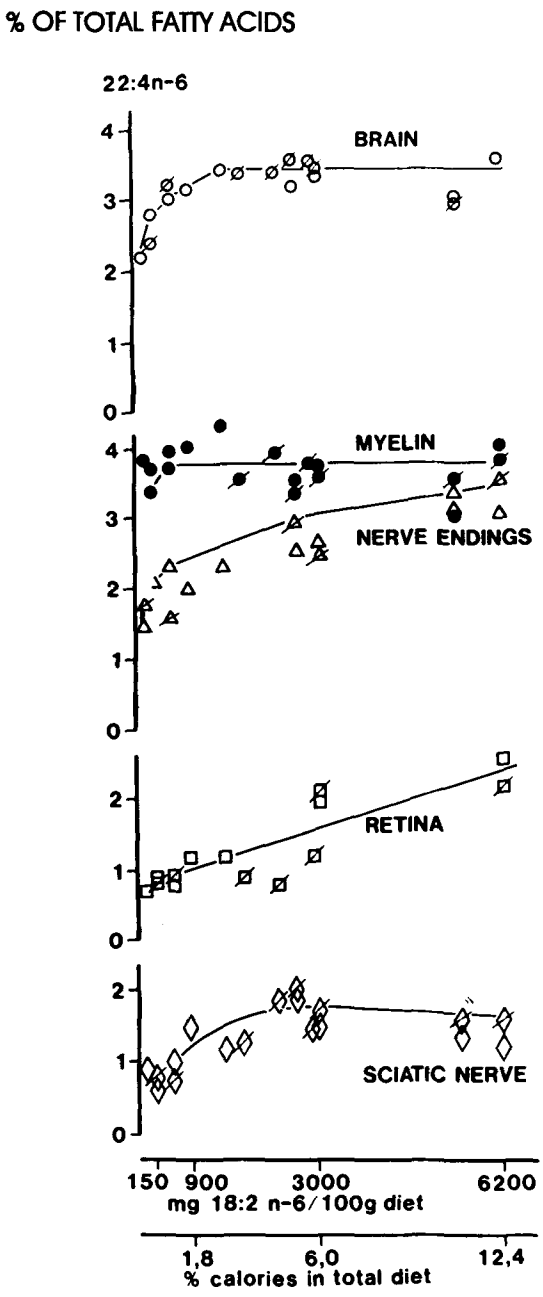

FIG. 5. Adrenic acid concentration in nervous tissues in relation to dietary linoleic acid content. Values are % of total fatty acids. Except for myelin, all variations were statistically significant ($p < 0.01$ for retina, nerve endings and brain; $p < 0.05$ for sciatic nerves). Open symbols, the dietary content of α-linolenic acid was 150 mg/100 g diet. Symbols with a bar, the dietary content of α-linolenic acid was 300 mg/100 g diet.

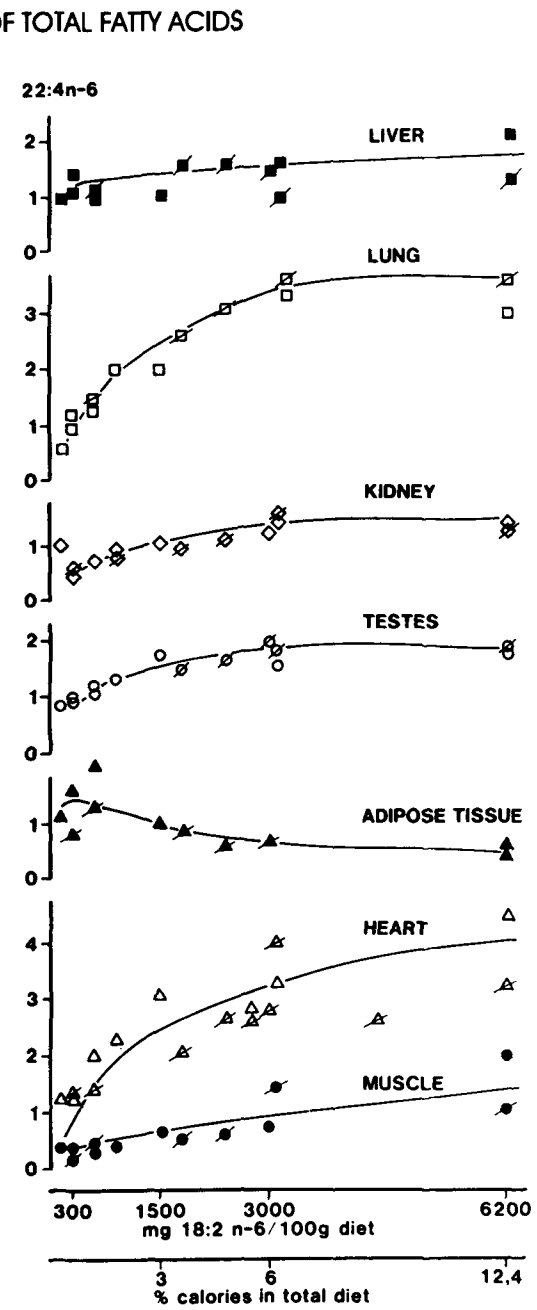

FIG. 6. Adrenic acid concentration in various organs in relation to dietary linoleic acid content. Values are % of total fatty acids. Except for liver and kidney, all variations were statistically significant ($p < 0.05$ for testes, adipose tissue and muscle, $p < 0.01$ for lung and heart). Open symbols, the dietary content of α-linolenic acid was 150 mg/100 g diet. Symbols with a bar, the dietary content of α-linolenic acid was 300 mg/100 g diet.

(Fig. 3). Above this concentration, 20:4n-6 levels remained stable in brain, retina, sciatic nerve, and synaptosomes. There was a slight increase in myelin. For other organs (Fig. 4), the quantity of 20:4n-6 increased parallel to dietary 18:2n-6 content until this reached 300 mg for testicle and muscle, 800 mg for kidney, and 1200 mg for liver, lung and heart. These values were independent of dietary 18:3n-3 content (150 or 300 mg/100 g diet), except for heart.

In adipose tissue, the increase was very small; excess dietary 18:2n-6 resulted in increased 18:2n-6 and not 20:4n-6.

Adrenic acid. In brain, myelin, and sciatic nerve, optimal levels of 22:4n-6 were attained at dietary 18:2n-6 levels of 1200 mg, 150 mg, and 900 mg/100 g food intake, respectively. For retina, there was a change in the slope at 150 mg (Fig. 5) and for synaptosomes at 900 mg. Tissue 22:4n-6 levels were independent of dietary 18:3n-3 (150

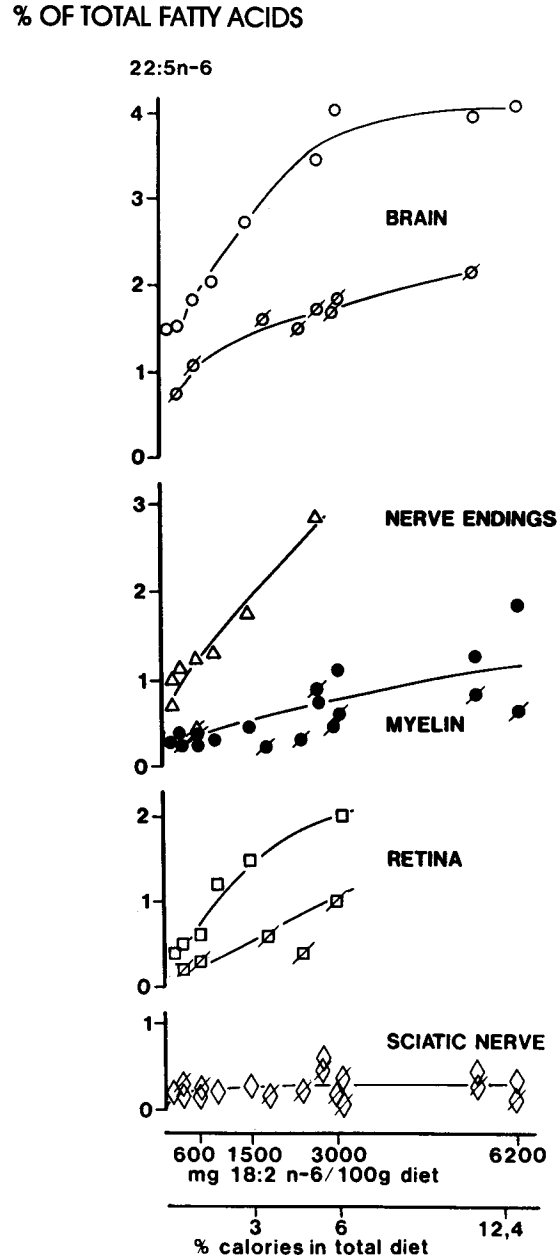

FIG. 7. Concentration of 22:5n-6 in nervous tissues as a function of dietary α-linolenic acid. Values are % of total fatty acids. Except for sciatic nerve, all variations were statistically significant ($p < 0.01$). Except for sciatic nerve values obtained with the 150 or 300 mg 183n-3/100 g diet are statistically significant ($p < 0.01$). Open symbols, the dietary content of α-linolenic acid was 150 mg/100 g diet. Symbols with a bar, the dietary content of α-linolenic acid was 300 mg/100 g diet.

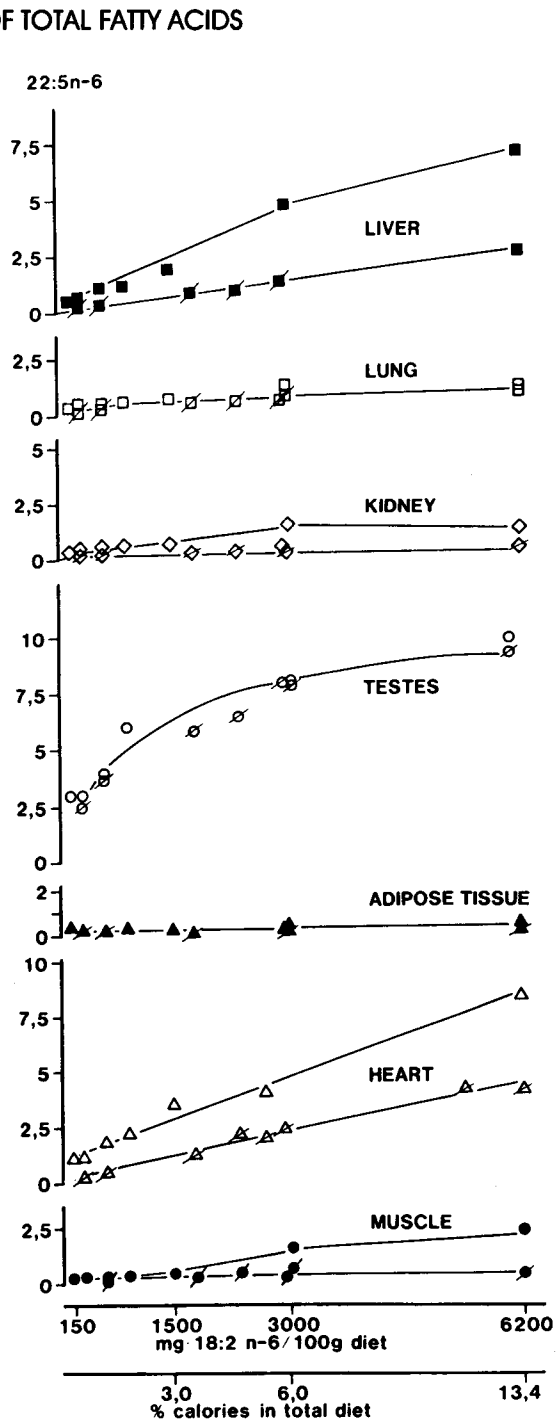

FIG. 8. Concentration of 22:5n-6 in various organs as a function of dietary α-linolenic acid. Values are % of total fatty acids. Except for adipose tissue and muscle (symbols with a bar), variations were statistically significant ($p < 0.01$). Except for testes, lung, and adipose tissue, values obtained with the 150 or 300 mg 18:3n-3/100 g diet were statistically different ($p < 0.01$) for dietary content of 18:2n-6 being equal or superior to 1500, 3000, 150, or 3000 mg 18:2n-6/100 g diet, in liver, muscle, heart, and kidney, respectively. Open symbols, the dietary content of α-linolenic acid was 150 mg/100 g diet. Symbols with a bar, the dietary content of α-linolenic acid was 300 mg/100 g diet.

or 300 mg/100 g diet). In other organs, the amounts of 22:4n-6 present were very small (Fig. 6).

Docosapentaenoic acid. In nervous tissue, this fatty acid accumulated when dietary 18:2n-6 was increased (Fig. 7). This accumulation depended on dietary 18:3n-3 content for brain and retina but not for sciatic nerve and myelin. With increasing amounts of dietary 18:2n-6, the other organs accumulated both 18:2n-6 (Fig. 2) and 22:5n-6 (Figs. 7 and 8). This accumulation depended on dietary 18:3n-3 content for liver, heart and muscle, but not for testicle (for lung and adipose tissue, values were too low to show a statistical difference).

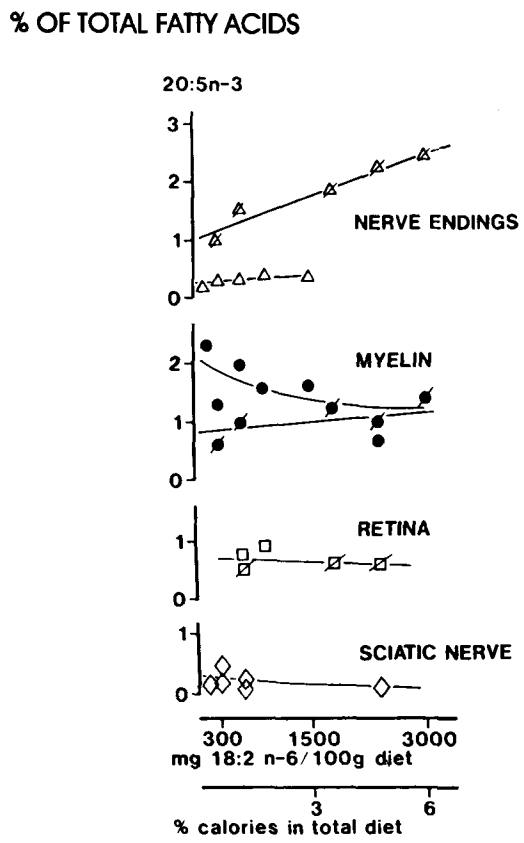

FIG. 9. Concentration of EPA in nervous tissues in relation to dietary linoleic acid content. Values are % of total fatty acids. Variations were statistically significant for nerve endings and myelin ($p < 0.05$) but not for retina and sciatic nerve. For nerve endings, all values obtained with 150 or 300 mg 18:3n-3 were statistically different ($p < 0.05$); for myelin it was statistically different only for 150 and 300 mg 18:2n-6/100 g diet. Open symbols, the dietary content of α-linolenic acid was 150 mg/100 g diet. Symbols with a bar, the dietary content of α-linolenic acid was 300 mg/100 g diet.

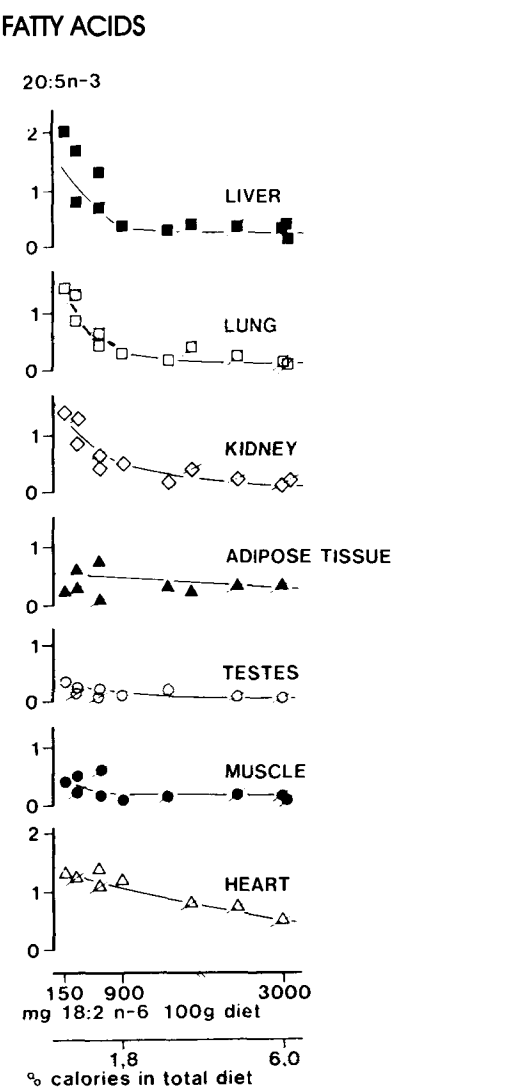

FIG. 10. Concentration of EPA in various organs in relation to dietary content of linoleic acid. Values are % of total fatty acids. Variations were statistically significant for liver, lung, kidney, heart ($p < 0.01$). Open symbols, the dietary content of α-linolenic acid was 150 mg/100 g diet. Symbols with a bar, the dietary content of α-linolenic acid was 300 mg/100 g diet.

Eicosapentaenoic acid. Levels of 20:5n-3 fatty acid were very low in all tissues examined (Figs. 9 and 10). An increase in dietary 18:2n-6 up to 900 mg/100 g decreased 20:5n-3 levels in liver, lung, and kidney. Above 900 mg, the decrease was small (Fig. 9). In heart, levels decreased in a linear manner. In retina, sciatic nerve, testicle, and muscle, the decrease was small. In total brain, the amount of 20:5n-3 was extremely low.

Docosapentaenoic acid and docosahexaenoic acid. Levels of 22:5n-3 were very low in all tissues examined, and the amount was independent of dietary 18:2n-6 content (data not shown). In contrast, a 4300% increase in dietary 18:2n-6 resulted in a slight decrease in 22:6n-3 levels in brain, synaptosomes, and retina (44, 25, 36%, respectively). On the other hand, myelin and sciatic nerve 22:6n-3 levels did not depend on dietary 18:2n-6 content. In liver, kidney, heart, muscle, lung, and testicle, the levels of 22:6n-3 were independent of dietary 18:2n-6 content (16).

DISCUSSION

Polyunsaturated fatty acids of the n-9 family were never detected in animals. This suggests that rats fed a low amount of linoleic acid in the presence of adequate amounts of α-linolenic acid are not deficient in essential fatty acids.

It was surprising to find that low levels of dietary linoleic acid (0.3% of calories, 150 mg/100 g food intake) did not have any effect on reproduction, gestation, perinatal mortality, body weight increase, or overall mortality. In fact, as long as the minimal quantity of α-linolenic acid (200 mg/100 g) was supplied, a linoleic acid intake of 150 mg/100 g covered the minimal requirements of certain organs and of certain fatty acids (meaning, for instance, that the level of arachidonic acid was constant and did not increase at a higher dietary content of linoleic acid). However, the minimal requirement of the organ most dependent on linoleic acid (the liver) was 1200 mg/100 g food intake (2.4% of calories). The level of the n-6 series polyunsaturated fatty acids in the brain varied with dietary linoleic acid content (19). The figure of 2.4% of calories is higher than that previously proposed (20) for male (1.3% of calories) or female rats (0.5% of

calories), but is the level generally accepted in human nutrition (21-24). In the human male adult, 1.3% of calories (25), in children 3% of calories (26), and in pigs 0.7% of calories (27) are the levels considered sufficient.

Brain linoleic acid requirements are very high in man during the perinatal period (28,29). However, it should be noted that high concentrations of linoleic acid during total parenteral nutrition in newborns alter the liver fatty acid profile as well as that of brain (30).

Excess dietary linoleic acid leads to a specific accumulation of 22:5n-6 which depends on the dietary linoleic acid levels. A deficiency of dietary α-linolenic acid leads to an accumulation of 22:5n-6, which then replaces the deficient 22:6n-3 (16,17). Dietary linoleic acid deficiency results in a reduction in arachidonic acid levels as well as in the level of 22:4n-6. There is a linear relationship between dietary linoleic acid content and the concentration of 20:4n-6 in membranes and other tissues up to a certain threshold. Above that level, the concentration of these fatty acids is constant regardless of dietary linoleic acid. It should be noted that in several organs (muscle, lung, kidney, liver, heart) as well as in myelin, a low level of dietary linoleic acid led to an incease in 20:5n-3. When dietary linoleic acid falls below the minimum requirement, 20:5n-3 (but not 22:5n-3 or 22:6n-3) increases. The 20:4n-6 deficiency is partially compensated for by 20:5n-3.

Mean fatty acid levels in human brain differ little from those of similar regions in rat brain. Human development involves a greater daily increase in brain mass over a longer period, and the ratio brain weight-to-total body weight is greater in man, even taking the 2/3 coefficient into account. Consequently, the minimal requirements in rat are *a fortiori* those in man. In any case, for obvious ethical reasons, it will not be possible to determine the effects of increasing dietary fatty acid levels on the composition of human cerebral membranes. Our study is the first to measure simultaneously the variations of all the polyunsaturated fatty acid levels in several organs as a function of variations in dietary linoleic acid content, while minimal α-linolenic acid requirements are being satisfied. In contrast to α-linolenic acid requirements, which are the same for all organs [200 mg/100 g food intake (16,17)], the linoleic acid requirement differs according to organ. The minimal requirements in man may, therefore, be taken as 1200 mg/100 g food intake (2.4% of calories) for linoleic acid and 200 mg/100 g food intake (0.4% of calories) for α-linolenic acid.

It should be noted that cultured nerve cells differentiate, multiply, the up and release neurotransmitters only if the medium contains 20:4n-6 and 22:6n-3, but not if it contains 182n-6 and 18:3n-3 (31,32). Hepatic desaturase must be functional for transformation of dietary precursors into longer chains. Δ6 Desaturase activity decreases very quickly after birth (33,34) and this might explain certain aspects of aging (35).

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Phospholipid Fatty Acid Composition of Various Mouse Tissues after Feeding α-Linolenate (18:3n-3) or Eicosatrienoate (20:3n-3)[1]

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The selective incorporation of dietary α-linolenate (18:3n-3) and its elongation product, eicosatrienoate (20:3n-3), into various phospholipids (PL) of mouse liver, spleen, kidney, and heart, was examined in a two-week feeding trial by assessing mol % changes in associated fatty acids. Mice were fed fat-free AIN 76A diets modified with either 2 wt% safflower oil (control); 1% safflower and 1% linolenate; or 1% safflower and 1% eicosatrienoate. After linolenate or eicosatrienoate feeding, 20:4n-6 was reduced by 36–50% in liver phosphatidylcholine (PC) and in liver and spleen phosphatidylethanolamine (PE). Linolenate was minimally incorporated into PL, but was desaturated and elongated to 20:5n-3, 22:5n-3, and 22:6n-3, with notable differences in the quantity of these n-3 derivatives associated with different tissues and PL. Eicosatrienoate was uniquely incorporated into the cardiolipin (CL) pool of all organs. There was also considerable retroconversion of 20:3n-3 to 18:3n-3 (PC,PE). Dietary eicosatrienoate may therefore affect metabolism in diverse ways—20:3n-3, which is retroconverted to 18:3n-3, may provide substrate for 20:5n-3 and 22:6n-3 syntheses, whereas intact 20:3n-3 may be incorporated into the CL pool. Acyl modifications of CL are known to affect the activity of key innermitochondrial enzymes, such as cytochrome c oxidase.
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Dietary fat has been recognized to alter physiological processes; however, the precise mechanisms by which these effects are realized are unknown. Our approach is to investigate the incorporation of dietary fatty acids, differing in chain length and in the position and number of double bonds, into specific phospholipid (PL) pools, by determining mol % changes in associated fatty acids. Most studies of this type have been done *in vitro. In vivo* studies are preferable because profound differences in fatty acid metabolism are seen *in vivo* as compared to *in vitro*. For example, 20:5n-3 is incorporated minimally into the phosphatidylinositol (PI) pool *in vivo* (1-4), but is readily incorporated into the PI pool of platelet and muscle PL *in vitro* (1,5,6). Likewise, the pronounced affinity of 18:2n-6 for cardiolipin (CL) described in this report is not demonstratable *in vitro*. When CL was reacted with phospholipase A_2 (PLA_2) and reacylated with fatty acyl CoA substrates and acyl transferases, there was no preference for linoleoyl CoA as compared to other acyl CoA derivatives (7,8). Similarly, cytidine diphosphate-dilinoleoylglycerol was not especially preferred in the *de novo* synthesis of CL as compared to other CDP species (9).

In the present investigation mice were fed purified 18:3n-3 and 20:3n-3 (a "dead-end" elongation product of 18:3n-3) (10,11) and the fatty acyl composition of six PL classes from various organs was then compared. There is interest in 18:3n-3, largely because of its role as a precursor of 20:5n-3 and 22:6n-3, the key fatty acids in fish oils (10,12-17). Only recently has evidence come forth to indicate that 18:3n-3 or its metabolites may be essential in humans (12,13,16,18) and monkeys (19).

Eicosatrienoate (20:3n-3) is a minor component of fish oil (0.8-1.4%; up to 4% after hydrogenation) (20), seed oils (0.7%) (21,22). [According to Lie Ken Jie *et al.* (22), *platycladus orientalis* (also known as *Biota or Thuja orientalis*; commonly known as Arbor-vitae) oil contains 4.9%, 11,14,17-20:3. Based on retention times of authentic 11,14,17-20:3, retention times of 5,11,14-20:3 obtained from *Ginkgo biloba seeds* (21), relative abundance, and mass spectral data, we determined that 11,14,17-20:3 represents 0.7% of the toal, and that the 5,11,14 isomer probably represents 4.4%. This 5,11,14 product is an abundant and common fatty acid in the seed oils of many gymnospermae (0.3-25%); by contrast, 11,14,17-20:3 is less common and less abundant (0.2-1.3%) (21)], and some PL (up to 0.8% in CL; present study). The *in vivo* incorporation of dietary 20:3n-3 into biological membranes has been examined on two previous occasions; however, incorporation into specific PL classes was not examined (23,24). *In vitro*, 18:3n-3 can be converted to 20:3n-3 by several cell types (25-28); and to 5,11,14,17-20:4 and 22:3n-3 by hepatoma culture cells (25,26).

The following aspects of fatty acid metabolism are of specific interest to us: (i) the extent to which intact 18:3n-3 and 20:3n-3 are incorporated into the various PL classes in different tissues, and the suitability of 20:3n-3 as an unmetabolized metabolic marker; (ii) the extent to which 18:3n-3 is converted to 20:5n-3, 22:5n-3, and 22:6n-3; (iii) the extent to which dietary 20:3n-3 is desaturated to 5,11,14,17-20:4, elongated to 22:3n-3, or retroconverted to 18:3n-3; and (iv) the respective abilities of 18:3n-3 and 20:3n-3 to depress the 20:4n-6 content of PL.

MATERIALS AND METHODS

Experimental animals and feeding protocol. The mice used for experimentation were specific pathogen-free, female, C57BL/6 mice (Trudeau Institute, Saranac Lake, NY) initially weighing 13-18 g. Mice received fat-free AIN 76A meal diets (29,30) modified with either 2% dry wt. safflower oil (control); 1% safflower and 1% linolenate; or 1% safflower and 1% eicosatrienoate, for a 2-week period. The steady state composition of most PL is known to be reached within 2 weeks in mice (31). AIN 76A meal diets

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Abbreviations: ANOVA, analysis of variance; CDP, cytidine diphosphate; CL, cardiolipin; FAME, fatty acid methyl ester; FID, flame ionization detector; GC, gas chromatograph(y); HETrE, hydroxyeicosatrienoate; H(P)ETE, hydro(peroxy)eicosatetraenoate; HPTCL, high performance thin-layer chromatography; LO, lipoxygenase; MS, mass spectroscopy; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, prostaglandin; PI, phosphatidylinositol; PL, phospholipid; PS, phosphatidylserine; SPH, sphingolmyelin.

and safflower oil were purchased from Dyets Inc. (Bethlehem, PA). Linolenate and eicosatrienoate free fatty acids were purchased from NuChek Prep (Elysian, MN) and found to be 99.95 and 97.77% pure, respectively, by gas chromatograph (GC). Free fatty acids were fed since fatty acid esters may be incompletely absorbed (32-35). To minimize autoxidation, 0.02% (w/w) *tert*-butylhydroquinone was added to the purified fatty acids (36).

The eight mice per treatment were housed in groups of four in 27 × 17 × 13 cm clear plastic cages. All 24 mice received the control diet for five days followed by 14 days of either the control, linolenate, or eicosatrienoate diets. Mice were fed approximately 25% of body weight/day (4 g feed/mouse/day), from 2000 to 0800 daily. Small amounts of food remained after the 12 hr feeding period, assuring that mice were fed *ad libitum*. Deionized water was also provided *ad libitum*.

Feed cups were cleaned daily, and cages and bedding were steam-cleaned and changed weekly. All mice were ear clipped for identification and individually weighed at the beginning and end of the experiment.

Diet preparation. The fatty acid and safflower oils were weighed, then dissolved in pentane and added to the basal diet, which was spun in a large commercial blender (model CB-5; Waring, Winsted, CT). The diets were then spread on trays and the pentane allowed to evaporate for 2 hr. Diets were sealed in plastic bags under N_2 in 100 g quantities and stored at -70°C until use. Total fat content of the diets was confirmed by reflux condensation with diethyl ether for 1 hr at 123°C; fatty acid composition was determined by GC (Table 1). The fatty acid composition of the safflower oil closely matched that of commercial high-linoleate-safflower oil (37).

Conclusion of experiment. On day 14, mice were killed by CO_2 intoxication (dry ice in a desiccator). The liver, spleen, kidney, and heart from each mouse were dipped in liquid N_2 and stored in plastic bags at -70°C for lipid extraction.

Lipid analysis. Tissue lipids were extracted by a method emplying sodium dodecyl sulfate (SDS; BDH Chemicals, Poole, England) (38), which resulted in a greater recovery of total PL than more traditional methods (e.g., Bligh and Dyer, ref. 39; also unpublished results). The SDS was extracted with heptane and methanol prior to use to remove any fatty acid contaminants. PL were separated into classes by high performance thin-layer chromatography (HPTLC) using a chloroform/methanol/acetic acid/water (50.0:37.5:3.5:2.0, by vol), solvent system (40) and 10 × 10 cm HPTLC pre-coated Silica Gel 60 plates (E. Merck, Darmstadt, Germany). Samples and PL standards [sphingolmyelin (SPH), phosphatidylcholine (PC), phosphatidylserine (PS), PI, phosphatidylethanolamine (PE), and CL] (Sigma Chemical Co., St. Louis, MO) were visualized by spraying plates with the reagent 8-hydroxyl-1,3,6-pyrenetrisulfonic acid trisodium salt (laser grade; 20 mg/100 mL methanol) (Eastman Kodak Co., Rochester, NY) (3). Three microgram quantities of lipid were detectable with this sensitive reagent. Lipids were then scraped and methylated in a one-step reaction employing acetyl chloride (41). Silica removal was not necessary in this procedure and yields were higher than those achieved with other methylation techniques (41). Pentadecanoate was used as an internal standard.

Fatty acid methyl esters (FAME) were separated with a Hewlett Packard GC Model 5890A (Hewlett-Packard Co., Palo Alto, CA), equipped with flame ionization detector (FID), model 3392A integrator, and a DB-225 capillary column (30 m × 0.25 μm i.d.; J&W Scientific, Folsom, CA). The make up gas was N_2, and the carrier gas was H_2 at a velocity of 1.3 mL/min (85KPa). The split was 30:1, and runs were done isothermally at 210°C (except in cases where material was limiting and splitless injection was necessary). The injection and detection temperatures were 250°C and 280°C, respectively, for split chromatography. FAME were identified by comparison with authentic standards (NuChek Prep). Lipids were extracted from *Platycladus orientalis* seeds and methylated to determine the retention times for 5,11,14-20:3 and 5,11,14,17-20:4. Failure to detect 20:3n-9 assured that essential fatty acid requirements had been met. In some cases, the identification of FAME was confirmed by mass spectroscopy (MS). Burdick and Jackson (Muskegon, MI) "High Grade" and Capillary GC/GC-MS grade solvents were used for all analyses. Fatty acids are expressed on a mol % basis (see Tables 2-5).

Statistics. A two-tailed (unpaired) t-test was used to evaluate weight differences between animals receiving the same experimental diet, but confined to different cages. Analysis of variance (ANOVA) was then used to compare weight differences between animals receiving different experimental diets. Three-way ANOVA was used to detect overall differences in fatty acid composition between diets, organs, and PL. Dietary effects were generally significant at $p < 0.05$, organ effects were significant at $p < 0.01$-0.001, and PL effects were significant at $p < 0.0001$. A fixed effects one-way ANOVA, combined with Fischer's Protected Least Significant Difference (42) was then used to detect statistical differences between dietary treatments (same organ and PL); and between different organs (same diet and PL), and different PL (same diet and organ). Between 3-5 animals per dietary treatment were randomly selected for individual fatty acid analysis of organs. For simplicity, Tables only include statistics for dietary effects.

TABLE 1

Fatty Acid Composition of Test Diets[a]

Fatty acid	Control	Linolenate	Eicosatrienoate
14:0	0.2	0.2	0.2
16:0	7.2	4.0	3.8
18:0	2.2	1.1	1.3
18:1n-9	12.0	6.2	5.9
18:2n-6	77.7	39.2	37.1
18:3n-3	0	49.1	0.4
20:0	0.3	0.2	0.2
20:1n-9	0.1	0	0.1
20:2n-6	0	0	0.4
20:4n-6	0	0	0.8
20:3n-3	0	0	49.5
22:0	0.3	0	0.2
22:1n-9	0	0	0.1

[a]Values are expressed as mol % and represent the mean of two determinations.

RESULTS

Weight gain. There were no significant differences in feed consumption, or in 2-week weight gain between replicates

TABLE 2

Fatty Acid Composition of Cardiolipin from Heart, Kidney, Liver, and Spleen[a]

	Heart			Kidney			Liver			Spleen		
FA	CON	LIN	EIC	CON	LIN	EIC	CON	LIN	EIC	CON	LIN	EIC
14:0	0.0	1.3	0.7	1.6	1.3	0.4	2.2	0.8	0.9	5.8	1.6	2.2
14:1n-5	0.4	0.9	0.3	0.1	0.1	1.3	0.0[c]	0.4[c]	3.6[d]	0.0	2.1	2.7
16:0	9.9	7.3	6.5	12.9[c]	7.0[d]	7.1[d]	11.8	9.8	9.4	27.2	19.8	18.7
16:1n-7	1.0	2.3	2.6	3.9	3.2	3.5	3.9	3.7	5.5	3.6	2.8	4.1
18:0	14.9	9.5	7.1	8.2	4.9	4.5	4.9	6.7	7.2	24.0	19.2	18.4
18:1n-7,9	20.1	22.8	21.0	27.5[c]	26.2[c]	23.0[d]	27.8	22.2	23.4	14.0	23.0	16.9
18:2n-6	42.2	40.3	49.5	37.4	42.3	44.2	41.6	44.4	38.7	23.9	21.4	20.5
18:3n-6	0.0	0.0	0.0	0.0	0.5	0.0	1.1	0.1	0.6	0.0	0.1	0.6
18:3n-3	0.0	3.8	1.3	0.0	2.1	0.8	0.0	3.0	2.6	1.5	2.8	2.2
20:2n-6	2.8	0.9	0.3	1.3	1.7	1.0	0.8	0.3	0.2	0.0	0.7	0.6
20:3n-6	4.8	2.2	2.7	1.4	2.1	1.8	2.7	2.0	1.7	0.0	0.4	0.8
20:4n-6	1.4	0.3	0.0	2.3	1.8	1.3	1.2	0.8	0.1	0.0	0.9	1.2
20:3n-3	0.0[c]	0.1[c]	4.1[d]	0.8[c]	1.0[c]	8.2[d]	0.3[c]	0.4[c]	4.1[d]	0.0[c]	0.8[c]	6.6[d]
20:5n-3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
22:4n-6	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.2	1.1
22:5n-3	0.0	0.1	0.0	0.3	0.2	0.0	0.0	0.0	0.0	0.0	0.4	0.0
22:6n-3	2.5	6.1	3.9	1.3[c]	3.9[d]	2.1[c]	0.4	2.9	1.4	0.0	1.5	0.6
20:0-24:0[b]	0.0	2.1	0.0	1.0	1.6	0.8	1.3	2.5	0.6	0.0	2.3	2.8

[a]Abbreviations are as follows: con, control-fed group; lin, linolenate-fed group; eic, eicosatrienoate-fed group. Results are expressed as mol % of each fatty acid in the phospholipid, and represent the mean values of 3-5 determinations.
[b]Includes: 20:0, 22:0, 24:0, 20:1n-9, 22:1n-9, 24:1n-9.
[c,d]Values sharing a superscript in common are not significantly different at $p > 0.05$ (Fisher's Protected Least Significant Difference).

TABLE 3

Fatty Acid Composition of Phosphatidylcholine from Heart, Kidney, Liver, and Spleen[a]

	Heart			Kidney			Liver			Spleen		
FA	CON	LIN	EIC	CON	LIN	EIC	CON	LIN	EIC	CON	LIN	EIC
14:0	2.8	0.1	0.1	0.5	0.2	0.2	0.2	0.2	0.1	1.3	0.8	0.8
16:0	35.2[c]	20.4[d]	23.7[d]	39.8	33.7	40.2	30.6	33.4	32.7	45.9	46.1	53.6
16:1n-7	2.1	0.3	0.2	0.7	0.5	0.4	1.5	2.0	1.8	4.5	3.6	3.0
18:0	28.7	25.2	23.7	13.6	14.1	13.7	18.7	16.1	16.1	15.4	9.1	10.1
18:1n-9	10.4	7.8	11.5	15.6	15.8	17.1	19.5	19.9	21.4	15.2	20.9	17.6
18:2n-6	5.4	3.9	5.2	7.2	7.2	7.3	11.5	13.0	13.1	7.1	9.1	6.8
18:3n-3	0.0	0.1	0.4	0.0	0.3	0.3	0.0	1.3	0.3	0.0	0.4	0.4
20:2n-6	0.0	0.0	0.0	0.2	0.1	0.0	0.3	0.1	0.3	0.4	0.2	0.0
20:3n-6	0.0	0.4	0.3	0.8	1.0	0.9	2.4	1.3	2.3	0.9	0.8	0.7
20:4n-6	8.4	9.3	11.3	12.4	10.4	9.5	10.6[c]	4.2[d]	5.7[c,d]	6.9	5.1	3.9
20:3n-3	0.0[c]	0.0[c]	2.0[d]	0.0[c]	0.1[c]	1.7[d]	0.0	0.0	0.5	0.0	0.1	1.4
20:5n-3	0.0	0.1	0.0	0.0[c]	2.1[d]	0.0[c]	0.0[c]	2.1[d]	0.6[c]	0.0	0.5	0.0
22:4n-6	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.1	0.0
22:5n-3	0.0[c]	2.6[e]	0.8[d]	0.0[c]	0.7[d]	0.0[c]	0.0	0.2	0.1	0.0	0.5	0.0
22:6n-3	7.0[c]	28.9[d]	20.6[d]	8.5	12.8	8.4	4.3	6.2	4.7	1.7	2.1	1.5
Others[b]	0.0	0.9	0.1	0.7	1.0	0.3	0.4	0.0	0.3	0.4	0.6	0.2

[a]See legend for Table 2 for explanation of abbreviations and statistical methodology.
[b]Includes: 14:1n-5, 18:3n-6, 20:0, 22:0, 24:0, 20.1n-9, 22:1n-9, 24:1n-9.
[c,d,e]See Table 2.

(two tailed t-test) or between different dietary treatments ($p > 0.05$) (data not shown).

Distribution of 20:3n-3. After eicosatrienoate feeding, 20:3n-3 was significantly incorporated into CL (all organs, 4.1-8.2%), PC (heart, kidney, 1.7-2.0%) and PE (spleen, kidney, heart, 0.8-2.0%); but not into PI, PS, or SPH (any organ, 0-1.4%) (Tables 2-5; data for SPH, PS, and the combined neutral fraction, NL, are not included in data Tables). Levels of 20:3n-3 in CL were greater than in all other PL, and the degree of incorporation varied with tissue ($p < 0.05$) (Fig. 1; kidney PL shown). As compared to other PL, CL was also unique in having the greatest mol % of 16:1n-7, 18:1n-7 and 18:2n-6. [18:1n-7 and 18:1n-9 were combined in the analyses. Generally, CL contained

TABLE 4

Fatty Acid Composition of Phosphatidylethanolamine from Heart, Kidney, Liver, and Spleen[a]

	Heart			Kidney			Liver			Spleen		
FA	CON	LIN	EIC	CON	LIN	EIC	CON	LIN	EIC	CON	LIN	EIC
14:0	2.8[c]	0.2[d]	0.2[d]	0.5	0.1	0.2	0.4	0.2	0.0	0.3	0.3	0.0
16:0	19.0[c]	8.5[d]	8.3[d]	12.2	11.1	12.3	20.2	21.1	19.8	12.1	13.9	12.2
16:1n-7	0.0	0.1	0.3	0.4	0.3	0.4	0.7	0.2	0.2	0.0	0.7	0.6
18:0	39.3[c]	29.8[d]	30.4[d]	27.7	25.0	27.7	27.3	25.8	25.5	26.5	22.9	23.9
18:1n-9	8.5	8.3	9.5	11.3	10.9	11.7	9.7	7.2	9.2	12.3	10.7	11.8
18:2n-6	2.0	2.9	4.2	2.8	3.1	3.3	4.2	3.7	4.1	3.6	4.3	4.2
18:3n-3	0.0	0.2	0.2	0.0	0.6	0.5	0.0	0.6	0.0	0.0	1.4	1.1
20:2n-6	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.1	0.1	0.0	0.3	0.0
20:3n-6	0.0	0.2	0.2	0.2	0.3	0.2	1.0[c]	0.6[d]	1.2[c]	0.7	1.1	1.2
20:4n-6	9.8	8.4	9.8	33.3	30.7	31.0	24.5[c]	15.8[d]	19.8[e]	26.8[c]	18.2[d]	22.2[c,d]
20:3n-3	0.0[c]	0.0[c]	0.8[d]	0.1[c]	0.0[c]	1.0[d]	0.0	0.0	0.1	0.0[c]	0.1[c]	2.0[d]
20:5n-3	0.0	0.2	0.0	0.1[c]	3.8[e]	0.9[d]	0.0[c]	3.9[e]	1.4[d]	0.0[c]	2.8[d]	0.6[c]
22:4n-6	0.5	0.2	0.7	0.9	0.4	0.4	0.6	0.1	0.3	5.3	2.0	3.1
22:5n-3	0.0[c]	2.0[e]	1.0[d]	0.0[c]	0.8[d]	0.1[c]	0.1[c]	1.4[d]	0.4[c]	0.3[c]	5.4[d]	2.3[c]
22:6n-3	18.1[c]	39.0[d]	33.9[d]	10.0	12.4	9.8	11.0[c]	19.0[d]	17.7[d]	11.9	14.4	14.3
Others[b]	0.0	0.0	0.4	0.5	0.5	0.5	0.3	0.3	0.2	0.2	1.5	0.5

[a]See legend for Table 2 for explanation of abbreviations and statistical methodology.
[b]Includes: 14:1n-5, 18:3n-6, 20:0, 22:0, 24:0, 20:1n-9, 22:1n-9, 24:1n-9.
[c,d,e]See Table 2.

TABLE 5

Fatty Acid Composition of Phosphatidylinositol from Heart, Kidney, Liver, and Spleen[a]

	Heart			Kidney			Liver			Spleen		
FA	CON	LIN	EIC	CON	LIN	EIC	CON	LIN	EIC	CON	LIN	EIC
14:0	0.0	0.8	1.0	1.3	0.5	0.3	1.2[c]	0.3[d]	0.3[d]	3.6	0.8	0.8
16:0	5.3	9.4	10.3	14.2	10.6	9.4	10.8[c]	8.1[d]	7.6[d]	13.2	10.1	10.4
16:1n-7	0.0	0.1	1.5	0.0	0.1	1.0	1.0	0.5	0.2	1.6	0.0	1.0
18:0	60.7	55.0	58.5	53.1	52.5	45.5	52.1	49.8	58.2	40.8	45.4	46.4
18:1n-9	5.9	3.5	3.4	3.8	3.2	5.4	4.2	3.6	3.4	7.0	6.4	6.7
18:2n-6	3.5	2.2	2.3	1.8	1.3	1.8	0.7	0.8	0.7	5.2	1.3	1.6
18:3n-3	0.0	1.0	1.0	0.0	1.6	2.4	0.0	0.9	0.0	0.5	2.5	3.8
20:2n-6	0.6	0.0	0.1	0.0	0.0	0.0	0.0	1.6	1.9	0.0	0.5	0.5
20:3n-6	3.6	1.1	1.1	1.0	1.7	1.6	1.1	1.0	1.5	0.5	1.5	1.6
20:4n-6	19.9	21.5	17.1	22.3	21.9	20.0	28.7	28.6	24.3	23.0	21.8	20.6
20:3n-3	0.0	0.0	0.2	0.1	0.1	1.7	0.0	0.1	0.4	0.6	0.1	1.4
20:5n-3	0.0	0.0	0.0	0.0[c]	1.0[d]	0.2[c]	0.0[c]	2.1[d]	0.5[c]	0.0[c]	1.4[d]	0.2[c]
22:4n-6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	1.1	1.0	0.3
22:5n-3	0.0	0.5	0.1	0.0	0.1	0.3	0.0	0.5	0.1	0.2[c]	1.7[d]	0.2[c]
22:6n-3	0.5[c]	3.3[d]	1.6[c]	1.8	4.2	8.9	0.0	1.1	0.4	0.7[c]	2.3[d]	1.0[c,d]
Others[b]	0.0	1.6	1.8	0.6	1.2	1.5	0.2	0.8	0.5	2.0	3.2	3.5

[a]See legend for Table 2 for explanation of abbreviations and statistical methodology.
[b]Includes: 14:1n-5, 18:3n-6, 20:0, 22:0, 24:0, 20:1n-9, 22:1n-9, 24:1n-9.
[c,d]See Table 2.

approximately equal amounts of 18:1n-7 and 18:1n-9].

Distribution of 18:3n-3. After linolenate feeding, there was generally more 18:3n-3 incorporated into PL than in the control-fed group, but differences were not significant (e.g., see CL data, Table 2). There were no statistically significant differences in the incorporation of 18:3n-3 among PL for any organ ($p > 0.05$).

Distribution of 20:5n-3. After linolenate feeding, there were significant increases in the 20:5n-3 content in PC (liver, kidney), PE and PI (liver, spleen, kidney) (Fig. 2) and PS (kidney) from 0.0% in the control to 1.4–3.8%; and in PE (liver, kidney, 0.9–1.4%), after eicosatrienoate feeding. After linolenate or eicosatrienoate feeding, 20:5n-3 levels were greater in PE than in other PL (all organs except heart); and 20:5n-3 was not detected in CL of any organ; or in PE, PI, or PS from the heart.

Distribution of 22:5n-3. Relative to the control, after linolenate feeding 22:5n-3 content significantly increased in PC (kidney, heart, 0.7–2.6%), PE (all organs, 0.8–5.4%; Fig. 2), PI (spleen, 1.7%), and PS (spleen, kidney, 1.1–3.3%). After eicosatrienoate feeding, 22:5n-3 increased in PE of heart to a level of 1.0%. In comparing the PE

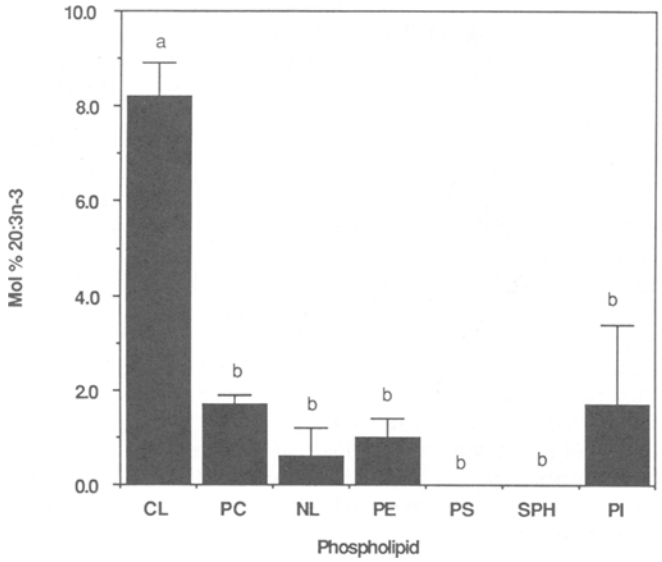

FIG. 1. Levels of 20:3n-3 in kidney PL after eicosatrienoate feeding. Error bars denote 1 SE. Bars sharing a letter in common are not significantly different at $p > 0.05$ (one-way ANOVA).

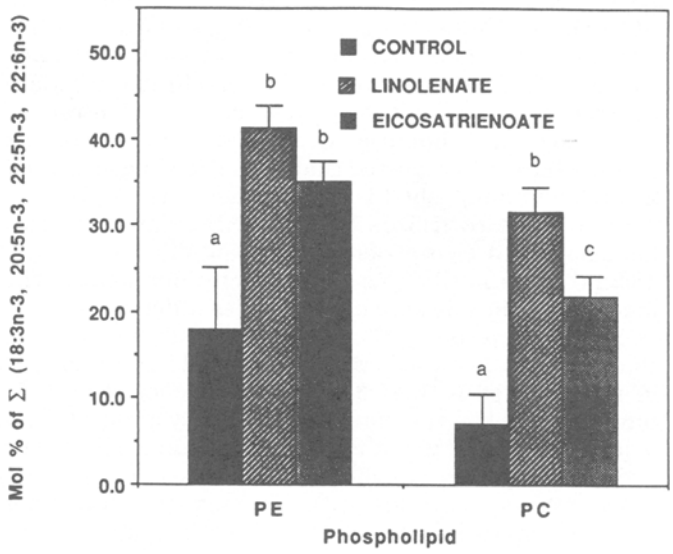

FIG. 3. Combined levels of 18:3n-3, 20:5n-3, 22:5n-3, and 22:6n-3 in heart PC and PE after feeding the three experimental diets. See Figure 1 for statistical symbolism.

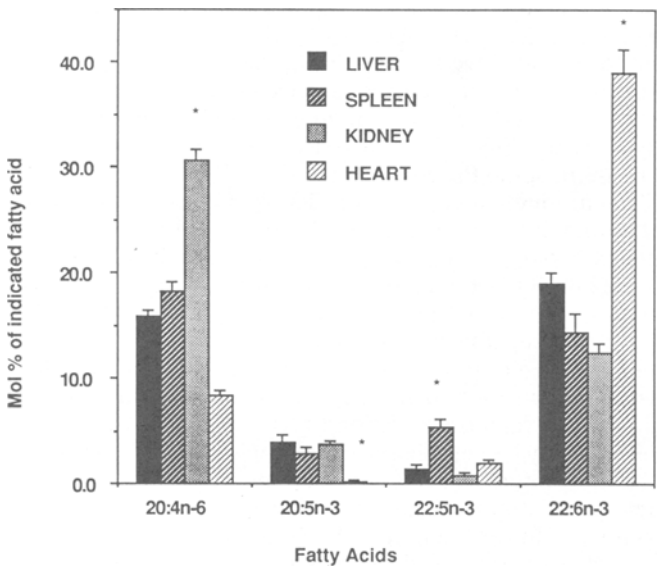

FIG. 2. Fatty acid composition of PE from different organs after linolenate feeding. An asterisk indicates a significant change ($p < 0.05$).

composition of different tissues, the spleen had the highest levels of 22:5n-3 (as well as 22:4n-6 and 20:3n-3) (Fig. 2).

Distribution of 22:6n-3. Generally, within organs, PE was the richest PL source of 22:6n-3, and within PL, the heart was the richest organ source of 22:6n-3 (Fig. 2).

Relative to the control, after feeding either linolenate and eicosatrienoate, there was a statistically significant increase in 22:6n-3 in all PL except SPH. Generally, these effects were more pronounced after linolenate feeding. In several cases there was a stastically significant and equivalent increase in 22:6n-3 content after feeding either linolenate or eicosatrienoate (PC heart, PE heart and PE liver), and the summed quantity of 18:3n-3 plus 20:5n-3 plus 22:5n-3 plus 22:6n-3 was increased by a factor of 1.6–3.1, relative to the control, indicating that retroconversion of 20:3n-3 to 18:3n-3 had occurred (Fig. 3). In PE of heart, 22:6n-3 replaced 16:0; and in PC of heart, 22:6n-3 replaced both 16:0 and 18:0. Docosahexaenoate was quantitatively the predominant n-3 metabolite accumulating in tissue.

Depletion of 20:4n-6. Depletion of 20:4n-6 is of quantitative importance in PI, PE, and PC, since PI and PE contain the greatest mol % of 20:4n-6 and PC is the most abundant membrane PL. Following linolenate or eicosatrienoate feeding, 20:4n-6 levels were significantly depressed in PC (liver) and PE (liver and spleen); and 20:4n-6 levels were slightly more depressed after feeding linolenate as compared to eicosatrienoate. Independent of diet, there was consistently more 20:4n-6 in the kidney PE pool than in other organ PE pools, illustrating tissue selectivity within PL classes (Fig. 2).

DISCUSSION

Uniqueness of cardiolipin acyl composition. CL is unique in containing large amounts of 18:2n-6 in mice (present study) and rats (up to 84%) (43); 18:1n-7, the elongation product of 16:1n-7 (44), in equal to or greater abundance than 18:1n-9 (8–13%) [present study and (45)]; and small amounts of the elongation products—20:3n-3 (0.8%; present study), 20:2n-6 in mice (present study) and rats (2–3%), and 5,11,14-20:3, the Δ5-desaturation product of 20:2n-6 (46).

Levels of 20:3n-3 in cardiolipin. We have demonstrated that 20:3n-3 is selectively incorporated into the CL pool of various organs at a level of 4.1–8.2% after two weeks feeding of 2 wt% fat diets to mice.

CL is an anionic PL found mostly in the inner-mitochondrial membrane (9); it is known to be necessary for maximal activity of a variety of mitochondrial enzymes, most notably cytochrome c oxidase (47–54). CL also has immunogenic activity (55–57).

Yamaoka *et al.* (43) found that in rats fed diets containing 16.8 wt% fish oil, 22:6n-3, 18:1n-7 and 18:1n-9 increased, and 18:2n-6 decreased in CL. Linoleate

decreased from 84% to 44% in heart CL, and from 61% to 21% in liver CL after 10 days of feeding; and from 84-14% after 30 days in heart CL. Their most striking finding was that after fish oil feeding, activity of cytochrome c oxidase decreased by 50%, and the oxygen consumption rate of rat heart mitochondria decreased. Other workers (58,59), have also reported that fish oil feeding increased 22:6n-3 and diminished 18:2n-6 in the heart CL pool.

In contrast to fish oil feeding in rats, in our study, linolenate and eicosatrienoate feeding did not depress 18:2n-6 levels in CL. This may reflect differences in the amounts of the oils fed and/or species differences.

Δ5-Desaturation of 20:3n-3. There was no evidence that 11,14,17-20:3 was desaturated to 5,11,14,17-20:4, as in rats injected with 20:3n-3 (24). Sprecher *et al.* (15) have shown that the elongation and acylation of fatty acids is not limiting, but that desaturation is limiting or occurs slowly. It is possible that mice have a less active Δ5-desaturase than rats (12), and thus 20:3n-3 was not desaturated to 5,11,14,17-20:4 (24). Confounding this interpretation is the fact that our animals were fed and not injected, with 20:3n-3, emphasizing the need for physiologically-conducted studies of lipid metabolism.

Retroconversion of 20:3n-3. Retroconversion is a physiologically-relevant peroxisomal process where chain shortening of fatty acids, which are poorly oxidized by mitochondrial β-oxidation (e.g., 22:4n-6, 22:6n-3), is believed to occur (60-63).

Our results (Fig. 3) suggest that 20:3n-3 was retroconverted to 18:3n-3 and that 18:3n-3 was elongated and desaturated to 20:5n-3, 22:5n-3, and 22:6n-3. 20:4n-3 (8,11,14,17-20:4), the precursor of 20:5n-3 (5,8,11,14,17-20:5), cannot be formed directly from 20:3n-3 (11,14,17-20:3) because animals (23,24,64-66) and plants (21,22) appear to lack the Δ8-desaturase. (In rat testes, there is some evidence of a Δ8-desaturase; ref. 67). In support of our findings, it has been demonstrated *in vitro*, that 11,14,17-ecosatrienoyl-CoA is a good substrate for peroxisomal oxidation enzymes (measured as fatty acyl-CoA-dependent NAD^+ reduction following incubation with clofibrate-proliferated rat liver peroxisomal fractions) giving V_m values of about 150% of that obtained with palmitoyl-CoA. K_m values were in the same range as the value obtained for palmitoyl-CoA (approximately 13 μM) (61).

Desaturation and elongation of 18:3n-3. Other workers have similarly found that desaturation and elongation products of 18:3n-3, but not dietary 18:3n-3 itself, are incorporated into PL (68,69); (for reviews, see refs. 34,35,70). There are several possibilities as to why this occurs: (i) Most 18:3n-3 is β-oxidized (12,71); (ii) in the *de novo* synthesis of PL (i.e., synthesis of CDP-DAG, or acylationof de-acylated precursors; ref. 72), or the reacylation of PL (73), the acyl transferases have greater affinity for longer chain derivatives of 18:3n-3; and (iii) phospholipases have more affinity for 18:3n-3 than longer chain derivatives (74,75).

For a given PL, the fatty acid patterns observed in different tissues probably reflect selective organ uptake rather than more active desaturases and elongases in the particular peripheral organ, since most desaturase and elongase activity is confined to the liver, testicle, and adrenal, at least based on results of *in vitro* studies (76,77). In our study, within the PE pool 20:5n-3 was lowest in the heart and highest in the liver and spleen; 22:5n-3 was highest in the spleen; and 22:6n-3 was highest in the heart (Fig. 3). The active uptake of 22:6n-3 in the heart (58,78), active uptake of 20:5n-3 in the kidney (79), and poor uptake of 20:5n-3 in the spleen (80,81) has been demonstrated in fish oil feeding experiments.

Depletion of 20:4n-6. The reductions in 20:4n-6 observed in some PL pools (e.g., PC and PE in liver and spleen) after linolenate or eicosatrienoate feeding may lead to significant changes in eicosanoid production. Others have shown that dietary 18:3n-3 decreased the amount of 18:2n-6 converted to 20:4n-6 by competing for the Δ6-desaturase (80,82), and the longer chain n-3 fatty acids in fish oil were recently shown to be more effective than 18:3n-3 in suppressing 20:4n-6 levels in liver, lung and plasma PL of rats (69,83). In agreement with other studies (1-4), we found the PI pool to be very resistant to changes in fatty acid compositon *in vivo*.

In summary, feeding studies with 20:3n-3 provide a unique opportunity to study fatty acid retroconversion processes because of the lack of a Δ8-desaturase in mammals. The importance of conducting such studies *in vivo* has been emphasized throughout this report.

Dietary 18:3n-3, or that derived from retroconversion of 20:3n-3, is not incorporated into PL in intact form, but rather is elongated and desaturated first, and then probably selectively taken up by tissues, accounting for the different levels of 20:5n-3, 22:5n-3 and 22:6n-3 observed in different organs. Eicosatrienoate, which is retroconverted to 18:3n-3, may compete for the Δ6-desaturase (80,82), leading to the observed reductions in 20:4n-6 in some PL pools.

The uniquely high levels of dietary 20:3n-3 incorporated intact into the CL pool, as compared to other PL, merits further study; and the incorporation of 20:3n-3 and 22:6n-3 (formed from retroconversion of 20:3n-3 to 18:3n-3) into the CL pool (e.g., in the heart) may affect the activity of key innermitochondrial enzymes (43).

From an eicosanoid perspective, the aforementioned reductions in 20:4n-6, in addition to possible inhibition of the cyclooxygenase by 20:3n-3 retroconverted to 18:3n-3 (69,82) may be significant physiologically. Furthermore, LTs and PGs cannot be formed from intact 20:3n-3 due to lack of a Δ8 double bond (84). (However, aborted cyclooxygenase side products may be formed; ref. 85). We have recently demonstrated the existence of a 15-LO product of 20:3n-3 [(11,13,17) 15-hydroxyeicosatrienoate; 15-HETrE] (German and Berger, unpublished results) which may down-regulate the 5-LO-production of 5-hydro(peroxy)eicosatrienoate (5-HPETE) analogously to other 15-hydroxylated fatty acids with 2-4 double bonds (86-88). By contrast to 20:3n-3, both 12-HETrE and 15-HETrE products are formed from 20:3n-6; and 12-HETrE may share biological potencies with 12-HETE, a pro-inflammatory agent (88).

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Synthesis and Characterization of Cytochrome P-450 Epoxygenase Metabolites of Eicosapentaenoic Acid

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Eicosapentaenoic acid, a major component of fish oil extracts, was recently shown to be metabolized to vicinal diol regioisomers by renal and hepatic cytochrome P-450 epoxygenases. The diol products were also found in the urine of people ingesting fish oil. In the present report, we developed a chemical method of making milligram amounts of the epoxide intermediates and their diol products. Eicosapentaenoic acid was reacted with 0.1 eq. *m*-chloroperoxybenzoic acid for 15 min. After normal- and reverse-phase high performance liquid chromatography plus capillary gas chromatography and electron impact mass spectrometry, the products were identified as 17,18-*cis*-epoxy-eicosa-5,8,11,14-tetraenoic, 14,15-*cis*-epoxy-eicosa-5,8,11,17-tetraenoic, 11,12-*cis*-epoxy-eicosa-5,8,14,17-tetraenoic, 8,9-*cis*-epoxy-eicosa-5,11,14,17-tetraenoic and 5,6-*cis*-epoxy-eicosa-8,11,14,17-tetraenoic acids. The total epoxide yield from eicosapentaenoate was 10% per incubation. By reincubating (cycling) the unused substrate 10-20 times, the total epoxide yield could be increased to 66-88%. Epoxide regioisomers were not produced in equal amounts; epoxygenation was facilitated as the distance between the target double bond and carbomethoxy group increased. Upon hydrolyzing the epoxides, the gas-chromatographic retention times and mass spectra of the diol products were found to match those of biological metabolites. In addition, each of these standards was rapidly and quantitatively synthesized in an amount (milligram) adequate for biological tests.
***Lipids* 25, 481-490 (1990).**

Fish oils are rich in two long-chain n-3 fatty acids, eicosapentaenoate (20:5n-3) and docosahexaenoate (22:6n-3). Two years of dietary supplements with fish oil appears to lower the incidence of fatal heart attacks (1), possibly by reducing platelet responsiveness and aggregation (2). The chronic antithrombotic effects are associated with a decrease in thromboxane A_2 production due to either reduced availability of arachidonic acid (20:4n-6) or inhibition of platelet cyclooxygenase (3). Thromboxane A_2, a potent aggregator of platelets, is formed from 20:4n-6 by platelet cyclooxygenase and thromboxane synthetase acting in tandem.

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Abbreviations: c:dn-a, "c" and "d" signify the number of carbon atoms and double bonds, respectively; the location of the last double bond is "a" carbons from the terminal methyl; DHEQ, dihydroxyeicosaquatraenoic acid; DHET, dihydroxyeicosatrienoic acid; ECL, equivalent chain length, EEQ, epoxyeicosaquatraenoic acid; EET, epoxyeicosatrienoic acid; GC-MS, gas chromatography-mass spectrometry; HPLC, high performance liquid chromatography; *m*-ClPBA, *m*-chloroperoxybenzoic acid; ODS, octadecasilyl; RP- and NP-HPLC, reverse-phase and normal-phase high performance liquid chromatography; TLC, thin-layer chromatography.

In a recent study, six days of dietary supplements with either 20:5n-3 or 22:6n-3 also inhibited platelet aggregation; however, the capacity of platelets to produce thromboxane was unaffected both *in vivo* and *ex vivo* (4). The dissociation between aggregation and thromboxane inhibition may be due to unknown metabolites of n-3 fatty acids (3); 20:5n-3 and 22:6n-3 added directly to platelet suspensions inhibited both aggregation and thromboxane production (5,6).

The cytochrome P-450 system oxidizes 20:5n-3 and 22:6n-3 along three major pathways: epoxygenation, ω-hydroxylation, and ω2-hydroxylation (7,8). The epoxygenase metabolites are, in turn, hydrolyzed to vicinal diols by microsomal or cytosolic hydrolases. Cytochrome P-450 monooxygenases also oxidize 20:5n-3 and 22:6n-3 to minor lipoxygenase-like metabolites (7,8). Thus, there are four distinct pathways by which cytochrome P-450 monooxygenases may oxidize long-chain n-3 fatty acids.

Most cytochrome P-450 metabolites of 22:6n-3 have been unequivocally identified by matching their mass spectra and chromatographic properties with those of synthesized standards (9,10). In addition, homologous diol, ω-hydroxy, ω2-hydroxy, and lipoxygenase-like metabolites from 20:5n-3 have been characterized by chromatography and mass spectrometry (7). Preliminary mass spectral studies involving negative chemical ionization also indicated that 20:5n-3 diols appear in the urine when people ingest fish oil (8a). However, the cytochrome P-450 epoxygenase metabolites of 20:5n-3 have yet to be characterized. The first objective of the present study was to synthesize standards for the structural identification of 20:5n-3 epoxides and diols. The second objective was to synthesize these standards in milligram amounts for the purpose of biological testing.

MATERIALS AND METHODS

Materials. Ethyl or unesterified 20:5n-3, 91% or >99% pure, was purchased from Nippon Chemical Feed Co. (Hokkaida, Japan) and Cayman Chemicals (Ann Arbor, MI); some was a gift from Dr. Takao Fujita (Nippon Suisan Kaisha Ltd., Tokyo, Japan). [1-^{14}C(U)]20:5n-3, purchased from New England Nuclear Corp. (Boston, MA), had a specific radioactivity of 63.4 Ci/mol but was only 97% radiopure as judged by reverse-phase high performance liquid chromatography (RP-HPLC) and thin-layer chromatography (TLC) (see below). Universol scintillation fluid was obtained from ICN Biomedicals (Costa Mesa, CA). 1,1'-carbonyldiimidazole, 3-pyridylcarbinol and technical grade (80.7% pure) *m*-chloroperoxybenzoic acid (*m*-ClPBA) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Dodecane (puriss), 10% rhodium on aluminum, and methyl-*N*-trimethylsilyl-trifluoroacetamide were obtained from Fluka Chemical Corp. (Ronkonkoma, NY), Eberhard Co. (Newark, NJ), and Pierce Chemical Co. (Rockford, IL), respectively. Epoxide standards (14,15-epoxy, 11,12-epoxy, 8,9-epoxy, and 5,6-epoxy-20:3) were provided by Dr. J.R. Falck (University of Texas Health Science Center, Dallas, TX).

Silica gel 60 plates and organic solvents (Omnisolve HPLC grade) were purchased from EM Science (Cherry Hill, NJ). The HPLC hardware and preparation of HPLC water and organic mixtures have been described previously (7). All HPLC columns were 25 cm long and contained 5-μm particles. The analytical columns (0.46 cm i.d.) were obtained from Beckman (San Ramon, CA; Ultrasphere Si) and Phenomenex (Rancho Palos Verdes, CA; Ultremex ODS). The semi-preparative (1.0 cm i.d.) and preparative (2.25 cm i.d.) silicic acid columns were purchased from Phenomenex (Ultremex Si). Fused-silica columns [0.25 mm i.d. $\times$ 15 or 60 m] coated with a dimethyl polysiloxane film (0.25 μm) were obtained from J&W Scientific (Rancho Cordova, CA; DB-1).

Preparation of methyl 20:5n-3. [^{14}C]20:5n-3 was diluted with unesterified 20:5n-3 to a specific radioactivity of 103 nCi/mol. After treatment with ethereal diazomethane (7), methyl 20:5n-3 was isolated in a retention volume of 128 mL using established RP-HPLC conditions (9). Based on RP-HPLC in which radioactivity and absorption at 192 and 237 nm were monitored, the methyl 20:5n-3 substrate was >99% pure both chemically and radiochemically.

Generation of epoxides. Methyl 20:5n-3 was treated with *m*-ClPBA, which converts *cis* double bonds to (+/-) *cis*-epoxides (11). In brief, 10 mg of radiolabeled methyl 20:5n-3 was dissolved in 0.5 mL CH_2Cl_2 and added to a vacuum hydrolysis tube containing a magnetic stirring bar. Then 0.1 eq. *m*-ClPBA, dissolved in 0.5 mL CH_2Cl_2, was added drop by drop over 1.5 min. Immediately thereafter, the hydrolysis tube was capped and its contents stirred at 30°C for 15 min. To remove the by-product *m*-chlorobenzoic acid, the reaction mixture was mixed vigorously with 1 mL ice-cold aqueous $NaHCO_3$ (2.4 mg/mL; pH 7.8) for 2 min and centrifuged. After discarding the aqueous phase, the organic phase was washed twice with aqueous $NaHCO_3$ and twice with equal volumes of deionized H_20 before being dried over anhydrous Na_2SO_4. CH_2Cl_2 was removed under a stream of nitrogen and the sample stored in CH_3OH at -80°C.

Isolation of epoxides. Components of the reaction mixture were separated by TLC using silica gel 60 plates and n-hexane/ethyl acetate/glacial acetic acid (65:35:0.1, v/v/v). The resolved compounds were visualized either by charring or a brief exposure to iodine fumes (quantitation studies). For charring, plates were sprayed with 8% (w/v) $CuSO_4$ in 8% (v/v) phosphoric acid (12) and placed in an oven at 160°C for 15 min.

After being suspended in the mobile phase, individual products were isolated using NP-HPLC and RP-HPLC. For NP-HPLC, aliquots of the methyl esters (50 or 500 μL) were injected onto a silicic acid column (analytical or preparative) and eluted in n-hexane/2-propanol (100:0.175, v/v) flowing at 1 or 18 mL/min, respectively. [To isolate the corresponding diols after acid-catalyzed hydrolysis (see below), a 50-μL aliquot was injected onto an analytical silicic acid column and eluted with hexane/2-propanol/glacial acetic acid (2000:21:3, v/v/v) at a flow rate of 1.5 mL/min)]. For RP-HPLC, methyl esters (50 μL) were injected onto an octadecasilyl (ODS) analytical column and eluted with CH_3CN/H_2O (56:44, v/v) at 1.5 mL/min. Components were collected over ice, concentrated under a vacuum, and stored in CH_3OH. During NP- and RP-HPLC, both radioactivity and absorption at 192 and 237 nm were monitored (9).

With or without further derivatization, the methyl esters were analyzed by capillary gas chromatography (GC). Using an on-column injector (J&W Scientific), each sample was suspended in 1 μL isooctane and applied to a 60-m capillary column coated with a non-polar adsorbent (dimethyl-polysiloxane). The temperature of the gas chromatograph (#5890, Hewlett-Packard, Palo Alto, CA) was programmed to increase instantly from 115°C to 230°C. Picolinyl esters were applied in similar fashion but in dodecane to a 15-m column and using a linear temperature program (4°C/min) to increase the oven temperature from 215°C to 295°C. The linear velocity of the helium carrier gas was 40 cm/second at the highest temperature used.

To characterize HPLC isolates and their derivatives, a plot of carbon number vs log (retention time) was generated using 20:0, 21:0, 22:0, 23:0, 24:0 and 26:0 methyl ester standards. Individual equivalent chain length (ECL) values were determined from interpolated retention times.

Identification of epoxides. Besides methyl esters, other derivatives were analyzed by electron impact (70 eV) mass spectrometry (quadrupole model 5970B, Hewlett-Packard). After saponification, 3-pyridinylmethyl (picolinyl) esters were generated using 1,1'-carbonyldiimidazole and 3-pyridylcarbinol (13). Methyl esters were also catalytically hydrogenated using 10% rhodium on aluminum (10). In addition, aliquots of the hydrogenated and non-hydrogenated derivatives were hydrolyzed in glacial acetic acid/water (1:1, v/v) stirred at 40°C for 16 hr. The products were dried under a vacuum and treated with ethereal diazomethane followed by methyl-*N*-trimethylsilyl-trifluoroacetamide (7).

Quantitation of epoxide yields. CH_2Cl_2 extracts, NP- and RP-HPLC fractions, and TLC scrapings were quantitated by liquid scintillation techniques (9). For TLC radioassays, the developed plates were air-dried and exposed to iodine fumes. Each fraction was outlined, scraped into a scintillation vial containing 1 mL H_2O, and mixed for 10 min. Scintillation fluid (10 mL) was then added and mixed vigorously for 10 min. All samples were counted for 50 min in a liquid scintillation counter (LS 350, Beckman) using an external standard for quench monitoring; counting efficiency was about 88%.

RESULTS AND DISCUSSION

Product analysis by TLC. Conditions cited for generating epoxides from unsaturated fatty acids vary widely with regard to incubation times and *m*-ClPBA equivalents (13-16). In preliminary TLC studies using 20:5n-3 and varying *m*-ClPBA equivalents (0.1-3.0), the yield of monoepoxides (A-spots, Fig. 1) was found to increase with *m*-ClPBA concentration; however, so did the production of other compounds (spots B-D and the point of application in the outermost lane, Fig. 1) which were more polar than the epoxides. But even after most of the 20:5n-3 had been consumed, the major products were not epoxides (A-spots), but rather more polar compounds (B-D spots in the outermost lane of Fig. 1). Using RP-HPLC, spots B-D were found to contain at least 7, 8 and 2 components, respectively; the expected number of regioisomers per group was 10 di-, 10 tri-, and 5 tetra-epoxide regioisomers. The order of (absorption at 192 nm)/cpm ratios was A>B>C>D. Since absorption at

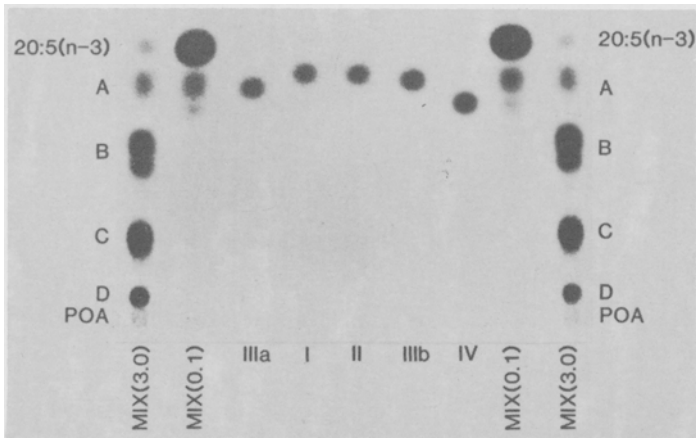

FIG. 1. Thin-layer chromatography of the epoxides derived from the methyl ester of 20:5n-3. Methylated 20:5n-3 was treated with either 3.0 or 0.1 eq. *m*-C1PBA and the mixtures applied to the outermost and next-to-outermost lanes, respectively. Individual products isolated by NP- and RP-HPLC were spotted in more medial lanes; the roman numerals and letters refer to their elution order by NP- and RP-HPLC, respectively. Upon development in n-hexane/ethyl acetate/glacial acetic acid (65:35:0.1, v/v), compounds were visualized by charring.

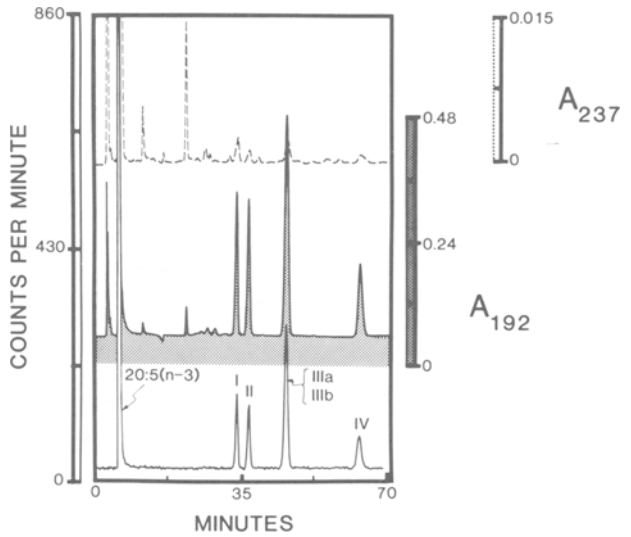

FIG. 2. Normal-phase high performance liquid chromatography of epoxides derived from the methyl ester of 20:5n-3. Methylated [^{14}C]20:5n-3 was treated with 0.1 eq. *m*-C1PBA and an aliquot of the mixture (23,000 dpm in 50 μL of mobile phase) injected onto a column [0.46 (i.d.) × 25 cm] containing 5-μm silica particles. Development with n-hexane/2-propanol (100:0.175, v/v) was carried out at 1.0 mL/min and 370 psig. The column effluent, monitored at 237 nm (upper tracing) and 192 nm (middle tracing), was collected in 0.4-min fractions for radioactivity determinations (lower tracing).

192 nm primarily reflects double bonds (17), the different absorptivities suggest that the number of double bonds differs: A>B>C>D. For these reasons, the polar products were tentatively identified as (B) di-, (C) tri-, and (D) tetra-epoxides, respectively. Analogous di- or tri-epoxides from 18:2n-6 and 18:3n-3 have also been described (18,19).

At the lowest equivalence studied (0.1), only monoepoxides were evident [A-spots of "Mix (0.1)", Fig. 1]. At 0.3 equivalence, traces of B-spots (di-epoxides) appeared. Based upon co-migrations of compounds isolated by NP-HPLC, the major (R_f 0.56) or minor (R_f 0.49) A-spots consisted of Compounds I, II, IIIa and IIIb or Compound IV, respectively. Thus TLC did not adequately resolve the reaction-mixture components even when simplified by the use of 0.1 eq. *m*-C1PBA.

Compounds I, II, IIIa, IIIb and IV were identified by mass spectrometry as 14,15-, 11,12-, 17,18-, 8,9-, and 5,6-epoxy-20:4, respectively (see below). Thus Figure 1 shows that the polarity increased (decreased migration or R_f) as the number of carbon atoms separating the oxirane ring from the carbomethoxy group decreased. As with epoxides from 18:1 (20), 18:3n-3 (21), 20:4n-6 (VanRollins, M., unpublished observations) and 22:6n-3 (9), it may be that the closer the oxirane ring to the carbomethoxy group, the greater the likelihood that both groups simultaneously form hydrogen bonds with surface silanols. As in the other fatty acid series, the R_f values of the ω3 and ω6 epoxides derived from 20:5n-3 were unusually low. Perhaps in these regioisomers, the oxirane ring is also close to the carbomethoxy group due to intramolecular arching (22). Thus, both the general and anomalous TLC properties of 20:5n-3 epoxides are very similar to those observed with other fatty acids; however, only the 5,6-regioisomer could be isolated by TLC.

Product analysis by NP-HPLC. Compared to TLC or RP-HPLC, NP-HPLC provided the maximum epoxide separation. By monitoring absorption at 192 nm, four peaks (I–IV) and the substrate were readily visualized (Fig. 2). Subsequent analysis by RP-HPLC indicated that peak III had two components, IIIa and IIIb. Thus, three of the five possible products were resolved at the outset, and unreacted substrate could readily be isolated for further epoxide synthesis. Because reactions with 0.1 eq. *m*-C1PBA resulted in virtually no by-products, there was no need to wash the NP column between runs, thereby avoiding the prolonged solvent re-equilibration characteristic of NP-HPLC. However, with repeated chromatography there was a danger that autoxidation contaminants would eventually be eluted. For this reason, absorption at 237 nm was routinely monitored. Upon identifying the five products by mass spectrometry, the NP-HPLC elution order was found to be (I), 14,15-; (II), 11,12-; (IIIa), 17,18-plus (IIIb), 8,9-; and (IV), 5,6-epoxy-20:4. Thus, NP-HPLC confirmed the anomalous elution of the 17,18-regioisomer found by TLC. In addition, NP-HPLC readily separated the 14,15-, 11,12- and 5,6-regioisomers.

Product analysis by RP-HPLC. 20:5n-3 products were resolved into three major peaks by RP-HPLC (Fig. 3). Only the first to elute (49.4 min) was baseline resolved. By adding NP-HPLC isolates to the 20:5n-3 mixture, it was found to contain IIIa, one of two compounds present in NP-HPLC peak III. The middle RP-HPLC peak (56.9 min), partly resolved from the last peak to elute, was found to contain NP-HPLC Compound I. The last and largest peak (58.4 min) was found to contain the second component of NP-HPLC peak III (IIIb) as well as NP-HPLC peaks II and IV. Because Compounds IIIa and IIIb were widely separated by RP-HPLC, the combination of NP and RP-HPLC resolved all five 20:5n-3 products. After product identification by mass spectrometry, the RP-HPLC elution order

was found to be 17,18-, 14,15-, and (11,12- plus 8,9- plus 5,6-epoxy-20:4). Thus, as with the epoxides derived from 22:6n-3 (9) and 20:4n-6 (14,23), there was a consistent pattern of elution: retention times increased as the distance between the oxirane ring and carbomethoxy groups decreased. In RP-HPLC the strength of van der Waals interactions probably depends upon the length of aliphatic tail available for intercalation with ODS chains.

Di-epoxides (A, Fig. 3) and autoxidation products (B, Fig. 3) were also shown by RP-HPLC. The latter were observed in unreacted fatty acid preparations, and their levels increased with repeated exposures of 20:5n-3 to air. Based on their polarities and high 237/192 absorption ratios (Fig. 3, upper and middle tracings), the autoxidation products may have been HEPEs (described in ref. 10), although they were not characterized further.

In summary, RP-HPLC resolved only the 17,18-regioisomer; however, it confirmed that 20:5n-3 incubations with 0.1 eq. *m*-ClPBA resulted primarily in the formation of epoxides, and that little 20:5n-3 was lost due to the formation of unwanted by-products.

Product analysis by capillary GC. Capillary GC was attempted with two different ester derivatives in an effort to resolve the five products. Methyl ester derivatives were examined using a 60-m capillary column under isothermal (230°C) conditions (Table 1). Fatty acid methyl esters with retention times bracketing those of the products were injected at the same time. As evident from the common ECL of 20.9, epoxide regioisomers were not resolved even with a 60-m column. Because this was also the ECL of 20:4n-6 epoxides, homologous epoxides were not resolved even when they differed by an additional double bond. Following hydrogenation, Compound IIIA was completely resolved, since its ECL shifted from 20.9 to 21.8. Because the ω3 regioisomer generally has the largest ECL in saturated-epoxide series (9,19,20), Compound IIIa was probably the 17,18-epoxy regioisomer.

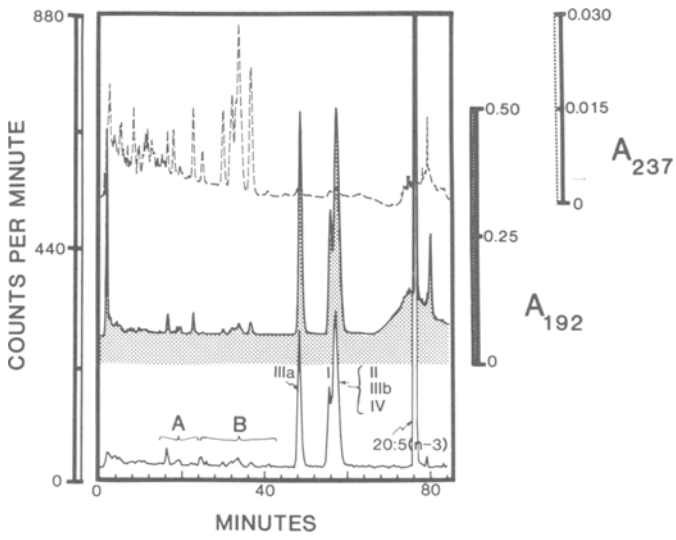

FIG. 3. Reverse-phase high performance liquid chromatography of epoxides derived from the methyl ester of 20:5n-3. Methylated [^{14}C]20:5n-3 was treated with 0.1 eq. *m*-ClPBA and an aliquot of the mixture (26,000 dpm in 50 μL methanol) applied to a column [0.46 (i.d.) × 25 cm] containing 5-μm ODS particles and resolved using CH_3CN/H_2O (56:44, v/v) flowing at 1.5 mL/min (1780 psig). At 63 min, a linear CH_3CN gradient was programmed to reach the 100% level 10 min later. The column effluent, monitored at 237 nm (upper tracing) and 192 nm (middle tracing), was collected in 0.4-min fractions for radioactivity determinations (lower tracing).

Hydrogenated Compounds I and II had the same ECL as the 14,15-epoxy-20:0 and 11,12-epoxy-20:0 standards (21.6). Therefore, the ECL data supported the mass spectral identifications of (I) 14,15-epoxy-20:4 and (II) 11,12-epoxy-20:4. Hydrogenated Compounds IIIb and IV

TABLE 1

Chromatographic Characterization of Products Derived from Methyl Eicosapentaenoate

	High performance liquid chromatography retention times (min)		Gas liquid chromatography retention times (equivalent chain length)	
Compound	Normal phase[a]	Reverse phase[b]	Methyl esters	Picolinyl esters
I	32.8	56.9	20.9 (21.6)[c]	28.1[d]
II	35.6	58.4	20.9 (21.6)	28.1
IIIA	44.6	49.4	20.9 (21.8)	28.2
IIIB	44.6	58.4	20.9 (21.5)	28.1
IV	61.6	58.4	20.9 (21.5)	28.2

[a]0.46 (i.d.) × 25-cm column containing 5-μm silica particles and n-hexane/2-propanol (100:0.175, v/v) flowing at 1 mL/min.

[b]0.46 (i.d.) × 25-cm column containing 5-μm ODS particles and CH_3CN/H_2O (56:44, v/v) flowing at 1.5 mL/min.

[c]0.25 mm (i.d.) × 60-m fused-silica column coated with a 0.25-μm film of methylpolysiloxane. Upon injection, the temperature was increased stepwise from 115°C to 230°C. Numbers in parentheses represent values for corresponding hydrogenated derivatives.

[d]15-m length of the column described in [c]. The oven temperature was programmed at 4°C/min from 215°C to 295°C.

had the same ECL as the 8,9-epoxy-20:0 and 5,6-epoxy-20:0 standards (21.5), respectively; thus,the GC data supported the mass spectral identifications of (IIIb) 8,9-epoxy-20:4 and (IV) 5,6-epoxy-20:4. In summary, after hydrogenation only the 17,18-regioisomer was completely resolved by capillary GC.

Compared to the methyl esters, picolinyl esters of the nonhydrogenated regioisomers were separated better by GC. Compounds IIIa (17,18-epoxy-20:4) and IV (5,6-epoxy-20:4), with oxirane rings at opposite ends of 20:5n-3, had the highest ECL (28.2). Furthermore, the ECL of each regioisomer was 0.1 unit higher than the corresponding 20:4n-6 isomer (not shown). In summary, capillary GC of the methyl or picolinyl esters did not completely resolve any regioisomer. Thus each of the five products had to be separated by NP- and RP-NPLC before it could be analyzed by mass spectrometry.

Product analysis by mass spectrometry. To identify each epoxide regioisomer, electron impact (70 eV) mass spectra were first generated using the original methyl esters. The resulting spectra contained only ions of weak intensity in the high-mass region, so that it was difficult to define the oxirane-ring positions unequivocally. However, by comparing their spectra with those of hydrogenated derivatives, the oxirane ring position and number of double bonds could be assigned with reasonable confidence. The spectrum of a novel derivative, the picolinyl ester, was used to confirm these assignments and unequivocally identify the double-bond positions.

Methyl esters of epoxides. The mass spectra generated from the five products (Table 2) had many of the following high-mass ions in common: 314 (M-H_2O), 303 (M-CH_3CH_2), 301 (M-OCH_3), 289 (M-$CH_3(CH_2)_2$), 285 (M-[CH_3CH_2+H_2O]), 271 (M-[$CH_3(CH_2)_2$+H_2O]), 257 (M-[$CH_3(CH_2)_2$+CH_3OH]) and 253 (M-[CH_3CH_2+CH_3OH+H_2O]). Such high-mass ions suggest a parent molecular weight of 332, which is 16 mass units heavier than methyl 20:5n-3 (316); thus a molecular weight of 332 indicates that each product possesses four double bonds and an oxirane ring. In addition, ions 303, 285 and 253 suggest that the terminal double bond is located between C-17 and C-18.

Mass spectra were also measured after hydrogenating the five products (Table 3). High-mass peaks at *m/z* 340 (M), 322 (M-H_2O), 309 (M-OCH_3), 297 (M-$CH_3(CH_2)_2$), 291 (M-[H_2O+OCH_3]), 279 (M-[$CH_3(CH_2)_2$+H_2O]), and 265 (M-[$CH_3(CH_2)_2HOCH_3$]) were present in all five spectra, consistent with a common molecular weight of 340. Thus, hydrogenation increased the molecular mass by 8 amu (from 332 to 340; Tables 2 and 3), which would indicate that four double bonds were originally present in each of the five compounds. The molecular weight 340 is 14 mass units greater than 326, the molecular weight of methyl 20:0, and is consistent with the presence of an oxirane ring. In addition, ions 74, 87, 143, 199 and 255 indicate the basic structure of a fatty acid methyl ester (25). Thus the hydrogenation studies suggested that all five compounds are oxirane derivatives of fatty acid methyl esters with four double bonds.

The electron impact mass spectrum of Compound IIIa (Fig. 4a) showed the presence of ions 303 (M-CH_3CH_2), 285 (M-[CH_3CH_2+H_2O]), 275 (M-CH_3CH_2CO), 273 (M-[CH_3CH_2C+H_2O]), 271 (M-[CH_3CH_2+CH_3OH]), 257 (M-[$CH_3(CH_2)_2$+CH_3OH]), 243 (M-[CH_3CH_2CO+CH_3OH]) and 71 (M-[CH_2CH=$CH]_4(CH_2)_3COOCH_3$). Ions 303, 285, 271 and 71 reflect α-cleavages to the oxirane ring, while ions 275, 273, 257 and 243 reflect transannular cleavages (for mechanisms, see Table 2 legends c-e). Except for some of the transannular cleavage ions, the above ions may also be seen following α-cleavages to the n-3 double bond present in the other regioisomers (Table 2). Thus, the mass spectrum of Compound IIIa was hardly definitive

TABLE 2

Summary of Ions in Mass Spectra of Products Derived from Methyl Eicosapentaenoate[a]

Compound I

55(63.0), 67(73.3), 69(25.0)[b], 79(100.0), 81(50.0), 91(86.1), 93(54.4)[b], 105(49.4), 111(6.2)[b], 117(25.2), 119(31.3), 131(15.6), 147(12.6), 161(6.7), 171(6.2), 185(3.5), 189(1.7)[b], 199(1.9), 203(1.6)[e], 206(1.9), 213(3.1), 217(0.8)[d], 220(3.1), 231(1.9)[b], 235(0.4)[e], 245(1.5)[b], 257(0.2), 263(0.5)[b], 271(0.4), 285(0.4), 289(0.2), 301(0.2), 303(0.2), and 314(0.1).

Compound II

55(42.8), 67(56.4), 79(100.0), 81(28.4), 91(42.9), 93(31.0), 106(25.1), 117(7.8), 119(10.7), 131(9.4), 133(7.0)[b], 149(3.4)[b], 151(1.8)[b], 161(3.4), 163(2.6)[e], 173(3.6), 180(3.5), 191(2.6)[b], 199(0.9), 205(1.1)[b], 209(0.3)[d], 211(0.3)[c], 217(0.4), 231(0.5), 245(0.6), 257(0.1), 259(0.2), 263(0.3), 271(0.2), 285(0.4), 289(0.1), 299(0.1), 301(0.1), 303(0.2), 314(0.05), and 332(0.05).

Compound IIIa

55(28.8), 57(28.4), 59(28.7)[c], 67(55.8), 71(10.0)[b], 79(93.2), 81(25.0), 91(100.0), 93(46.3), 105(41.8), 117(28.5), 119(22.7), 131(23.1), 145(11.6), 159(5.8), 173(3.6), 180(3.5), 187(1.6), 199(1.6), 206(2.1), 213(1.4), 217(0.3), 231(0.4), 243(0.2)[e], 245(0.9), 253(0.2), 257(0.1)[d], 260(0.3), 271(0.4)[b], 273(0.2)[c], 275(0.2)[e], 285(0.4)[b], 301(0.1), and 303(0.1)[b].

Compound IIIb

55(53.4), 67(61.2), 79(100.0), 81(36.6), 91(49.9), 93(30.9), 95(31.0), 105(28.3), 117(12.5), 119(15.6), 123(6.7)[e], 131(8.6), 137(1.8)[d], 139(6.8)[c], 141(1.5)[b], 151(3.3)[b], 169(0.8)[d], 171(2.8)[c], 173(3.8)[b], 183(0.5)[b], 191(1.2)[b], 199(0.9), 205(0.8), 213(0.8), 217(0.4), 231(0.4), 245(0.8), 248(0.5), 257(0.1), 271(0.2), 285(0.2), 289(0.1), 301(0.1), and 303(0.1).

Compound IV

55(50.2), 67(50.4), 79(100.0), 81(20.2), 91(51.0), 93(37.4), 99(25.6)[c], 101(6.0)[b], 105(25.1), 108(21.8), 111(12.7)[b], 117(22.2), 119(15.0), 129(10.1)[d], 131(20.7)[c], 143(9.7)[b], 159(3.1), 165(1.8), 171(2.1), 173(3.0), 185(1.3), 187(0.9), 189(0.7)[b], 197(1.1), 199(0.9)[d], 213(1.4)[b], 217(0.3)[d], 231(0.6)[b], 245(0.4), 257(0.1), 271(0.1), 285(0.2), 289(0.1), and 303(0.1).

[a]Electron impact (70 eV) mass spectra were generated from Compounds I-IV isolated from peaks labeled in Figures 2 and 3. Values in parentheses represent intensities as a percentage of the major ion 79 or 91.

[b]This ion reflects simple α cleavage to the oxirane ring (24). As with [c-e], it may be associated with concomitant losses of H_2O or CH_3OH.

[c]This ion reflects transannular cleavage with a hydrogen transfer from a carbon α to the oxirane ring, yielding R-CH=CH + HO$^+$=CH-R' (24).

[d]This ion reflects transannular cleavage with a hydrogen transfer between carbon atoms of the oxirane ring, generating R-C=O$^+$ + CH_2-R' (20).

[e]This ion reflects cleavage similar to [d] except that the charge is localized to the aliphatic fragment $^+CH_2$-R' (20).

TABLE 3

Summary of Ions in Mass Spectra of Hydrogenated Products Derived from Methyl Eicosapentaenoate[a]

Compound I

55(100.0), 57(48.0), 69(49.2), 74(79.9), 83(46.1), 87(48.3), 95(24.9), 97(24.3), 109(12.4), 111(11.1), 113(21.4)[b], 123(9.0), 125(7.2), 143(12.2), 197(5.8), 199(1.2), 207(2.8), 209(3.0)[e], 219(0.7), 223(2.70)[d], 225(2.7)[c], 237(5.0)[b], 241(8.5)[e], 251(1.2)[b], 265(0.8), 269(10.8)[b], 273(0.2), 279(0.1), 291(0.5), 292(0.5), 297(0.4), 309(0.6), 322(0.2), and 340(0.1).

Compound II

55(100.0), 57(48.8), 69(78.8), 74(79.1), 81(44.4), 83(45.8), 87(48.6), 95(24.8), 97(21.8), 111(7.1), 124(10.7), 143(2.7), 149(10.1), 155(24.1), 167(6.0)[e], 177(3.0)[b], 181(3.1)[d], 183(21.4)[c], 195(2.5)[b], 199(12.2)[e], 209(2.6)[b], 215(1.5)[c], 227(6.2)[b], 249(0.2), 255(0.3), 265(0.2), 267(0.2), 273(0.4), 279(0.2), 291(0.7), 297(0.2), 309(0.6), 322(0.1), and 340(0.3).

Compound IIIa

55(100.0), 57(62.0), 59(31.4)[c], 69(54.2), 71(15.5)[b], 74(62.8), 81(29.3), 83(31.0), 87(53.8), 95(23.0), 97(26.4), 99(15.2), 109(12.5), 111(12.4), 123(7.6), 125(5.3), 143(7.8), 167(3.4), 199(3.9), 213(1.5), 223(1.6), 233(1.9), 237(0.8)[b], 239(3.8), 248(1.7), 251(3.5)[e], 255(0.3), 261(0.8)[b], 265(3.8)[d], 279(3.9)[b], 283(3.2)[e], 291(0.6), 293(1.0)[b], 297(0.4)[d], 309(0.8), 311(7.6)[b], 322(0.1), and 340(0.2).

Compound IIIb

55(100.0), 57(50.2), 69(49.7), 74(49.7), 83(49.1), 87(47.7), 95(43.4), 97(24.9), 111(11.0)[b], 125(20.0)[e], 139(23.5)[d], 141(50.6)[c], 153(6.2)[b], 157(12.5)[e], 171(3.1)[d], 173(6.2)[c], 183(2.2)[d], 185(10.0)[b,c], 197(10.9)[b], 213(0.4), 223(0.4), 225(0.4), 237(0.3), 251(0.6), 265(2.1), 273(0.4), 280(0.2), 291(0.6), 297(0.4), 309(0.4), 322(0.1) and 340(0.1).

Compound IV

55(91.1), 57(49.3), 69(29.0), 71(28.9), 74(11.4), 83(49.2), 87(3.3), 99(100.0)[c], 111(12.2), 115(28.9)[e], 131(27.3)[c], 143(10.7)[b], 158(6.7), 181(3.4), 195(0.8), 208(0.8), 213(1.3), 225(1.1)[d], 239(7.4)[b], 248(0.3), 251(0.7), 265(1.7), 273(0.5), 281(1.3), 291(0.9), 297(0.4), 309(0.4), 322(0.2) and 340(0.1).

[a]Electron impact (70 eV) mass spectra were generated after hydrogenation of Compounds I-IV isolated from peaks labeled in Figures 2 and 3. Values in parentheses represent intensities as a percentage of the major ion 55 or 99.

[b]This ion reflects simple α cleavage to the oxirane ring (24). As with [c-e], it may be associated with concomitant losses of H_2O or CH_3OH.

[c]This ion reflects transannular cleavage with a hydrogen transfer from a carbon α to the oxirane ring, yielding R-CH=CH + HO^+=CH-R' (24).

[d]This ion reflects transannular cleavage with a hydrogen transfer between carbon atoms of the oxirane ring, generating R-C=O^+ + CH_2-R' (20).

[e]This ion reflects cleavage similar to [d] except that the charge is localized to the aliphatic fragment $^+CH_2$-R' (20).

in assigning oxirane ring position. Compound IIIa was tentatively identified as 17,18-epoxy-20:4.

The mass spectrum of the hydrogenated Compound IIIa (Fig. 4b) contained ion peaks at 311 (M-CH_3CH_2), 293 (M-[CH_3CH_2+H_2O]), 283 (M-CH_3CH_2CO), 279 (M-[CH_3CH_2+CH_3OH]), 265 (M-[$CH_3(CH_2)_2$+CH_3OH]), 261 (M-[CH_3CH_2+H_2O+CH_3OH]), and 251 (M-[CH_3CH_2CO+CH_3OH]). These ions, reflecting α-cleavages, indicate an oxirane ring at C-17 and C-18. Ions 255 (M-$CH_3CH_2CH(O)CHCH_2$) and 223 (M-[$CH_3CH_2CH(O)CHCH_2$+CH_3OH]), reflecting β-cleavages, also suggest that the oxirane ring is at C-17 and C-18. Hydrogenation did not shift the α-cleavage fragment 71 (Figs. 4a and 4b). Therefore, there are no double bonds between the methyl terminus (C-20) and the oxirane ring (C-17 and C-18). In contrast, hydrogenation shifted the α-cleavage fragments 303, 285 and 271 and the transannular cleavage fragments 275, 257 and 243 by 8 amu (Figs. 4a and 4b), indicating four double bonds between the oxirane ring and the $COOCH_3$ group. In summary, the mass spectra of hydrogenated vs non-hydrogenated methyl esters suggest that the double-bond positions remained intact; therefore, Compound IIIa was tentatively identified as 17,18-*cis*-epoxy-eicosa-5,8,11,14-tetraenoic acid. After similar interpretations of the other spectra (Tables 2 and 3), the other four compounds were tentatively identified as I, 14,15-*cis*-epoxy-eicosa-5,8,11,17-tetraenoic; II, 11,12-*cis*-epoxy-eicosa-5,8,14,17-tetraenoic; IIIb, 8,9-*cis*-epoxy-eicosa-5,11,14,17-tetraenoic; and IV, 5,6-*cis*-epoxy-eicosa-8,11,14,17-tetraenoic acids. Thus spectra from non-hydrogenated and hydrogenated methyl ester derivatives established the oxirane-ring position and the presence of four double bonds. Although supportive, such spectra did not specifically define double-bond positions.

Picolinyl esters of epoxides. Spectra of the picolinyl esters readily confirmed the molecular weight of the regioisomers. For instance, the spectrum of the 17,18-isomer (Fig. 5) contained a molecular ion (409) of 10% intensity, as well as ions 408 (M-H) and 394 (M-CH_3). The molecular ions of the four other regioisomers ranged from 6 to 11% intensity (Table 4). Together, these ions and ion 366 (M-43) established a molecular weight of 409 for each regioisomer (Table 4), which is 16 mass units heavier than *m/z* 393, the molecular ion of picolinyl 20:5n-3. The presence of an oxirane ring and four double bonds was thereby confirmed.

Spectra of picolinyl esters also confirmed the assigned positions for the oxirane rings. Diagnostic high-intensity, high-mass ions are normally generated when the positively charged pyridyl nitrogen abstracts allylic hydrogens (26). The presence of an oxirane ring further intensifies the hydrogen rearrangements (13). In the spectrum of the 17,18-isomer, ions 380 (M-CH_3CH_2) and 338 (M-$CH_3CH_2CH(O)CH$) reflect α-cleavages to the oxirane ring. Moreover, ions 368 (M-CH_3CH_2C), 352 (M-CH_3CH_2CO) and 350 (M-[CH_3CH_2C+H_2O)] reflect transannular cleavages. Both sets of ions established an oxirane ring at C-17 and C-18. For the 8,9-, 11,12- and 14,15-regioisomers, the most intense ion above 200 amu (i.e., *m/z* 260, 300, and 340, respectively) reflects α-cleavages in which the charged fragment contains the oxirane ring. The most intense ion above 200 amu in the spectrum of the 5,6-isomer (*m/z* 208) reflects transannular cleavage. Since the most intense ion above 200 amu (13.9-48.0%) for all five regioisomers reflects either α- or transannular cleavages, the oxirane position was readily confirmed.

Unlike the methyl esters, the spectra of picolinyl esters unequivocally established the position of double bonds which were repeated every 40 mass units (-CH=CH-CH_2-). For instance, in the spectrum of the 17,18-isomer, ions 204, 244, 284, and 324 reflect α-cleavage on the omega side of Δ5, Δ8, Δ11, and Δ14, respectively, whereas ions *m/z* 178, 218, 258 and 298 reflect α-cleavage on the carboxyl side (Fig. 5). Double-bond positions were likewise identified for the other four regioisomers (Table 4). For each regioisomer, ions reflecting α-cleavage to the double bond on the omega side tended to be more intense

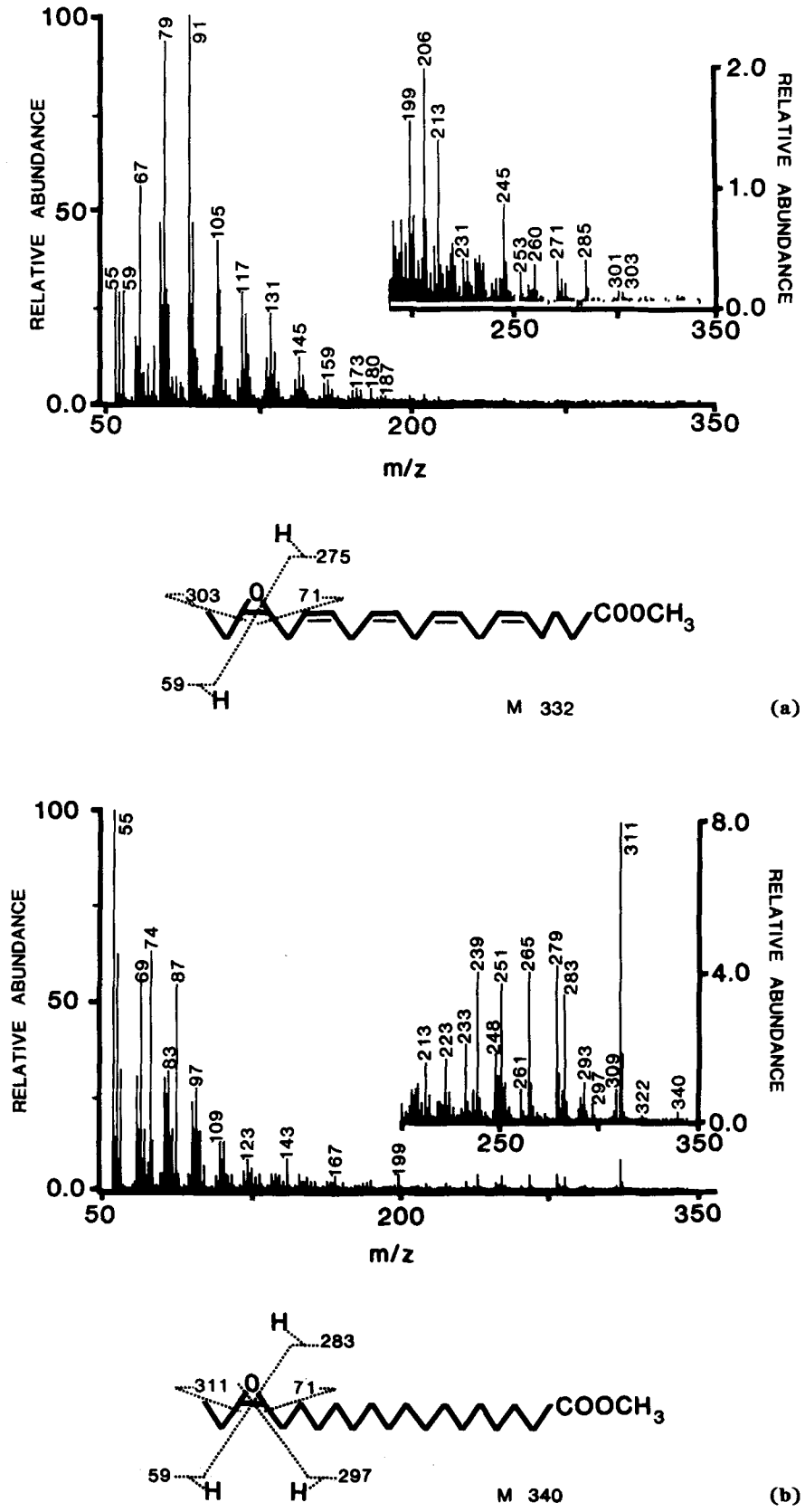

FIG. 4. Electron impact (70 eV) mass spectra of (a) non-hydrogenated and (b) hydrogenated Compound IIIA.

TABLE 4

Summary of Ions in Mass Spectra of Picolinyl Esters of Epoxides Derived from Eicosapentaenoate[a]

Epoxide position	Oxirane-ring cleavages (α or transannular)	α Cleavages to double bond on ω side	α Cleavages to double bond on carboxyl side	M	(M-1)	(M-15)	(M-43)
17,18-	380(25.9), 368(2.4), 352(3.0), 338(4.2)	324(3.0) 284(12.7) 244(4.2) 204(1.4)	298(2.0), 258(6.0), 218(5.0) 178(1.8)	10.5	4.4	2.9	ND
14,15-	340(48.0), 328(2.6), 312(2.7), 298(13.3)	380(5.8) 284(5.6) 244(7.5) 204(1.9)	354(1.7), 258(6.1), 218(4.1) 178(2.1)	11.2	6.0	1.5	3.0
11,12-	300(21.1), 288(5.2), 272(4.9), 258(19.4)	380(6.5) 340(12.2) 244(2.4)	354(7.9), 314(2.5), 218(4.7)	5.6	6.6	3.0	3.0
8,9-	260(13.9), 248(13.0), 232(7.0), 218(11.6)	380(8.1) 340(9.0) 300(4.7)	354(7.3) 314(4.6) 274(1.8)	7.0	7.6	2.7	3.4
5,6-	220(6.4), 208(19.8) 192(1.3), 178(2.4)	380(6.0) 340(11.7) 300(6.1)	354(3.0), 314(2.9), 260(3.0)	6.2	5.9	2.9	2.6

[a]Values in parentheses represent intensities as a percentage of the major ion 92. In addition to 92 (C_6H_6N), ions common to all epoxide regioisomers were: 108(C_6H_6NO), the McLafferty rearrangement 151($C_8H_9NO_2$), and 164 ($C_9H_{10}NO_2$).

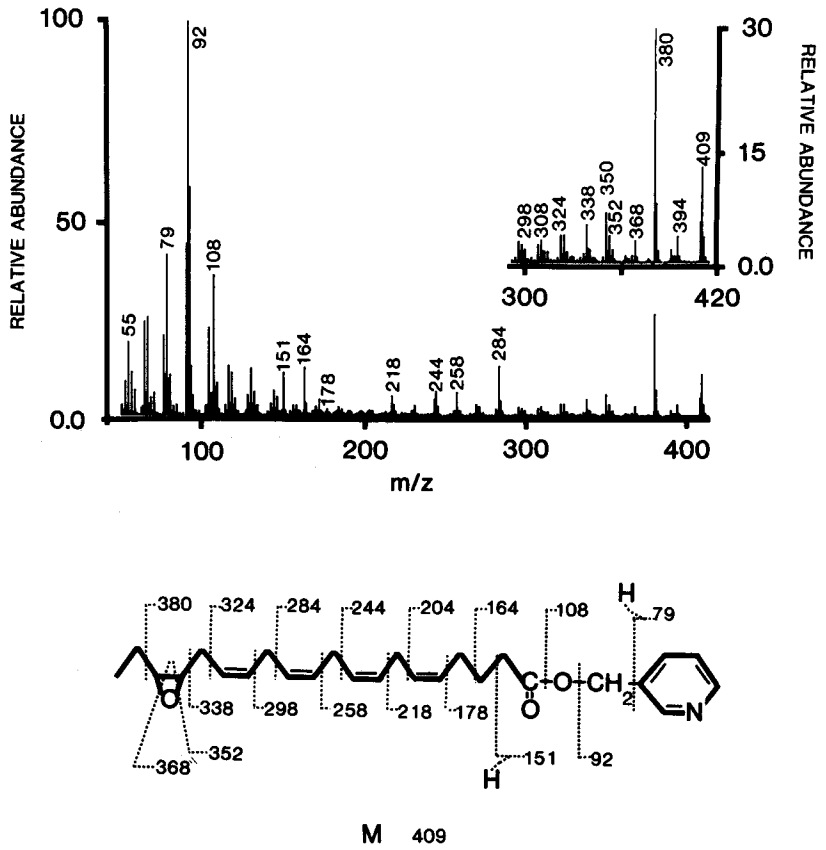

FIG. 5. Electron impact (70 eV) mass spectrum of the picolinyl ester of Compound IIIA.

TABLE 5

Epoxide Yield from Methyl Eicosapentaenoate

Compound	Radioactivity (%)[a]
20:5n-3	86.3 ± 0.6 (85.5 ± 0.2)
Monoepoxides	
17,18-	3.3 ± 0.1
14,15-	1.9 ± 0.1
11,12-	1.7 ± 0.1
8,9-	1.7 ± 0.1
5,6-	1.5 + 0.05
Total	10.1 ± 0.4 (11.8 ± 0.2)
Diepoxides[b]	0.5 ± 0.02

[a]Data represent the mean % ± S.D. (n = 4) of total radioactivity recovered after RP-HPLC. Corresponding TLC values are shown in parentheses. The total radioactivity collected represented 99.5 ± 6.2% (RP-HPLC) and 92.0 ± 1.1% (TLC) of the amount applied, respectively.

[b]Other minor components were probably autoxidation artifacts (HEPEs, see ref. 7) and represented 0.4 ± 0.05% of total radioactivity.

(6.4 ± 0.8% S.E.M., n = 17; Table 4) than similar cleavage on the picolinyl side (3.9 ± 0.5%). Such cleavages readily established the integrity of the double bonds. Therefore, the structures of the five regioisomers were unequivocally identified as: IIIa, 17,18-epoxy-eicosa-5,8,11,14-tetraenoic; I, 14,15-epoxy-eicosa-5,8,11,17-tetraenoic; II, 11,12-epoxy-eicosa-5,8,14,17-tetraenoic; IIIb, 8,9-epoxy-eicosa-5,11,14,17-tetraenoic; and IV, 5,6-epoxy-eicosa-8,11,14,17-tetraenoic acids. In summary, use of the novel picolinyl ester derivatives confirmed the oxirane-ring position and the presence of four double bonds. Moreover, by identifying the position of each double bond, this derivative established that the original double bonds remained intact.

Diols. To identify the diol products, each regioisomer was converted to a *bis* (trimethylsilyl ether) methyl ester, whereupon its spectrum was found to match the corresponding 20:5n-3 metabolite (7). Thus they were established as IIIa, 17,18-; I, 14,15-; II, 11,12-; IIIb, 8,9-; and IV, 5,6-dihydroxy-20:4, respectively. Based on the established EET (epoxyeicosatrienoic acids) and DHET (dihydroxyeicosatrienoic acids) nomenclature for 20:4n-6 metabolites, we suggest that the 20:5n-3 epoxygenase metabolites be differentiated as EEQs (epoxyeicosaquatraenoic acids) and DHEQs (dihydroxyeicosaquatraenoic acids).

Determination of yields. There was 99.2 ± 0.4% (n=4) of incubated radioactivity recovered following extractive isolation, bicarbonate and aqueous washes. Recovery was unaffected by prolonging incubation up to 16 hr or using up to 3.0 eq. *m*-ClPBA. Thus, as with 22:6n-3 (9), the extraction procedure was quantitative.

Individual and total EEQ yields were assessed radiometrically. Since the summed EEQ was the theoretical 10% value, the reaction was complete within 15 min (Table 5). As noted for 22:6n-3 and 20:4n-6 epoxides (9,14,16), *m*-ClPBA favors formation of the polar regioisomers, i.e., those with more carbon atoms between the oxirane ring and carboxyl groups (Table 5). Likewise, cytochrome P-450 epoxygenases favor formation of polar EEQs (7) as well as polar epoxides derived from 22:6n-3 and 20:4n-6 (10,27). The same preference is also seen with the hydrolysis products or vicinal diols, which are formed during incubation with microsomes and 18:2n-6 (28), 18:3n-3 (28), 20:4n-6 (14,29), 20:5n-3 (7), and 22:6n-3 (8). Thus, both chemical and enzymatic synthesis favor formation of the more polar regioisomers, which occurs independently of the fatty acid substrate. Because such preferences contrast with the tendency of intact cells to epoxygenate fatty acids close to their carboxyl ends (30), microsomal preparations and isolated isoenzymes may not reflect normal substrate specificities.

Minor products were also quantitated by HPLC. Small amounts of diepoxides (A group, Fig. 3; 0.5%) and autoxidation products (B group, Fig. 3; 0.4%) were evident. The reaction mixture exhibited the same concentration of autoxidation products as non-incubated 20:5n-3. Thus, incubation for 15 min did not increase the formation of autoxidation products.

Comparison of TLC and HPLC values indicates excellent agreement between the two techniques. The more rapid TLC should prove useful in finding *m*-ClPBA equivalences to maximize the yield of polar products for structural identification. However, there are two problems. First, total epoxide values tended to be higher with TLC, due partly to a 1-2% trailing of fatty acid into the monoepoxide region on the TLC plate (Fig. 1). Second, total recovery was slightly lower with TLC, probably due to adsorption losses and lack of two-π counting conditions caused by the silica gel deposit at the bottom of the scintillation vial.

Use of 0.1 eq. *m*-ClPBA has an advantage in the synthesis of radiolabeled or deuterated standards, because the maximum theoretical yield (10%) is achieved with minimal loss of substrate caused by side reactions. The overall yield from isotopically labeled 20:5n-3 may be improved by cycling techniques because of the quantitative recovery of unused substrate, going from 10% to 66% and 88% within 10 or 20 cycles, respectively. Such yields are significantly higher than the 36% found with [$^2H_8$]20:4n-6 and 1.05 eq. *m*-ClPBA (14). In that study, most deuterated 20:4n-6 was converted to (unidentified) polar by-products. Using 0.1 eq. *m*-ClPBA and [$^{10}H_2$]20:5n-3, analogous standards may now be efficiently synthesized to assess EEQ and DHEQ tissue concentrations following the ingestion of fish oils. Analogous radiolabeled standards may prove useful in studying the incorporation of EEQs and DHEQs into phospholipids and acylglycerides (31). Finally, the synthesis of large amounts of EEQs and DHEQs for pharmacological studies may readily be achieved when an NP-HPLC column of high capacity is used. By scaling up the incubation volume (85 mg/3.5 mL CH_2Cl_2) and using a preparative column (2.25 cm i.d.), more than10 mg of each EEQ regioisomer has been isolated within a three-day period.

In summary, five epoxide regioisomers were synthesized from 20:5n-3 and characterized by TLC, RP-HPLC, NP-HPLC and capillary GC. Using electron impact mass spectrometry with methyl- and picolinyl-ester derivatives, the positions of the oxirane ring and double bonds were unequivocally established. Epoxide synthesis was quantitative due to efficient extraction and the absence of unwanted by-products. Use of cycling techniques increases yields up to 80% of the initial substrate, and more than 10 mg of each EEQ was synthesized in three

days. Thus, all ten EEQ plus DHEQ regioisomers were rapidly synthesized in amounts adequate for biological testing

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The Response to Endotoxin in Guinea Pigs After Intravenous Black Currant Seed Oil

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The influence on the metabolic response to endotoxin of three days of total parenteral nutrition with lipids high in gammalinolenic acid (18:3ω6, GLA) compared to soy oil (SO) was examined in acute operatively stressed guinea pigs. GLA is the precursor of dihomogammalinolenic acid (DHLA), the substrate for synthesis of "1" series prostaglandins such as PGE_1, which have previously been shown to be protective in endotoxin lung injury and traumatic shock. Guinea pigs fed an intravenous diet containing black currant seed oil (BCO) emulsion (20% GLA) or soy oil emulsion (0% GLA) for 2.5 days had their arterial pH, pCO_2, pO_2, and bicarbonate measured at baseline and hourly during a 7-hr infusion of endotoxin (lipopolysaccharide (LPS), 2mg/kg) or saline. Plasma lactate and fatty acid profile analyses were performed at the end of the LPS infusion. Increased levels of GLA and DHLA were present in the plasma phospholipid fraction of animals fed the black currant seed oil diet, while soy-fed animals had only trace amounts of GLA. In addition, the ratio of DHLA to arachidonate was higher in animals receiving the black currant seed oil total parenteral nutrition (TPN). After 2 hr of LPS infusion, all animals exhibited the typical shock response resulting in metabolic acidosis characterized by a significant ($p<0.05$) drop in pH from 7.34±.02 (SO) and 7.39±.02 (BCO) at baseline to 7.14±.05 and 7.22±.04 by 7 hr for SO and BCO groups, respectively. Plasma lactate values at the end of the infusion were significantly elevated compared to saline in both groups ($p<0.05$). Guinea pigs fed GLA showed no improvement over soy-fed animals, and actually exhibited a more pronounced metabolic acidosis and increased mortality (50% vs 0%) over the 7 hr of LPS infusion. No beneficial effects of a diet high in GLA were observed in the present study.

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Gram-negative sepsis is seen in a significant number of postoperative, burn, and trauma patients. Endotoxic shock, caused by the lipopolysaccharide (LPS) from the cell membranes of gram-negative bacteria, is characterized by metabolic acidosis, respiratory alkalosis and tachypnea, tachycardia, and shock, and is often fatal (1). The combination of these events results in the harmful redistribution of blood away from vital tissues (2,3).

Since levels of arachidonic acid (AA, 20:4ω6) metabolites such as thromboxane A_2 (TxA_2), prostaglandin E_2 (PGE_2), and prostacyclin (PGI_2) are elevated in endotoxemia (2) and play a crucial role in the pathogenesis of endotoxic shock (3), several investigators have explored various means of reducing the levels of arachidonic acid and its metabolites. Cook *et al.* (4) demonstrated that fatty acid-deficient animals show decreased levels of TxA_2 and are more resistant to endotoxin. The eicosapentaenoic acid (EPA, 20:5ω3) found in fish oil is thought to compete with arachidonic acid for binding sites on cyclooxygenase and to yield prostaglandins of the "3" series with attenuated potency compared with those of the "2" series from arachidonate. Providing EPA as a substrate decreases prostanoid metabolites from AA *in vitro* (5,6) and *in vivo* (7), in addition to blocking fever (8) and reducing lactic acidosis in response to endotoxin (9). Mascioli *et al.* (10,11) have demonstrated improved survival in a model of endotoxic shock following both enteral and parenteral administration of fish oil.

Gammalinolenic acid (GLA) is found in relatively large amounts in evening primrose oil (EPO, 9%), borage oil (BO, 25%), and black currant seed oil (BCO, 15%). Although GLA is a precursor of arachidonic acid and the "2" series prostaglandins, GLA is also shunted into an alternate pathway resulting in production of prostaglandins of the "1" series that exhibit effects different from those of the "2" series. Feeding of GLA-enriched diets increases levels of GLA and dihomogammalinolenic acid (DHLA) in plasma (12), epidermal (13), and liver phospholipids (14) and results in elevations of PGE_1 (15). DHLA, the elongation product of GLA, may compete with arachidonic acid for cyclooxygenase when the ratio of DHLA to arachidonate is high. Administered exogenously in man, DHLA enhances platelet PGE_1 production and potentiates antithrombotic changes in hemostatic function (16). Prostaglandin E_1 in turn increases peripheral blood flow (17), decreases blood pressure (18), inhibits platelet aggregation (19) and arachidonic acid metabolism (20), and has been shown to be protective in models of endotoxic (21) and traumatic shock (22). Shunting the prostanoid pathway away from arachidonic acid metabolites toward those from DHLA would seem to be beneficial in endotoxic shock, the pathophysiology of which is largely mediated by arachidonic acid metabolites.

The effects of GLA are similar to those of fish oil which have shown benefit in this model. We, therefore, tested the hypothesis that GLA feeding would ameliorate the effects of endotoxin. The present study examines the influence of three days of total parenteral nutrition (TPN) high in GLA from black currant seed oil in a model of endotoxic shock in guinea pigs.

MATERIALS AND METHODS

Animal procedures. Thirty-seven male guinea pigs (350-400 g) of the Hartley strain (Elm Hill Breeding Lab, Chelmsford, MA) were fed standard guinea pig chow

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Abbreviations: TPN, total parenteral nutrition; LPS, lipopolysaccharide (endotoxin); GLA, gammalinolenic acid; DHLA, dihomogammalinolenic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; PGE_1, prostaglandin E_1; PGE_2, prostaglandin E_2; $PFG_{2\alpha}$, prostaglandin $F_{2\alpha}$; TxA_2, thromboxane A_2; ANOVA, analysis of variance; BCO, black currant seed oil; SO, soy oil.

(Prolab, Agway Inc., Syracuse, NY) for 5-7 days until they weighed 375-450 g. At that time, on day 1, animals were anesthetized with 100 mg/kg ketamine (Parke-Davis, Morris Plains, NJ) and 40μg/kg atropine (Elkins Sinn Inc. Cherry Hill, NJ) and were given preoperative subcutaneous injections of trimethoprim/sulfamethoxazole (30 mg/kg, Lyphomed, Rosemont, IL) to prevent infection and 1% lidocaine as a local anesthetic. A silastic catheter (0.02″ i.d. × 0.037″ o.d., American Scientific Products, McGaw Pk, IL) was inserted into the left internal jugular vein, and a polyethylene catheter (0.023″ i.d. × 0.038″ o.d., Clay Adams, Parsippany, NY) was placed in the left carotid artery. Both lines were tunneled subcutaneously to an exit site between the animals' shoulders, and the venous line was attached to a swivel apparatus allowing free movement. The carotid line was filled with heparin (1,000 U/mL) and capped. Animals were placed in stainless-steel cages and allowed to recover from surgery for several hr while receiving an infusion of 0.9% saline at 1 mL/hr.

Diets and feeding. Guinea pigs were randomized to receive 200 kcal/kg/day of TPN, including 50 kcal/kg of protein, 75 kcal/kg of carbohydrate, and 75 kcal/kg of fat as either a 20% soybean oil emulsion (Intralipid, Kabi Vitrum, Alameda, CA), or a 20% black currant seed oil emulsion (Clintec Nutrition, Deerfield, IL) for 66 hr. Diets were supplemented with 30 mEq/L of sodium chloride, sodium acetate, and potassium chloride, 25 mEq/L of potassium acetate, 15 mEq/L of potassium phosphate, 8.5 mEq/L of calcium gluconate, 8 mEq/L of magnesium sulfate, 2mL/L of multivitamins (MVI-12, USV Laboratories, Tuckahoe, NY), and 10mL/L of trace minerals (MTE-5, Lyphomed, Rosemont, IL). Amino acid and dextrose solutions were compounded by the New England Deaconess Hospital pharmacy, and fresh lipid was added by the investigators twice daily. TPN infusion was begun 3 hr after surgery on day 1 at $^2/_3$ the full rate to allow for adaptation to the diet for 18 hr. At 9 a.m. on day 2, the rate was increased to full for 48 hr and continued until day 4.

Endotoxin infusion. On the morning of day 4, animals were weighed and randomized to receive, in the TPN, either 2 mg/kg of endotoxin (*E. coli* 026:B6, Difco, Detroit, MI) or the same volume of saline, at a rate of 3.4 mL/hr for the last 7 hr of infusion, bringing the total time receiving TPN to 73 hr. Prior to the start of the endotoxin infusion, two baseline arterial pH, pCO_2, pO_2, and bicarbonate measurements were performed 10 min apart on a Corning 168 Blood Gas Analyzer (Corning Glass, Medfield, MA), and diastolic and systolic blood pressure and heart rate were measured on a Grass Model 79D polygraph (Quincy, MA). After the start of endotoxin infusion, these measurements were performed hourly for 7 hr. Approximately 0.2 mL of blood was drawn for the blood gas measurements every hr. At 7 hr, 5-8 mL of blood was drawn through the arterial line and mixed with 3.8% citrate in a blood/citrate (9:1) ratio for lactate and plasma fatty acid profile analyses. Animals were then sacrificed by an intravenous injection of ketamine (200 mg/kg).

Sample analysis. Blood was spun at 1,000×g for 10 min and the plasma separated from the red cells. Aliquots of plasma were frozen under nitrogen at -20°C for lactate and fatty acid profile analyses. Fatty acid profiles were measured on a Sigma 2000 gas chromatograph (Perkin Elmer, Norwalk, CT) as previously described (23). Reliable limits of detectability of the gas chromatograph were 0.001μmoles/mL. Plasma lactate was determined on a YSI Model 23L lactate analyzer (Yellow Springs Instrument Co. Inc., Yellow Springs, OH).

Statistical analysis. Eight control animals receiving saline-soy oil (SO), n=3; BCO n=5—were grouped for statistical analysis (BMDP Statistical Software, Los Angeles, CA) of blood gases, heart rate, and blood pressure since the two groups were not significantly different at any time point after two-way analysis of variance (ANOVA). Subsequent one-way ANOVA and t-tests with the Bonferroni correction were performed at each time point comparing the saline control to the two experimental endotoxin groups to discern specific intergroup differences. Repeated measures analyses were performed over time on hr 0 through 5. Animal mortality after hr 5 prevented repeated measures analysis including hr 6 and 7. Fatty acid profiles were analyzed by two-way ANOVA, and no significant effect of endotoxin treatment was observed. A subsequent one-way ANOVA by diet followed. All data are represented as mean ± standard error of the mean unless otherwise indicated. Type 1 error was set at 0.05.

RESULTS

Animals in both the soy and black currant seed oil groups lost weight during TPN. The weights dropped from 407 ± 31 g to 360 ± 24 g in the soy group and 424 ± 32 g to 369 ± 25 g in the black currant seed group. Neither weights nor weight changes in the two diet groups were significantly different. Several animals in both diet groups expired during the feeding period due to technical problems with the infusion catheters or due to infection. Of the 22 guinea pigs remaining after the feeding period, (SO, n=9; BCO, n=13), 6 in the SO group and 8 in the BCO group received endotoxin. One of the animls in the BCO group expired by hour 5, and 3 more by hour 6, resulting in fifty percent mortality in black currant-fed animals receiving endotoxin.

Table 1 shows the percentages of triglyceride and phospholipid fatty acids in the black currant seed and soy bean oil emulsions used in the present study. The phospholipid fraction, which accounts for only 6% of total fatty acids received by the animals, is provided for comparison with the plasma phospholipid profile. Animals were fed intravenously, and the presence of emulsion in the plasma must be considered.

The fatty acid profiles of the plasma phospholipid fractions are shown in Table 2. Plasma from SO animals contained only trace amounts of GLA, whereas the BCO group had incorporated significantly higher proportions of GLA and DHLA into their plasma phospholipid, ($p<0.001$ and $p<0.05$, t-test). Arachidonate levels were not different between the diet and treatment groups, but the ratio of DHLA to AA, which may affect fatty acid utilization in prostaglandin synthesis, was higher in BCO animals, 0.52 ± 0.05 vs 0.38 ± 0.10 (not statistically significant).

Figure 1A illustrates arterial pH measured at baseline and during the 7-hr endotoxin infusion. After two hours of infusion, BCO-fed guinea pigs receiving LPS exhibited a significant metabolic acidosis ($p<0.01$ vs saline), while the pH of the soy group had not decreased significantly.

TABLE 1

Fatty Acid Composition of the Guinea Pig Diets (relative %)

Diet	Soy bean		Black currant	
Fraction Fatty acid	TG[a]	PL[b]	TG	PL
14:0	0.1	—	0.4	—
16:0	9.8	31.3	6.7	28.9
16:1ω7	0.1	1.3	—	1.3
18:0	3.9	14.8	1.4	13.1
18:1ω9	22.2	27.2	14.1	24.9
18:2ω6	53.7	11.6	43.4	15.4
18:3ω3	7.3	—	13.4	2.1
18:3ω6	—	—	15.3	1.5
20:3ω6	—	—	—	—
20:4ω6	0.4	4.1	—	3.5
22:6ω3	—	8.5	—	8.1

[a]Triglyceride fraction.

[b]Phospholipid fraction; accounts for only 6% of total fatty acids in diet.

Arterial pH values at 2 hr were 7.27 ± .07 (SO) and 7.22 ± .08 (BCO). This metabolic acidosis was sustained or worsened throughout the period of study. By hour 4, the pH of the BCO group was significantly lower than that of the soy group receiving endotoxin ($p<0.05$). Repeated measures analysis over hours 0-5 indicated that the BCO group had a more pronounced decline in pH in response to endotoxin than did the soy-fed animals, $p<0.05$. By hour 6, 50% of the BCO group had died, causing a rise in the mean pH suggesting that the remaining animals in that group were probably more resistant to the endotoxin. No animals in the soy group died during endotoxin infusion.

Arterial pCO_2 levels are also shown in Figure 1B. At hour 2, the black currant group showed a significant drop in pCO_2 compared to saline and soy endotoxin guinea pigs, ($p<0.05$), due to an attempted respiratory compensation of the metabolic acidosis. Both soy and BCO endotoxin groups maintained slightly lowered pCO_2 levels for the remainder of the infusion. Repeated measures analysis indicated no differences in changes in pCO_2 among the diet groups over time.

TABLE 2

Plasma Phospholipid Fatty Acid Profiles

Diet Fatty acid	Soy bean[a]	Black currant[a]
14:0 (μmole/mL)	0.01 ± 0.00	0.01 ± 0.00[b]
(percentages)	0.32 ± 0.03	0.34 ± 0.03
16:0	0.24 ± 0.01	0.25 ± 0.02
	15.43 ± 0.56	15.95 ± 0.53
16:1ω7	0.02 ± 0.00	0.02 ± 0.00
	1.12 ± 0.05	1.16 ± 0.08
18:0	0.29 ± 0.02	0.26 ± 0.02
	18.47 ± 0.46	16.78 ± 0.17[c]
18:1ω9	0.31 ± 0.02	0.33 ± 0.03
	20.16 ± 0.58	20.86 ± 0.64
18:2ω6	0.29 ± 0.03	0.31 ± 0.04
	18.59 ± 0.76	19.34 ± 0.88
18:3ω3	0.12 ± 0.02	0.09 ± 0.01
	7.91 ± 1.61	6.03 ± 0.95
18:3ω6	trace	0.07 ± 0.00[d]
	trace	4.30 ± 0.19[d]
20:3ω6	0.026 ± 0.01	0.034 ± 0.01[e]
	1.74 ± 0.24	2.23 ± 0.15
20:4ω6	0.08 ± 0.01	0.07 ± 0.01
	4.99 ± 0.32	4.59 ± 0.20
22:6ω3	0.06 ± 0.01	0.06 ± 0.01
	3.87 ± 0.31	3..43 ± 0.20
sum	1.54 ± 0.10	1.56 ± 0.11
20:3ω6/20:4ω6	0.38 ± 0.10	0.52 ± 0.05
22:6ω3/20:4ω6	0.78 ± 0.02	0.77 ± 0.02

[a]Soy bean n=5, black currant n=8.

[b]Mean ± SEM

[c]$P<0.005$ ANOVA; $p<0.01$ vs soy bean oil by Bonferroni t-test.

[d]$p<0.005$ ANOVA; $p<0.01$ vs soy bean oil by Bonferroni t-test.

[e]$p<0.05$ ANOVA; $p<0.05$ vs soy bean oil by Bonferroni t-test.

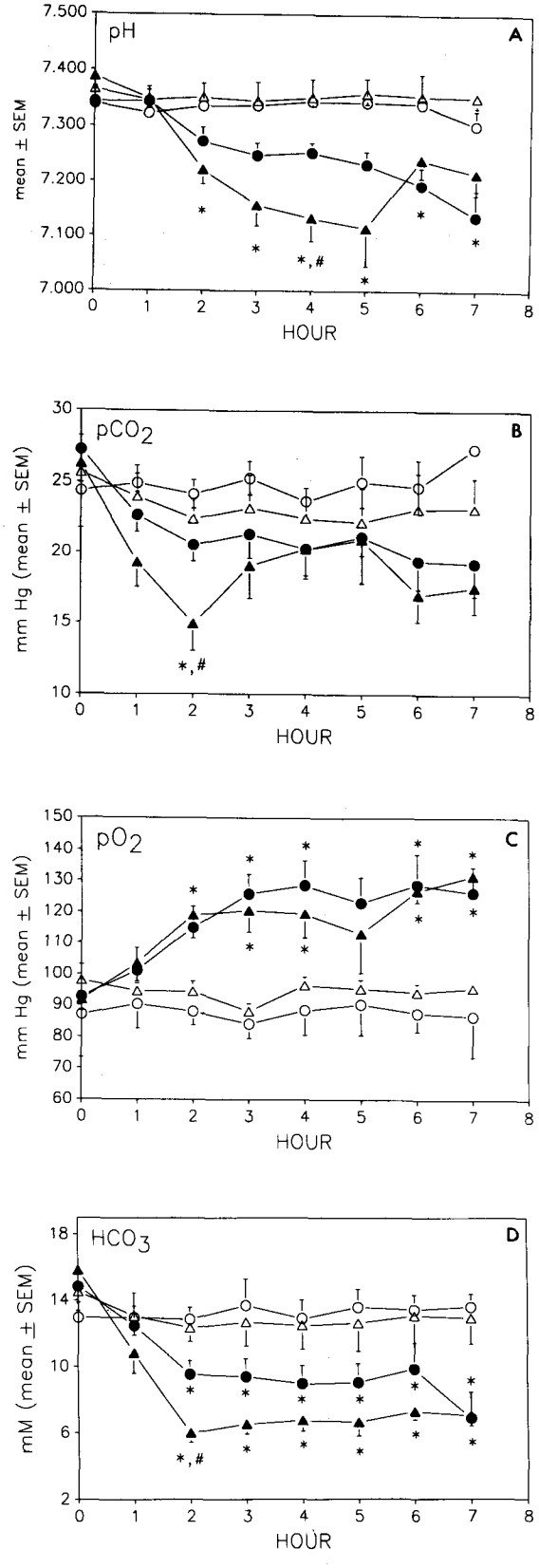

FIG. 1(A-D). Arterial pH (A), pCO_2(B), pO_2 (C), and bicarbonate (HCO_3^-) (D) before and during a 7-hr infusion of endotoxin (dark symbols) or saline (open symbols) in guinea pigs receiving TPN with soy bean (circles) or black currant seed oil (triangles) emulsion. Values are expressed as mean ±SEM. *p<0.05 vs saline, #p<0.05 vs soy endotoxin group by t-test with Bonferroni correction.

The attempted respiratory compensation of the metabolic acidosis resulted in a rise in pO_2 levels by hour 2 in the BCO group, and by hour 3 in the SO group (Figure 1C, $p<0.01$). Levels remained elevated for the rest of the infusion. There were no differences in changes over time among the diet groups determined by repeated measures analysis.

Figure 1D indicates that bicarbonate levels in both the soy and black currant seed oil groups fell significantly by hour 2 ($p<0.05$) in response to the endotoxin, and remained depressed for the duration of the infusion. Bicarbonate levels of the BCO endotoxin group were significantly lower than those of the SO animals at hour 2 ($p<0.005$), and repeated measures determinations indicated a greater drop in bicarbonate over time in the BCO groups compared to SO ($p< 0.05$).

Plasma lactate levels determined after 7 hr of endotoxin infusion indicated that both the soy and black currant group had a significant lactic acidemia as shown in Figure 2. The two endotoxin groups were not significantly different from each other.

Heart rate and blood pressure were also measured hourly during the endotoxin infusion and are illustrated in Figures 3 and 4, respectively. At hour 4, the BCO group exhibited a significantly lower heart rate ($p<0.05$) than the saline control, and both diet groups had significantly lower heart rates than the saline group at hour 7. The mean arterial blood pressure of the BCO group was significantly higher than the SO group after 2 hr of endotoxin infusion ($p<0.01$).

DISCUSSION

Several studies have demonstrated the successful alteration of arachidonic acid (AA) metabolites through manipulation of dietary lipids. Terano *et al.* (6) employed EPA feeding in models of acute inflammation and demonstrated decreased concentrations of PGE_2 and TxB_2 in inflammatory exudates. Similarly, peritoneal macrophages from mice fed menhaden oil stored less arachidonic acid in cellular lipids and secreted significantly less PGE (the authors did not diifferentiate between series "1" and "2") and TxB_2 when stimulated *in vitro* (24). A study by Croft *et al.* (25) examining plasma and tissue phospho-

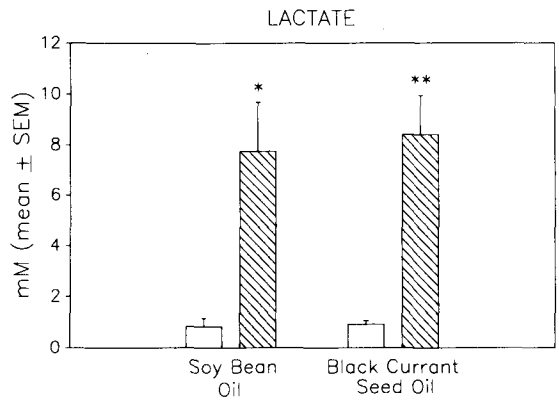

FIG. 2. Plasma lactate levels following endotoxin (dark bars) or saline (open bars) infusion in guinea pigs fed TPN with soy bean or black currant seed oil emulsion. Values are expressed as ± SEM. *,p<0.05, <0.005 vs saline by Student's t-test.**

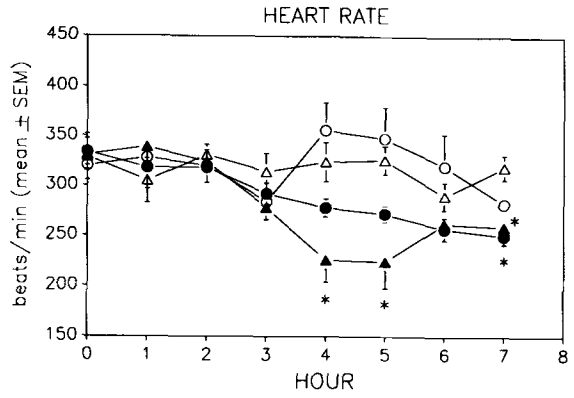

FIG. 3. Heart rate at baseline and during a 7-hr infusion of endotoxin (dark symbols) or saline (open symbols) in guinea pigs receiving TPN with soy bean (circles) or black currant seed oil (triangles) emulsion. Values are expressed as mean ± SEM. *p<0.05 vs saline by t-test with Bonferroni correction.

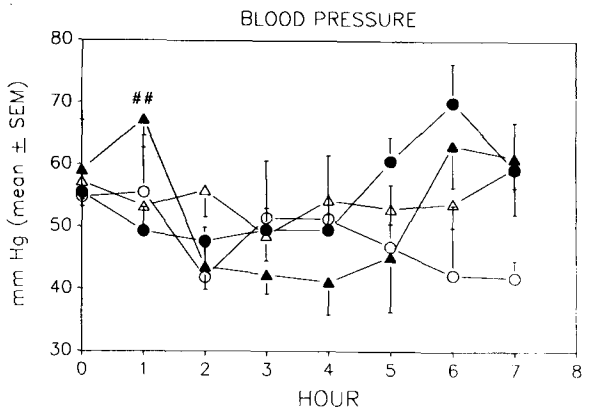

FIG. 4. Mean arterial blood pressure (2/3 diastolic plus 1/3 systolic) at baseline and during a 7-hr infusion of endotoxin (dark symbols) or saline (open symbols) in guinea pigs receiving TPN with soy bean (circles) or black currant seed oil (triangles) emulsion. Values are expressed as mean ±SEM. *p<0.05 vs saline, **p<0.01 vs soy endotoxin group by t-test with Bonferroni correction.

lipids and whole blood thromboxane and prostacyclin in rats fed EPA showed similar results. In guinea pigs, six weeks of oral feeding with a fish oil-supplemented diet significantly blunted the febrile response induced by recombinant interleukin-1 (8), and Mascioli *et al.* (10,11) have shown enhanced survival after endotoxin challenge following oral as well as intravenous administration of fish oil.

It has been suggested that these effects are due to the ability of EPA to competitively inhibit the oxidation of arachidonate by cyclooxygenase (26), thus decreasing the amount of "2" series prostaglandins and thromboxanes but also resulting in the synthesis of "3" series metabolites which may be of attentuated potency.

Providing GLA, the precursor for prostaglandins of the "1" series, might shift the production of prostaglandins toward that of the "1" series and away from the "2" as with fish oil. PGE_1has been shown to decrease TxA_2 levels in endotoxin-induced lung injury (27) and improve survival in experimental traumatic shock (21). Furthermore, PGE_1 has antiinflammatory properties when administered both orally and subcutaneously in models of acute and chronic inflamation (28). PGE_1 may function in these models by altering intracellular cyclic adenosine 5′-monophosphate (AMP) levels in specific inflammatory cell populations. Direct oral administration of DHLA has been shown to increase the DHLA/AA ratio in plasma and platelets, with an enhancement in the ability of platelets to produce PGE_1 (16).

In the present study, guinea pigs fed the black currant seed oil diet had significantly higher levels of GLA and DHLA in the plasma phospholipid fraction than the soy-fed group. Similar results, showing no difference in arachidonic acid levels but a higher ratio of DHLA to arachidonate in liver lipids, were found in guinea pigs fed an oral diet supplemented with 10% GLA (14). In addition, animals in the present study received TPN during the endotoxin infusion to maintain high levels of DHLA since it is rapidly metabolized. However, BCO animals showed no improvement in resistance to the development of endotoxic shock when compared with soy-fed animals, and in fact exhibited a more rapid decline in pH and appreciable mortality.

It is doubtful that the marked response to the endotoxin and the 50% mortality in the BCO group are due to increased PGE_1 production since this prostaglandin has been shown to be beneficial in a variety of models in which it was administered directly (21, 28). Reliable assays for "1" series prostaglandins are not readily available, so only assumptions can be made regarding the relative presence of "1" or "2" series prostaglandins. However, several other studies on GLA feeding may be helpful in explaining the results of the present experiment.

In a study by Huang *et al.* (29), the effect of feeding with GLA in comparison with safflower oil on aspirin-induced gastric hemorrhage was examined. In safflower-fed rats, aspirin treatment induced hemorrhage and reduced the ratio of AA to linoleate, possibly due to Δ 6 desaturase inhibition by aspirin (29,30). In rats fed with GLA, which bypasses the Δ 6 desaturase, aspirin neither caused hemorrhage nor changed the AA to linoleate ratio. Another study by Diel (31) showed similar results. The authors hypothesize that providing GLA in these studies (29,31) was beneficial presumably due to its ability to bypass Δ 6 desaturation and to supply the substrate for the formation of AA acid and its "2" series prostanoid metabolites.

In a study by Miller and Ziboh (13), guinea pigs were fed for eight weeks with safflower oil or borage oil, which contains 25% GLA. Evaluation of epidermal fatty acids and prostanoids showed that in addition to increased GLA, DHLA, and PGE_1 levels in the borage oil group, there were also significant elevations of arachidonic acid and all its derived prostaglandins, $PGF_2\alpha$, PGE_2, and PGD_2. These prostanoid changes would not be beneficial in a model of endotoxic shock mediated by arachidonic acid metabolites.

An interesting hypothesis would be to investigate the synergy of simultaneous feeding of both an oil rich in GLA as well as an oil rich in EPA. The EPA would serve to decrease the formation of "2" series prostanoids and the GLA would serve to increase the "1" series prostanoids. This combination of oil feeding may well be more potent than that of EPA alone.

In summary, acute TPN with GLA-rich black currant seed oil that produced weight loss in 400-g guinea pigs led

to expected changes in fatty acid phospholipid profiles and DHLA/AA ratios but failed to improve the response to endotoxin and, in fact, worsened it. This suggests that either the dietary requirements, length, or route of administration were not adequate and/or that this lipid may be adverse for this model of endotoxin sepsis. It should also be considered that other eicosanoids may be involved in the endotoxin response. GLA may not be protective systemically in endotoxic shock, presumably due to its ability to produce more arachidonate-derived eicosanoids such as thromboxane A_2 and leukotriene B_4.

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Uptake, Incorporation and Metabolism of (^{3}H)Triolein in the Isolated Perfused Rabbit Heart[1]

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The purpose of these experiments was to study the uptake and metabolism of exogenous triglyceride in the isolated perfused rabbit heart. When infused into the rabbit heart, [9,10-^{3}H(N)]triolein was retained and incorporated into a lipid fraction that had the chromatographic mobility of authentic triolein. Incorporation of labeled triolein was not likely to be the result of a lipoprotein lipase-mediated lipolysis/resynthesis cycle, since: (i) The distribution of radioactivity following administration of [^{3}H]oleic acid was markedly different from the distribution of radioactivity following the administration of [^{3}H]triolein; (ii) heparin was administered to the rabbits at the time of sacrifice; and (iii) the hearts were perfused with a protein-free buffer for 20 min prior to the labelling period. When isoproterenol was administered to hearts labelled with [^{3}H]triolein, there was an increased output of total radioactivity, composed of labelled free fatty acids, diacylglycerol and monoacylglycerol. In these same hearts, there was an increased output of glycerol in response to isoproterenol. However, following the administration of bradykinin or angiotensin II, neither the radioactivity nor the glycerol content of the perfusate was changed. These data suggest that [^{3}H]triolein is selectively incorporated into the triglyceride pool of the isolated perfused rabbit heart. Furthermore, this [^{3}H]triolein is available to hormonally-activated lipolytic enzymes.
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Cardiac triacylglycerol serves as an important source of free fatty acids, the preferred metabolic fuel of the heart (1-3). While glycerol ouput is generally considered to be an index of triglyceride mobilization (4,5), there is some disagreement as to its significance. Some investigators have suggested that glycerol ouput is a poor indicator of the metaboalism of fatty acids derived from triacylglycerol (6); others have suggested that the concentration of glycerol in the effluent from isolated perfused hearts is more closely related to the coronary flow than to either lipolysis or fatty acid utilization (7,8). In some species, such as the rabbit, the basal output of glycerol from the heart is so low that its use as an index of triglyceride mobilization is limited. An adjunct approach to study cardiac triglyceride metabolism might be to label the endogenous triaclyglycerol pool, which is readily achieved in perfused organs by the infusion of a labelled fatty acid (9). When organs labelled in this fashion are are challenged with a lipolytic stimulus, the release of radioactivity might then be related to the metabolism of triglycerides. However, labelled fatty acids are incorporated not only into the triglyceride pools, but they are also incorproated into phospholipids, even when a saturated fatty acid is used (9). Thus, any increase in radioactivity in the effluent cannot be specifically attributed to release of fatty acids from the triacylglycerol pool. There are reports that phospholipids can be taken up intact and incorporated into cellular membranes (10-13). Moreover, it has been demonstrated that phosphatidylcholine labelled at *SN*-2 position with [^{3}H]arachidonic acid was taken up by the rabbit heart and incorporated selectively into the phospholipid pool that was accessible to hormonally-activated phospholipase A_2 (13). Although the uptake of exogenous triglycerides is generally believed to occur by a lipoprotein lipase-mediated hydrolysis/resynthesis cycle (2,14-18), there are reports indicating alternative mechanisms of uptake of intact or partial glycerolipids by the heart and bovine aortic endothelial cells (19-23). Therefore, if the triacylglycerol pool could be enriched with [^{3}H]triolein, then it might be possible to study horomonally stimulated lipolysis in the rabbit heart by measuring the release of radiolabelled products. Moreover, it could allow the determinatin of whether mono- and diglycerides, as well as free fatty acids, are released in response to various hormonal stimuli. To test this hypothesis, we have investigated: (i) The uptake and incorporation of [^{3}H]triolein into the lipids of the isolated Krebs-Henseleit buffer perfused rabbit heart; (ii) the lipolytic effect of isoproterenol (ISO), a β-adrenergic receptor agonist; and (iii) the effects of bradykinin (BK) and angiotensin II (AII) on cardiac triglyceride metabolism.

MATERIALS AND METHODS

The isolated heart preparation. The hearts were removed from heparinized (1000 U/kg) male, New Zealand White rabbits (1.5-2.5; kg, Myrtle's Rabbitry, Thompson Station, TN) under Na+ pentobarbital anesthesia and immediately placed in Krebs-Henseleit buffer (114 mM NaCl, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 4.7 mM KCl and 5.5 mM glucose; KHB) containing 10 U/mL of heparin. They were mounted on a Langendorff apparatus and perfused with KHB at 37°C and equlibrated with 95% O_2/5% CO_2. The flow rate was maintained at 18 mL/min with a Harvard peristaltic pump (model 1203, Harvard Instruments, Dover, MA). The coronary perfusion pressure was measured with a Statham pressure transducer (P2306; Statham Medical Instruments, Los Angeles, CA) attached to the aortic cannula. Myocardial tension was measured with a Grass FT.03D force-displacement trans-

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Abbreviations: AII, angiotensin II; BK, bradykinin; BSA, bovine serum albumin; DG, diglyceride; FAF, fatty acid free; FFA, free fatty acids; ISO, isoproternol; MG, monoglyceride; PL Phospholipid; TG, triglyceride; TLC, thin-layer chromatography; VEH, vehicle.

ducer (Grass Instruments Co., Quincy, MA) connected to a stainless-steel hook attached to the left ventricular apex, and adjusted to provide a diastolic tension of 2 g. The heart rate was measured with a tachometer triggered by the ventricular contractions. All hearts were allowed to perfuse for a 20 min stabilization period. Any heart failing to develop at least 8 grams of tension at this time was discarded.

Labeling procedures. Tritiated triolein ([9,10-^{3}H(N)] triolein, 20 μCi, 26.8 Ci/mmol; New England Nuclear Corp. Boston, MA; 0.76 nmol triolein, equal to 2.28 nmol oleic acid) was sonicated for 10 min in 10 mL 50 mM phosphate buffer, pH 7.4, containing 10 mg/mL fatty acid free (FAF) bovine serum albumin (BSA), as described by Wise and Jungus (24). Ten mL of the emulsion was infused (Sage infusion pump, model 341, Sage Instruments, Div. of Orion Research, Cambridge, MA) into the arterial circuit at 1.2 mL/min for 8 min, while the buffer persusion rate remained at 18 mL/min. The final concentration of [^{3}H]triolein in the perfusate was 4.8 nM, while the concentration of FAF-BSA was 9.5 μM. As a control, a second series of hearts was labelled in an identical manner with 20 μCi [^{3}H]oleic acid (9 Ci/mmol, New England Nuclear Corp.; 2.24 nmol) prepared as described for [^{3}H]triolein. The final concentration of [^{3}H]oleic acid in the perfusate was 14 nM. After the infusion of label, hearts were perfused with KHB for 12 min to wash out unincorporated label.

Experimental protocol. One group of hearts was frozen *in situ* after 46 min of perfusion (18 min after the end of the labelling period) using Wollenberger clamps cooled in liquid N_2. Another group of hearts received a single bolus dose of ISO (100 ng), BK (3 μg) or AII (100 ng) contained in 100 μL of KHB or their vehicle (100 μL of KHB). The agonist was administered at 41 min of perfusion of the heart. The effluent was collected at 30 seconds (9 mL) intervals for 1 min before and for 5 min after administration of the drug. Immediately following the collection of the last sample, the hearts were frozen as described. All the hearts were stored at -70°C, and lipids were extracted within two days. The radioactivity was measured in a 1-mL portion of each effluent sample by liquid scintillation spectroscopy. Glycerol was measured as described below in a 2-mL portion of each effluent sample.

Lipid extraction and analysis. The frozen hearts were pulverized in a porcelain mortar and pestle pre-cooled in liquid N_2. The lipids were extracted from the powdered tissue as described (25). The dried extract was weighed and redissolved in 1 mL of $CHCl_3$, and a 100 μL aliquot was plated on a thin-layer chromatography (TLC) plate (silica gel 60-F-254, Alltech Associates, Deerfield, IL). Lipid classes were separated as described previously (26), and visualized in I_2 vapor. The zones co-migrating with authentic standards were scraped and the radioactivity measured by liquid scintillation spectroscopy. The Rf values of the standards were: phospholipids, 0; monoglycerides, 0.1-0.15; diglycerides, 0.3-0.35; free fatty acids, 0.5-0.55; triglycerides, 0.75-0.85; and cholesterol esters, 0.9-0.95.

In a separate series of experiments, the triglyceride content of unlabeled rabbit hearts was determined. Total lipid extracts were separated into lipid classes as described (26); the zone corresponding to authentic triglyceride was scraped into a glass-wool stoppered chromatography column and eluted with 20 mL $CHCl_3$. A portion of the eluate was analyzed for triglyceride as described (27).

The radioactivity in the basal and peak isoproterenol-stimulated effluent samples was further analyzed. The lipids in the effluent of stimulated hearts were extracted by a modification of the method used for the hearts. Samples (5 mL) of the effluent were pipetted into 100-mL silanized centrifuge tubes containing 4 mL of imidazole buffer (10 mM EGTA, 10 mM imidazole, 100 mM KCl, pH adjuted to 7.4 with KOH), 10 mL $CHCl_3$, and 20 mL MeOH. The tubes were capped and placed on a shaker platform at high speed for 30 seconds. After the addition of 10 mL of $CHCl_3$ to each tube, the shaking process was repeated. Finally,a 10 mL of imidazole buffer was added and the shaking process repeated again. The phases were allowed to separate, the upper (aqueous) phase was aspirated and discarded, the lower (organic) phase was evaporated to dryness, and the residue redissolved in 500 μL of $CHCl_3$/MeOH (1:1, v/v). A portion (400 μL) of each was subjected to TLC as described for the tissue lipid extracts.

Metabolic indices. Since the respiratory index is generally considered to be a reliable indicator of the metabolic state of the heart, this value was derived for a series of 20 (unlabeled) hearts. The 0_2 consumption and CO_2 generation were measured by determining pO_2 and pCO_2 of arterial (taken immediately above the heart) and venous (taken from near the coronary sinus) perfusate samples. The samples were simultaneously drawn in gas-tight syringes and immediately analyzed with a Corning blood gas analyzer. As an additional index of the metabolic state of the perfused rabbit heart, the lactate/pyruvate ratio was measured at 20 min in an additional series of hearts. Lactate was measured by mixing 1 mL of perfusate with 1 mL of an assay cocktail containing (per mL) 0.85 mmol glycine, 0.85 mmol hydrazine hydrate, 2.5 μmol NAD and 7.5 U lactate dehydrogenase. Following incubation at room temperature for 20 min, the absorbance at 340 nm was determined and the NADH concentration was calculated from the molar extinction coefficient (6.2×10^3). Pyruvate was measured by mixing 1 mL of perfusate with 1 mL of an assay cocktail containing (per mL) 85 μmol KH_2PO_4, 2 U lactate dehydrogenase and 0.25 μmol NADH. After a 20 min incubation at room temperature, the absorbance at 340 nm was determined. The decrease in the NADH concentration was calculated from the molar extinction coefficient.

Glycerol assay. The glycerol in the perfusate was measured by the method utilizing glycerol kinase (28). Briefly, 2 mL of the effluent was mixed with 100 μL of an assay cocktail containing (per 100 μL) 1 U glycerol kinase (Sigma Chemical Co., St. Louis, MO G4509), 10 U horseradish peroxidase (Sigma, P8375), 4 U L-α-glycerophosphate oxidase (Boehringer Mannheim, Indianapolis, IN; 775 797), 0.8 μmol disodium ATP, 4 μmol $MgCl_2$, 0.8 μmol 3,5-dichloro-2-hydroxybenzene sulfonic acid, 0.4 μmol 4-aminoantipyrene, dissolved in 46 mM tris buffer, pH 7.6. The absorbance at 520 nm was determined following incubation at 37°C for 15 min. Samples were compared to authentic glycerol standards containing 0.5 to 32 nmol/mL.

RESULTS

The mechanical performance of each heart was monitored during the course of each experiment. The average

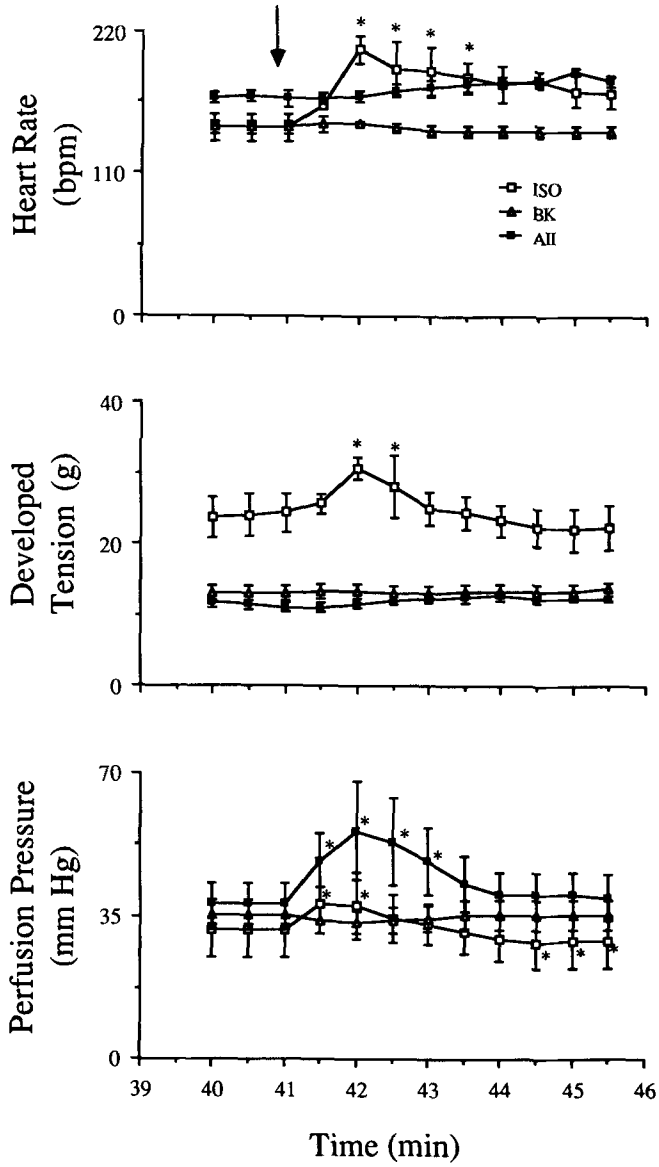

FIG. 1. Effect of isoproterenol (ISO), bradykinin (BK) or angiotensin II (AII) administered at the time indicated by the arrow on heart rate (upper panel), developed tension (middle panel), and perfusion pressure (lower panel). Data for each drug were analyzed by two-way analysis of variance. n = 3 For each drug; * indicates $p < 0.05$ as compared to the pre-drug values.

values at 20 min did not differ between experimental groups and were: Heart rate, 168 ± 3 beats per min; developed tension, 14.8 ± 0.75 grams; and perfusion pressure, 38.2 ± 1.1 mm Hg. During the infusion of triolein, there were modest drops in the heart rate (about 22 beats per min) and developed tension (about 2 grams). These effects could be mimicked by the infusion of phosphate buffer. The administration of ISO, AII or BK produced changes in the mechanical function of the heart as shown in Figure 1. Isoproterenol significantly increased the heart rate and developed tension; there was a biphasic effect (a significant increase followed by a significant decrease) on the perfusion pressure. Angiotensin II significantly increased the perfusion pressure, but was without effect on heart rate and developed tension. Bradykinin had no effect on any of the mechanical functions. The administration of vehicle had no effect on the mechanical parameters.

TABLE 1

Distribution of Radioactivity in Hearts Labelled with [3H]Triolein and [3H]Oleic Acid

	[3H]Triolein	[3H] Oleic acid
Total extractable Lipid (mg)	155 ± 14.7	182 ± 14.9
Wet weight (grams)	4.9 ± 0.15	5.6 ± 0.19[b]
mg Lipid per g Wet weight	31.6 ± 2.77	32.5 ± 2.24
Radioactivity Incorporated (n Ci/mg lipid)	3.59 ± 0.4	7.12 ± 0.55[b]

[a]Data were analyzed by Student's t-test for unpaired data.
[b]Indicates $p < 0.05$.

Uptake and distribution of radioactivity in the heart. The incorporation of [3H]triolein and [3H]oleic acid into the extractable lipids of the rabbit heart is shown in Table 1. After labelling with [3H]triolein, the radioactivity retained by the heart represented about 2-3% of the administered dose. Neither the inclusion of FAF-BSA in the post-labelling perfusion buffer nor reduction of the perfusion temperature to 22°C during labelling reduced the amount of radioactivity incorporated into the heart (data not shown).

The appearance of radioactivity in the effluent was followed in five unstimulated hearts from the beginning of the labelling period (20 min of perfusion) to 80 min of perfusion by collecting a 10 second (3 mL) sample of perfusate each minute. These data are presented in Figure 2. When the infusion of [3H]triolein is stopped, the radioactivity appearing in the effluent declined with a half-time of 2.28 ± 0.36 minutes, suggesting that > 97% of unincorporated material had been washed out prior to agonist administration. The residual radioactivity appearing in the effluent declined steadily over the course of the experiment with an average half-time of 24.7 ± 2.34 min.

In hearts labelled with [3H]oleic acid, the incorporation of radioactivity was significantly greater, representing about 6-7% of the administered dose. Although the average wet weight of the hearts labelled with [3H]triolein was significantly less than that of the hearts labelled with [3H]oleic acid, there were no significant differences in the total amount of extractable lipid or in the ratio of extractable lipid to wet weight. The distribution of the incorporated radioactivity is shown in Figure 3. In the lipid extract from hearts labelled with [3H]triolein, more than 92% (corresponding to 3.33 ± 0.4 nCi/mg lipid) of the radioactivity in TLC plates co-migrated with authentic triglyceride, and less than 3% (corresponding to 0.099 ± 0.0068 nCi/mg lipid) co-migrated with phospholipid. In contrast, less than 25% (corresponding to 1.83 ± 0.356 nCi/mg lipid) of the radioactivity extracted from hearts labelled with [3H]oleic acid co-migrated with authentic triglyceride and nearly 65% (corresponding to 4.52 ± 0.307 nCi/mg lipid) co-migrated with phospholipid.

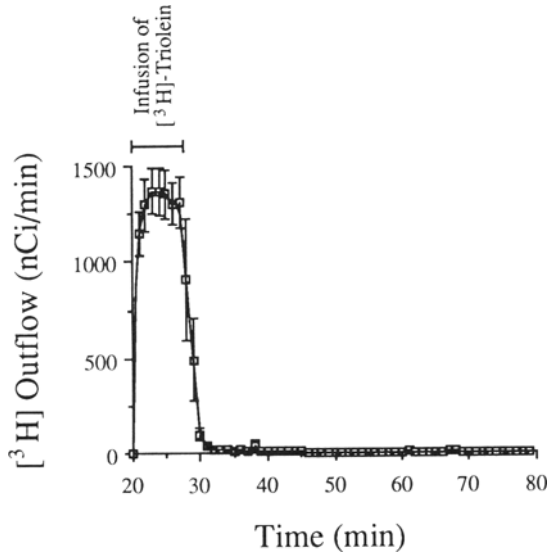

FIG. 2. Profile of the effluent from the beginning of the labelling period to 80 min of perfusion. The data for each heart were fitted manually to a two compartment pharmacokinetic model. Immediately after cessation of the infusion of [^{3}H]triolein, the radioactivity in the effluent declined with a half-time of 2.28 ± 0.36 min. The residual radioactivity declined steadily over the course of the experiment, with a half-time of 24.7 ± 2.45 minutes. Each point represents the mean ± S.E.M. of five experiments.

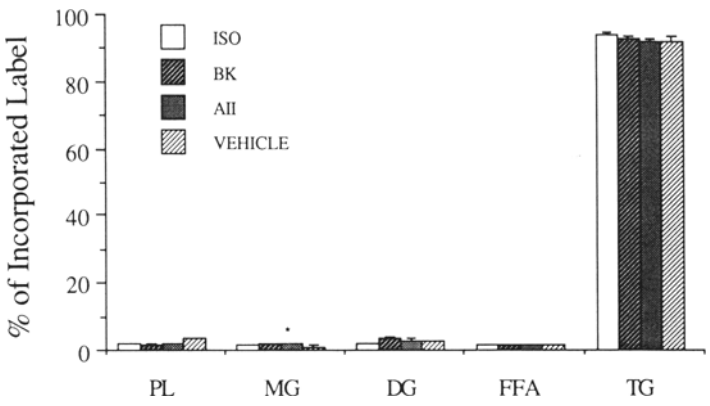

FIG. 4. Output of radioactivity in the effluent following administration of isoproterenol (ISO), angiotensin II (AII), bradykinin (BK) or vehicle (VEH) from hearts labelled with [^{3}H]triolein. The basal outflow of radioactivity was: ISO, 2.29 ± 0.54 nCi/30 sec; BK, 2.96 ± 0.97 nCi/30 sec; AII, 1.6 ± 0.40 nCi/30 sec; and VEH, 10.9 ± 3.91 nCi/30 sec. After the collection of the basal samples, drugs or vehicle were administered as indicated by the arrow. Only ISO administration resulted in increased outflow of radioactivity. Data for each drug were analyzed by two-way analysis of variance. n = Hearts for each drug and 3 for vehicle; * indicates $p < 0.05$ as compared to basal values.

Effect of ISO, BK and AII induced release of radioactivity in hearts prelabelled with [^{3}H]triolein. Administration of ISO, but not BK or AII, produced a significant increase in the outflow of radioactivity from hearts prelabelled with [^{3}H]triolein. The data are presented in Figure 4. The lipids were extracted from the effluent of the hearts stimulated with ISO, and the lipid classes separated by TLC. The administration of ISO significantly increased the outflow of radioactivity co-migrating with free fatty acids (from 510 ± 227 to 2007 ± 647 dpm/30 seconds), as well as increasing the outflow of monoacylglycerol (from 119 ± 61 to 275 ± 70 dpm/30 seconds) and diacylglycerol (from 98 ± 47 to 356 ± 83 dpm/30 seconds.

Following the administration of the agonists, the hearts were frozen, and the lipids extracted as described above. As shown in Figure 5 , the distribution of radioactivity in the tissue lipids extracted from hearts following agonist administration was not significantly different from the distribution of radioactivity following administration of vehicle.

Effect of IOS, BK and AII on glycerol output from rabbit hearts. Isoproterenol, the agent which increased the release of radioactivity, also significantly increased the outflow of glycerol (Fig. 6). The increase in glycerol output occurred within 2 min following the administration of ISO, and was still elevated 5 min after the administration of agonist. There was no increase in the glycerol content of the effluent following the administration of BK, AII or their vehicles. Total triglyceride, an index of the amount of substrate available for hydrolysis to glycerol and free fatty acids, was 11.1 ± 2.23 μmol/g wet wt (n = 6).

Metabolic indices. In a separate series of experiments, the pO_2 and pCO_2 were measured at 20 min of perfusion. These data were used to calculate the O_2 consumption, CO_2 generation and respiratory quotient. These values (n = 22) were: O_2 consumption, 32.9 ± 2.9 μL/min/g wet wt; CO_2 generation, 22.2 ± 4.4 μL/min/g wet wt; and respiratory quotient, 0.733 ± 0.14. In an additional series of experiments, the lactate/pyruvate ratio was measured at 20 min and was 2.67 ± 0.912 (n = 12).

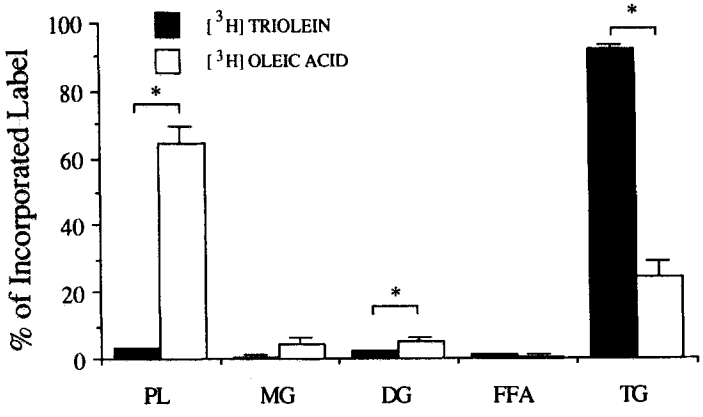

FIG. 3. Distribution of radioactivity among the lipid classes in hearts labelled with [^{3}H]triolein or [^{3}H]oleic acid. Following labelling with [^{3}H]triolein, the lipids extracted from the hearts were subjected to TLC. Almost all of the label co-migrated with authentic triglyceride, while the hearts labelled with [^{3}H]oleic acid, about 65% of the label co-migrated with phospholipid, and less than 25% co-migrated with triglyceride. n = 6 For each group; * indicates $p < 0.05$ by Student's t-test for unpaired data. PL, phospholipids; MG, monoglycerides; DG, diglycerides; FFA, free fatty acids; TG, triglycerides.

DISCUSSION

Triglyceride metabolism in the heart is an important physiological process, as free fatty acids derived from triglyceride hydrolysis are thought to be the preferred energy substrate in this organ (1-3). Glycerol output is generally considered to be a reliable index of lipolysis in

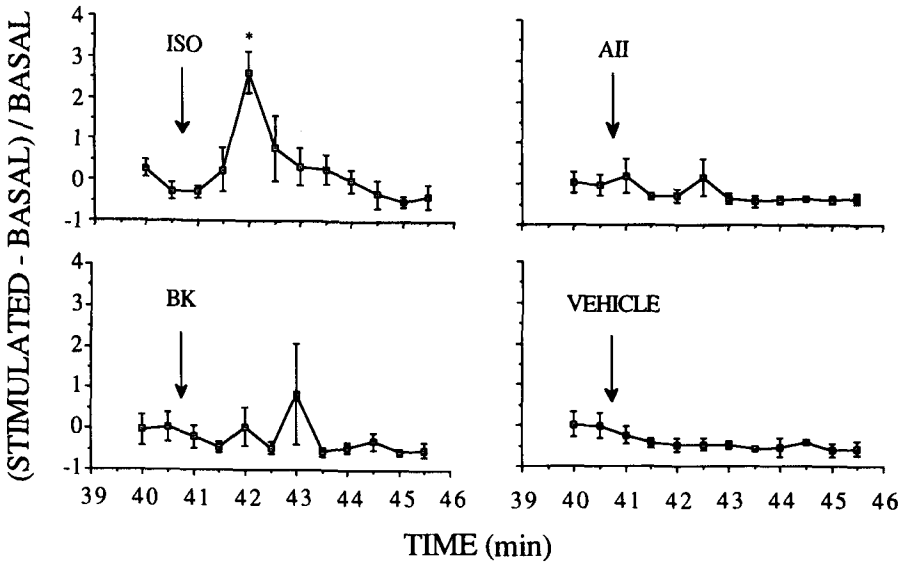

FIG. 5. Distribution of radioactivity in hearts labelled with [3H]triolein following the administration of isoproterenol (ISO), angiotensin II (AII), bradykinin (BK) or vehicle (VEH). The amount of radioactivity remaining in the hearts at the end of the experimental period was not significantly different among the four groups, as shown by a one-way analysis of variance. The average of all groups was 503 ± 49.1 nCi per heart. There were no statistically significant differences between experimental groups for the radioactivity contained in TG, PL, DG, or FFA. The monoglyceride fraction extracted from hearts treated with AII contained significantly more radioactivity when compared to the control group (1.88 ± 0.51% for the AII treated hearts vs 0.97 ± 0.42% for control; $p < 0.01$). n = 6 For each drug or vehicle.

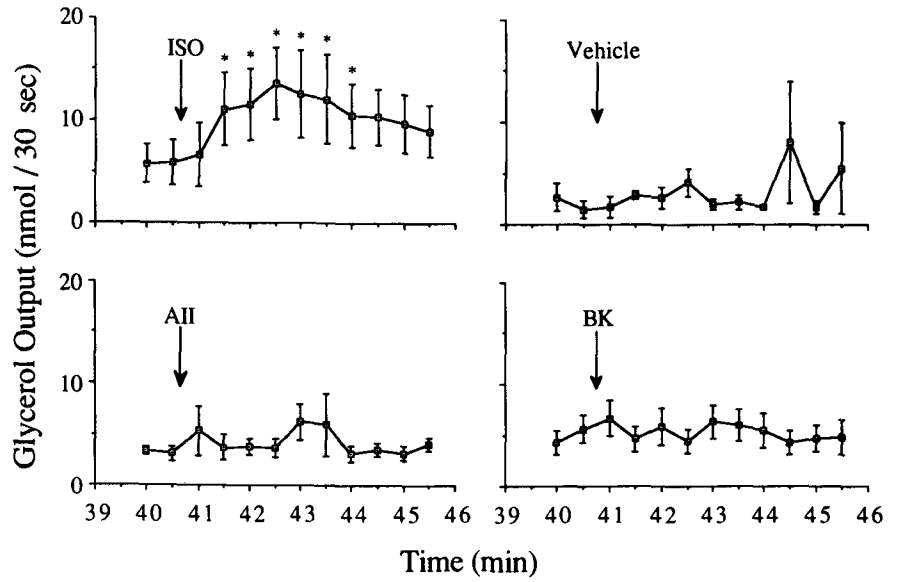

FIG. 6. Glycerol output following the administration of isoproterenol (ISO), angiotensin II (AII), bradykinin (BK), or vehicle (VEH) to hearts labelled with [3H]triolein. After the collection of basal samples, drugs were administered at the times indicated by the arrow. Data for each drug were analyzed by two-way analysis of variance. n = 3 Hearts for each drug and 3 hearts for vehicle; $p < 0.05$ as compared to basal values.

the rat heart and adipose tissue (29), and as such is commonly used as an index of lipolysis under basal and hormonally-stimulated conditions. However, our data show that the basal output of glycerol in the rabbit heart is very low, less than 2 nmol/min/g wet wt, as compared to about 15 nmol/min/g wet wt in the rat heart (30). Nevertheless, the rabbit heart respiratory quotient of 0.733 ± 0.14 is consistent with the notion that the rabbit heart is making extensive use of fatty acids as a source of metabolic fuel. It is unlikely that the lack of glycerol in the effluent of perfused rabbit hearts is due to the lack of endogenous triglyceride substrate, since our data indicate that the rabbit heart contains more than 10 μmoles of triglyceride/g wet wt. This triglyceride content is greater than that reported for rat heart (8). This discrepancy might indicate that the rabbit heart has relatively

high glycerol kinase activity, although the activity of this enzyme in the more widely studied rat heart is very low (29).

The present study, which was undertaken to determine if exogenous [^{3}H]triolein can be utilized to study triglyceride metabolism in the heart, indicates that [^{3}H]triolein is taken up and incorporated intact by the isolated perfused rabbit heart. The current notions of uptake and metabolism of circulating triglycerides would presuppose that the [^{3}H]triolein is first hydrolyzed by lipoprotein lipases present in the vascular endothelium to free fatty acids, that these are transported across the plasma membrane and used for the resynthesis of triglyceride. The data presented here, however, are inconsistent with this explanation for a number of reasons. The pre-treatment of the hearts with heparin (a process known to release lipoprotein lipase from the vascular endothelium) followed by perfusion for 20 min with KHB is most likely to remove some extracellular lipoprotein lipase from the vascular endothelium, so that less of this enzyme is available for hydrolysis of the labelled triolein. Although there is an intracellular component of lipoprotein lipase that may become extracellular, it is dependent upon apoprotein C-II for activation (31). Even if the FAF-BSA used in the preparation of the label were contaminated with this protein, it is still unlikely that lipoprotein lipase plays a significant role in the uptake and incorporation of [^{3}H]triolein, as clearly demonstrated by the significant differences in the distribution of radioactivity following labelling with [^{3}H]triolein and [^{3}H]oleic acid. Nearly all of the radioactivity extracted from hearts labelled with [^{3}H]triolein exhibited the chromatographic characteristics of authentic triglyceride, while less than 3% showed the chromatographic profile of phospholipids. In contrast, only about 25% of the radioactivity in hearts labelled with [^{3}H]oleic acid co-migrated on TLC plates with triglyceride, while about 65% co-migrated with phospholipids. If the [^{3}H]triolein were being hydrolyzed and re-synthesized during the process of incorporation, then it would be expected that the distribution of label following [^{3}H]triolein would be indistinguishable from that following [^{3}H]oleic acid administration. However, this was clearly not the case.

Others (21) have found that only 4% of incorporated labelled oleic acid was recovered in the phospholipids of rat heart, whereas in our study of the rabbit heart, 65% of the incorporated [^{3}H]oleic acid was found in the phospholipid. Although species differences cannot be dismissed, we believe that differences in methodology might account for this discrepancy. We infused 2.24 nmol of oleic acid for 10 min in a non-recirculating system. Tambouli *et al.* (8) infused 8 μmol for 45 min in a recirculating system. The 3500-fold reduction in dose, coupled with a brief single-pass system, might well account for the differences in uptake and distribution of oleic acid in our system.

An alternative explanation for the retention of label following the infusion of [^{3}H]triolein is the non-specific adsorption of the material onto the luminal surface of the vascular endothelium, since the retention of label does not appear to be temperature dependent. Although we cannot exclude the possibility of passive adsorption, it appears to be unlikely because the infusion of FAF-BSA in the buffer during washing period after the termination of [^{3}H]triolein did not alter the amount of label retained in the heart. Since BSA is known to bind lipids, it would be expected that BSA would bind triglyceride non-specifically adsorbed to the vascular endothelium.

From these observations it follows that [^{3}H]triolein infused into the heart is retained almost exclusively in the triglyceride pool most likely as an intact molecule. Supporting this view is the finding that, at least under some circumstances, intact or partial glycerides may be taken up by the heart (19-23). The precise mechanism by which triglycerides are taken up by the cardiac cells is not known. There are reports indicating the presence in some tissues of specific proteins for the transport of various lipid classes including fatty acids, phospholipids and triglycerides (32-34). Whether a similar process in the uptake of [^{3}H]triolein in the cardiac cells is involved remains to be determined. Since [^{3}H]triolein was emulsified with BSA during the labelling period, the possibility that the uptake of the labelled triglyceride might be mediated by a cardiac albumin receptor cannot be excluded (35). Our present data do not permit us to discern the cardiac cell types which are labelled with [^{3}H]triolein. Triglyceride-containing lipid droplets have been demonstrated in the cardiac myocytes (36) and the rabbit heart contains adipose tissue adjacent to the coronary arteries. Since both the vascular endothelium and smooth muscle are involved in the uptake and metabolism of lipoproteins (37,38), these sites could also be the targets for the uptake of [^{3}H]triolein in the heart.

An important finding in the present study was that ISO, but not BK or AII, stimulated lipolysis (as measured by the release of glycerol), and enhanced the release of labelled free fatty acids, monoglycerides and diglycerides from hearts prelabelled with [^{3}H]triolein. These observations suggest that, when [^{3}H]triolein is incorporated into the heart, it is available to hormonally-activated lipolytic enzymes. Since the release of tritium elicited by ISO was associated with mono- and diglycerides, it appears that the glycerol output alone is a poor indicator of hormonally-stimulated triglyceride metabolism, assuming that [^{3}H]triolein is incorporated into the same pool of endogenous triglycerides that are hydrolyzed by ISO to generate glycerol. The release of tritium in response to ISO was not due to changes in the mechanical activity of the heart because AII, which also produced significant changes in the mechanical function, failed to alter the output of radioactivity or glycerol in the hearts prelabelled with [^{3}H]triolein.

In conclusion, this study demonstrates that triglycerides can be taken up intact and retained in the isolated perfused rabbit heart, and that they are accessible to hormonally-induced lipases. The labelling of the endogenous triglyceride pools of the heart with [^{3}H]triolein may represent a new and valuable procedure for the study of cardiac triglyceride metabolism and the activity of lipolytic enzymes under physiological conditions in an intact organ.

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Quantitative Effects of Dietary Polyunsaturated Fats on the Composition of Fatty Acids in Rat Tissues

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A method combining data on fatty acid composition into subsets is used to illustrate general relative competitive selectivities in the metabolic and transport events that maintain fatty acid compositions in tissue lipids and to minimize differences among tissues or species in the amount of individual fatty acids. Fatty acid compositions of triglycerides and phospholipids in several tissues of the rat were maintained with simple relationships between the exogenous n-3 and n-6 dietary polyunsaturated fatty acids and the endogenous n-7 and n-9 types of fatty acid. The general pattern of fatty acids in triglycerides was similar for liver, plasma and adipose tissue, averaging about 30% as saturated acids, 67% as 16- and 18-carbon unsaturated acids and only about 2% as 20- and 22-carbon highly unsaturated acids. The tissues maintained a linear relationship between the amount of 18-carbon polyunsaturated fatty acids in the diet and in the tissue triglycerides, with the proportionality constant for 18:3n-3 being 60% of that for 18:2n-6. The total phospholipids of liver, plasma and red blood cells maintained about 45% of the fatty acids in the form of saturated fatty acids and 20–30% as 20- and 22-carbon highly unsaturated fatty acids irrespective of very different proportions of n-3, n-6 and n-9 types of fatty acids. In all three tissues, the 20-carbon highly unsaturated fatty acids of the n-3, n-6 and n-9 type were maintained in a competitive hyperbolic relationship with apparent EC_{50} values for dietary 18:2n-6 and 18:3n-3 near 0.1% of dietary calories. The consistent quantitative relationships described in this study illustrate an underlying principle of competition among fatty acids for a limited number of esterification sites. This approach may be useful in predicting the influence of diet upon tissue levels of the substrates and antagonists of eicosanoid biosynthesis.
Lipids 25, 505–516 (1990).

Many published reports provide detailed lists of the abundances of the different fatty acids that are maintained in rat tissues. The reports provide evidence that different enzymes act together to maintain steady state compositions of fatty acids in cellular lipid (reviewed in ref. 1). The esterifying enzymes tend to place saturated fatty acids (SFA) at the *sn*-1 position of glycerolipids. Therefore, the saturated fatty acids (SFA) at the *sn*-1 position constitute nearly one-half of the fatty acids in diacylglycerophospholipids (*ca.* 40–45%) and about one-third of the fatty acids in triacylglycerols (*ca.* 25–35%). In contrast, the 16- and 18-carbon unsaturated fatty acids (UFA) are esterified at the *sn*-2 position during *de novo* synthesis and the 20- and 22-carbon highly unsaturated fatty acids (HUFA) at the *sn*-2 position during the retailoring process. Slightly different activities of the *de novo* and retailoring enzymes in different tissues appear to produce different proportions of UFA and HUFA in different tissues. As a result, the fatty acid composition in each tissue can reflect both the selectivity of the enzymes and the relative abundances of the different acids available to the enzymes (2). These general enzyme selectivities appear to be similar qualitatively for either rat or human tissue. Although alterations in dietary fat can influence the maintenance levels, a strong influence of the general esterification selectivity with the endogenous fatty acids seems likely to cause the strikingly similar overall compositional patterns for fatty acids in lipids of human tissues world-wide (3), and for the similarity of the fatty acid patterns in the lipids of rats and humans.

In the absence of any dietary fat, tissue lipids have small amounts of n-3 and n-6 fatty acids (apparently acquired from maternal supplies), and most of the fatty acids in tissue lipids (about 90%) are those synthesized from acetate: 14:0, 16:0, 18:0, 16:1n-7, 18:1n-7 and 18:1n-9 (4,5). In addition to these acids, rat tissues can also accumulate and maintain significant amounts of 20- and 22-carbon highly unsaturated fatty acids (HUFA), 20:3n-9, 20:3n-7 (6) and 20:4n-7 (7,8). The pattern that is maintained among these fatty acids is created by the relative rates of synthesis of the various acids from their carbohydrate and amino acid precursors (which customarily comprise the major source of dietary calories and cellular acetyl-coenzyme A). Continued hydrolysis and replacement of tissue lipid esters maintains the proportions of saturated (14:0, 16:0, 18:0), unsaturated (16:1n-7, 18:1n-7 and 18:1n-9) and highly unsaturated fatty acids (20:3, 20:4) in accord with the general selectivities for esterification and oxidation during fatty acid and glycerolipid metabolism (1).

When the diet includes polyunsaturated fatty acids of either the n-3 or n-6 type (which cannot be synthesized *de novo* in animal tissues), there is a marked displacement of the "endogenous" type of HUFA (20:3n-9, 20:4n-7) by new HUFA of the n-3 type (20:5n-3, 22:5n-3, 22:6n-3) or the n-6 type (20:3n-6, 20:4n-6, 22:4n-6, 22:5n-6) derived from dietary 18:3n-3 and 18:2n-6, respectively. For example, with a rat chow diet (9), a pattern of tissue acids is maintained which includes relative amounts of the endogenous fatty acids (14:0, 16:0, 18:0, 16:1n-7, 18:1n-7 and 18:1n-9) which are rather similar to those seen with a fat-free diet, but with 18:2n-6 apparently displacing 18:1n-7 and 18:1n-9 and with 20:4n-6 displacing 20:3n-9, 20:3n-7 and 20:4n-7. The early recognition (10) that the dietary n-3 acid (18:3n-3) can inhibit the elongation and desaturation of 18:2n-6 to 20:4n-6 was extended to a thorough demonstration of competitive metabolic interactions between polyunsaturated fatty acids of the n-3, n-6 and n-9 types (4,5).

Because the 20-carbon highly unsaturated fatty acids (HUFA) play important roles as precursors and

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Abbreviations: BHT, butylated hydroxytoluene; CE, cholesterol esters; GLC, gas-liquid chromatography; HUFA, highly unsaturated fatty acids; NEFA, non-esterified fatty acids; PL, phospholipids; SFA, saturated fatty acids; TG, triglycerides; TLC, thin-layer chromatography; UFA, unsaturated fatty acids.

antagonists of eicosanoid biosynthesis (11,12), altering the abundance of dietary precursors of these HUFA seems certain to influence a tissue's capacity to form eicosanoids. An altered capacity seems likely to affect the frequency and severity of eicosanoid-related disorders (13). Such a possible influence makes it important to understand in more detail how tissues maintain their typical levels of n-3 and n-6 HUFA and to understand how much those levels may be influenced by different amounts of dietary polyunsaturated fatty acids. If we can understand the basic processes and selectivities by which rat tissues maintain their characteristic patterns of fatty acids, we can then test the extent to which that understanding could be applied to interpreting the patterns of fatty acids in lipids of humans.

There were two reasons that caused us to examine the degree to which the pattern of n-3/n-6 fatty acids maintained in the glycerolipids of plasma and red cells of the rat reflected the n-3/n-6 patterns maintained in two other related tissues, liver and adipose: i) the glycerolipids and non-esterified fatty acids of plasma mediate metabolic and transfer interactions among the diet, liver and adipose; and ii) the eventual parallel diet studies with humans can be conducted more easily with blood samples than with tissue biopsies.

This report describes the results of dietary studies with rats that establish quantitative relationships among dietary and endogenous tissue fatty acids for these experimental animals. The results are expressed in the form of algebraic equations that may be useful for estimating the different compositions of fatty acids in tissues which result from altered dietary intakes. Further experience with the equations may eventually permit using analyses of compositions of fatty acids in tissues to estimate the average dietary intakes of n-3 and n-6 acids.

MATERIALS AND METHODS

General maintenance of animals. Pregnant Sprague-Dawley rats (Sasco, Inc., Oregon, WI) were maintained at 70–72°C, 40% humidity and 12 hr light-dark cycle in the Biological Resources Laboratory of the University of Illinois at Chicago. They were fed one of the defined diets described below from the time of their arrival in the facility until after the weaning period when the pups were placed in separate cages and maintained on the same diet as their parent. Pups were selected for uniform body size and sex and maintained with three rats or less per cage, with water and diet provided *ad libitum.* During growth studies, they were weighed weekly until they reached approximately 500 g (males) or approximately 300 g (females).

Alternatively, female rats being maintained on a specific diet were bred with an unrelated male (previously maintained on rat chow) for one week and then separated. After delivery, the nursing females were kept for three weeks with their pups and then sacrificed at the time of weaning. In this way, the polyunsaturated fatty acid composition found in tissues more closely reflected that of the experimental diets rather than fatty acids acquired from mothers fed on chow. At three weeks after delivery, the pups were selected for uniform body size, separated (three animals per cage) according to sex and fed *ad libitum* (10–30 g/day) until they reached 20 weeks in age. Replicate analyses of samples from two or three animals of each sex were used to obtain the averages reported for each type of tissue lipid.

Diet. Diet materials obtained from Teklad (a Harlan-Sprague Dawley Inc. Company, Madison, WI) were either a pelleted fat-deficient "complete" diet (#TD85514), which is designated in this report as diet O, or a powdered fat-omitted, carbohydrate-reduced basal diet mixture (#TD86446) that contained per kilogram the antioxidant, ethoxyquin (20 mg); casein, high protein (400 g); DL-methionine (6.0 g); sucrose (203.98 g); corn starch (200 g); cellulose fiber (100 g); mineral mix, AIN-76 Teklad #170915 (70 g); vitamin mix, Teklad #40060 (20 g). The latter basal mix was blended at a rate of 500 g/Kg of diet with desired amounts of carbohydrate and fat (with an additional 10 mg antioxidant per 100 g fat) to prepare a wide range of diets.

Flaxseed oil was obtained from Hain Pure Food Co. (City of Commerce, CA), olive oil from Sigma Diagnostics (St. Louis, MO), a low-linoleate, high-oleate fat (Trisun HB95) from SVO Enterprises (Eastlake, OH) and corn oil from Columbus Foods Co. (Chicago, IL). These oils contained the following percentages of 18:2n-6 and 18:3n-3, respectively: flax, 18.3 and 50.2; olive, 5.4 and 0; Trisun, 1.9 and 0 and corn, 58.8 and 0.83. To ensure proper mixing of the solid Trisun HB95 with the other fats, weighed amounts of the appropriate fats were melted together in a beaker at 70–90°C with 10 mg *t*-butyl hydroquinone (Aldrich Chemical Co., Milwaukee, WI) per 100 g of fat. Thereafter, the liquid fat was mixed quickly in an electric blender with a mixture of 150 g of starch and 250 g of basal diet and then blended thoroughly in the plastic pan using a handheld electric mixer with 200 g of sucrose plus 20 g of glycerol (to improve the final texture of the diet) and 250 g of basal diet and 100 g of starch. We prepared three major groups of diets: 0 en% fat, 10 en% fat and 20 en% fat. Subgroups within these three major groups had different ratios of n-3 and n-6 fatty acids and/or a different total amount of linoleic acid. In calculating caloric contents, starch, sucrose and glycerol were considered to provide 4.1 Cal/g and the fats, 9.3 Cal/g. Gas chromatographic analysis of the methyl esters formed by the official AOAC method #7.063 indicated that each gram of starch contained 4.1 mg of total fatty acid which included 1.8 mg 18:2n-6.

The diets (except diet O) were prepared fresh each week and stored at 4°C in large, wide-mouth, plastic jars. Food was replaced daily (discarding the unused food), and the fatty acid composition of the various diets was periodically confirmed by gas chromatographic analysis (see Table 1). The percent of calories as 18:2n-6 (en% 18:2n-6) ranged from 0–11. The observed values routinely matched those predicted from the measured fatty acid compositions of the starch and fats within the limits of the analytical procedure, and there was no evidence of decomposition of dietary polyunsaturated fatty acids indicating that there was a sufficient amount of antioxidant.

Tissue preparation. The animals were anesthetized in a chamber using diethyl ether. Immediately after opening the chest, blood samples (*ca.* 4 mL) were taken from the right ventricle using a 25 × 3/4 butterfly with 12″ tubing to facilitate phlebotomy into a 10-mL plastic syringe containing 0.5 mL of 3.2% sodium citrate and 0.4% of citric acid (Abbott Hospitals, Inc., North Chicago, IL).

TABLE 1

Fatty Acid Composition of the Diets

Component	Weight %						
	0	A	B	C	D	Fl	Co
18:1n-9	0.00	80.17	77.40	70.95	48.56	21.20	28.38
18:2n-6	0.00	3.67	4.88	6.01	13.12	18.68	58.46
18:3n-3	0.00	0.00	2.37	8.08	25.03	51.18	1.13
Other acids	0.00	16.15	15.36	14.96	13.29	8.94	12.02
Diet (n-3/n-6)	0.00	0.00	0.49	1.34	1.91	2.74	0.02
en%[a] 18:2n-6	0.00	0.33	0.44	0.55	1.19	4.11	12.86
en%[a] 18:3n-3	0.00	0.00	0.22	0.73	2.28	11.26	1.25
en%[a] PUFA	0.00	0.33	0.66	1.28	3.47	15.37	13.11

[a]en% is the percent of daily energy provided in the diet.

One mL blood samples were spun in a Brinkmann Eppendorf Centrifuge (Brinkmann Instruments, Inc., Karlsruhe, West Germany) for 4 min at #8 speed. The red cells and plasma were separated and stored at −40°C. The liver and adipose tissue were quickly removed, rinsed in cold saline, frozen in liquid N_2, pulverized in stainless steel mortar (4″ diameter) and lyophilized for 50–70 hr. Powdered dried tissue samples of about 50 mg were then weighed and kept at −20°C in 3 mL of a mixture of chloroform/methanol (1:1, v/v) containing 0.010% (w/v) butylated hydroxytoluene (BHT) in Teflon-lined screw-cap tubes until the lipids were analyzed.

Lipid extraction from tissues. A standard solution of lipid carriers and internal standards (containing BHT; di-12:0 phosphatidylcholine, di-17:0 phosphatidylcholine; 13:0, 17:1, 19:1, 22:3 free fatty acids; tri-12:0, tri-17:0, tri-19:1 triglycerides; and 13:0, 17:0 cholesterol esters) was added to the tissue samples in chloroform/methanol (1:1, v/v). The 22:3 provided a valuable internal reference for recovery of HUFA in the overall procedure, and it was also added to the separated thin-layer chromatography (TLC) bands of cholesterol esters, triglycerides and phospholipids (CE, TG and PL) for further quality control to confirm that peroxidative losses of HUFA did not occur during extraction and analysis.

Tissue samples were then homogenized for 30 seconds at 4°C with a Polytron homogenizer (Beckman Instruments, Westbury, NY) and centrifuged at 2000 rpm in a Sorvall RT6000 refrigerated centrifuge at 4°C for 10 min. This step was repeated twice. Three mL of chloroform and 2 mL of water were added to the combined solvent layers to facilitate the separation. The organic phase was separated by centrifugation, transferred, concentrated under N_2 and redissolved in an appropriate amount of chloroform/methanol (1:1, v/v). The extracted lipid solution containing internal standards was applied to the pre-absorbent silica gel G TLC plate (Cat. #02689; Analtech, Newark, DE). After the application, solvent completely dried, the plate was fully developed to 18 cm in hexane/diethyl ether/acetic acid (80:20:1, by vol.). The lipid classes were visualized by spraying the plate with rhodamine 6G (0.02% in ethanol) and exposure to ultraviolet light. All of the silica of each band was scraped into 10-mL, screw-capped tubes (Teflon-lined caps).

Lipid extraction from plasma. ISTD standard mixture (20 μL) was spotted on a 2 cm wide lane of the TLC plate, and then 100 μL of plasma was applied onto the top of it. The plates were developed first in methanol to approximately 1.5 cm above the pre-adsorbent border. After removing the plate and evaporating the methanol for several min, the plate was then developed to 1.5 cm with chloroform/methanol (1:1, v/v) to complete the extraction of lipids from the plasma protein at the origin. After the solvent had completely evaporated, the plate was fully developed to 15 cm above the adsorbent band in hexane/diethyl ether/acetic acid (80:30:1, by vol.). The resulting lipid bands were visualized by spraying the plate with rhodamine and viewing the plate in ultraviolet light. Rf values were noted and the bands were then scraped into respective screw-capped tubes.

Lipid extraction from red blood cells. Samples of 100 μL red blood cells were taken using disposable micro-pipettes (Cat. #1095-J SMI Scientific, Berkeley, CA) and 20 μL of the RBCP standard were added (containing 10.33 μg di-12:0 and, 3.98 μg di-17:0 phosphatidylcholine, and 3.00 μg 22:3n-3). Then the lipids were extracted by adding in sequence (with rinsing of the micro-pipette and vigorous mixing after each addition) 2 mL methanol, 1 mL chloroform (containing 100 μg BHT/100 mL), 1.5 mL water and 1.0 mL of chloroform. After vigorous mixing, the tubes were left for several minutes, vortexed, centrifuged and the lower layer was removed. Two more 1.5 mL chloroform extractions were made from the aqueous layer, and the combined extracts were evaporated under a stream of nitrogen. One mL of BF_3 reagent was added, and samples were transmethylated as described below. Methyl docosanoate (22:0) or tricosanoate (23:0) was added to help calculate recoveries of the sample and internal standards, and the methyl esters were extracted using 1.5 mL hexane and 3.0 mL water. The hexane extract was concentrated under nitrogen, redissolved in 200 μL hexane, and then passed over a 2 cm (1 mL) column of florosil with a Pasteur pipette to remove cholesterol. The column was rinsed with petroleum ether/diethyl ether (9:1), collecting the first 3 mL for analysis of the methyl esters, which was then concentrated under a stream of nitrogen.

Derivatization and gas-liquid chromatography. To each tube containing silica gel (and also several blank control tubes), 1 mL of methanol (with 50 μg BHT/mL) was added, and to estimate recovery of HUFA, 22:3 was added to the phospholipid and triacylglycerol fractions. All sample tubes wre treated with 1 mL of BF_3 reagent (Supelco, Bellefonte, PA), tightly capped and placed on a heating block (or a boiling water bath) at 100°C for 30 min. The tube was withdrawn from the block, cooled, and either 22:0 or 23:0 methyl ester was added as an internal standard for gas-liquid chromatography (GLC). The fatty acid methyl esters were extracted by adding 1 mL of hexane (with 50 μg BHT/mL). Three mL of water was added to each tube and, after vortexing and standing, the hexane extract was removed and concentrated as necessary under a stream of nitrogen for GLC analysis.

For GLC, a Packard Model 430 gas chromatograph was fitted with a dropping needle injection system and a flame ionization detector. Chromatography was in a flexible fused capillary column (30 m × 0.25 mm I.D.; 0.25 microns thickness) with a bonded stationary phase of either Supelcowax-10 (Supelco) or Durabond-225 (J&W Scientific, Folsom, CA), using hydrogen at 100 psi as the

carrier gas. The column temperature was programmed to be initially at 140°C, increase at a rate of 20°C/min to a temperature of 180°C, then increase with a rate of 3.0°C/min to a final temperature of 240°C, which was held for 3 min. The injection port temperature was 250°C and the detector was 260°C. Retention times in minutes of major fatty acids (on Supelcowax-10 and Durabond-225 columns respectively) were: 16:0 (4.3, 5.3); 18:0 (5.9, 8.0); 18:2, (6,6 8.8); 20:4, (10.0, 13.3); 20:5, (11.0, 14.3); and 22:6, (15.5, 19.3). The presence of several internal standards (obtained from Nucheck Prep, Elysian, MN) in each fraction assayed provided extensive quality control evidence of the performance of the analytical methods, indicating that the average recovery of the easily oxidized HUFA, 22:3n-3, was 101 ± 21% (mean ± SD; n = 61) relative to the stable internal standard, 17:1, and a consistent recovery of each tissue lipid class with the different diets employed. Liver samples from males and females had an average of 35 μg TG/mg dry wt and 52 μg PL/mg dry wt, whereas adipose had 700 μg TG/mg wet wt. Blood cells contained an average of 2.4 μg PL/μL packed cells, whereas plasma averaged 860 μg PL/mL, 900 μg TG/mL and 180 μg non-esterified fatty acids (NEFA)/mL.

RESULTS

Weight gain. During a preliminary study with four diets (A, B, C, and D) that were provided at the time of weaning, male rats grew from 120 g at 35 days after birth to 300 g at 75 days and 430 g at 130 days with no apparent effect due to the differences in dietary polyunsaturated fat. Similarly, the female littermates maintained on these diets grew from 110 g at 35 days to 220 at 75 days and 280 at 130 days (although with slightly lower weights for the animals fed Diet B). Because there were no significant differences among the animals with the four diets, female rats raised on these diets were bred to produce a second generation in which the maternal lipids provided to the pups reflected those of the special diets rather than the standard rat chow. Also, three additional diets (0 en% fat, 20 en% flax oil, and 20 en% corn oil) were included to provide a wider range of dietary fatty acid compositions and a wider range of physiological responses. The weights of male rats in this second diet study increased from 135 g at 35 days to 300 g at 75 days with diets A, B, C, D and flax oil; but at 75 days the animals with corn oil weighed more (345 g), and those with the fat-deficient diet (diet O) weighed only 250 g. At 130 days, the animals with diet O weighed about 300 g, whereas the rest weighed about 420 g, with those on diet C and corn oil weighing slightly more than those on diets A and D. The weights of the female rats in this second diet study increased as it did in the first study: from 90–120 g at 35 days to 175–235 g at 75 days and 210–300 g at 130 days. Within these ranges, females with diet O and diet A gained the least weight, whereas those with diets C and D and flax and corn oil gained the most, and intermediate values occurred with diet B.

Fatty acid composition of triglycerides. The average overall fatty acid compositions maintained in the triglycerides of three different tissues (plasma, liver, adipose) were similar to each other irrespective of the sex of the animals, averaging about 25% saturated fatty acids (SFA), 70% 16- and 18-carbon unsaturated fatty acids (UFA), and 2–5% HUFA. Table 2 provides results for liver, adipose and plasma triglycerides of female rats to illustrate the similar influence of diet upon the composition of individual fatty acids in the triglycerides of these different tissues (Tables 2A–C). With the fat-deficient diet, the n-7 acids were about 38% of triglyceride UFA, whereas they were about 25% of UFA with 10 en% fat, and only about 5% of UFA with 20 en% fat (Tables 2A–C). The proportion of triglyceride UFA in the form of n-7 acids was similar for all three tissues, and it was slightly higher in the triglycerides of amel rats (results not shown). For plasma triglycerides (Table 2A), the female rats maintained about 30% of the fatty acids as saturated fatty acids (SFA) irrespective of the influx of dietary fat. This was slightly higher than the SFA in the triglycerides of adipose (Table 2C) or male plasma (values not shown). Only at the highest level of dietary polyunsaturated fat were appreciable amounts of HUFA accumulated in the plasma (Table 2A) and liver (Table 2B) triglycerides. The approximate value for 20:5n-3 maintained by 10 en% dietary 18:3n-3 was about 3%, and that for 20:4n-6 maintained by 12 en% dietary 18:2n-6 was about 5%. In contrast, adipose tissue of males and females (Table 2C) consistently maintained very low amounts of HUFA in triglycerides with all diets, reaching maximal values of about 1 with diets Fl and Co.

With higher amounts of 18:2n-6 in the diet, the rat tissues maintained progressively higher levels of 18:2n-6 in triglycerides (Tables 2A–C). The linear trend was similar for plasma, liver and adipose, with no significant difference between males and females. Thus, all six sets of data resembled each other. Over the range of 0 to 12 en% dietary 18:2n-6, these tissues maintained the triglycerides with a weight percent of 18:2 that was approximately equal to 3 times the dietary en% of 18:2 in accordance with Equation [1a]. Similarly, the tissues maintained proportionately higher levels of 18:3 in the triglyceride fraction with higher influxes of dietary 18:3n-3, in accordance with Equation [1b].

$$\text{Triglyceride wt\% 18:2n-6} = 2.95 \times (\text{dietary en\% 18:2n-6}) + 1.9[r^2 = 0.97;\ n = 94] \quad [1a]$$

$$\text{Triglyceride wt\% 18:3n-3} = 1.84 \times (\text{dietary en\% 18:3n-3}) + 0.3[r^2 = 0.95;\ n = 94] \quad [1b]$$

These consistent linear trends for the maintenance levels of 18:2n-6 and 18:3n-6 in triglycerides appeared to be independent of the amounts of other fatty acids in the diets or the proportion of total calories as fat.

Fatty acid composition of phospholipids. The general pattern of fatty acids was similar for the phospholipids of plasma (Table 3A), liver (Table 3B) and erythrocytes (Table 3C), and the values for males (Table 3) and females (values not shown) were also similar; averaging approximately 42% SFA, 32% UFA and 25% HUFA. The HUFA were less abundant in the phospholipids of plasma (20%) than in red cells (28%) or liver (31%). Greater amounts of UFA in plasma phospholipids (Table 3A) accompanied the lower HUFA, maintaining the customary balance of nearly equal amounts of total saturated and unsaturated fatty acids that occur with the two esterified hydroxyls of phosphoglycerides.

TABLE 2

Fatty Acid Composition of Triglycerides from Female Rats[a]

	A. PLASMA							B. LIVER							C. ADIPOSE						
Acid	O	A	B	C	D	Fl	Co	O	A	B	C	D	Fl	Co	O	A	B	C	D	Fl	Co
	weight %							weight %							weight %						
14:0	1.22	2.51	2.65	2.71	0.97	0.25	2.71	1.18	0.70	0.45	0.56	0.37	0.74	0.09	1.36	1.34	1.46	1.29	1.23	1.01	0.99
16:0	21.62	23.96	29.24	29.84	21.02	11.94	23.62	25.77	21.54	23.66	28.91	25.92	24.56	20.43	24.60	20.33	22.67	22.31	24.48	18.27	19.28
16:1n-7	17.05	12.89	13.45	7.79	3.66	1.66	1.67	14.07	9.71	9.51	9.10	5.50	3.38	1.14	14.85	9.71	9.65	6.61	7.52	2.85	3.37
18:0	3.72	5.03	3.59	4.74	4.55	5.75	4.31	2.70	3.16	3.55	3.28	2.67	3.68	2.63	2.10	2.78	2.70	3.40	2.93	3.67	3.08
18:1n-9	48.99	43.55	39.33	40.26	45.96	36.77	24.65	43.35	57.14	51.66	52.90	48.00	31.73	25.17	45.50	56.54	54.45	55.82	46.69	32.58	31.82
18:1n-7	4.90	7.86	8.08	5.32	2.01	0.00	1.38	8.91	4.25	7.60	0.00	3.87	3.11	2.32	10.29	7.16	6.48	4.47	3.81	2.75	2.97
18:2n-6	1.75	2.69	2.40	4.24	8.05	14.11	35.17	1.86	2.25	2.17	2.72	6.20	13.57	40.76	0.84	1.85	1.93	4.11	5.72	16.64	36.09
18:3n-6	0.00	0.00	0.00	0.00	0.00	0.00	0.94	0.00	0.00	0.00	0.00	0.00	0.00	1.16	0.00	0.10	0.11	0.05	0.15	0.04	0.19
18:3n-3	0.21	0.26	0.44	2.50	8.08	24.14	0.88	2.16	0.00	0.38	1.39	5.47	16.76	0.26	0.27	0.13	0.50	1.76	6.59	20.38	1.12
20:3n-9	0.44	1.25	0.60	0.00	0.00	0.00	0.00	0.22	0.00	0.00	0.00	0.00	0.00	0.00	0.15	0.06	0.05	0.04	0.00	0.04	0.00
20:2n-6	0.00	0.00	0.00	0.00	0.00	0.00	0.67	0.00	0.18	0.26	0.10	0.09	0.00	0.17	0.00	0.00	0.00	0.00	0.00	0.08	0.13
20:3n-6	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.07	0.14
20:4n-6	0.10	0.00	0.00	0.25	0.59	0.40	2.62	0.00	0.50	0.29	0.21	0.21	0.39	3.40	0.00	0.00	0.00	0.04	0.06	0.13	0.61
22:4n-6	0.00	0.00	0.00	0.00	0.00	0.00	0.65	0.00	0.00	0.00	0.00	0.00	0.00	0.69	0.00	0.00	0.00	0.01	0.00	0.00	0.14
20:5n-3	0.00	0.00	0.00	0.32	1.90	2.69	0.00	0.00	0.00	0.00	0.10	0.61	0.94	0.00	0.00	0.00	0.00	0.03	0.11	0.28	0.00
22:5n-3	0.00	0.00	0.00	0.31	1.43	1.15	0.00	0.00	0.00	0.00	0.08	0.48	0.55	0.00	0.00	0.00	0.00	0.00	0.32	0.57	0.01
22:6n-3	0.00	0.00	0.22	1.71	1.78	1.13	0.72	0.34	0.54	0.48	0.64	0.61	0.58	1.51	0.04	0.00	0.00	0.06	0.36	0.46	0.07
DIET(n-3/n-6)	0.00	0.00	0.43	1.28	1.94	2.74	0.02	0.00	0.00	0.43	1.28	1.94	2.74	0.02	0.00	0.00	0.43	1.28	1.94	2.74	0.02
HUFA(n-3/n-6)	0.00	0.00	0.00	9.35	8.36	12.45	0.17	0.00	0.71	0.86	2.95	5.76	5.40	0.33	0.00	0.00	0.00	1.84	13.67	5.27	0.08
UFA(n-3/n-6)	0.12	0.10	0.18	0.59	1.00	1.71	0.02	1.17	0.00	0.17	0.51	0.88	1.23	0.01	0.32	0.07	0.25	0.42	1.12	1.22	0.03
SFA as %TOTAL	26.56	31.50	35.48	37.29	26.54	17.94	30.65	29.42	25.41	27.66	32.75	28.97	28.98	23.15	28.05	24.45	26.83	27.01	28.63	22.95	23.35
UFA as %TOTAL	72.90	67.25	63.70	60.12	67.77	76.69	64.69	67.59	73.36	71.32	66.12	69.05	68.56	70.82	71.76	75.49	73.12	72.81	70.47	75.24	75.55
HUFA as %TOTAL	0.54	1.25	0.82	2.59	5.69	5.37	4.67	2.99	1.23	1.02	1.13	1.99	2.46	6.03	0.18	0.06	0.05	0.18	0.90	1.81	1.10
n-7 as %UFA	30.11	30.85	33.81	21.82	8.37	2.16	4.71	32.67	19.04	23.99	13.77	13.57	9.47	4.89	35.03	22.34	22.06	15.22	16.08	7.45	8.40

[a] Results from males were similar

TABLE 3

Fatty Acid Composition of Phospholipids from Male Rats[a]

	A. PLASMA							B. LIVER							C. RED BLOOD CELLS						
Acid	O	A	B	C	D	Fl	Co	O	A	B	C	D	Fl	Co	O	A	B	C	D	Fl	Co
	weight %							weight %							weight %						
14:0	0.62	0.77	0.84	0.38	0.68	0.37	0.39	0.14	0.29	0.11	0.24	0.20	0.17	0.19	0.75	0.80	0.64	0.71	0.55	1.46	0.51
16:0	21.17	19.47	19.44	19.76	26.80	16.13	25.38	13.35	14.68	13.24	15.97	18.95	20.19	19.65	28.84	29.76	29.38	31.79	30.15	33.20	32.27
16:1n-7	9.07	3.59	3.41	2.92	2.75	1.06	0.68	5.69	4.52	3.23	2.90	3.43	1.35	0.96	5.68	2.55	3.14	3.34	2.02	1.48	0.76
18:0	18.34	16.91	18.20	21.10	19.95	32.62	18.89	22.33	14.51	17.01	20.28	16.77	19.69	16.78	12.18	10.96	11.56	12.89	13.69	14.63	14.06
18:1n-9	22.09	22.42	18.89	17.21	14.06	6.84	4.57	16.30	21.22	20.36	16.18	11.16	4.92	3.33	17.26	17.42	13.84	13.64	14.78	8.54	5.96
18:1n-7	9.29	9.27	8.52	6.58	5.94	1.80	3.51	13.55	10.57	10.40	7.07	6.50	3.33	4.42	8.64	7.66	7.11	5.52	3.80	2.48	2.97
18:2n-6	1.48	11.64	11.00	11.62	13.68	16.26	19.55	1.18	8.79	9.65	8.68	11.67	13.90	13.89	0.57	5.96	5.22	6.19	8.56	10.95	13.18
18:3n-6	0.00	0.00	0.00	0.00	0.00	0.00	0.31	0.00	0.00	0.00	0.00	0.00	0.00	0.16	0.00	0.00	0.00	0.00	0.00	0.00	0.00
18:3n-3	0.00	0.00	0.00	0.00	0.86	1.81	0.00	0.00	0.00	0.00	0.00	0.56	1.29	0.00	0.00	0.00	0.00	0.49	0.70	1.89	0.00
20:3n-9	15.35	4.80	3.62	0.00	0.00	0.00	0.00	17.65	5.37	2.87	1.28	0.30	0.00	0.00	16.21	4.18	2.85	0.92	0.50	0.99	0.50
20:2n-6	0.00	1.44	1.17	1.67	1.52	0.65	0.67	1.12	0.92	0.00	0.00	0.00	0.00	0.85	0.00	0.00	0.00	0.00	0.00	0.00	0.36
20:3n-6	0.00	1.41	1.21	1.58	1.03	0.88	0.69	0.26	2.15	1.42	1.50	1.51	1.43	0.81	1.37	1.24	0.86	0.86	0.95	0.48	0.41
20:4n-6	1.24	5.77	5.47	4.91	4.50	5.95	20.79	6.64	11.43	8.65	6.36	8.29	13.04	30.84	6.95	16.63	15.86	10.57	10.16	13.17	23.45
22:4n-6	0.00	0.00	0.00	0.00	0.00	0.00	0.90	0.00	2.03	0.00	0.00	0.00	0.00	0.94	0.00	0.43	0.74	0.00	0.00	0.16	2.26
22:5n-6	0.00	1.39	0.59	0.00	0.00	0.00	0.86	0.93	0.00	0.48	0.00	0.00	0.00	1.31	0.00	0.26	0.14	0.00	0.00	0.00	0.26
20:5n-3	0.00	0.00	1.01	4.01	4.49	8.29	0.00	0.00	0.19	2.29	6.36	10.86	9.45	0.00	0.00	0.27	1.86	4.54	6.81	4.52	0.00
22:5n-3	0.00	0.00	0.00	0.79	1.04	1.67	0.51	0.00	0.16	0.70	1.16	2.21	3.37	0.80	0.00	0.25	1.42	2.23	3.29	3.15	0.66
22:6n-3	1.36	1.11	6.61	7.48	2.70	5.68	2.30	0.98	3.16	9.70	12.26	7.89	8.05	5.26	0.70	1.01	4.61	5.70	3.28	2.22	1.73
DIET(n-3/n-6)	0.00	0.00	0.43	1.28	1.94	2.74	0.02	0.00	0.00	0.43	1.28	1.94	2.74	0.02	0.00	0.00	0.43	1.28	1.94	2.74	0.02
HUFA(n-3/n-6)	1.10	0.12	0.90	1.51	1.16	2.21	0.12	0.12	0.21	1.21	2.51	2.10	1.44	0.17	0.09	0.08	0.46	1.09	1.20	0.68	0.09
UFA(n-3/n-6)	0.00	0.00	0.00	0.00	6.25	11.11	0.00	0.00	0.00	0.00	0.00	0.05	0.09	0.00	0.00	0.00	0.00	0.08	0.08	0.17	0.00
SFA as %TOTAL	40.12	37.16	38.49	41.24	47.43	49.11	44.66	35.83	29.48	30.36	36.49	35.91	40.05	36.63	41.77	41.52	41.58	45.40	44.39	49.30	46.84
UFA as %TOTAL	41.93	46.93	41.83	38.32	37.29	27.77	28.63	36.72	45.09	43.64	34.83	33.32	24.79	22.77	32.15	33.58	29.32	29.19	29.86	25.34	22.88
HUFA as %TOTAL	17.95	15.91	19.68	20.44	15.28	23.12	26.71	27.59	25.43	26.11	28.91	30.76	35.34	36.40	26.08	24.90	29.10	25.42	25.74	25.23	30.28
n-7 as %UFA	43.79	27.42	28.52	24.77	23.29	10.30	14.65	52.40	33.45	31.25	28.63	29.81	18.89	23.65	44.56	30.38	34.98	30.38	19.47	15.63	16.32
x as % n-6 HUFA																					
20:2n-6	0.00	14.39	13.89	20.42	21.49	8.71	2.80	12.51	5.59	0.00	0.00	0.00	0.00	2.44	0.00	0.00	0.00	0.00	0.00	0.00	1.33
20:3n-6	0.00	14.07	14.34	19.40	14.66	11.78	2.87	3.28	13.80	13.42	19.12	15.37	9.89	2.38	16.46	6.70	4.90	7.50	8.52	3.45	1.54
20:4n-6	100.00	57.66	64.75	60.18	63.85	79.51	86.99	84.81	73.20	82.00	80.88	84.63	90.11	90.98	83.54	89.60	90.10	92.50	91.48	95.37	87.68
22:4n-6	0.00	0.00	0.00	0.00	0.00	0.00	3.76	0.00	13.01	0.00	0.00	0.00	0.00	2.79	0.00	2.32	4.22	0.00	0.00	1.18	8.47
22:5n-6	0.00	13.88	7.02	0.00	0.00	0.00	3.59	11.91	0.00	4.58	0.00	0.00	0.00	3.85	0.00	1.38	0.78	0.00	0.00	0.00	0.97
y as % n-3 HUFA																					
20:5n-3	0.00	0.00	13.23	32.65	54.58	53.00	0.00	0.00	5.45	18.08	32.15	51.81	45.28	0.00	0.00	17.76	23.58	36.38	50.92	45.68	0.00
22:5n-3	0.00	0.00	0.00	6.45	12.69	10.66	18.10	0.00	4.65	5.52	5.88	10.56	16.13	13.16	0.00	16.55	18.01	17.90	24.58	31.86	27.78
22:6n-3	100.00	100.00	86.77	60.90	32.73	36.34	81.90	100.00	89.91	76.40	61.97	37.63	38.59	86.84	100.00	65.69	58.40	45.72	24.50	22.46	72.22

[a]Results from females were similar.

In plasma phospholipids, the n-7 type of acids were almost one-half of the UFA with diet O, whereas they were about 25% with 10 en% fat and only 12% with 20 en% fat (Table 3A). Although the general pattern of SFA, UFA and HUFA was relatively constant, wide differences occurred in the relative proportions of n-9, n-6 and n-3 fatty acids that were maintained in the phospholipids. For example, 20:3n-9 decreased from about 17% of the acids in liver phospholipid (Table 3B) with diet O to undetectable levels with diets Fl and Co. Alternatively, 20:5n-3 varied from 0–13% and 20:4n-6 varied from 6–30%.

Plasma, liver and red cells all tended to maintain n-3/n-6 ratios in phospholipid HUFA that reflected the ratio of the diet being fed. In contrast, the relative proportion of 20:4n-6 within the n-6 HUFA of phospholipid tended to remain constant for each tissue, independent of the widely varied influx of n-3 and n-6 dietary fatty acids. Liver maintained the 20:4n-6 at about 80% of the n-6 HUFA in the phospholipids (Table 3B). In contrast, 20:4n-6 was only about 74% of n-6 HUFA in plasma phospholipids (Table 3A), whereas it averaged about 90% of the n-6 HUFA in red cell phospholipids (Table 3C). Significant proportions of 22:4n-6 and 22:5n-6 in the n-6 HUFA of red cells were maintained only with diets A and Co, which provided the precursor, 18:2n-6, with little competition from dietary n-3 fatty acids. With many of the diets, the precursor of monoenoic prostaglandins, 20:3n-6, represented 15–24% of the n-6 HUFA of liver and plasma. Unexpectedly, the proportion of 20:3 in the n-6 HUFA of red cell phospholipids was less than in liver and plasma, and it decreased with an increased influx of the dietary precursor, 18:2n-6. All three tissues maintained the lowest percent of 20:3n-6 in the n-6 HUFA when the Co diet was fed. This effect of 18:2n-6, coupled with an inability to maintain the 22-carbon n-6 HUFA during dietary influx of 18:3n-3, caused the n-6 HUFA to be almost exclusively in the form of 20:4n-6 with diet Fl.

The relative proportion of 22:6n-3 that was maintained within the n-3 HUFA of tissue phospholipids decreased with an increased dietary influx of the n-3 fatty acid, 18:3n-3. The decrease was accompanied by increased proportions of 20:5n-3 and 22:5n-3 that tended to reflect the dietary supply of the precursor, 18:3n-3. Thus, with diets O, A and Co that contained negligible amounts of 18:3n-3, plasma phospholipids maintained 22:6n-3 almost exclusively as the n-3 HUFA (Table 3A), whereas the level dropped progressively to about 33% with increased dietary influx of 18:3n-3. Similar changed proportions were observed for the n-3 HUFA in liver phospholipids (Table 3B), and an even more striking decrease to about 22% was noted for 22:6n-3 among the n-3 HUFA of red cells (Table 3C).

A competitive hyperbolic relationship. Rather than fitting a linear relationship with dietary precursors as seen for 18:2n-6 and 18:3n-3 in tissue triglycerides, the amounts of n-3 and n-6 HUFA that were maintained in phospholipids fitted a "saturable" hyperbolic relationship to the dietary supply of precursors which resembled the competitive hyperbolic relationship commonly used to describe saturable rate-limiting processes.

$$Response = \frac{V_{max}}{1+\frac{K_m}{en\%S}\left(1+\frac{en\%I}{K_i}\right)} \qquad [2]$$

Trial and error fitting of the data in Tables 3A–C to Equation [2] indicated that the hyperbolic equation was fitted with a very small EC_{50} for the dietary fatty acid (*ca.* 0.1 en%). Further trial and error fitting of these data led us to add a term, C_o, to describe the influence of other dietary fatty acids upon the resultant tissue HUFA contents. Also, another term, K_s, was added to modify the shape of the hyperbola to better fit the observed shape. With these modifications, we used the three forms of Equation [3] to examine the degree to which dietary n-3 and n-6 nutrients could predict the observed fatty acid compositions. In these equations C_3, C_6 and C_o function analogous to K_m or K_i values for n-3, n-6, and other types of dietary fatty acids, whereas the dimensionless constant, K, in equation [3c] is a selected value of the ratio of [C_9/en%9] that fits the equation to 20:3n-9 in the tissue. The fitted value of K was 0.2 for plasma and 0.3 for erythrocytes of males and females, whereas for liver phospholipids it was 0.4 for males and 0.5 for females.

$$n\text{-}3\ as\ \%HUFA = \frac{100}{1+\frac{C_3}{en\%3}\left[1+\frac{en\%6}{C_6}+\frac{en\%0}{C_o}+\frac{en\%3}{K_s}\right]} \qquad [3a]$$

$$n\text{-}6\ as\ \%HUFA = \frac{100}{1+\frac{C_6}{en\%6}\left[1+\frac{en\%3}{C_3}+\frac{en\%0}{C_o}+\frac{en\%6}{K_s}\right]} \qquad [3b]$$

$$n\text{-}9\ as\ \%HUFA = \frac{100}{1+K\left[1+\frac{en\%3}{C_3}+\frac{en\%6}{C_6}\right]} \qquad [3c]$$

The ability of Equation [3] to fit the wide range of observed compositions of n-3, n-6 and n-9 among the HUFA of tissue phospholipids is illustrated in Figures 1, 2 and 3. For each tissue, a single set of values for the constants was selected which permitted the appropriate version of Equation [3] to fit the compositions of the n-9, n-6 or n-3 HUFA which were observed for that tissue. Figure 1A illustrates that as the total amount of dietary polyunsaturated fatty acid increased, the amount of 20:3n-9 maintained in plasma phospholipid HUFA decreased similarly for males and females (closed and open diamonds, respectively). The decrease was quantitatively fitted by Equation [3] (hatched bars) using the values for C_6, C_3, C_o and K_s shown in panel 1B. These values were selected by trial and error to permit the hyperbolic equation to also fit the patterns observed for the n-6 acids (20:3 plus 20:4; Fig. 1B) and n-3 acids (20:5 plus 22:5; Fig. 1C) in liver phospholipids of males and females. Differences among the metabolic selectivities of the three tissues in maintaining their tissue-specific patterns of fatty acids are reflected in the differences in the values for the constants selected to fit the data. These differences are summarized in Table 4. The average values for C_6 and C_3 are less than 0.1% of the dietary calories, whereas the value for C_o is greater than 1%.

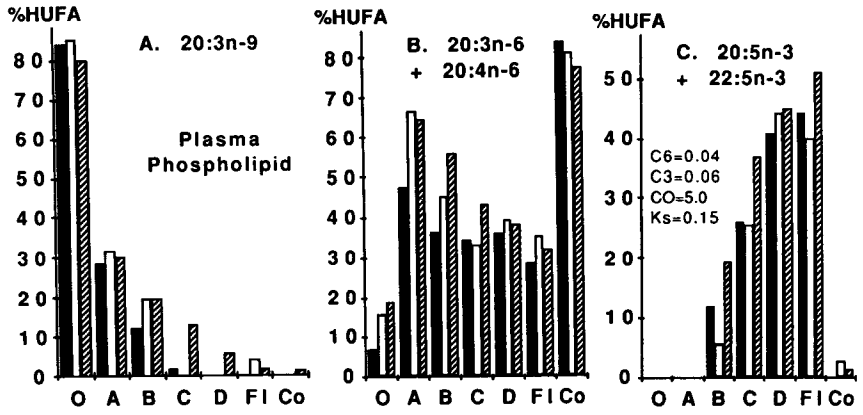

FIG. 1. Highly unsaturated fatty acids maintained in phospholipids of rat plasma. Curves represent values for males (open bars), females (solid bars) and those predicted by the equation (hatched bars) using the constants shown in C. A, 20:3 n-9 in HUFA; B, 20:3n-6 plus 20:4n-6 in HUFA; and C, 20:5n-3 plus 22:5n-3 in HUFA.

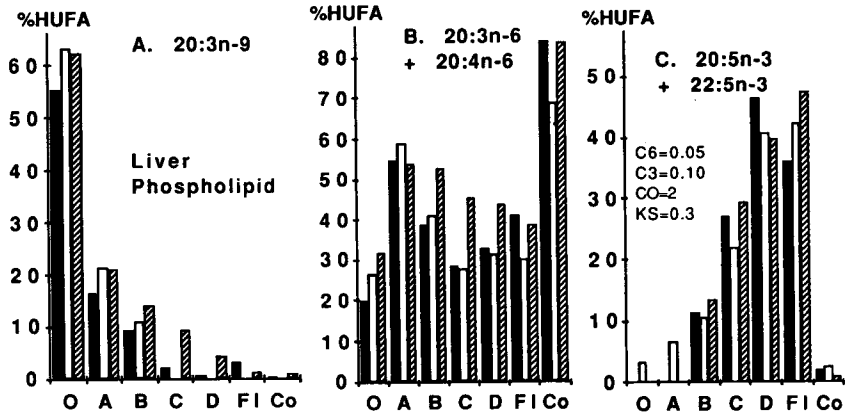

FIG. 2. Different levels of highly unsaturated fatty acids maintained in phospholipids of rat liver. Bars represent types of values as described in Figure 1.

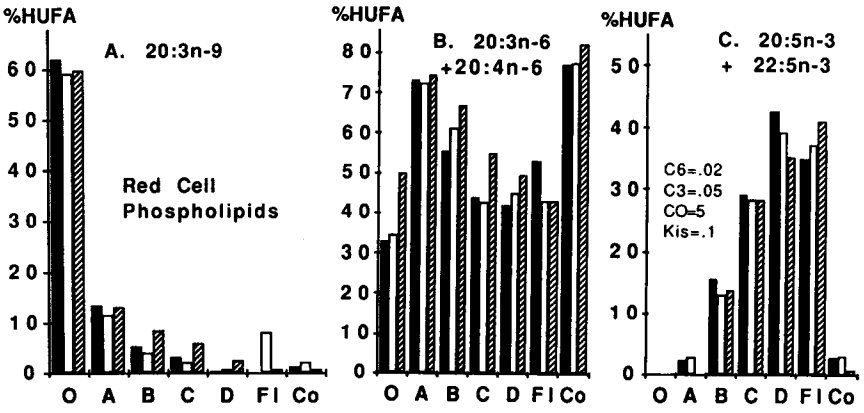

FIG. 3. Different levels of highly unsaturated fatty acids maintained in phospholipids of rat red blood cells. Bars represent types of values as described in Figure 1.

TABLE 4

Summary of Fitted Constants[a]

	C6 (en%)	C3 (en%)	CO (en%)	Ks (en%)	K
Liver	0.05	0.10	2.00	0.30	0.40[b]
Plasma	0.04	0.06	5.00	0.15	0.20
RBC	0.02	0.05	5.00	0.10	0.30
Average	0.036	0.07	4.00	0.18	0.30

[a]The values for the constants (C_6, C_3, C_0, K_S and K) are derived from results shown in Figures 2, 3 and 4 as defined for equations [3a], [3b] and [3c].

[b]A value of 0.50 fitted results for female rats.

Fatty acid composition of non-esterified fatty acids. The composition of NEFA in plasma (Table 5A) contained an average of about 37% SFA, 54% UFA and 3% HUFA, and resembled that of the NEFA in liver (Table 5B), which averaged about 38% SFA, 57% UFA and 5% HUFA. Values for the NEFA maintained in males (provided in Table 5) are similar to those for females (results not shown). Both tissues maintained n-3/n-6 ratios in NEFA that reflected the supplies in the different diets, although the n-3/n-6 ratios in UFA tended to be less than those of the diets and the n-3/n-6 ratios in HUFA tended to be greater. Although the n-7 acids as a percent of UFA were maintained at the greatest level with the fat-deficient diet and tended to be lowest with the diets containing 20 en% fat, the trends were not as consistent (especially for liver NEFA) as those observed with the plasma phospholipids (Table 3A) and triglycerides (Table 2A).

DISCUSSION

General categories of fatty acids. To interpret the data in this study, we employed a hypothesis that the general metabolic properties of fatty acids could be a useful means of grouping the many different varied fatty acids that are esterified in tissue lipids. With this priority system, the categories were initially defined according to the general selectivity of the enzymes that catalyze the formation of ester bonds (SFA, UFA and HUFA) rather than to some consequent physical property of the product or some eventual physiological consequence that might occur subsequently. As a result, the recognized difference between the n-3 and n-6 HUFA in the formation and function of eicosanoids (12,14) was subordinated to the finding of little *in vitro* evidence for differences of esterification selectivity between these two types of acid (15). The relative lack of evidence for any unique specificity for esterification of any of the different types of fatty acids

TABLE 5

Fatty Acid Composition of NEFA from Male Rats[a]

	A. PLASMA							B. LIVER						
Acid	O	A	B	C	D	Fl	Co	O	A	B	C	D	Fl	Co
	weight%							weight%						
14:0	3.70	5.40	2.54	4.00	2.70	2.29	2.78	1.05	1.61	1.54	1.78	1.81	2.48	1.08
16:0	30.96	30.17	24.22	30.22	29.79	24.76	24.92	25.19	29.55	28.97	31.59	25.10	33.71	29.64
16:1n-7	11.47	6.52	5.62	8.13	8.74	3.01	2.79	12.52	14.14	12.71	14.13	11.92	9.72	6.72
18:0	9.05	11.04	10.67	15.56	10.05	12.94	12.43	7.31	3.19	3.67	3.21	5.65	8.90	7.74
18:1n-9	27.83	29.65	44.42	32.15	26.17	25.65	23.54	36.64	38.86	34.88	33.99	28.73	15.24	16.36
18:1n-7	12.74	8.64	6.20	2.07	5.82	0.00	0.00	11.03	8.48	12.06	7.78	7.41	3.89	2.92
18:2n-6	1.96	3.79	3.85	4.17	5.42	10.22	24.17	2.13	2.29	3.02	1.89	6.16	8.16	25.49
18:3n-6	0.00	0.00	0.00	0.00	0.00	0.00	0.18	0.00	0.00	0.00	0.00	0.00	0.00	0.30
18:3n-3	1.03	1.47	0.85	0.68	5.95	17.49	1.58	0.00	0.00	0.67	1.05	5.91	11.01	1.54
20:3n-9	0.93	0.66	0.00	0.00	0.00	0.00	0.00	1.19	0.38	0.00	0.00	0.00	0.00	0.00
20:2n-6	0.00	0.00	0.00	0.00	0.00	0.00	0.28	1.94	0.00	0.21	0.56	0.30	0.74	0.98
20:3n-6	0.00	0.00	0.00	0.00	0.00	0.00	0.26	0.00	0.00	0.00	0.00	0.00	0.00	0.00
20:4n-6	0.00	1.72	1.03	0.68	1.32	0.91	6.58	0.00	0.73	0.63	0.43	0.68	0.61	3.58
22:4n-6	0.33	0.94	0.60	0.46	0.47	0.00	0.49	0.00	0.30	0.22	0.00	0.54	0.00	1.27
22:5n-6	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
20:5n-3	0.00	0.00	0.00	1.61	2.92	1.99	0.00	0.00	0.00	0.30	0.47	2.68	2.03	1.12
22:5n-3	0.00	0.00	0.00	0.27	0.65	0.74	0.00	0.00	0.00	0.12	0.09	1.24	1.84	0.00
22:6n-3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.99	0.48	0.97	3.04	1.85	1.66	1.63
DIET(n-3/n-6)	0.00	0.00	0.43	1.28	1.94	2.27	0.02	0.00	0.00	0.43	1.28	1.94	2.74	0.01
HUFA(n-3/n-6)	0.00	0.00	0.00	1.65	2.00	3.01	0.00	0.51	0.50	1.49	3.78	3.98	6.75	0.40
UFA(n-3/n-6)	0.53	0.39	0.22	0.16	1.10	1.71	0.07	0.00	0.00	0.22	0.56	0.96	1.35	0.06
SFA as %TOTAL	43.70	46.60	37.43	49.78	42.54	39.99	40.13	33.54	34.36	34.18	36.58	32.57	45.09	38.46
UFA as %TOTAL	55.03	50.08	60.93	47.21	52.11	56.37	52.26	62.33	63.76	63.34	58.83	60.14	48.03	53.34
HUFA as %TOTAL	1.26	3.32	1.63	3.01	5.35	3.64	7.61	4.13	1.88	2.42	4.59	7.29	9.94	8.37
n-7 as %UFA	43.99	30.28	19.39	21.61	27.95	5.34	5.34	37.79	35.47	39.11	37.23	32.14	28.35	18.08

[a]Results from females were similar.

(n-3, n-6, n-7, n-9) does not diminish the importance of the physiological consequences of their subsequent metabolism. Rather, it helps emphasize the vital importance of the initial selection of the proportions of the acids as nutrients. Once dietary selections are made, the tissue fatty acid composition tends to be defined by competitive metabolism, and the resulting physiological consequences tend to become more inescapable. The present approach to comparing relative proportions of categories of fatty acids emphasizes relative metabolic interrelationships and minimizes the differences in absolute amounts of specific fatty acids among tissues which are evident in the different constants of Table 4.

Competitive metabolic interactions among the n-3, n-6 and n-9 types of fatty acids in maintaining tissue HUFA compositions have been recognized for a long time (5,10). The maintenance levels of a particular fatty acid in a tissue lipid reflects a steady-state balance in synthesis, degradation, transport, esterification and hydrolysis. By describing the amounts of the different individual competing HUFA as a percentage of the total esterified HUFA in a given tissue, the present report provides a single general algebraic relationship (*e.g.*, Equation [3b]) that quantitatively estimates the magnitude of those competitive interactions. The assignment of a single set of constants to describe the maintenance of n-3, n-6 and n-9 HUFA in each tissue also provides a simple way to help define the tissue-specific differences that reflect genetically-controlled enzyme activities. The observed similarities of these constants among different tissues probably indicates that the competitive interactions among HUFA are fairly similar in the different tissues even though relatively small differences occur in the constants, and the total amount of HUFA in a specific type of lipid being examined may differ appreciably among tissues. This report provides comparisons among the different fatty acids (and among different tissues of rats) so that subsequent studies of lipid metabolism in rats can use the quantitative steady-state information base to interpret the kinetics of tissue reponse to dietary changes.

A recent discussion of the fatty acid composition in human plasma lipids (3) emphasized the similarity of fatty acid compositions of plasma and adipose lipids reported for individuals from all over the world. This similarity was discussed in terms of possible similar supplies of fatty acids in the diet of the individuals tested. In the present report, the fatty acid compositions of plasma lipids from rats can be recognized to be very similar to the worldwide averages reported for humans (3), with endogenous fatty acids (16:0, 18:0, 16:1, 18:1) constituting the majority of fatty acids in the lipids of plasma (16) and adipose (17) and with similar proportions of SFA, UFA and HUFA for rats and humans. Wide variations in the composition of the dietary fatty acids were created in the present study of rats, and still the relative proportions of the endogenous fatty acids maintained in plasma and adipose lipids remained similar. The qualitative similarity of the compositions maintained by rats and humans suggested that (for individuals from all over the world) relatively similar proportions of endogenous fatty acids are supplied from carbohydrate and amino acid precursors by the fatty acid synthetic system. The major source of acetate for the biosynthesis of these acids would be carbohydrates (50–75% of dietary calories), which may provide relatively similar proportions of endogenous fatty acids to the esterifying enzymes that synthesize the plasma and adipose lipids. In this way, similar compositions for rats and humans reflect similar general selectivities in the formation and esterification of endogenous fatty acids.

Marginal supplies of essential fatty acids. Each set of animals in this study was provided a single diet throughout the lifetime of the experiment. To ensure minimum confounding variables, the mothers of the experiment animals also were maintained on the same diet for several months prior to conception. This protocol minimized the kinetics of altered dietary compositions (which are so common with human studies) and also minimized the impact of the maternal n-6 and n-3 fatty acids that are supplied to the animals prior to delivery and weaning. Preliminary experiments produced barely discernable symptoms of essential fatty acid deficiency only after prolonged feeding of the fat-deficient diet that contained 70% of calories as sucrose. Diets that contained appreciable starch (with *ca.* 0.4 en% 18:2n-6) apparently provided the very small amounts (*ca.* 0.2 en%) of needed 18:2n-6. Although we had expected to see physiological signs of a deficiency of essential fatty acids with several of the diets in this study, the older literature (4,5) provides clear evidence of asymptomatic animals when they obtained at least 0.3% of dietary calories as 18:2n-6. Growth curves for rats with limited nutrient supply of essential fatty acids showed a hyperbolic response to dietary polyunsaturated fatty acid with a half-maximal stimulation in the range of about 0.1% of dietary calories as 18:2n-6 (4,5). Marginal growth conditions for rats with limited 18:2n-6 (0.2 en%) were associated with the maintenance of nearly equal amounts of 20:3n-9 and 20:4n-6 in liver lipids (4,5). Similar metabolic interactions of HUFA also occurred in humans for whom dietary 18:2n-6 at 0.5 en% produced no deficiency symptoms while maintaining plasma 20:3n-9/20:4n-6 ratios near 1 (18). In contrast, lower dietary 18:2n-6 (0.07 en%) that produced deficiency symptoms in about one-half of the babies studied maintained the plasma 20:3 n-9/20:4n-6 ratios near 1.4 (19). Both results on 20:3n-9/20:4n-6 ratios and on the midpoint amount needed for growth indicate again that the general metabolic characteristics of the metabolism of HUFA and glycerolipid are similar in rats and humans. The similar hyperbolic response to dietary 18:2n-6 of growth and tissue 20:4n-6 [based on data of Mohrhauer and Holman (4) as discussed by Lands (21)] supports the concept that the principal role of the essential n-6 dietary acid is in maintaining an adequate amount of 20:4n-6 in tissues to serve as precursor for eicosanoid biosynthesis. Apparently, when adequate levels of 20:4n-6 are not maintained, then adequate physiology is not maintained. Further support for this concept comes from the ability of Equation [3] to predict thrombotic occlusion times (20; as discussed in ref. 21) thromboxane formation by platelets (22,23; as discussed in ref. 21), and tumor proliferation (24; as discussed in ref. 21). The hyperbolic relationship of these physiologic events to the amounts of dietary n-3 and n-6 precursors suggests that the percent of tissue HUFA as n-6 fatty acid may be a useful index of the capacity of the tissue to form eicosanoids when stimulated. Furthermore, most of these hyperbolic relationships were also fitted with a half-maximal stimulation in the range of 0.1% of dietary calories as 18:2n-6 (21).

Dietary fat and endogenous n-7 and n-9 acids. The decreased amount of n-7 fatty acids (as a percentage of UFA) maintained in tissue lipids in response to increased fat in the diet provides a metabolic indicator of the relatively decreased influx of dietary carbohydrate which accompanied the increase in dietary lipid. Analyses of n-7 acids in plasma lipid fractions, therefore, might be considered as helpful in assessing the relative proportions of daily calories ingested as fat and carbohydrate. However, the results in this study indicate that the useful range for such an assay would most likely be with fat comprising from 0 to 20% of the daily calories. In this range, the n-7 acids as % UFA in plasma phospholipids may have the following approximate relationship to dietary fat:

$$n\text{-}7 \text{ as } \%PL\text{-}UFA = \frac{100}{1+1.5(1+en\%fat/5)} \quad [4]$$

This relationship predicts that the plasma phospholipid would maintain about 40, 18, 12, 9 and 7% of the UFA as n-7 acids when the diets contained 0, 10, 20, 30 and 40 en% fat, respectively. Consequently, gas chromatographic analyses of the n-7 acids in plasma phospholipids would be expected to give low values that are difficult to interpret when the fat content of the diet exceeds 20% of total calories. Nevertheless, this analytical approach to estimating the percent of fat calories might be useful in estimating the compliance in dietary studies with relatively low-fat diets. Evidence that this relationship may occur in humans is found in a recent report of malabsorption (25).

The amount of 20:3n-9 (as a percentage of the HUFA) that was maintained in tissue lipids decreased in a hyperbolic response to increased polyunsaturated fat in the diet. The quantitative nature of the response confirms the results of Mohrhauer and Holman (4,5) as discussed elsewhere (21). The low value of 0.2 for K in Equation [3c] fits higher proportions of 20:3n-9 in plasma HUFA and indicates a corresponding lower competitive effect of the dietary en% of n-3 and n-6 fatty acids than seen with liver (*i.e.*, K = 0.5 for females). Similarly, the value of 0.4 for K in male liver indicates a greater tendency in males than in females for the n-9 derivatives to predominate somewhat. Such a comparative difference was reported for liver phospholipids following fasting and refeeding, with a stronger entry of 18:1n-9 into the liver lecithin of males than females (26).

Synthesis, transport and storage of triglycerides. Movement of fatty acids from adipose to liver triglycerides occurs rapidly via plasma NEFA and the return flow is provided by plasma triglycerides secreted by the liver (which are then converted to plasma NEFA by lipoprotein lipase). When dietary fatty acid compositions differ greatly from that of adipose tissue, the liver would be presented with different supplies of fatty acids throughout the day as the NEFA derived from intermittent eating blends with the continual supply of plasma NEFA from adipose. The corresponding continual export of triglycerides from the liver maintains a supply of plasma triglyceride fatty acids that will mix intermittently with dietary supplies (depending on the amount of fat in the diet) and provide a blend of fatty acids for incorporation into adipose tissue triglycerides. In the experimental protocol of this study, the tissues were given optimal opportunity to reach steady state by keeping the composition of the dietary influx relatively constant throughout the lifetime of the rats. This protocol avoided the large dietary variations commonly encountered in human nutrition, and it permitted comparisons of fatty acid composition which illustrate the basic relationships between the fatty acids of diet and tissue. The close similarities of the fatty acid composition of plasma, liver and adipose triglycerides illustrate the close metabolic interactions of these three triglyceride pools. Also, the linear (non-hyperbolic) relationship to dietary supplies illustrates the unlimited supply of triglyceride ("element variable") esterification sites, which contrast to the limited supply of phospholipid ("element constant") esterification sites.

Recent studies (27) of the oxidation of 18:2 and 18:3 by liver mitochondria provide an explanation for the two-fold greater entry into triglycerides by 18:2n-6 as compared to 18:3n-3. As the dietary polyunsaturated fatty acids enter the cell in the form of non-esterified fatty acids (NEFA), the mitochondria may convert the 18:3n-3 to acid-soluble products and CO_2 at a rate twice that for 18:2n-6 (27). This selective removal of 18:3n-3 could cause the nearly two-fold difference in slopes noted for Equations [1a] and [1b] by maintaining the 18:3/18:2 ratio at one-half that provided by the diet. The overall *in vivo* selectivity in this study fits results reported for NEFA rather than the coenzyme A esters (27), and it supports the concept of a coupled action of the ligase and transferase rather than a transfer of acids for oxidative metabolism from a general pool of cellular acyl-coenzyme A esters.

Maintenance of HUFA in phospholipids. The general metabolic and transport selectivities for forming rat plasma phospholipids produced an average composition of about 43% SFA, 36% UFA and 20% HUFA. It seems likely that similar selectivities may also prevail in humans who have average values of about 42% SFA, 35% UFA and 20% HUFA [calculated from data of Holman (16) and Nikkari (28)]. The experimental diets in this study provided over a hundred-fold range of n-3/n-6 ratios (from 0.02 to 2.74), and the ratios for HUFA in tissue phospholipid were similar to the relative abundances of the n-3/n-6 nutrients. This similarity supports an earlier suggestion (3) that the elongation of 18 carbon n-3 and n-6 fatty acids to 20-carbon derivatives may occur with no appreciable discrimination between the two types of fatty acids. The hyperbolic relationship of phospholipid HUFA to dietary precursors illustrates an important underlying principle of competitive interactions for a limited number of esterification sites.

Plasma phospholipids are primarily formed and secreted by the liver, and some selectivity in the secretion process has been reported. For example, humans, rats, dogs and oxen were all shown to maintain somewhat less stearate and arachidonate in biliary lecithin as compared to plasma lecithin (29,30). Apparently, the liver may have either functionally distinct pools of lecithin or it may engage in selective secretion from a single heterogeneous pool. The lower total HUFA in phospholipids of rat plasma (ca. 20%) as compared to that in liver (ca. 30%) noted in the present study also reflects a selective transfer into plasma. In this case, it was less highly unsaturated lipids than the average liver phospholipid. This difference

in transfer and metabolism is reflected by the differences among the fitted constants for liver and plasma in Table 4.

Examining the relative proportions of the n-3 HUFA provided useful insight to the metabolic selectivities. The weight percent of 22:6n-3 in plasma phospholipids was maintained at a higher average level with diets B and C than with D and Fl, even though the latter diets contained even greater nutrient supplies of 18:3n-3. The greater influx of 18:3n-3 from the diet may permit tissues to more rapidly synthesize 20:5 and 22:5 in amounts that could suppress by competitive interactions the esterification of 22:6n-3. Alternatively, greater amounts of available n-3 precursor may suppress the putative Δ4 desaturase activity. Whatever the mechanism, it is important to recognize that 22:6n-3 does not respond to dietary precursors in the way that 20:5n-3 and 22:5n-3 do, and the n-6 acids did not have the pattern of response of the n-3 acids. The vigorous retention of 22:6n-3 when there is limited supply of n-3 precursors creates a problem in studying selective effects of dietary polyunsaturated fats on tissue HUFA. For example, our results confirm and extend the report by Iritani and Fujikawa (31) of a relatively constant sum of "polyunsaturated fatty acids with more than three double bonds" (*i.e.*, HUFA) in phospholipids of plasma (18–20%) and liver (31–35%). However, their results were biased by the protocol of feeding for only two weeks, which was not long enough to develop a steady-state maintenance level for 22:6n-3. This was evident in the high values for this acid (*ca.* 27% of HUFA) in liver phospholipid even when no n-3 acid was in the diet for two weeks. Eight weeks on the n-3 deficient diet led to 6.7% of HUFA (31), closer to our result of about 3% following 20 weeks of the diet.

An earlier analysis (3) of the extensive data provided by Mohrhauer and Holman (4,5) emphasized that the elongation from 20- to 22-carbon HUFA appeared more facile with n-3 than n-6 acids. The present results extend the insight into factors influencing 22:6n-3 formation, showing that the elongation to 22:5n-3 proceeded with ease, but the step commonly attributed to a Δ4-desaturase may not be capable of increased flux in response to increased supplies of precursor. The competition did not appear to prevent elongation of 20:5n-3 to 22:5n-3, since both of these acids increased as dietary 18:3n-3 increased. The paradoxically lower tissue contents of 22:6n-3 with increased dietary 18:3n-3 made it difficult to fit Equation [3] to values for this fatty acid. In contrast, the sum of 20:5n-3 plus 22:5n-3 fit the same constants used in the Equations to describe the maintenance levels for the n-6 and n-9 acids.

Inhibition by n-3 acids of the conversion of 20:4n-6 to 22-carbon derivatives seemed to permit very little 22-carbon n-6 HUFA being maintained in tissue phospholipids [except with the diets (0, A, Co) that contained very low amounts of 18:3n-3]. The combined effects of high levels of dietary fatty acids to make 20:4n-6 the predominant n-6 HUFA results in optimal conversion of dietary 18:2n-6 into the major eicosanoid precursor. This effect may favor more vigorous formation of eicosanoids when a tissue is stimulated.

Two important trends are evident from the present study of quantitative nutrition—rat tissues tend to maintain fatty acid compositions that may be quantitatively predicted from the composition of dietary fatty acids; and the general selectivities in the metabolism that maintains the composition of fatty acids in the plasma and red cell glycerolipids of rats appears to be very similar to that in humans. After sufficient experimentation has confirmed the linear and hyperbolic relationships described in this report, it may be possible to employ these equations with analytical values for fatty acids in plasma and erythrocyte lipids to estimate the average intake of nutrient n-3 and n-6 fatty acids.

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Tissue Phospholipid Fatty Acid Composition in Genetically Lean (Fa/-) or Obese (fa/fa) Zucker Female Rats on the Same Diet

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The fatty acid composition of serum total lipids, of phospholipids of various organs (liver, heart, kidney), and of nervous structures (brain, retina, sciatic nerve, myelin, synaptosomes) have been compared in lean (Fa/-) and genetically obese (fa/fa) Zucker female rats. Both received a standard commercial diet including 37% of 18:2n-6 and 5% of n-3 polyunsaturated fatty acids (PUFA), 1.7% of which were in the form of 20:5n-3 and 22:6n-3. In comparison with lean rats, the results for the obese rats pointed out (i) no difference in the fatty acid composition of nervous structures; (ii) a decrease of 18:2n-6 (from -8% to -35%) and of 20:4n-6 (from -9% to -49%) in serum, liver and in kidney; this was compensated for by an increase in 20:3n-6 (from +30% to +320%) and in total n-3 PUFA (from +68% to +76%); (iii) a decrease of 20:4n-6 (-18%) and of 22:6n-3 (-24%) in heart compensated for by an increase in 18:2n-6 (+39%) and in 20:3n-6 (+233%); and (iv) constant levels of total PUFA (n-6 and n-3) in the various fractions studied, except in serum where this level decreased (-23%). Finally, except for the nervous structures, tissue phospholipids of obese rats included a lower proportion of 20:4n-6 and a higher proportion of 20:3n-6. This resulted in a significant reduction in the 20:4n-6/20:3n-6 ratio; by contrast, the 20:3n-6/18:2n-6 ratio increased. The results suggest that in Zucker rats, the obese character (fa/fa) affects the desaturation-elongation process of 18:2n-6 to 20:4n-6 by specifically decreasing Δ5-desaturase activity.

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The proportion and nature of polyunsaturated fatty acids (PUFA) acylated in the *sn*-2 position β of phospholipids determine, in part, the physical and functional properties of biological membranes (1-3). PUFA belong to two non-interconvertible series (n-6 and n-3). Due to processes of successive elongation and desaturation, PUFA are obtained from the two key acids, linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3).

The conversion process involves liver microsomal desaturases (4,5) and, in particular, two enzymes whose activity, in a defined nutritional context, determines the quantity of long-chain PUFA available to the organism: (i)Δ6-desaturase that permits the conversion of 18:2n-6 to γ-linolenic acid (18:3n-6) and the conversion of 18:3n-3 to stearidonic acid (18:4n-3); this enzyme is generally considered the rate limiting step in PUFA desaturation (6-8); and (ii) Δ5-desaturase that permits the conversion of dihomo-γ-linolenic (20:3n-6) to arachidonic acid (20:4n-6) and of 18:4n-3 to eicosapentaenoic acid (20:5n-3).

Certain factors, such as experimental diabetes (9-12), alcohol and aging (8), and dietary cholesterol (13) more or less specifically inhibit Δ6- and/or Δ5-desaturase activities. Insulin seems to be specifically implicated in the simultaneous regulation of the two enzyme activities (14). Thus, rats made diabetic by streptozotocin injection show physiopathological and biochemical characteristics comparable to those accompanying total PUFA deficiency; insulin administration causes these symptoms to disappear rapidly (15).

Genetically obese Zucker rats (fa/fa) are hyperlipemic and hyperinsulinemic (16,17). In these animals, the proportion of 20:4n-6 in phospholipids (PL) of serum, platelets, liver and heart is significantly decreased as compared to the values seen in lean controls (Fa/-) (18-21). Surprisingly, the reverse process, i.e., an increase in the conversion of 18:2n-6 to 20:4n-6, has been observed in genetically obese mice (ob/ob) (22,23).

In recent studies in Zucker rats, we have confirmed the above observations and shown that in obese rat hearts, phospholipids are low in n-3 PUFA (24) while phospholipids of adipocyte plasma membranes are, by contrast rich in n-3 PUFA (25).

The aim of the present study was to obtain complete fatty acid data on serum, liver and heart in these animals and to add (in addition to kidney), nervous structures, such as brain, retina, sciatic nerve, myelin and synaptosomes, which are known to contain high amounts of n-3 PUFA in their phospholipids (26).

MATERIAL AND METHODS

Six obese (fa/fa) and six lean (Fa/-) Zucker female rats (three-months-old) were used. They received a commercial standard diet; the lipid content and the fatty acid composition of the diet are shown in Table 1. The diet contained 28% of saturated fatty acids (SFA), 30% of monounsaturated fatty acids (MUFA) and 37% of n-6 PUFA in the form of 18:2n-6 and about 5% of n-3 PUFA, 1.7% of which were in the form of 20:5n-3 and 22:6n-3.

The rats were fasted for 15 hr, and then killed by decapitation. The serum was recovered for assay of total cholesterol, triglycerides and phospholipids (Boehringer methods). Liver, heart, kidneys, brain, retina and sciatic nerves were quickly excised, rinsed and deep-frozen at -80°C. A fraction of the brain was used to prepare synaptosomes and myelin as described previously (27). Serum lipids and various freeze-dried tissues were extracted according to Folch *et al.* (28) in the presence of butylhydroxytoluene (BHT) (0.02%, w/v). The phospholipids of liver, heart, kidney, brain and retina were then separated by thin-layer chromatography on silica gel (60 G Merck) using hexane/diethyl ether/formic acid

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Abbreviations: BHT, butylhydroxytoluene; GLC, gas-liquid chromatography; FFAP, free fatty acid phase; MUFA, monounsaturated fatty acids; PL, phospholipids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; TSL, total serum lipids.

TABLE 1

Dietary Fatty Acid Composition[a]

Fatty acids	%
Saturated	
14:0	0.9
16:0	20.0
18:0	6.7
Total	27.6
Monounsaturated	
16:1n-7	3.1
18:1n-9	26.7
Total	29.8
n-6 Polyunsaturated	
18:2n-6	36.6
20:4n-6	0.2
Total	36.8
n-3 Polyunsaturated	
18:3n-3	3.1
20:5n-3	0.9
22:6n-3	0.8
Total	4.8
n-6 plus n-3	41.6
n-6/n-3 ratio	7.67

[a]The dietary lipid content is 7.3%.

(80:20:1, v/v/v) containing 0.02% of BHT (w/v) (29). Finally, the fatty acid composition (% of total fatty acids) of the various lipid fractions was determined by gas-liquid chromatography (GLC) of the methyl esters using a glass capillary column coated with free fatty acid phase (FFAP) (inner diameter, 0.3 mm; length, 50 m; detection by flame ionization).

Statistical analyses were done by Student's t-test. In fatty acid analysis, as percentage data was not normally distributed, statistical analyses were realized using the variance-equalizing transformation (arc-sine transformation) according to Zar (30).

RESULTS

Organ weight and lipid content. For heart, kidney and brain, the weight and total lipid contents were independent of genotype. However, weight and total lipid content were notably increased (+36% and +28%, respectively) in the livers of obese animals; the amount of total liver lipids was increased by 74% in these animals (Table 2).

Serum lipids. As reported previously, the serum of obese rats had a considerably higher lipid content; this was particularly due to the increase in triglyceride levels which were increased seven-fold. The level of phospholipids was doubled, but cholesterol levels were only moderately increased (Table 3).

Total serum lipids (TSL). The level of SFA in TSL was slightly but significantly higher (+8%; $p < 0.05$) in the obese rats due to a marked increase in palmitic acid levels. The difference between total MUFA was quite apparent, since MUFA levels were almost two times higher in obese than in lean rats (23% *vs* 12%), due to the concomitant doubling of palmitoleic and oleic acid levels (Table 4).

In the obese rats, the total proportion of n-6 PUFA dropped by 36% due to a moderate decrease (-19%) in linoleic acid and a considerable decrease (-48%) in arachidonic acid. There also was a significant increase in 20:3n-6, reaching 0.8% in obese vs 0.3% in lean rats. On the other hand, the level of total n-3 PUFA was higher in obese rats (+68%); this increase was due to all fatty acids of this series with 22:6n-3 (DHA) being by far the major contributor. However, the increase in the level of n-3 PUFA did not compensate for the decrease in the amount of n-6 PUFA, so that the level of total PUFA (n-6 plus n-3) was 23% lower in the obese rats. The decrease in n-6 PUFA and

TABLE 3

Serum: Lipid Class Content in Zucker Female Rats—Comparison of Lean (Fa/-) and Obese (fa/fa) Animals[a]

n=6 Lipids	Cholesterol (g/L)	Triglycerides (g/L)	Phospholipids (g/L)
Lean	0.64 ± 0.08	0.39 ± 0.03	1.15 ± 0.15
Obese	0.82 ± 0.11[b]	2.83 ± 0.58[c]	2.06 ± 0.04[c]

[a]Results are means ± S.D. of six animals.

[b]$p < 0.5$.

[c]$p < 0.05$.

TABLE 2

Weights and Lipid Contents of Different Organs in Zucker Female Rat. Comparison of Lean (Fa/-) and Obese (fa/fa) Animals[a]

	Liver		Heart		Kidney		Brain	
	Lean[b]	Obese[c]	Lean[b]	Obese[c]	Lean[b]	Obese[c]	Lean[b]	Obese[c]
Weight (g)	7.08 ± 1.55	9.61[d] ± 0.67	0.79 ± 0.16	0.85 ± 0.06	1.78 ± 0.44	1.76 ± 0.06	1.62 ± 0.13	1.45 ± 0.05
Lipids/g of weight (mg)	58.9 ± 1.7	75.6[d] ± 4.0	63.0 ± 12.9	51.4 ± 7.3	48.3 ± 17.9	46.1 ± 2.7	109.1 ± 2.7	112.7 ± 15.1
Lipids/organ (mg)	417 ± 54	726[d] ± 80	49.8 ± 9.8	48.7 ± 6.2	86 ± 25.4	81.1 ± 4.7	177 ± 9	163.4 ± 21.9

[a]Results are means ± S.D. of six animals.

[b]Live weight lean: 270 ± 21 g.

[c]Live weight obese: 350 ± 42 g.

[d]$p < 0.01$.

TABLE 4

Serum, Liver, Kidney and Heart: Fatty Acid Composition of Total Serum Lipids and Liver, Kidney and Heart Phospholipids in Zucker Female Rats. Comparison Between Lean (Fa/-) and Obese (fa/fa) Aanimals[a]

	Serum		Liver		Kidney		Heart	
Fatty acids n=6	Lean	Obese	Lean	Obese	Lean	Obese	Lean	Obese
16:0	17.0 ± 1.2	20.3 ± 1.4[c]	16.4 ± 1.5	12.6 ± 0.9[c]	18.5 ± 0.8	19.2 ± 0.6	10.1 ± 0.4	10.5 ± 0.4
18:0	12.1 ± 1.5	11.0 ± 1.1	21.0 ± 2.2	26.2 ± 2.2[c]	17.4 ± 0.7	15.0 ± 2.0	22.1 ± 1.2	19.4 ± 1.8
ΣSFA	30.4 ± 1.4	32.8 ± 1.5[b]	38.5 ± 1.8	39.8 ± 1.8	36.9 ± 0.6	35.5 ± 2.2	32.9 ± 1.2	30.8 ± 1.4
16:1n-7	1.2 ± 0.1	3.2 ± 0.5[c]	0.4 ± 0.1	0.8 ± 0.2[c]	0.6 ± 0.2	0.2 ± 0.1[c]	0.3 ± 0.2	0.5 ± 0.1[b]
18:1n-9	8.8 ± 0.6	17.6 ± 1.4[c]	3.9 ± 0.6	3.0 ± 0.3[b]	7.4 ± 0.4	8.8 ± 0.4[c]	2.7 ± 0.2	4.0 ± 0.3[c]
18:1n-7	1.7 ± 0.2	1.9 ± 0.2	1.9 ± 0.3	1.5 ± 0.2[b]	2.5 ± 0.3	3.0 ± 0.4[c]	3.2 ± 0.2	3.4 ± 0.2
ΣMUFA	12.3 ± 0.8	23.1 ± 1.5[c]	6.4 ± 0.8	5.5 ± 0.4	10.9 ± 0.6	13.5 ± 0.5[c]	6.4 ± 0.4	8.0 ± 0.4[c]
18:2n-6	18.4 ± 1.7	14.8 ± 0.5[c]	14.9 ± 1.2	9.7 ± 0.7[c]	15.3 ± 0.6	14.0 ± 1.2[b]	18.7 ± 0.8	26.0 ± 1.5[c]
20:3n-6	0.3 ± 0.1	0.8 ± 0.2[c]	0.5 ± 0.1	1.6 ± 0.2[c]	0.5 ± 0.2	0.3 ± 0.1	0.7 ± 0.2	0.5 ± 0.1
20:4n-6	30.2 ± 1.6	15.3 ± 1.9[c]	28.7 ± 0.7	24.9 ± 0.7[c]	31.2 ± 1.2	28.4 ± 1.4[b]	22.3 ± 0.5	18.3 ± 0.8[c]
Σn-6 PUFA	50.3 ± 1.2	32.3 ± 1.8[c]	44.7 ± 1.1	36.9 ± 1.1[c]	48.5 ± 1.0	44.3 ± 1.8[c]	42.6 ± 0.8	46.2 ± 0.8[c]
20:5n-3	1.0 ± 0.2	2.3 ± 0.3[c]	0.3 ± 0.1	1.2 ± 0.2[c]	0.6 ± 0.1	1.6 ± 0.2[c]	0.2 ± 0.1	0.5 ± 0.2
22:5n-3	0.7 ± 0.1	1.5 ± 0.2[c]	0.9 ± 0.1	1.2 ± 0.2[c]	0.4 ± 0.1	0.7 ± 0.1[c]	1.9 ± 0.2	2.1 ± 0.1
22:6n-3	4.8 ± 0.4	7.1 ± 0.3[c]	9.1 ± 1.3	15.3 ± 1.0[c]	2.7 ± 0.3	4.3 ± 0.4[c]	16.0 ± 0.6	12.2 ± 0.8[c]
Σn-3 PUFA	6.9 ± 0.6	11.6 ± 0.4[c]	10.4 ± 1.3	17.7 ± 1.1[c]	3.8 ± 0.3	6.7 ± 0.7[c]	18.1 ± 0.4	14.9 ± 0.8[c]
n-6 plus n-3	57.2 ± 0.9	43.9 ± 1.8[c]	55.1 ± 1.1	54.6 ± 1.6	52.2 ± 1.4	51.0 ± 2.4	60.8 ± 0.7	61.1 ± 0.4
n-6/n-3	7.3 ± 0.7	2.8 ± 0.2[c]	4.3 ± 0.6	2.1 ± 0.2	12.9 ± 1.3	6.7 ± 0.4[c]	2.4 ± 0.1	3.1 ± 0.2[c]
20:3n-6/18:2n-6 × 100	1.6	5.4	3.3	16.5	6.5	9.3	1.6	2.7
20:4n-6/20:3n-6	100.6	19.1	57.4	15.6	31.2	21.8	74.3	26.1

[a]Results are means ± S.D. of six animals.

[b]$p < 0.5$.

[c]$p < 0.01$.

Minor fatty acids are not reported in the table: 15:0 (0-0.3%); 17:0 (0.4-0.8%); 16:1n-9 (0-1.3%); 18:3n-6 (0-0.4%); 20:2n-6 (0.2-1.3%); 22:4n-6 (0-0.4%); 22:5n-6 (0-0.2%); and 18:4n-3 (<0.1%).

increase in n-3 PUFA resulted in a considerable decrease in n-6/n-3 ratio in the obese animals (2,6). In these animals, the 20:3n-6/18:2n-6 ratio (index of Δ6-desaturase activity) was 3.3 times higher; by contrast, the 20:4n-6/20:3n-6 ratio (index of Δ5-desaturase activity) decreased five-fold (Table 4).

Liver phospholipids. The level of total SFA of liver phospholipids was of the same order (39%) in both types of rat. However, this equivalence resulted from a compensation between palmitic acid (more abundant in lean rats) and stearic acid (more abundant in obese rats). There was no significant difference between total MUFA, and only low proportions of these fatty acids were found.

On the other hand, the levels of n-6 and n-3 PUFA showed marked differences. As was observed for serum total lipids, liver phospholipids of obese rats included less n-6 PUFA than in lean rats (-17%; $p < 0.01$) due to lower levels of 18:2n-6 and 20:4n-6. By contrast, 20:3n-6 increased considerably in the obese animals consistent with what was observed in TSL. The overall decrease in the amount of n-6 PUFA was quantitatively compensated for by an increase in the level of n-3 PUFA (+70%) with a particular contribution from 22:6n-3. Thus, the amount of total PUFA (n-6 plus n-3) was identical in both cases and represented 55% of total fatty acids; however, the n-6/n-3 ratio was two times lower in the obese animals (2.1 vs 4.3). In the obese animals, the 20:3n-6/18:2n-6 ratio increased five times, while the 20:4n-6/20:3n-6 ratio decreased 3.7 times, similar to what was observed in serum lipids (Table 4).

Kidney phospholipids. Kidney phospholipids showed identical levels of total SFA, independent of the genotype. The levels of total MUFA were higher in the PL of obese rats (+26%; $p < 0.01$) as the levels of fatty acids in this series were higher. Kidney PL of the obese animals contained significantly less total n-6 PUFA than controls (-9%) largely due to the decrease of 18:2n-6 and 20:4n-6; as in liver and serum, the level of total n-3 PUFA increased considerably (+76%); this increase compensated for the deficit in total n-6 PUFA, so that the sum of n-6 plus n-3 was very similar in both representing 51-52% of total fatty acids. As in the liver, the n-6/n-3 ratio was decreased two-fold in the obese animals. The 20:3n-6/18:2n-6 ratio was higher and the 20:4n-6/20:3n-6 ratio decreased, but less than in the total serum lipids and liver phospholipids (Table 4).

Heart phospholipids. The levels of total SFA of heart PL were equal in obese and lean rats. As in serum and kidney, the level of MUFA was notably increased in the obese animals (+25%; $p < 0.01$) due to an increase in oleic acid (about +50%) (Table 4). In contrast to what was observed for serum, liver and kidney, the proportion of total n-6 PUFA in the heart was increased in the obese animals in spite of the decrease in arachidonic acid (-18%; $p < 0.01$) observed in heart. The overall gain in the proportion of n-6 PUFA in the liver and the kidney was essentially due to

TABLE 5

Brain, Retina, Synaptosomes, Myelin and Sciatic Nerve: Fatty Acid Composition of Phospholipids in Zucker Female Rats. Comparison Between Lean (Fa/-) and Obese (fa/fa) Animals

	Brain[a]		Retina[b]		Synaptosomes[c]		Myelin[c]		Sciatic nerve[c]	
Fatty acids	Lean	Obese	Lean	Obese	Lean	Obese	Lean	Obese	Lean	Obese
16:0	20:6 ± 0.8	21.6 ± 1.6	15.8	17.3	22.2	22.9	15.6	16.8	28.6	29.0
18:0	19.1 ± 0.4	18.3 ± 0.8	17.7	17.8	20.7	19.8	17.4	17.6	8.2	9.5
Σ SFA	40.6 ± 0.6	41.0 ± 1.5	37.7	37.3	43.9	44.3	35.1	36.5	41.2	43.1
16:1n-7	0.4 ± 0.1	0.5 ± 0.1	1.5	1.6	0.8	1.0	1.2	1.4	9.0	8.1
18:1n-9	19.9 ± 0.6	20.0 ± 0.8	11.5	11.9	14.8	15.4	34.5	35.6	33.6	32.9
18:1n-7	4.3 ± 0.3	4.3 ± 0.2	3.3	3.6	3.8	3.3	5.2	4.9	2.9	3.2
20:1n-9	1.9 ± 0.3	1.8 ± 0.3	0.4	0.2	0.6	0.5	2.6	2.6	0.5	0.4
20:1n-7	0.6 ± 0.1	0.5 ± 0.1	—	—	0.2	0.2	0.9	0.8	0.2	0.1
Σ MUFA	27.7 ± 1.2	27.7 ± 1.2	17.6	17.7	20.6	21.2	44.8	44.6	47.2	45.4
18:2n-6	0.8 ± 0.1	0.8 ± 0.1	5.6	5.0	1.1	1.0	0.8	0.9	2.3	2.3
20:3n-6	0.4 ± 0.1	0.5 ± 0.1	0.4	0.5	0.3	0.4	0.7	0.8	0.4	0.3
20:4n-6	11.0 ± 0.5	10.4 ± 0.7	12.0	11.8	13.5	15.1	10.1	9.0	3.7	4.0
22:4n-6	3.4 ± 0.3	2.8 ± 0.3	1.4	1.6	3.3	2.5	3.8	3.6	1.0	0.8
22:5n-6	0.6 ± 0.1	0.5 ± 0.1	0.3	0.4	1.4	0.8	0.8	0.5	1.0	0.8
Σ n-6 PUFA	16.2 ± 0.6	15.1 ± 0.8	19.9	19.5	19.8	20.1	16.3	15.0	8.8	8.4
22:5n-3	0.2 ± 0.1	0.2 ± 0.1	1.1	1.4	—	—	0.2	0.3	0.4	0.3
22:6n-3	15.2 ± 0.4	15.7 ± 1.3	23.2	23.6	15.7	14.4	3.6	3.6	1.8	2.0
Σ n-3 PUFA	15.4 ± 0.4	16.1 ± 1.3	24.6	25.3	15.7	14.4	3.8	3.9	2.8	3.1
n-6 plus n-3	31.6 ± 0.9	31.2 ± 2.1	44.5	44.8	35.5	34.5	20.1	18.9	11.6	11.5
n-6/n-3	1.1 ± 0.1	0.9 ± 0.1	0.81	0.77	1.26	1.40	4.29	3.85	3.14	2.70
20:3n-6/18:2n-6 × 100	50.0	62.5	7.1	10.0	27.3	40.0	87.5	88.9	17.4	13.0
20:4n-6/20:3n-6	27.5	20.8	30.0	23.6	45.0	37.7	14.4	11.2	9.2	13.3

[a]Measurements carried out on six rats (values are means ± S.D.).

[b]Measurements carried out on a pool of six rats.

[c]Measurements carried out on two pools of three rats.

Minor fatty acids are not reported in the table: 15:0 (0-0.4%); 17:0 (0-0.4%): 20:0 (0.1-0.9%); 22:0 (0-0.4%); 16:1n-9 (0-1.0%); 22:1n-9 (0-0.9%); 24:1n-9 (0-0.3%); 18:3n-6 (0-0.4%); 18:3n-3 (<0.1%).

an increase in linoleic acid (+39%; $p < 0.01$). As in serum, liver and kidney, the level of 20:3n-6 was very high in obese rats. Total n-3 PUFA (particularly 22:6n-3) was lower in obese fats resulting in total PUFA (n-6 plus n-3) being maintained at essentially the same level (21%) in both types of rats. The n-6/n-3 ratio was higher in obese animals, contrary to what was observed in serum, liver and kidney. The 20:3n-6/18:2n-6 ratio was higher, in spite of the considerable amounts of 18:2n-6 present, and the 20:4n-6/20:3n-6 ratio was decreased three-fold in the hearts of obese animals (Table 4).

Lipids of nervous structures. No differences between obese and lean rats were observed in the complex (brain, retina, sciatic nerve) or subcellular (synaptosomes, myelin) neural structures studied by any of the criteria used (Table 5).

DISCUSSION

As previously reported (21,31,32), we observed hyperlipemia in genetically obese Zucker female rat (fa/fa) mostly due to an increase in triglyceride levels. The hypertriglyceridemia was accompanied by a marked increase in the total amount of liver lipids. There was also an accumulation of saturated fatty acids, and especially of monounsaturated fatty acids (mainly 18:1n-9 and 16:1n-7), in serum, indicating an increase in Δ9-desaturase activity and in *de novo* lipogenesis (18,19,33). These perturbations in the *de novo* synthesis of fatty acids in obese rats were expressed by an increase in the levels of 18:0 in liver phospholipids and of 18:1n-9 in kidney and heart phospholipids.

n-6 PUFA. The present study shows a dwindling of the desaturation processes which permit the conversion of 18:2n-6 to 20:4n-6. Except for the neural structures, in which the n-6 PUFA levels were remarkably independent of genotype, the tissue phospholipids of obese rat typically included much less 20:4n-6 (from -9% to -49%) than in controls.

Contrary to what has been reported in experimental diabetes (12), 18:2n-6 was accumulated only in heart phospholipids of obese rats. On the other hand, the accumulation of 20:3n-6 was more general and relatively high. Thus, in obese rats, the 20:3n-6/18:2n-6 ratio, which is an index of Δ6-desaturase activity, was always higher (including in the heart), while the 20:4n-6/20:3n-6 ratio, an index of Δ5-desaturase activity, was always lower (Table 6). This suggests that inhibition of the conversion

TABLE 6

Comparisons of the 20:3n-6/18:2n-6 Ratio (Index of Δ6-Desaturase Activity) and of the 20:4n-6/20:3n-6 Ratio (Index of Δ5-Desaturase Activity) in Tissues of Lean and Obese Zucker Female Rats

Tissue	Serum	Liver	Kidney	Heart	Brain
Δ6-Desaturase index (20:3n-6/18:2n-6 × 100)					
• Lean	1.6	3.3	6.5	1.6	50.0
• Obese	5.4	16.5	9.3	2.7	62.5
• Obese/lean	3.37	5.00	1.43	1.68	0.80
Δ5-Desaturase index (20:4n-6/20:3n-6					
• Lean	100.6	57.4	31.2	74.3	27.5
• Obese	19.1	15.6	21.8	26.1	20.8
• Obese/lean	0.19	0.27	0.70	0.35	1.32

of 18:2n-6 to 20:4n-6 did not result from a decrease of Δ6-desaturation, but rather of Δ5-desaturation. The results confirm the recent data of Blond *et al.* (34) obtained by direct measurement of Δ6- and Δ5-desaturase activities in liver microsomes of Zucker rats. In heart phospholipids, the accumulation of 18:2n-6 occurred concomitantly with a reduction in 22:6n-3 levels. The increase in 18:2n-6 may suggest a change in the proportion of different phospholipids in favor of cardiolipins. These diphosphatidylglycerols, which occur mainly in mitochondrial membranes (35), contain remarkably high levels of this fatty acid (60-90%) (36,37). In the artificially diabetic rat, the heart is also the organ in which the highest levels of 18:2n-6 are observed (12). The fact that the differences seen between obese and lean rats do not extend to nervous tissue emphasizes the ability of these structures to incorporate the various PUFA in a very controlled fashion because neural tissues do not have Δ6- or Δ5-desaturase activity (38). Liver is known as the major site of Δ6- and Δ5-desaturation in mammals (39).

n-3 PUFA. The obese genotype is characterized by higher than normal levels of n-3 PUFA in serum lipids and in liver and kidney phospholipids, thus compensating for the drop in the level of n-6 PUFA and maintaining the sum n-6 plus n-3. This increase in n-3 PUFA, particularly in respect to 22:6n-3 and 20:5n-3, may result from preferential acylation of these acids as compared to n-6 PUFA due to their presence in dietary lipids rather than to activation of α-linolenic acid conversion (21). These C_{20} and C_{22} fatty acids represent about 2% of the total fatty acids in the diet (118 mg/100 g of diet) and alone cover a large portion of n-3 PUFA requirements in the rat (40,41). In order to elucidate this point, further, it would be necessary to experiment with a diet containing n-3 PUFA in the form of α-linolenic acid only.

As noted above, preferential incorporation of n-3 PUFA into phospholipids is not a general mechanism in obese rats because there is also a decrease in the relative level of 22:6n-3 in heart phospholipids. The drop in the level of 20:4n-6 and rise in 20:3n-6, which was already reported for obese Zucker rats at one month of age (24), could cause lowered production of PGI_2 and PGE_2, representing physiological conditions which favor the cardiovascular complications typical of obesity.

In respect to the nervous structures, and in parallel with n-6 PUFA, the level of n-3 PUFA was maintained in all the cellular or subcellular structures considered. The n-6/n-3 ratio was unchanged and remained between 1 and 2 in the brain, retina and synaptosomes; it remained close to 4 for myelin and sciatic nerve (27,42). This homeostasis of n-3 PUFA composition in nervous structures has already been noted in rats, even when receiving long-chain n-3 PUFA in the form of fish oil supplied in the diet (43,44), except when excessive amounts of fish oil were provided for the animals (45).

The obese genotype in Zucker rats is characterized by abnormalities in PUFA composition of tissue phospholipids. These anomalies may correspond to changes in the proportion of phospholipid classes, as reported in some membranes (25). The whole of the changes of a genetic disorder occur in obese mice (ob/ob) (22,23) or in some cases of human obesity (46), even if they are different from those which characterize Zucker rats. The changes cause biophysical (fluidity) and functional (activities of Na^+K^+ATPase and adenylate cyclase, number of insulin receptors, glucose transport) alterations in cellular membranes (46).

According to Horrobin (8), decreased Δ6-desaturase activity could be one of the keys to the problem of aging. To compensate for this decrease, dietary lipids could be supplemented with 18:3n-6, which would bypass this metabolic deficiency. However, Δ5-desaturase activity seems to also decrease in certain physiological states, such as experimental diabetes (12) and hypercholesterolemia artificially induced by an excessive intake of dietary cholesterol (13). Takahashi and Horrobin (47) have recently shown that such a process is characteristic of aging in mammals. According to these authors, the level of 20:3n-6, as well as the 20:3n-6/20:4n-6 ratio, increases with age in liver phospholipids and platelets in rats. Thus, supplementing dietary lipids with γ-linolenic acid would not always permit a normal level of 20:4n-6 in tissue phospholipids and would lead to an increase of 20:3n-6 (48). Therefore, it seems that a diet directly supplying C_{20} and C_{22} PUFA (20:4n-6, 20:5n-3, 22:6n-3) would be advisable, as soon as the relative requirements of these two families of fatty acids have been defined.

In any case, the genetically obese Zucker rat (fa/fa) is a good model for studies aimed at preventing or nutritionally treating enzyme deficiencies in regard to fatty acid desaturation.

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Fatty Acid Content of Marine Oil Capsules

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The use of dietary ω3 fatty acid capsules has been associated with a decrease in plasma triglyceride levels. In addition, populations consuming diets rich in fish appear to have a decreased incidence of cardiovascular disease. Eicosapentaenoic acid (EPA, 20:5ω3) and docosahexaenoic acid (DHA, 22:6ω3) are major fatty acids in fish oils. It is believed that fish oils exert their biologic effect through these fatty acids. Many individuals are currently taking fish oil capsules to lower lipids, increase bleeding time, and possibly decrease cardiovascular risk. These capsules also have been classified as food additives with less stringent controls on content. We assessed the fatty acid, cholesterol, and vitamin A and E content of eight commercially available capsules along with cod liver oil. The content of EPA was found to range from 8.7-26.4% (wt %) with a mean of 17.3% (82.4% of labeled content), and that of DHA from 8.9-17.4% with a mean of 11.5% (90.0% of labeled content) as assessed by capillary column gas-liquid chromatography. The mean content of the polyunsaturated ω3 fatty acids was 31.9%, and that of the ω6 fatty acids was 1.4%. The content of saturated fatty acids was 32.0%, and that of monounsaturated fatty acids was 25.1%. Cholesterol content was low, with a range of 0.7-8.3 mg/g, the α-tocopherol range was 0.62-2.24 mg/g, and the range of retinyl esters was 0.4-298.4 μg/g. Cod liver oil had substantially more retinyl esters (2450.1 μg/g) than did fish oil capsules. Our data serve as an independent guide to fish oil capsule fatty acid content upon single lot analysis, and indicate that these capsules contain as much saturated fat as they contain ω3 fatty acids.
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The major ω3 fatty acids in plasma are α-linolenic acid (18:3ω3) and its derivatives eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). α-Linolenic acid can be obtained from plant sources (rapeseed oil and soybean oil), while EPA and DHA are abundant in fish and fish oil products. Interest in fish oil products was stimulated by initial observations that Eskimos have a low incidence of atherosclerosis despite having a diet high in total fat. However, much of their excess fat intake is due to increased intake of marine oils. Subsequent studies showed that increased fish oil in the diet was associated with substantial decreases in plasma triglycerides as well as an increase in bleeding time (1-4). It is known that ω3 fatty acids serve as precursors for thromboxane B_3 as well as thromboxane A_3, prostaglandin E_3, I_3 and leukotriene B_5 (5-7). These substances all have important effects with regard to immune and platelet function. Functionally important effects of ω3 fatty acids in humans that have been reported include an increased bleeding time, a reduction in platelet aggregation stimulated by adenosine diphosphate (ADP) or collagen, and a reduction in blood pressure (8-11). Moreover these latter effects of fish oils have been related to decreased blood pressure responses to angiotensin II and norepinephrine. In addition, fish oil use has been associated with a reduction in polymorphonuclear leukocyte chemotaxis and a reduced inflammatory response (12). For these reasons, a large number of patients in the United States are currently using fish oil capsules for a variety of disorders including hyperlipidemia, hypertension, arthritis, and autoimmune disorders. In some cases controlled clinical studies have documented some efficacy of fish oil capsules in the treatment of these disorders.

The precise fatty acid composition of many of the commercially available products used to supplement patients with ω3 fatty acids has not been widely published (13,14). The purpose of our investigation was to assess the fatty acid composition of commercially available fish oil capsules and of cod liver oil using sensitive and reproducible capillary-column gas-liquid chromatographic techniques. In addition, we assessed the cholesterol and vitamin A and E content of these capsules, as well as of cod liver oil.

METHODS AND ANALYSIS

Fish oil capsules were obtained from pharmacies and health food stores in the Boston area. All capsules were in a gelatin form. All analyses were carried out in triplicate. Six individual capsules of each brand from the same lot were analyzed for fatty acid content; 9 capsules of each brand, also from the same lot, were used for vitamin and cholesterol analysis. The following capsules were analyzed: Mega EPA-1000, General Nutrition Corporation, Pittsburgh, PA; Promega, Parke-Davis Consumer Health Products Group, Warner Lambert Company, Morris Plains, NJ; Protochol, E.R. Squibb and Sons, Inc., Princeton, NJ; Discovery, Carter Products, Division of Carter-Wallace Inc., New York, NY; MaxEPA GNC, General Nutrition Corporation, Pittsburgh, PA; Super MaxEPA, Twin Laboratories Inc., Ronkonkoma, NY; MaxEpa, R.P. Scherer Inc., North America, Clearwater, FL; SuperEpa, Bronson Pharmaceuticals, LaCanada, CA; Norwegian Cod Liver Oil, General Nutrition Corporation, Pittsburgh, PA.

The retail cost to us in 1988 was $3.39 for 237 mL of cod liver oil, and ranged from $0.09 per capsule for SuperEpa to $0.28 per capsule for Promega. We did not analyze for the chemical forms (triglycerides, ethyl/methyl esters) in the products we studied, and this information was not available from product labeling at time of analysis.

Fatty acid analysis. A modification of LePage's method was used for lipid extraction and transesterification of

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Abbreviations: EPA, eicosapentaenoic acid; DHA docosahexaenoic acid; FID, flame ionization detector; GC, gas chromatograph; FAME, fatty acid methyl esters.

fatty acids (15). Briefly, fish oil was removed from capsules, weighed precisely (10-15 mg) and dissolved in 5 mL of isooctane. One hundred μL samples along with 40μg of internal standard (margaric acid, 17:0) were mixed in 13 × 100 mm glass tubes. Two mL of a methanol/benzene (4:1 v/v) solution was added, as was 200 μL of acetyl chloride with slow stirring. The tubes were flushed with nitrogen gas, sealed with Teflon tape, and tightly closed with Teflon-lined caps. The content was then methanolyzed at 100°C for 1 hr in a Reacti-Therm heating/stirring block. Tubes were then cooled in water, 1 mL of isooctane was added, followed by 5 mL of 6% potassium carbonate to neutralize the mixture. Tubes were centrifuged at 1500 *g* for 15 min, and the clear upper phase containing fatty acid methyl esters (FAME) was transferred to a 1-mL autosampler vial. After overlayering with anhydrous sodium sulfate (1 mm layer thick), vials were filled with nitrogen, sealed with crimp caps, and readied for injection.

An automated system was used for analysis, which consisted of a Hewlett Packard (HP, Hewlett-Packard Co., Avondale, PA) 7673A automatic sampler, an HP 5890 gas-liquid chromatograph (GC), an HP 3393A integrator, and a Nelson chromatography data system (Nelson Analytical, Inc., Cupertino, CA) which collects, reintegrates and stores the data. Samples in the autosampler tray were cooled to keep sample temperature at approximately 10°C and to minimize FAME oxidation. The column used for analysis was a 0.32-mm (interior diameter), 30-m (length) Supelcowax 10 (Supelco, Inc., Bellefonte, PA) capillary column. A flame ionization detector (FID) was used. The GC was run using H_2 as the carrier gas with a head pressure of 9 psi and a flow rate of 4 mL/min. Other conditions were as follows: flame H_2 - 20- psi at 25 mL/min and air - 40 psi at 385 mL/min; make up N_2 - 27 mL/min; temperature program, first ramp 150-190°C with increases of 4°C/min, second ramp 190-210°C for 10 min; third ramp 210-250° with increases of 10°C/min; and then the temperature was held at 250°C for 6 min. Both the injector and detector temperatures were maintained at 270°C. The signal range and attenuation were both 0. Samples (0.5 μL) were injected into the GC in the splitless mode. Standard FAME obtained from Nuchek Prep (Elysian, MN), Sigma (St. Louis, MO), and Supelco were used for collaboration and peak identification. FAME concentrations were calculated by the Nelson data system based on comparisons with the concentration of the internal standard (17:0, margaric acid), and the percentage report was based on fatty acid amount/total oil amount.

Sterol analysis. Sterols were extracted from fish oils according to Method B for lipid extracts (with minor modifications) as described by Kovacs *et al.* (16). About 50 mg of lipid extract was saponified in a tightly capped (Teflon-lined) 16 × 125 mm screw cap test tube containing 0.5 mL 50% potassium hydroxide (KOH) and 2 mL 95% ethanol. The tube contents were boiled on a hot plate for 1 hr while being continuously stirred with a magnetic bar. After cooling, 1.5 mL of distilled water was added and the unsaponifiable material was extracted 4-times with hexane (2.5 mL each). The combined extracts were dried under nitrogen, suspended in 500 μL hexane, and analyzed for free sterols by gas chromatography. A Varian 6500 Gas Liquid Chromatograph (Varian Associates Inc., Walnut Creek, CA) was used. The glass column was 10-foot by 4 mm with a 2-mm interior diameter and was packed with 1% OV-210/2% SE-30 on acid-washed Gas-Chrom P. The column temperature was 240°C, the injector temperature was 275°C, and the detector (FID) temperature was held at 240°C. The carrier gas was helium at a flow rate of 30 mL/min. The Nelson (Nelson Analytical, Inc., Cupertino, CA) software package was used to integrate peaks.

Vitamin analysis. Retinyl esters were measured by normal phase high pressure liquid chromatography as previously described (17). Vitamin E (α- and γ-tocopherols) were measured after alkaline hydrolysis by reverse phase high pressure liquid chromatography as previously described (18).

RESULTS

Table 1 shows the brand name and manufacturing company for each product tested, as well as their labeled EPA, DHA, cholesterol, vitamin A, and vitamin E content. According to label information, EPA content ranged from 18% to 30% (wt %), while DHA content ranged from 12% to 20%. According to the label, cholesterol content of fish oil capsules ranged from 0-12 mg per 1 g capsule, vitamin A content from less than 66 to 10,000 IU/g retinol equivalents, and vitamin E from 0-0.6 mg/g.

In Table 2 we have listed the concentrations of the major saturated fatty acids found in the various capsules. Many of the capsules contained substantial amounts of saturated fat. Myristic acid (14:0) content ranged from

TABLE 1

Fish Oil Supplement Label Content[a]

Brand name	Company	EPA	DHA	Cholesterol	Vitamin A	Vitamin E
MegaEpa	GNC	180	120	12	<2% RDA	6% RDA
Promega	Parke-Davis	280	120	<1	n.p.[b]	1 IU
Protochol	Squibb	180	120	5	<2% RDA	n.p.
Discovery	Carter	180	120	n.p.	n.p.	1 IU
MaxEpa	GNC	180	120	6	NG	6% RDA
SuperMaxEpa	Twin Lab	187.5	125	n.p.	<2% RDA	n.p.
MaxEpa	Scherer	186	158	n.p.	<10% RDA	n.p.
SuperEpa	Bronson	300	200	1	n.p.	n.p.
Cod liver oil	GNC	n.p.	n.p.	n.p.	10,000 IU	n.p.

[a]Values provided in mg/g, unless otherwise indicated.

[b]Data not provided.

17.8 mg/g for SuperEpa to 77.1 mg/g for MaxEpa, with a mean of 75.7 mg/g or about 7.6% of content by weight. The palmitic acid (16:0) content ranged from 109.0 mg/g for Promega to 187.9 mg/g for MaxEpa, with a mean of 138.9 mg/g or 13.9% of content. The stearic acid (18:0) content ranged from 5.4 mg/g for SuperEpa to 95 mg/g for Promega, with a mean content of 75 mg/g or 7.5%. The content of 20:0 ranged from 0 or undetectable for a number of products to 17.6 mg/g for Promega, with a mean of 10.7 mg/g or 1.07%. The content of 22:0 ranged from 9.7 mg/g for Protochol to 85.3 mg/g for MaxEpa, with a mean of 34.7 mg/g or 3.5%. Therefore the mean content of both 14:0 and 16:0 was 214.6 mg/g or 21.5%, and that of total saturated fatty acids in fish oil capsules was 320 mg/g or 32.0%. The saturated fat content of cod liver oil was 371.2 mg/g or 37.1%, being rich in 18:0 and 22:0.

Data on polyunsaturated fatty acids in fish oil capsules and cod liver oil are presented in Table 3. The content of linoleic acid (18:2ω6) ranged from 10.7 mg/g for Super MaxEpa to 27.0 mg/g for MaxEpa GNC, with a mean content of 14.4 mg/g or 1.4%. The content of α-linoleic acid (18:3ω3) ranged from 14.0 mg/g for Super MaxEpa to 48.2 mg/g for Promega, with a mean content of 31.2 mg/g or 3.1%. The content of EPA (20:5ω3) ranged from 140.0 mg/g for MaxEpa to 263.9 mg/g for Promega, with a mean content of 172.9 mg/g or 17.3%. The content of DHA ranged from 89.7 mg/g for MaxEpa to 173.7 mg/g for SuperEpa, with a mean content of 114.8 mg/g or 11.5%. The EPA content of all capsules was somewhat lower than label information, ranging from 75.3-94.3% of labeled content, with a mean of 82.4%. DHA content ranged from 57% to 115% of labeled content, with an average of 90%.

Data on the monounsaturated fatty acid content of fish oil capsules is provided in Table 4. The content of myristoleic acid (14:1ω5) ranged from 4.5 mg/g for SuperEpa to 75.3 mg/g for Promega, with a mean of 14.9 mg/g, while the content of palmitoleic acid (16:1ω7) ranged from 5.4 mg/g for SuperEpa to 95.0 mg/g for Promega, with a mean of 75.0 mg/g. The content of oleic acid (18:1ω9) ranged from 107.5 for MegaEpa to 206.8 for SuperEpa, with a mean of 129.4 mg/g. The content of eicosenoic acid (20:1ω9) ranged from 15.2 mg/g for Promega to 55.5 mg/g for SuperEpa, with a mean value of 25.9 mg/g. Cod liver oil had a very high 20:1ω9 content at 107.9 mg/g. The content of cetoleic acid (22:1ω11) ranged from undetectable for Promega and MaxEpa to 16.5 mg/g for SuperEpa, with a mean value of 6.0 mg/g. While we did not have a standard for this fatty acid at the time of the analysis, it is most probably 22:1 fatty acid, and logically 22:1ω11 rather than 22:1ω9. The latter fatty acid is found in plant oils, while the former is known to be present in marine oils.

Data in Table 5 indicate that the marine oil capsules studied contained 75.3-94.3% (mean of 82.4%) of labeled

TABLE 2

Saturated Fatty Acids in Fish Oil Capsules[a]

Brand name	14:0	16:0	18:0	20:0	22:0
MegaEpa	74.0 ± 2.1	160.0 ± 13.2	80.3 ± 1.3	u.l.[b]	13.8 ± 1.1
Promega	66.6 ± 3.6	109.0 ± 42.0	95.0 ± 7.9	17.6 ± 3.6	22.9 ± 6.2
Protochol	71.6 ± 10.0	165.2 ± 15.6	80.5 ± 3.9	u.l.	9.7 ± 1.5
Discovery	75.0 ± 2.5	150.6 ± 2.7	85.2 ± 2.7	u.l.	59.9 ± 2.7
MaxEpa GNC	74.1 ± 8.2	169.7 ± 7.8	84.6 ± 3.1	4.5 ± 0.9	22.1 ± 0.8
SuperMaxEpa	75.3 ± 4.0	157.2 ± 11.0	80.7 ± 4.6	10.0 ± 4.4	29.2 ± 2.2
MaxEpa	77.1 ± 12.6	187.9 ± 53.0	88.6 ± 11.1	u.l.	85.3 ± 8.5
SuperEpa	17.8 ± 7.9	111.7 ± 55.2	5.4 ± 3.9	u.l.	34.9 ± 1.0
Cod liver oil	54.3 ± 9.4	110.5 ± 15.4	102.8 ± 4.6	20.6 ± 1.8	83.0 ± 5.1

[a]Data provided as mean of triplicate determinations ± SD in mg/g.

[b]Undetectable levels (u.l.).

TABLE 3

Polyunsaturated Fatty Acids in Fish Oil Capsules[a]

Brand name	18:2ω6	18:3ω3	EPA	DHA
MegaEpa	13.1 ± 1.5	18.9 ± 4.4	139.5 ± 6.4	130.6 ± 1.7
Promega	13.8 ± 2.3	48.2 ± 6.7	263.9 ± 11.4	118.3 ± 5.5
Protochol	12.9 ± 1.0	30.5 ± 15.6	145.5 ± 5.7	138.0 ± 4.5
Discovery	12.9 ± 1.0	35.1 ± 6.7	141.4 ± 4.1	90.1 ± 2.3
MaxEpa GNC	27.0 ± 1.8	26.4 ± 9.6	156.4 ± 1.4	111.4 ± 0.9
SuperMaxEpa	10.7 ± 0.4	14.0 ± 10.0	150.7 ± 5.4	106.9 ± 3.2
MaxEpa	12.8 ± 2.6	32.6 ± 10.5	140.0 ± 6.9	89.7 ± 3.4
SuperEpa	11.7 ± 0.4	44.1 ± 17.8	245.6 ± 12.8	173.7 ± 9.6
Cod liver oil	18.6 ± 1.3	25.4 ± 15.9	86.6 ± 5.9	89.0 ± 5.2

[a]Data provided as mean of triplicate determinations ± SD in mg/g.

TABLE 4

Monounsaturated Fatty Acids in Fish Oil Capsules[a]

Brand name	14:1ω5	16:1ω7	18:1ω9	20:1ω9	22:1ω11
MegaEpa	6.7 ± 0.5	80.3 ± 1.3	107.5 ± 1.5	17.5 ± 1.5	4.9 ± 1.3
Promega	75.3 ± 19.0	95.0 ± 7.9	112.6 ± 39.3	15.2 ± 5.6	u.l.[b]
Protochol	8.0 ± 1.1	80.5 ± 3.9	109.5 ± 16.3	20.8 ± 3.5	6.7 ± 2.1
Discovery	4.9 ± 1.1	85.2 ± 2.7	117.2 ± 9.6	31.3 ± 2.4	8.8 ± 1.9
MaxEpa GNC	6.2 ± 1.4	84.6 ± 3.1	125.2 ± 14.9	21.7 ± 1.7	5.8 ± 1.3
SuperMaxEpa	6.1 ± 0.9	80.7 ± 4.6	109.4 ± 9.3	20.3 ± 2.4	5.4 ± 1.5
MaxEpa	7.8 ± 1.6	88.6 ± 11.1	147.0 ± 2.2	24.9 ± 2.9	u.l.
SuperEpa	4.5 ± 3.1	5.4 ± 3.9	206.8 ± 88.0	55.5 ± 6.6	16.5 ± 3.3
Cod liver oil	8.8 ± 2.0	102.8 ± 4.6	173.4 ± 17.6	107.9 ± 612.3	8.7 ± 2.0

[a]Data provided as mean of triplicate determinations ± SD in mg/g.

[b]Undetectable levels (u.l.).

TABLE 5

Comparison Between Label and Assays for EPA and DHA[a]

Brand Name	Label	EPA assay	%	Label	DHA assay	%
MegaEpa	180	140	77.8	120	131	109.2
Promega	280	264	94.3	120	118	98.3
Protochol	180	146	81.1	120	138	115.0
Discovery	180	141	78.3	120	90	75.0
MaxEpa GNC	180	156	86.7	120	111	92.5
SuperMaxEpa	188	151	83.9	125	107	85.6
MaxEpa	186	140	75.3	158	90	57.0
SuperEpa	300	246	82.0	200	174	87.0

[a]Data provided as mg/g.

EPA content. The content of DHA ranged from 57.0-115.0% (mean 90.0%). The contents of cholesterol, α-tocopherol, γ-tocopherol, and retinyl esters of the marine oil capsules studied are provided in Table 6. The cholesterol content was low and ranged from 0.49 mg/g for Promega to 8.34 mg/g for Protochol (mean 3.29 mg/g) (14). The α-tocopherol content ranged from 0.78 mg/g for MaxEpa GNC to 2.24 mg/g for SuperEpa (mean 1.17 mg/g). The γ-tocopherol content ranged from 0-0.53 mg/g (Discovery), with a mean of 0.31 mg/g. The retinyl ester content ranged from 0.4 μg/g for Promega to 298.4 μ g/g for SuperEpa (mean of 47.0). Cod liver oil contained very high levels of retinyl ester which were eight-fold higher than the content of SuperEpa. One gram of the cod liver oil product tested contained about 2.4 times the recommended daily allowance of retinol equivalents (RE). Therefore one teaspoon contained over 12 times (12,000 RE) the recommended daily allowance (RDA) of retinol equivalents. At levels over 10 times the RDA (1000 μg RE) toxic symptoms can occur (19).

DISCUSSION

The use of marine oil capsules is quite widespread in the United States. They are available as over-the-counter food additives in health food stores and drug stores. Some authorities have recommended their use in the prevention of atherosclerosis and the treatment of hyperlipidemia. Some animal studies have indicated that diets high in marine oil along with saturated fat and cholesterol cause less atherosclerosis than high cholesterol, high saturated fat diets alone (20,21). The American Heart Association has recently taken the position that marine oil capsules should not be routinely used for the treatment of hyperlipidemia. The purpose of our studies was to assess the fatty acid, cholesterol, α-tocopherol, and retinyl ester content of commercially available marine oil capsules. To our knowledge this information has not been previously published.

One surprising feature of our analysis was the relatively high content of saturated fatty acids in marine oil capsules. The average combined content of myristic and palmitic acid was 21.5%, while the mean content of total saturated fatty acids was 32.0% (chicken 4.2%, beef 10%, fish 1.2%). The mean total ω3 fatty acid content of the marine oil capsules studied was very similar at 31.9%, with the mean EPA content being 17.3% and the mean DHA content being 11.5%. The mean total polyunsaturated fat content of fish oil capsules was 33.3% (chicken 3.2%, beef 1%, fish 1.8%), while that of monounsaturated fatty acids was 25.1% (chicken 6.1%, beef 11.6%, fish 1.2%). These values only represent approximately 90% of total fat content because about 10% of the fatty acid peaks within marine oils have not been identified. Therefore our data clearly indicate that commercially available marine oil capsules contain as much saturated fat as they contain ω3 fatty acids. It is well known that fish with a high content of ω3 fatty acids (1.3-2.1 g/3.5 oz portion) such as mackerel, salmon, and herring also have approx-

TABLE 6

Amount of Cholesterol, α-Tocopherol, γ-Tocopherol and Retinyl Ester in Fish Oil[a]

Brand name	Cholesterol	α-Tocopherol	γ-Tocopherol	Retinyl esters
MegaEpa	3.60 ± 0.61	1.31 ± 0.12	n.d.[b]	1.3 ± 0.1
Promega	0.49 ± 0.01	1.12 ± 0.05	n.d.	0.4 ± 0.1
Protochol	8.34 ± 0.33	0.62 ± 0.10	n.d.	16.0 ± 0.5
Discovery	3.84 ± 0.20	0.64 ± 0.04	0.53 ± 0.0	1.5 ± 0.4
MaxEpa GNC	5.09 ± 0.18	0.78 ± 0.05	n.d.	26.2 ± 4.1
SuperMaxEpa	3.55 ± 0.24	1.31 ± 0.05	n.d.	8.3 ± 2.1
MaxEpa	0.80 ± 0.02	1.37 ± 0.05	0.18	23.8 ± 4.2
SuperEpa	0.60 ± 0.03	2.24 ± 0.02	0.23	298.4 ± 8.0
Cod liver oil	4.69 ± 0.59	0.18 ± 0.00	n.d.	2450.1 ± 175.6

[a]Values are the mean ± SD of triplicate determinations except for cholesterol determinations, which were done in duplicate.

[b]n.d.—not detectable.

[c]SuperEpa capsules analyzed were from different lot numbers than those used for the fatty acid, α- and γ-tocopherol, and retinyl ester assays.

imately 4.2 (2.5%), 1.9 (2%), and 2.6 (3%) g, respectively, of saturated fat per 3.5 ounces of cooked fish. A serving of fatty fish such as mackerel contains about 3 g of ω3 fatty acids (22).

The mean EPA content of marine oil capsules was 82.4% of the labeled content, while that of DHA was 90.0%. These differences may be due to fatty acid oxidation and degradation during storage, processing or analysis. Moreover, our methodology for fatty acid methylation and separation may have differed substantially from that used by the various manufacturers. For example, packed columns, which are often still used for analysis, may not adequately separate EPA from other fatty acid peaks.

The cholesterol content of all marine oil capsules was quite low in some supplements ranging from 0.5 to 8.3 mg/g. Therefore individuals taking as many as 10 capsules per day would on average only ingest an additional 33 mg of cholesterol. All marine oil capsules contained detectable amounts of α-tocopherol. One concern is that individuals taking large amounts of marine oil capsules may have higher requirements for dietary α-tocopherol. Such has been found to be the case in animal studies (23). However, recent studies in normal human subjects taking 18 capsules per day of MaxEpa from our institution indicate that α-tocopherol levels increased over 8 weeks of administration (24,25). Therefore the additional tocopherol in these capsules offsets any excess dietary tocopherol requirement. The retinyl ester content in all marine oil capsules was very low compared with cod liver oil.

The data presented serves as a guide to the fatty acid, cholesterol, tocopherol, and retinyl ester content of commercially available marine oil capsules. Their differing fatty acid content may explain some of the significant differences reported for effects of fish oil capsules on plasma lipoproteins, especially low density lipoproteins (LDL) (25-28). For example capsules containing higher amounts of myristic and palmitic acid would be predicted to have more adverse effects on plasma LDL cholesterol levels (especially if 18 capsules or more per day are used) than capsules containing more stearic acid and more monounsaturated fatty acids (29-32). Our current recommendation is that consumers should not indiscriminantly use these capsules, but only take them if recommended by a physician for triglyceride lowering and/or some other medically sound indication.

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Exchange of Free Cholesterol Between Plasma and Erythrocytes from Hyperthyroid and Hypothyroid Rats *in Vitro*

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In our previous studies, we found that circulating thyroid hormone levels alter cholesterol partition between plasma and erythrocytes by changing the phospholipid content of erythrocytes (Ruggiero, F.M., *et al.* (1984) *Horm. Metabol. Res. 16*, 37-40; Ruggiero, F.M., *et al.* (1987) *Lipids 22*, 148-151). As an extension of this work, we now followed the exchange of free cholesterol between plasma and erythrocytes in control, hyperthyroid and hypothyroid rats under various experimental conditions *in vitro*. In control rats, erythrocytes incubated with plasma at 37°C for 4 hr lose 10% of cholesterol which was esterified by lecithin:cholesterol acyltransferase (LCAT) present in the plasma. In hyperthyroid rats, erythrocytes incubated with plasma lose 30% of cholesterol within the same time. By contrast, in the case of hypothyroid rats incubation for 4 hr was necessary to transfer 24% of free cholesterol from plasma to erythrocytes. Inhibition of cholesterol esterification did not affect the loss of erythrocyte cholesterol in control and in hyperthyroid rats. Ca^{2+} increased the LCAT activity in the plasma of these rats. The findings shed light on the role of thyroid hormones in regulating cholesterol levels in plasma through active cholesterol transfer between plasma and erythrocytes.
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Cholesterol has an asymmetric distribution in the erythrocyte membrane with most of it being located in the inner membrane leaflet (1). Cholesterol is known to affect membrane stability and membrane contour in human erythrocytes and to prevent endocytosis (2,3). Several studies have shown that unesterified cholesterol of plasma lipoproteins exchanges with cholesterol in the erythrocyte membrane both *in vivo* and *in vitro* (4-6).

It has also been reported that thyroid hormones affect plasma cholesterol concentration, hepatic cholesterol metabolism, and cholesterol synthesis (7-9). Recently, we have shown that changes are induced by thyroid hormones in the lipid composition of plasma and of erythrocyte membranes (10,11) and in the lipid patterns of mitochondria and microsomes of liver, heart and brown adipose tissue of the rat (12-14). We reported that circulating thyroid hormone levels in blood regulate cholesterol distribution between the plasma and erythrocytes by changing the phospholipid content of erythrocytes (10,11). Therefore, in hyperthyroid rats, cholesterol decreases in plasma and increases in erythrocytes (10), whereas in hypothyroid rats, cholesterol increases in plasma and decreases in erythrocytes (11).

The exchange of unesterified cholesterol has been studied *in vitro* (15,16), as it is difficult to study this exchange under *in vivo* conditions. It is known that incubation of plasma causes cholesterol esterification and that heating of plasma at 57°C for 30 min inhibits the process of esterification (15). So far, no data are available on the exchange of cholesterol between plasma and erythrocytes from hyperthyroid and hypothyroid rats and on the effect of Ca^{2+} on these exchange processes. The purpose of the present work was to study the exchange of free cholesterol between plasma and erythrocytes in control, hyperthyroid and hypothyroid rats under suitable conditions *in vitro*. The results reported here give further insight into the role of thyroid hormones in regulating cholesterol levels in plasma through active cholesterol exchange between plasma and erythrocytes.

MATERIALS AND METHODS

Animals. Male Wistar rats, 200-250 g in weight, fed *ad libitum* with a standard diet, were used and divided into three groups: group 1, control rats; group 2, rats made hyperthyroid by daily intraperitoneal administration of 3,3',5-triiodo-L-thyronine (T_3) (30 μg/100 g body weight) for six consecutive days (17) (**hyperthyroid**); group 3, rats made hypothyroid by 6-n-propyl-2-thiouracil (PTU) administration (0.05% in drinking water) for 3-4 weeks (18) (**hypothyroid**). The animals were killed by decapitation 24 hr after the last administration. Blood was collected directly into heparinized beakers and centrifuged at 750 × g for 7 min. The plasma was removed by careful suction, and the cells were washed four times with a five-fold volume of buffer containing NaCl 140 mM, KCl 5 mM, $MgSO_4$ 1 mM, $CaCl_2$ 1mM, NaH_2PO_4 1 mM, Tris 10 mM, and glucose 5 mM, pH 7.4.

Incubations conditions. In order to test the *in vitro* exchange of free cholesterol between plasma and erythrocytes from control, hyperthyroid and hypothyroid rats, 0.6 mL of plasma from i) control, ii) hyperthyroid and iii) hypothyroid rats was incubated with 0.4 mL of erythrocytes from the respective groups. The hematocrit levels in normal, hyperthyroid and hypothyroid rats were 40% (data not reported). The incubation for measuring only the exchange of free cholesterol between plasma and erythrocytes was carried out with plasma incubated at 57°C for 30 min with stirring to destroy lecithin: cholesterol acyltransferase (LCAT) (heat-treated plasma) (15). Experiments with plasma and erythrocytes in the presence of 5 mM $CaCl_2$ were also performed. The incubations were carried out at 37°C in plastic centrifuge tubes (1.5 mL capacity) with gentle shaking for 2,4 or 6 hr. Incubations never exceeded 6 hr in order to prevent osmotic lysis. At the end of incubation, erythrocytes were separated from plasma by centrifugation at 3,000 × g for 15 min and then washed once with the buffer used previously for washing the erythrocytes.

High performance liquid chromatographic (HPLC) analysis of cholesterol and determination of phospholipids. Cholesterol was analyzed by HPLC using a Beckman

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Abbreviations: HPLC, high performance liquid chromatography; LCAT, lecithin:cholesterol acyltransferase.

gradient liquid chromatograph. To determine cholesterol (free and total), an Altex ultrasphere-ODS, reverse phase chromatographic column (4.6 × 250 mm; Beckman, Palo Alto, CA) was used. Free cholesterol was determined in plasma by the method of Duncan *et al.* (19). The mobile phase was 2-propanol/acetonitrile/water (60:30:10, v/v/v) at a flow rate of 1 mL/min. For total cholesterol determinations, both plasma and erythrocytes were saponified with alcoholic KOH for 60 min at 45°C, and the mixture was extracted with hexane. Then the extract was evaporated and the residue dissolved in 2-propanol, an aliquot of which was injected into the column. The mobile phase was 2-propanol/acetonitrile (50:50, v/v) at a flow rate of 1 mL/min (19). Total lipids from plasma and erythrocytes were extracted with chloroform/methanol by the procedure of Bligh and Dyer (20). A portion of this extract was digested with perchloric acid at 180°C, and lipid phosphorus was determined by the Nakamura method (21).

RESULTS

HPLC was used to measure the exchange of free cholesterol between plasma and erythrocytes in control, hyperthyroid and hypothyroid rats under various experimental conditions *in vitro*. This chromatographic technique has proven accurate for serum cholesterol analysis as it measures only the absorbance at 200 nm due to the double bond present (19). Table 1 shows the cholesterol content of plasma and erythrocytes before and after incubation at 37°C for 2,4 or 6 hr. The results at time 0 are in perfect agreement with those reported in the literature (10,11,15). The data confirm that the amount of cholesterol lost by erythrocytes was esterified by LCAT present in plasma (15). In the presence of heat-treated plasma (to destroy LCAT activity), the cholesterol lost from erythrocytes remained in plasma as free cholesterol which increased by 22%. No change in esterified cholesterol was noted. Erythrocytes incubated with plasma in the presence of 5 mM Ca^{2+} showed a small increase in cholesterol loss. In addition, increased LCAT activity caused a 28% decrease of cholesterol in plasma and a 29% increase in esterified cholesterol.

In a previous paper it was reported that in hyperthyroid rats cholesterol content decreases in plasma and increases in erythrocyte membranes (10). Table 2 shows that erythrocytes from hyperthyroid rats incubated for 4 hr at 37°C with plasma from the same animals lost 30% of their cholesterol. It is interesting to note that the cholesterol content both of plasma and erythrocytes is similar to that reported for control rats after the same time of incubation. Heat-treatment of plasma from hyperthroid rats suppresses LCAT activity and the cholesterol lost by erythrocytes (18%) remains in the plasma as free cholesterol which increases by 74%. Ca^{2+} increases the loss of erythrocyte cholesterol and LCAT activity.

The experiments have been performed with plasma and erythrocytes from hypothyroid rats because in these animals cholesterol content increases in plasma and decreases in erythrocyte membranes when compared to control rats (11). When plasma from these animals was incubated for 4 hr at 37°C with erythrocytes from the same animals, 24% of the free cholesterol was transferred from the plasma to the erythrocytes (Table 3).

Lastly, Table 4 reports the phospholipid content and the ratio of free cholesterol *versus* phospholipids before and after incubation for 4 hr at 37°C in plasma, and in erythrocytes from control, hyperthyroid and hypothyroid rats. The phospholipid content increases in hyperthyroid rats and decreases in hypothyroid rats both in plasma

TABLE 1

Changes in Cholesterol Content Under Various Experimental Conditions in Control Rats

Incubation time (hr)	Experimental conditions	Cholesterol content[a]			
		Plasma		Erythrocytes	
		Free	Esterified	Free	Total
0	Plasma and erythrocytes	0.32 ± 0.05	0.92 ± 0.10	1.20 ± 0.15	2.44
2	Plasma plus erythrocytes	0.31 ± 0.04	0.97 ± 0.09	1.14 ± 0.11	2.42
4	Plasma plus erythrocytes	0.30 ± 0.03	1.03 ± 0.11[b]	1.08 ± 0.10[c]	2.41
6	Plasma plus erythrocytes	0.31 ± 0.03	1.05 ± 0.12[b]	1.10 ± 0.10[c]	2.46
4	Heat-treated plasma plus erythrocytes	0.39 ± 0.04[b]	0.93 ± 0.13	1.12 ± 0.09	2.44
4	Plasma plus 5 mM Ca^{2+} plus erythrocytes	0.23 ± 0.03[d]	1.19 ± 0.12[d]	0.98 ± 0.14[d]	2.40

[a]Each value represents the mean obtained from 16 experiments with 4 rats each ± S.E. Free and esterified cholesterol are expressed as μmoles/mL of blood. In each experiment 0.6 mL of plasma was incubated with 0.4 mL of erythrocytes. (Normal rat hematocrit level : 40).

[b]$p < 0.01$.

[c]$p < 0.02$.

[d]$p < 0.001$.

TABLE 2

Changes in Cholesterol Content Under Various Experimental Conditions in Hyperthyroid Rats

Incubation time (hr)	Experimental conditions	Cholesterol content[a]			
		Plasma		Erythrocytes	
		Free	Esterified	Free	Total
0	Plasma and erythrocytes	0.23 ± 0.03	0.64 ± 0.11	1.58 ± 0.21	2.45
2	Plasma plus erythrocytes	0.27 ± 0.04	0.85 ± 0.15[b]	1.32 ± 0.17[c]	2.44
4	Plasma plus erythrocytes	0.30 ± 0.04[b]	1.04 ± 0.20[b]	1.10 ± 0.19[b]	2.44
6	Plasma plus erythrocytes	0.31 ± 0.03	1.03 ± 0.16[b]	1.11 ± 0.16[b]	2.45
4	Heat-treated plasma plus erythrocytes	0.40 ± 0.05[b]	0.70 ± 0.15	1.30 ± 0.18[c]	2.40
4	Plasma plus 5 mM Ca^{2+} plus erythrocytes	0.23 ± 0.05	1.18 ± 0.16[b]	1.02 ± 0.23[b]	2.43

[a]Each value represents the mean obtained from 16 experiments with 4 rats each ± S.E. Free and esterified cholesterol are expressed as μmoles/mL of blood. In each experiment 0.6 mL of plasma from hyperthyroid rats was incubated with 0.4 mL of erythrocytes from hyperthyroid rats.

[b]$p < 0.001$.

[c]$p < 0.01$.

TABLE 3

Changes in Cholesterol Content Under Various Experimental Conditions in Hypothyroid Rats

Incubation time (hr)	Experimental conditions	Cholesterol content[a]			
		Plasma		Erythrocytes	
		Free	Esterified	Free	Total
0	Plasma and erythrocytes	0.41 ± 0.06	1.20 ± 0.17	0.84 ± 0.13	2.45
2	Plasma plus erythrocytes	0.35 ± 0.03[b]	1.19 ± 0.15	0.90 ± 0.12	2.44
4	Plasma plus erythrocytes	0.31 ± 0.04[c]	1.16 ± 0.20	0.97 ± 0.10[b]	2.44
6	Plasma plus erythrocytes	0.30 ± 0.05[c]	1.18 ± 0.18	0.98 ± 0.10[b]	2.46
4	Heat-treated plasma plus erythrocytes	0.32 ± 0.05[c]	1.18 ± 0.19	0.95 ± 0.11[c]	2.45
4	Plasma plus 5 mM Ca^{2+} plus erythrocytes	0.25 ± 0.05[c]	1.27 ± 0.15	0.95 ± 0.09[d]	2.47

[a]Each value represents the mean obtained from 16 experiments with 4 rats each ± S.E. Free and esterified cholesterol are expressed as μmoles/mL of blood. In each experiment 0.6 mL of plasma from hypothyroid rats was incubated with 0.4 mL of erythrocytes from hypothyroid rats.

[b]$p < 0.01$.

[c]$p < 0.001$.

[d]$p < 0.02$.

and in erythrocytes *in vivo* (10,11). It remains unchanged after incubation of plasma with erythrocytes in all three types of rats. The free cholesterol *versus* phospholipid ratio changes in plasma and in erythrocytes from hyperthyroid and hypothyroid rats after incubation for 4 hr.

DISCUSSION

The results reported here show that the use of HPLC for measuring cholesterol levels in plasma and in erythrocytes provides the basis of a useful method for studying cholesterol exchange between plasma and erythrocytes. The present study was stimulated by our recent observa-

TABLE 4

Phospholipid Content in Plasma and in Erythrocytes from Control, Hyperthyroid and Hypothyroid Rats

Animals	Incubation time (hr)	Phospholipids		Ratio free cholesterol vs phospholipids	
		Plasma	Erythrocytes	Plasma	Erythrocytes
Control	0	0.81 ± 0.10	1.29 ± 0.18	0.39 ± 0.04	0.93 ± 0.10
Control	4	0.78 ± 0.15	1.20 ± 0.15	0.38 ± 0.03	0.90 ± 0.09
Hyperthyroid	0	1.15 ± 0.20[b]	1.63 ± 0.24[b]	0.20 ± 0.02[b]	0.97 ± 0.11
Hyperthyroid	4	1.10 ± 0.18[b]	1.60 ± 0.20[b]	0.27 ± 0.02[b]	0.69 ± 0.08[b]
Hypothyroid	0	0.45 ± 0.08[b]	0.90 ± 0.15[b]	0.91 ± 0.08[b]	0.93 ± 0.11
Hypothyroid	4	0.48 ± 0.10[b]	0.85 ± 0.14[b]	0.65 ± 0.05[b]	1.14 ± 0.12[b]

[a]Each value represents the mean obtained from 8 experiments with 4 rats each ± S.E. Phospholipids are expressed as μmoles lipid Pi/mL of blood. P values vs control rats were calculated by Student's t-test.

[b]$p < 0.001$.

tion that cholesterol exchange between plasma and erythrocytes *in vivo* does depend, among other factors, on circulating thyroid hormone levels, *i.e.* cholesterol exchange increases in hyperthyroid rats, but is much less pronounced in hypothyroid rats (10,11). The determining factor for free cholesterol partition between plasma and erythrocytes *in vivo* is apparently the phospholipid content of erythrocytes which increases in hyperthyroid rats and decreases in hypothyroid rats in respect to control animals (10,11). The purpose of this seems to be to maintain an acceptable cholesterol *versus* phospholipid molar ratio (22). In fact, Lange *et al.* (2,3) have reported that erythrocyte cholesterol is maintained *in vivo* below a critical level beyond which important membrane changes would occur.

It has been reported that free cholesterol of plasma lipoproteins exchanges with cholesterol in erythrocyte membranes *in vitro* (15,16). We found that the cholesterol content of plasma and erythrocytes is similar in control and in hyperthyroid rats after incubation for 4 hr at 37°C. The data demonstrate that erythrocytes from hyperthyroid rats suffer a greater loss of cholesterol when incubated with plasma from the same animals. In hypothyroid rats, cholesterol exchange between plasma and erythrocytes, which is inhibited by the accumulation of free and esterified cholesterol in the plasma *in vivo*, continues until equilibration is reached *in vitro*. In these animals, cholesterol content in plasma and in erythrocytes is similar in control and in hypothyroid rats because only the unesterified cholesterol is exchangeable between plasma and erythrocytes. These findings suggest that cholesterol, which in hyperthyroid and in hypothyroid rats is not in equilibrium between plasma and erythrocytes *in vivo*, reaches equilibrium during the 4-hr incubation. This equilibrium is independent of phospholipid content of both plasma and erythrocytes.

It is known that incubation of plasma brings about cholesterol esterification. D'Hollander and Chevallier (15) have reported that erythrocyte cholesterol is the sole precursor of esterified cholesterol in plasma, and that there is absolutely no relation between loss of erythrocyte cholesterol and esterification. Data reported in Table 1 confirm that cholesterol esterification was inhibited when LCAT activity was destroyed without affecting the loss of erythrocyte cholesterol. The inhibition of cholesterol esterification was also found in hyperthyroid and in hypothyroid rats. In this condition, loss of erythrocyte cholesterol was not affected.

Ca^{2+} has been shown to be an activator of lecithinase activity (22) and of polyphosphoinositide phosphodiesterase in erythrocyte membranes (23). Giraud *et al.* (24) have reported that cholesterol exchange between erythrocytes and lipid vesicles requires Ca^{2+} at least in trace amounts. In fact, in all three types of erythrocytes, we noted a small increase in cholesterol loss from erythrocytes in the presence of 5 mM Ca^{2+}. In these experiments we have unexpectedly noted that Ca^{2+} increases the reaction of esterification by LCAT in the plasma from control and hyperthyroid rats.

It therefore appears that cholesterol exchange between plasma and erythrocytes depends on the phospholipid content of erythrocytes *in vivo*, but it is an exchange process which reaches an equilibrium between plasma and erythrocytes *in vitro*.

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The Differential Effect of Eicosapentaenoic Acid and Oleic Acid on Lipid Synthesis and VLDL Secretion in Rabbit Hepatocytes

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The suppression of plasma very low density lipoprotein (VLDL) triglyceride levels by dietary fish oils rich in polyunsaturated n-3 fatty acids has been attributed to decreased hepatic VLDL secretion. To investigate the effect of n-3 fatty acids on lipid metabolism and VLDL secretion in a tissue culture system, we incubated rabbit hepatocytes with oleic acid and eicosapentaenoic acid (EPA) and examined [^{3}H]glycerol and [^{14}C]fatty acid incorporation into hepatocyte triglyceride and phospholipid and into media VLDL. Glycerol incorporation studies showed that EPA failed to stimulate VLDL triglyceride secretion from hepatocytes as occurred with oleic acid ($P<0.05$). Oleic acid preferentially enhanced hepatocyte triglyceride synthesis while EPA stimulated significantly phospholipid synthesis ($P<0.01$). Varying the relative concentrations of oleic acid and EPA at a constant total fatty acid concentration corroborated preferential triglyceride synthesis from oleic acid. Synthesis shifted predominantly to phospholipids with increasing concentrations of EPA and lower levels of oleic acid. Incorporation of the [^{14}C]fatty acids (800 μM) followed similar patterns: 87% of [^{14}C]oleic acid was incorporated into hepatocyte triglyceride and 44% of [^{14}C]EPA was assimilated in hepatocyte phospholipid ($p<0.001$). Fatty acids at trace concentrations (53 nM) showed a more divergent pattern of lipid incorporation: 60% of [^{14}C]oleic acid was incorporated into triglyceride while 91% of [^{14}C]EPA was incorporated into phospholipid ($p<0.001$). We conclude that in primary rabbit hepatocyte culture, which appears to be a useful model to study lipid metabolism and VLDL secretion, EPA is avidly incorporated into phospholipid while oleic acid predominantly becomes esterified in triglyceride. In addition, EPA, unlike oleic acid, fails to stimulate hepatocyte VLDL secretion. These divergent effects on hepatocyte lipid metabolism are, at least in part, likely to be responsible for fish oil induced suppression of plasma triglycerides.
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The low incidence of hyperlipidemia and coronary artery disease in Greenland Eskimos, despite their consumption of a diet high in animal fat and cholesterol, has stimulated investigation of potential protective factors contained in their principal foods of seal, whale and fish (1–4). The fat of these marine mammals and fish contains high concentrations of the long chain, polyunsaturated n-3 family of fatty acids consisting largely of eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) (4). Recent studies in normal subjects and in patients with hypertriglyceridemia have demonstrated that polyunsaturated fish oils have potent metabolic effects distinct from those of polyunsaturated vegetable oils (5). Diets containing large amounts of fish oil fed to normal volunteers and hyperlipidemic patients effectively lowered plasma cholesterol and triglyceride (6,7). Later studies using smaller doses of fish oil supplements confirmed their hypotriglyceridemic effect, but showed that there was little effect on total plasma cholesterol (8).

Three potential mechanisms have been proposed to explain the hypotriglyceridemic effects of dietary marine oils: suppression of VLDL synthesis and of VLDL secretion by the liver with a resultant decrease in low density lipoprotein (LDL) synthesis, enhanced clearance of VLDL from the plasma, and enhanced excretion of fecal sterols (7). Several lines of evidence suggest that the most important mechanism is suppression of hepatic VLDL synthesis: i) dietary fish oils block carbohydrate-induced hypertriglyceridemia which results from increased synthesis of VLDL (9); ii) in human lipoprotein turnover studies, dietary fish oils inhibit VLDL synthesis (10,11); and iii) in perfused rat liver and in cultured rat hepatocytes, VLDL secretion is suppressed by n-3 fatty acids compared to n-6 polyunsaturated dietary fatty acids of vegetable origin (12–14).

We studied the differential effects of the n-3 fish oil fatty acid, EPA, and the monounsaturated fatty acid oleic acid (18:1n-9) upon hepatocyte lipid metabolism and VLDL secretion in cultured rabbit hepatocytes. Whereas rat hepatocytes in primary culture have been used to examine VLDL secretion (15,16), the rabbit model, while widely studied with respect to cholesterol metabolism, has seldom been used to investigate hepatocyte triglyceride synthesis and secretion. However, the lipoprotein pattern of the rabbit more closely resembles that of man—rabbit VLDL contains almost entirely apo B-100; the rabbit has an active mechanism for transferring core lipids among lipoproteins; and, the rabbit converts an appreciable fraction of apo B-100 of VLDL to LDL (17). Furthermore, HDL is the predominant lipoprotein in the rat, which is not the case in the rabbit.

In addition to VLDL secretion, we studied the differential effects of oleic acid and EPA upon the synthesis of hepatocyte triglyceride and phospholipid. Unlike oleic acid, EPA shares an essential role in prostanoid biosynthesis and structural similarities with arachidonic acid, a polyunsaturated fatty acid primarily incorporated into phospholipids rather than triglyceride. We speculated

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Abbreviations: CL, cardiolipin; DHA, docosahexaenoic acid; DME, Delbecco's Modified Eagles; EPA, eicosapentaenoic acid; HDL, high density lipoprotein; LDL, low density lipoprotein; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; TLC, thin-layer chromatography; VLDL, very low density lipoprotein.

that in hepatocyte culture, EPA may preferentially incorporate into hepatocyte phospholipid rather than stimulate hepatocyte triglyceride synthesis and secretion, as previously demonstrated with oleic acid (15). Such differential hepatocyte metabolism of dietary fatty acids may play a role in their different effects on plasma lipoproteins.

MATERIALS AND METHODS

Hepatocyte preparation. Rabbit hepatocytes were isolated and cultured by a modification of the method of Seglen developed for rat hepatocytes (18). Male New Zealand White rabbits (1.0–2.5 Kg) were fed a chow diet *ad libitum* (Purina Rabbit Chow, Ralston Purina Company, St. Louis, MO; 4.4% fat; fatty acid composition: 19.2% saturated, 26.9% monounsaturated, 45.7% total n-6, 7.7% total n-3). After the animals were anesthetized with intravenous sodium pentobarbital and heparinized, the liver was pre-perfused with 100–200 mL of calcium- and magnesium-free buffer. A recirculating perfusion was then established for 12–16 min with 0.20 g of collagenase (Worthington, Freehold, NJ) in 500 mL of magnesium-free HEPES buffer through which oxygen was bubbled. The parenchyma of the resected liver was gently minced with scissors and filtered through nylon mesh. After settling at 4°C for 10 min, the supernatant was discarded, and the hepatocytes were washed twice in HEPES/Krebs-Henseleit buffer containing 2% bovine serum albumin. After filtration through 253 micron and 64 micron nylon mesh, cells were counted in the presence of 0.25% trypan blue (>90% viable) and placed into 60-mm culture dishes in 3 mL of Delbecco's Modified Eagles (DME) media (GIBCO, Grand Island, NY) at a concentration of 3×10^5 cells/mL; DME media contained 10% fetal calf serum and gentamicin (50 μg/mL), and had a glucose concentration of 450 mg/dL. After four hr incubaton at 37°C, the media were replaced once with DME containing fetal calf serum, and experiments were conducted on the following day.

Experimental procedures. Triglyceride synthesis as determined by [^{3}H]water incorporation or by mass measurement of VLDL has been shown to give results similar to values obtained by [^{3}H]glycerol incorporation as used in this study (16,19). Incorporation of [^{14}C]oleic acid and [^{14}C]EPA into triglyceride and phospholipid was determined in hepatocytes incubated with the respective unlabeled fatty acid to investigate differential incorporation of these fatty acids into hepatocellular lipids.

Twenty hr after the cells were plated, the culture media were removed and the hepatocytes were washed twice with phosphate-buffered saline. New culture media, free of fetal calf serum, were supplemented with [^{3}H]glycerol (0.66 μCi/mL) and 0.25 mM fatty-acid free bovine serum albumin (Sigma Chemical Co., St. Louis, MO) complexed to either 800 μM oleic acid (NuCheck Prep, Elysian, MN) (oleic acid media), to 800 μM eicosapentaenoic acid (EPA media) (NuCheck Prep) or to no fatty acid (control media). Complexing of fatty acid to albumin was done according to the method of Spector and Hoak (20). For each of the four rabbits in the initial [^{3}H]glycerol (NEN, Boston, MA) incorporation experiment, two plates were incubated with each of the three experimental media for the 2-, 5-, and 8-hr periods. During the 8-hr incubation, approximately 4% of the [^{3}H]glycerol was taken up by hepatocytes from the media supplemented with fatty acids, while approximately 95% of the [^{3}H]glycerol originally added remained in the aqueous phase. More than 70% of the [^{3}H]glycerol taken up by cells was recovered from the lipid phase, and more than 95% of lipid counts were present in cellular triglyceride and phospholipid. More than 90% of [^{3}H]glycerol incorporated into media triglyceride was present in the VLDL fraction, and minimal [^{3}H]glycerol was incorporated into media phospholipid. To examine differential fatty acid assimilation in the lipids of the cells, ^{14}C-labeled fatty acids (NEN) at supplemented (800 μM) and trace (53 nM) concentrations were incubated with hepatocytes for six hr in the presence of [^{3}H]glycerol as noted above. Approximately 10% of labeled fatty acids were taken up by cells and essentially all were in the intracellular lipid phase. Less than 3% of ^{14}C radioactivity in the media after 6 hr was in water soluble oxidation products; volatile $^{14}CO_2$ was not measured.

Lipid analysis. Culture media were centrifuged at 2500 rpm for 5 min to pellet residual cells before decanting the media supernatant. After saline (density 1.006 g/L) was layered over the media, the VLDL fraction was separated by ultracentrifugation at 50000 rpm for 11 hr using a Beckman 50.3 Ti rotor (Beckman Instruments, Fullerton, CA). After washing the hepatocyte monolayer twice with cold, phosphate-buffered saline, cells were scraped, washed in saline and sonicated. An aliquot was removed for protein determination (Biorad Chemical Division, Richmond, CA). Lipid extraction of both the VLDL media fraction and the hepatocyte sonicate was by the method of Bligh and Dyer (21). The lipid phase was then washed with 1:5 volume of 10 mM glycerol in 0.5% NaCl, dried under nitrogen and spotted onto Silica G thin-layer chromatographic (TLC) plates (Supelco, Bellefonte, PA). The TLC plates were run in a hexane/diethyl ether/chloroform/acetic acid (80:10:10:1.5, by vol) solvent system, lipid classes were made visible with iodine vapor, and the triglyceride and phospholipid bands were scraped off. To identify phospholipid subclasses, the lipids were applied onto activated K-6 silica gel plates (Supelco) and separated by the technique of Gilifillan *et al.* (22). By this technique, phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and cardiolipin (CL) were separated into distinct bands, while the pairs phosphatidylinositol plus phosphatidylserine (PI+PS) and lysophosphatidylcholine plus sphingomyelin (LPC+SM) tended to co-migrate. [^{3}H]Glycerol and [^{14}C]fatty acid assimilation in phospholipid and triglyceride was determined by liquid scintillation spectrometry and was expressed as DPM/mg cell protein.

To determine the fatty acid composition of individual lipid classes, bands in thin-layer chromatography were identified with fluorescein spray, scraped off, and the constituent lipids were transmethylated with boron trifluoride. Relative fatty acid composition was determined by gas-liquid chromatography of the methyl ester derivatives using a bonded silica microcapillary column (30 m; SP2330-1; Supelco) under isothermal conditions (170°C) as previously described (23).

Statistical methods. The significance of the effect of fatty acids and time on VLDL triglyceride secretion and

incorporation of [^{3}H]glycerol into lipids of hepatocytes was tested by analysis of variance using a treatment-by-treatment-by-subjects design (Tables 1, 2, and 3; and Fig. 1) (24). Significance of differences between the specific fatty acids was tested by Scheffé's analysis. Analysis of variance was used to test the significance of divergent patterns of [^{3}H]glycerol and [^{14}C]fatty acid incorporation into lipids after incubations at trace and mass concentrations, and also differences in fatty acid composition of lipid classes.

RESULTS

Very low density lipoprotein triglyceride secretion. EPA (800 μM) markedly suppressed VLDL triglyceride secretion from hepatocytes compared to incubation with oleic acid (800 μM) or with fatty-acid free control media. Table 1 summarizes mean [^{3}H]glycerol incorporation from oleic acid, EPA and control media into VLDL triglyceride secreted by rabbit hepatocytes into the medium. Oleic acid media stimulated VLDL secretion three-fold over control levels with linear increases over 8 hr of incubation. In contrast, media supplementation with an equimolar concentration of EPA resulted in only a small increase in VLDL secretion above control levels at 2 hr, and suppression of media VLDL secretion below control values at 5 and 8 hr (Table 1). The differences in VLDL secretion with oleic acid media compared to EPA and control media were statistically significant by Scheffé's analysis. However, VLDL secretion with EPA and control media were not statistically different.

Incorporation of [^{3}H]glycerol into triglyceride and phospholipid synthesized by hepatocytes. As shown in Table 2, triglyceride synthesis in hepatocytes incubated with oleic acid (800 μM), as indicated by [^{3}H]glycerol incorporation, was greater than in cells incubated with EPA (800 μM). However, EPA stimulated greater cellular triglyceride synthesis than the fatty-acid free control media. Scheffé's analysis showed that these differences were statistically significant.

In contrast to the relatively modest stimulation of cellular triglyceride synthesis by EPA, hepatocytes incubated with this n-3 fatty acid incorporated significantly more [^{3}H]glycerol into phospholipid than when exposed to oleic acid (Table 3). Moreover, cells incubated with EPA incorporated [^{3}H]glycerol into phospholipid at a level three times that of hepatocytes in the control media (Table 3). Of note, while EPA induced a greater proportion of [^{3}H]glycerol incorporation in phospholipid than did oleic acid (Table 3), most [^{3}H]glycerol assimilated in cellular lipid after EPA incubation was present in triglyceride (Tables 2 and 3). Analysis of variance of [^{3}H]-glycerol incorporation into phospholipid by the treatment-by-treatment-by-subjects design revealed statistical significance for type of fatty acid, time, and fatty acid-time interaction. The fatty acid-time interaction represents the slowing of [^{3}H]glycerol incorporation between 5 and 8 hr in the EPA incubation, which was not apparent in the control or oleic acid incubations.

To further explore the divergent effects of EPA and oleic acid on the incorporation of [^{3}H]glycerol into hepatocellular lipid, we varied the relative concentrations of a mixture of oleic acid and EPA in the culture media while maintaining the total molar concentration of fatty

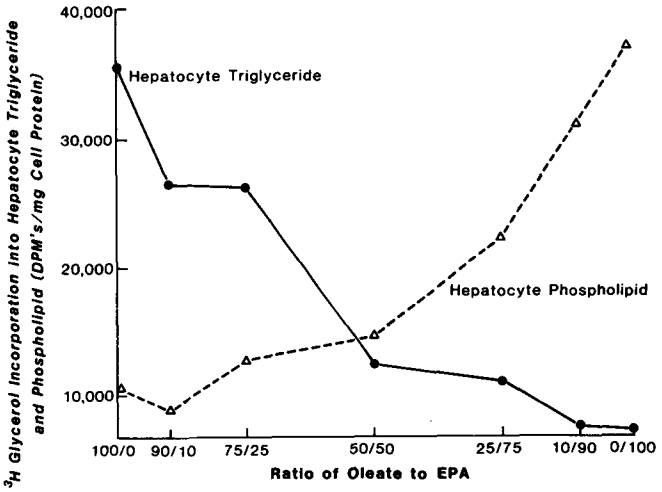

FIG. 1. The effect of varying the molar ratio of oleic acid to EPA while keeping the total fatty acid concentration constant (800 μM) upon the corporation of [^{3}H]glycerol into hepatocyte triglyceride and phospholipid. Data represent a 6-hr incubation of hepatocytes from one rabbit. Each data point represents the mean of four plates.

acid constant (800 μM). Figure 1 displays a profound shift of cellular [^{3}H]glycerol incorporation from triglyceride into phospholipid during a 6-hr incubation with hepatocytes as the relative fatty acid composition was varied from 100% oleic acid to 100% EPA.

Differential incorporation of [^{14}C]fatty acid into the lipids of hepatocytes. Paralleling the differences in [^{3}H]-glycerol incorporation described above, ^{14}C-labeled oleic acid and EPA entered hepatocyte triglyceride and phospholipid in a divergent pattern. A dual label experiment evaluated the synchronous uptake of the [^{3}H]glycerol tracer and [^{14}C]fatty acids at supplemental (800 μM) and trace (53 nM) concentrations into *de novo* synthesized hepatocyte triglyceride and phospholipid. With the respective 800 μM fatty acid supplementation, triglyceride accounted for 87% of [^{14}C]oleic acid incorporated into hepatocyte lipid, while phospholipid contained 44% of the [^{14}C]EPA present in cellular lipid ($p < 0.001$) (Table 4). Less than 20% of ^{14}C-label of either fatty acid was present in free fatty acid cholesterol ester, or water-soluble oxidation product fractions of hepatocytes. In the presence of the supplemental fatty acids, glycerol followed the previously described pattern of preferential incorporation into triglyceride (82% of incorporation in lipid) with oleic acid (800 μM) incubation and prominent assimilation into phospholipid (50% of incorporation into lipid) with EPA (800 μM) incubation (data not shown in Table 4). Trace concentrations of the [^{14}C]fatty acids (53 nM) were added to the control media to determine whether oleic acid and EPA were metabolized differently by hepatocytes under control conditions. Trace concentrations of the fatty acids accentuated the divergent pattern of fatty acid incorporation into hepatocyte lipid—60% of [^{14}C]oleic acid was incorporated into triglyceride as compared to 4% of [^{14}C]EPA (<0.001). Ninety-one percent of the [^{14}C]EPA was incorporated in phospholipid compared to 32% of [^{14}C]oleic acid ($p<0.001$) (Table 4). We observed a higher percentage of both [^{14}C]oleic acid and [^{14}C]EPA incorporated into triglyceride when hepatocytes were incubated with 800 μM fatty acid as compared to control

TABLE 1

Incorporation of [^{3}H]Glycerol into VLDL Triglyceride in Medium[a]

	Control			Oleate (800 μM)			EPA (800 μM)		
Animal No.	2 hr	5 hr	8 hr	2 hr	5 hr	8 hr	2 hr	5 hr	8 hr
1	67	254	667	293	803	1280	253	87	102
2	59	284	554	352	431	2940	78	96	80
3	131	364	754	836	1470	2510	239	201	140
4	217	238	556	613	627	723	160	145	246
Mean ± SE	118	285	633	524	832	1860	182	132	142
	37	28	48	125	225	517	40	26	37

[a]Expressed in DPM/mg cell protein. Measurements represent mean data from four culture plates per rabbit at each time interval. Oleate > Control and Oleate > EPA: $p < 0.05$.

TABLE 2

Incorporation of [^{3}H]Glycerol into Triglyceride in Hepatocytes[a]

	Control			Oleate (800 μM)			EPA (800 μM)		
Animal No.	2hr	5 hr	8 hr	2 hr	5 hr	8 hr	2 hr	5 hr	8 hr
1	16200	18300	16200	36900	42700	37700	35700	41400	42200
2	5660	8260	8600	27800	31500	28100	14400	29200	26600
3	8500	10400	9500	40800	69100	60000	17600	26900	20800
4	6790	8200	11600	29700	44200	58000	20200	29900	52100
Mean ± SE	9290	11300	11500	33800	46900	48500	22000	31800	35400
	2380	2400	1690	3100	7920	6100	4740	3260	7160

[a]Expressed in DPM/mg cell protein. Measurements represent mean data from four culture plates per rabbit at each time interval. Oleate > Control, EPA > Control and Oleate > EPA: $p < 0.01$.

TABLE 3

Incorporation of [^{3}H]Glycerol into Phospholipid in Hepatocytes[a]

	Control			Oleate (800 μM)			EPA (800 μM)		
Animal No.	2 hr	5 hr	8 hr	2hr	5hr	8hr	2 hr	5 hr	8 hr
1	3600	3950	4820	4640	5970	6460	9950	12900	16800
2	3620	6340	8350	6360	8870	12600	7950	19900	23600
3	4280	4850	6320	9010	14800	14000	15700	32300	28300
4	3320	3790	4920	3780	5400	6870	8010	12700	19200
Mean ± SE	3700	4730	6100	5950	8890	9990	10400	19400	21900
	202	584	824	1150	2100	1940	1820	4600	2530

[a]Expressed in DPM/mg cell protein. Measurements represent mean data from four culture plates per rabbit at each time interval. EPA > Control and EPA > Oleate: $p < 0.01$.

incubations with trace concentrations (53 nM) ($p<0.001$) (Table 4). Such enhanced accumulation of fatty acid in hepatocyte triglyceride at high fatty acid concentration is consistent with the depot function of intracellular triglyceride.

Triglyceride and phospholipid fatty acid composition. In the control incubation, the fatty acid content of hepatocyte triglyceride consisted of 30% oleic acid and essentially no EPA, while the phospholipid subclasses contained 11% to 18% oleic acid and <1.5% EPA (see Table 5). Incubation for six hr with media containing either oleic acid (800 μM) or EPA (800 μM) resulted in an approximate 40% enrichment of hepatocyte triglyceride content by the respective fatty acid as compared to control (70% compared to 30% for oleic acid, $p<0.001$; and 42% compared to trace for EPA, $p<0.001$). Despite comparable incorporation of [^{14}C]EPA into hepatocyte triglyceride and phospholipid in this experiment (Table 4), the EPA incubation resulted in a 9.6% or less increase in the percent EPA content of the phospholipid subclasses above the control fatty acid content (Table 5). Similarly, oleic acid incubation enhanced oleic acid

TABLE 4

Incorporation of ^{14}C-Labeled Eicosapentaenoic and Oleic Acids into Phospholipid and Triglyceride of Rabbit Hepatocytes at Tracer (53.6 nM) and 800 μM Concentrations[a]

Experiment	Phospholipid	Triglyceride
	(DPM × 10^3 per mg cell protein)	
Tracer (53 nM)		
Oleate	15.6 ± 0.3 (32%)	29.2 ± 0.1 (60%)
EPA	105.8 ± 4.7 (91%)	5.0 ± 0.1 (4%)
800 μM		
Oleate	8.9 ± 1.6 (11%)	73.0 ± 1.3 (87%)
EPA	45.8 ± 2.1 (44%)	49.0 ± 3.5 (47%)

[a]Hepatocytes were incubated for 6 hr; each data point represents mean ± SE of three culture plates from one rabbit.

composition of the phospholipid subclasses by less than 7%. As noted in Table 5, under the conditions of our experiment, chain elongation of EPA (20:5n-3) to the related 22:5n-3 and 22:6n-3(DHA) fatty acids was modest.

DISCUSSION

Our studies are the first to demonstrate the differential effects of dietary fatty acids on lipid metabolism on the cultured rabbit hepatocyte model. We extend the earlier observations in the rat of Bell-Quint and Forte (15), that media supplemented with oleic acid enhances hepatocyte triglyceride synthesis and VLDL secretion. We found, by contrast, that EPA fails to stimulate hepatocyte VLDL secretion and, as compared to oleic acid, that EPA is preferentially incorporated into phospholipid within the hepatocyte. Wong *et al.* (14) reported decreased fatty acid esterification and triglyceride secretion by rat hepatocytes incubated with EPA and docosahexaenoic acid (22:6n-3) as compared to cells incubated with oleic acid, and suggested that the n-3 fatty acids primarily enhanced fatty acid oxidation. Nossen *et al.* (25) recently demonstrated in cultured rat hepatocytes that the secretion of triglyceride was inhibited 60% by 1 mM EPA. In addition, they showed that incorporation of [^{3}H]glycerol into cellular triglyceride was stimulated by 30% while 1 mM oleic acid incubation resulted in a 450% enhancement in triglyceride secretion and a seven-fold increase in cellular triglyceride incorporation (25). However, they founrd a similar three- to four-fold enhancement of hepatocyte phospholipid synthesis with both EPA and oleic acid rather than the differential effect of these fatty acids on cellular phospholipid metabolism that we have observed.

Our finding that EPA suppressed VLDL secretion below the control level at 5 and 8 hr cannot be accounted for by decreased hepatocyte triglyceride synthesis alone, since EPA stimulated cellular triglyceride synthesis above the control level, although not to the degree observed with oleic acid. These observations suggest that this long chain n-3 polyunsaturated fatty acid not only

TABLE 5

Fatty Acid Content (Percent of Total Fatty Acids) of Triglyceride and Phospholipid[a]

Fatty acids	Triglyceride			Phosphatidylcholine			Phosphatidylethanolamine			Phosphatidylinositol + phosphatidylserine		
	Control	Oleate	EPA	Control	Oleate	EPA	Control	Oleate	EPA	Control	Oleate	EPA
16:0	31.0 ± 1.6	13.0 ± 0.2	16.1 ± 1.0	19.7 ± 2.0	20.8 ± 1.6	8.4 ± 3.2	7.1 ± 3.0	4.4 ± 1.3	4.3 ± 1.7	3.8 ± 0.9	4.7 ± 1.9	5.4 ± 3.5
18:0	11.5 ± 0.4	2.9 ± 0.2	3.7 ± 0.3	25.3 ± 3.9	20.5 ± 0.4	25.5 ± 1.6	25.1 ± 1.5	25.7 ± 1.1	26.9 ± 1.7	35.7 ± 2.0	39.1 ± 1.5	36.5 ± 2.0
18:1n-9 (oleate)	30.2 ± 0.6	70.2 ± 0.1	13.0 ± 1.7	17.8 ± 1.6	20.2 ± 0.6	15.4 ± 0.6	12.7 ± 1.0	17.5 ± 0.8	12.0 ± 1.1	11.2 ± 1.7	17.5 ± 3.1	10.1 ± 1.3
18:2n-6	13.6 ± 0.3	4.8 ± 0.1	7.2 ± 0.3	24.6 ± 1.8	22.3 ± 0.8	20.7 ± 1.2	16.5 ± 1.5	16.8 ± 1.2	14.9 ± 2.1	12.1 ± 3.0	8.6 ± 0.8	8.2 ± 0.6
20:2n-6										3.0 ± 0.6	3.3 ± 0.8	3.5 ± 0.9
20:3n-6										2.3 ± 0.2	2.3 ± 0.6	2.4 ± 0.7
20:4n-6				6.4 ± 1.2	4.9 ± 0.3	8.0 ± 1.4	11.8 ± 3.0	14.1 ± 2.0	9.8 ± 1.0	12.2 ± 0.4	9.5 ± 2.1	9.5 ± 2.5
20:5n-3 (EPA)			41.9 ± 2.7			9.6 ± 2.0			6.3 ± 0.4	1.2 ± 0.9		4.8 ± 1.0
22:5n-3			2.5 ± 0.2			1.0 ± 0.3	1.5 ± 1.0	2.4 ± 0.5	2.2 ± 0.2			1.4 ± 0.8
22:6n-3 (DHA)						1.0 ± 0.3	2.2 ± 0.7	2.1 ± 0.7	2.5 ± 0.4			
Other	13.7	9.1	15.6	6.2	11.3	10.4	23.1	17.0	21.0	18.5	15.0	18.3

[a]Data represent mean values ± SE of three culture plates from one rabbit after a 6-hr incubation with control and oleate (800 μM) media.

causes less intracellular triglyceride synthesis than oleic acid but also may suppress hepatocyte triglyceride secretion by an additional mechanism, possibly by inhibiting VLDL assembly or release. The early enhancement of VLDL secretion (at 2 hr incubation) by EPA relative to control may be attributable to a transient stimulation of VLDL triglyceride synthesis before an EPA-induced block of VLDL secretion develops.

We hypothesized that EPA deposition in hepatocyte lipid, unlike the predominant incorporation of oleic acid into triglyceride, paralleled the preferential incorporation of the long chain PUFA arachidonate into the *sn*-2 position of hepatocyte phospholipid (26). Consequently, we examined the effects of oleic acid and EPA supplemented media on incorporation of [^{3}H]glycerol and [^{14}C]fatty acid into the glycerol backbone and the fatty acyl moiety, respectively, of hepatocyte triglyceride and phospholipid. [^{3}H]glycerol incorporation experiments showed that oleic acid modestly stimulated intracellular phospholipid synthesis; EPA, however, stimulated phospholipid synthesis to a greater extent than oleic acid, though cellular triglyceride synthesis remained predominant. Supplementation of culture media with varying molar ratios of oleic acid and EPA at a constant fatty acid concentration confirmed a shift from stimulation of cellular triglyceride synthesis to phospholipid synthesis as oleic acid was replaced by EPA in the media (Fig. 1). In parallel with the preferential assimilation of the glycerol backbone into phospholipid with EPA incubation, a large portion of [^{14}C]EPA was incorporated into phospholipid (Table 4). This pattern was distinctly different from the deposition of the bulk of [^{14}C]oleic acid into triglyceride which paralleled enhanced triglyceride synthesis as indicated by higher glycerol incorporation in the oleic acid-supplemented media (Table 2).

The lack of identical relative incorporation of [^{3}H]-glycerol and [^{14}C]fatty acid into triglyceride and phospholipid, when incubated with supplemental fatty acids, may be attributable to the different rates of turnover of the glycerol and fatty acyl moieties of the triglyceride and phospholipid molecules. In addition, differences in the pool sizes of metabolic active triglyceride and phospholipid in hepatocytes could modulate the extent of incorporation of labeled fatty acids and glycerol into lipid fractions. As we did not quantify lipid pool size or turnover rates in this study, the impact of such effects on the observed incorporation data is not clear. These factors may, in part, also account for the variations in relative incorporation of labeled glycerol and fatty precursors into triglyceride and phospholipid among experimental animals (Tables 2 and 3; Fig. 1). A significant increase in oxidation of the n-3 fatty acid into water-soluble oxidation products did not account for the decreased esterification into triglyceride. One may speculate that the differential effects of these dietary fatty acids on hepatocyte lipid metabolism may be due to divergent involvement in phospholipid and triglyceride synthetic pathways based on their structure (i.e., degree of desaturation or chain length).

To establish whether oleic acid and EPA are metabolized differently by the hepatocyte in the absence of a high fatty acid load, we examined their lipid deposition under control conditions in which the labelled fatty acids were present at trace concentrations (53 nM). Trace amounts of EPA entered phospholipid with a decided preference, in strong contrast to the selective uptake of trace amounts of oleic acid into triglyceride. When both fatty acids were presented to hepatocytes in high concentrations (800 μM), their deposition shifted into the liver's major fatty acid storage lipid, triglyceride, as one would anticipate. However, EPA and oleic acid incorporation had only modest effects on the fatty acid content of the phospholipid subclasses studied (PC, PE, and PI plus PS), likely due to the large mass of phospholipid fatty acids in the cell, as compared to the approximate 40% incorporation of both fatty acids into less abundant hepatocyte triglyceride (Table 5). Table 5 also indicates that in the presence of supplemental EPA, the synthesis of both 22:5n-3 and 22:6n-3 occurred in the hepatocyte. The synthesis of 22:6n-3 occurred only in the phospholipid fractions, particularly in PC, and not in PI plus PS. Presumably some of this synthesis occurred from 20:5n-3 by chain elongation and the action of the Δ4 desaturase enzyme. It is possible that longer incubation of hepatocytes with n-3 fatty acids, or long-term feeding experiments, would result in more extensive phospholipid fatty acid replacement by n-3 fatty acids, including the chain-elongation products of EPA. In summary, these experiments, utilizing labeled glycerol and fatty acid, demonstrate that oleate is largely deposited into hepatocyte triglyceride, whereas a greater portion of EPA is incorporated into hepatocyte phospholipid in a manner dependent on the media fatty acid concentration.

We conclude that rabbit hepatocyte metabolism of the polyunsaturated n-3 fatty acid EPA and the monounsaturated fatty acid oleic acid varies fundamentally. These studies demonstrate that isolated rabbit hepatocytes provide a useful model for the study of hepatocyte lipid metabolism—at low and high fatty acid concentrations, EPA is avidly incorporated into phospholipid while oleic acid is predominantly incorporated into triglyceride. These findings are not unexpected in view of the known selective enrichment of long-chain n-3 polyunsaturated fatty acids, i.e., docosahexaenoic acid (22:6n-3), into the phospholipids of tissues such as the rod outer segments of the retina (27,28). Moreover, while a high concentration of oleic acid stimulates hepatocyte VLDL secretion several-fold, EPA fails to stimulate VLDL secretion from the cell. These *in vitro* results parallel the observed hypotriglyceridemic effects of diets rich in n-3 fatty acid containing fish oils observed in normal subjects and in hyperlipidemic patients. These data obtained from rabbit, an animal whose VLDL metabolism closely resembles that of man, suggest that the divergent effects of dietary fatty acids on hepatocyte lipid metabolism and secretion are, at least in part, responsible for fish oil-induced suppression of plasma triglyceride. Additional studies of hepatocytes in primary culture may further delineate the biochemical mechanisms of these differences, the effects of other dietary fatty acids (saturated, monoenoic and dienoic) on hepatocyte lipid metabolism, and elucidate the structural characteristics of fatty acids responsible for these divergent effects.

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Reduction in Microalbuminuria in Diabetics by Eicosapentaenoic Acid Ethyl Ester

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Eicosapentaenoic acid (EPA) ethyl ester (1.8 g/d) was administered to 16 diabetic patients (5 insulin-dependent and 11 noninsulin-dependent diabetics) for 6 mon. EPA in total plasma fatty acids increased from 4.0 ± 2.4 mol% (mean ± SD) to 7.5 ± 3.1 mol% ($p<0.001$). Albumin excretion, measured with spot urine, was significantly reduced from 65 to 36 mg/g creatinine (geometric means, $p<0.001$). Fasting blood sugar levels, glycohemoglobin, body weight and blood pressure did not change significantly during the study. There were also no significant changes in serum levels of creatinine, urea nitrogen, total cholesterol and triglycerides. Although no overt hemorrhage was observed in the patients, hematocrit was reduced from 42.6 ± 2.8% to 41.0 ± 3.9% ($p<0.02$). Ten other similar diabetic patients (4 insulin-dependent and 6 noninsulin-dependent diabetics) were followed as a reference group, not concomitantly, for 6 mon with neither EPA ethyl ester nor placebo. The parameters mentioned above were not changed significantly in this group during 6 mon. EPA administration might retard the appearance of overt diabetic nephropathy.
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Diabetes mellitus (DM) is a major cause of chronic renal insufficiency. The appearance of microalbuminuria in diabetics is an early sign of diabetic nephropathy (1), and the reduction in microalbuminuria probably prevents or slows the development of diabetic nephropathy.

There are a few approaches to management of microalbuminuria. Strict control of blood glucose levels for a long period seems to reduce microalbuminuria (2,3), although a one-year period of strict control may not be long enough for improvement of microalbuminuria (4). Treatment of hypertension is very important for reduction in albuminuria (5), and effective even in overt diabetic nephropathy (6). Angiotensin converting enzyme-inhibitors are probably one of the promising antihypertensive drugs, because they have been shown to diminish albuminuria in a short-term trial in patients with early overt nephropathy (7). A low protein diet may be another approach (8). Other drugs, such as aldose-reductase inhibitors, somatostatin analogues, glycosylation inhibitors and anti-platelet drugs, may also reduce microalbuminuria (9). In the present study we examined whether or not eicosapentaenoic acid (EPA) ethyl ester could reduce microalbuminuria in diabetic patients.

MATERIALS AND METHODS

Ninety-percent pure EPA ethyl ester, which had been obtained as previously described (10), was encapsulated in gelatin. Each capsule contained 300 mg of EPA ethyl ester with 0.2% α-tocopherol. Major impurities were arachidonic acid ethyl ester (4%) and 18:4n-3 fatty acid ethyl ester (2%).

Sixteen diabetic patients with microalbuminuria from the Outpatient Clinic of Kurobe City Hospital, Toyama, Japan, were used as subjects. Informed consent was obtained from all patients. They were asked to take 6 capsules of EPA ethyl ester (1.8 g of EPA) per day for 6 mon (EPA group) and to return to the clinic at monthly intervals for a routine checkup of blood sugar and blood pressure. Doses of oral hypoglycemics, insulin and antihypertensives were not changed during the study period. They were also asked to continue their usual lifestyle and to maintain their diet during the study. For comparison of albumin excretion rates, spot urine samples were collected just before and at the end of the study. The second urine of the sampling day, which was collected in the clinic, was used as the spot urine sample. (The first urine excreted at home after awakening was not used in the study.) There was about 2.5 hr between the first and second urine for all patients. Only two urine samples (before and at the end of the study) were checked for albumin. Fasting blood samples were collected at the same time as urine samples for the albumin measurement.

Ten other similar diabetics (Reference group) were followed exactly the same way as the EPA group for 6 mon except that neither EPA ethyl ester capsules nor placebo capsules were administered. Patients in this group were not followed concomitantly with the EPA group. Table 1 shows the details of the patients of both groups.

The spot urine sample was analyzed for albumin by radioimmunoassay (RIA) with a commercial kit (Albumin RIA, Pharmacia, Tokyo). The values were normalized by urinary creatinine levels. A part of the blood sample was collected in the presence of ethylenediaminetetraacetate

TABLE 1

Details of EPA Group and Reference Group

Type of DM	Age (Y)	Sex (F/M)	Duration of DM[a] (Y)	Retinopathy (Scott) (0/I/II/III/cataract)
		(EPA group)		
IDDM[b]	62 ± 10	3/2	12 ± 4	0/1/1/2/1
NIDDM[c]	66 ± 5	8/3	8 ± 3	8/2/1/0/0
Total	65 ± 7	11/5	9 ± 4	8/3/2/2/1
		(Reference group)		
IDDM	56 ± 10	2/2	12 ± 3	1/0/2/1/0
NIDDM	66 ± 5	2/4	14 ± 5	2/1/1/2/0
Total	62 ± 9	4/6	13 ± 4	3/1/3/3/0

[a]DM, diabetes mellitus.
[b]IDDM, insulin-dependent diabetes mellitus.
[c]NIDDM, noninsulin-dependent diabetes mellitus.

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Abbreviations: DM, diabetes mellitus; EPA, eicosapentaenoic acid; HbA_{1c}, glycosylated hemoglobin A_{1c}; IDDM, insulin-dependent diabetes mellitus; NIDDM, noninsulin-dependent diabetes mellitus.

(EDTA) (1.5 mg/mL) and used for the measurement of whole blood viscosity and hematocrit. Blood viscosity was measured with a cone-and-plate type viscometer (E-type, Tokyo-Keiki, Tokyo) at a shear rate of 375/sec at 37°C. Viscosity obtained at the end of the study was standardized to the hematocrit levels obtained at the start of the study. Standardization was performed according to the correlation line between hematocrit and viscosity obtained from more than 130 normal people in our laboratory (11). Hematocrit was measured by microcentrifugation. Plasma was obtained from EDTA-anticoagulated blood and frozen at −20°C until fatty acid analysis was performed (12). Briefly, total plasma lipids were extracted from plasma with chloroform/methanol (2:1, v/v); the extract was saponified and acidified to give free fatty acids, which were then methylated by methanolic boron trifluoride and separated by gas chromatography (GC). Serum total cholesterol, triglycerides, urea nitrogen and creatinine, and urinary creatinine were measured with an autoanalyzer (Model 726, Hitachi, Tokyo). Blood glucose levels were determined enzymatically. Glycosylated hemoglobin A_{1c} (HbA_{1c}) was measured by high pressure liquid chromatography (HPLC).

Data were compiled according to insulin-dependent diabetes mellitus (IDDM) or noninsulin-dependent diabetes mellitus (NIDDM) patients within the same group, and combined data were also calculated if there were no significant differences between these two subgroups. The data are expressed as means ± SD or means (means − SD, means + SD) after log transformation. Statistical analysis was performed using a paired t-test within the same group; $p < 0.05$ was taken as significant. No statistical comparison was made between the EPA and the Reference group, because these two groups were not studied concomitantly.

RESULTS

During the administration of EPA, no serious side effects were observed. Compliance with EPA capsules was checked by EPA content in plasma. In the EPA group, mol% of EPA in plasma total fatty acids of each patient increased more than 25% above the base-line value, and did not change in the Reference group (Table 2). Other fatty acids, including arachidonic acid, did not change in either of the groups (data not shown). There were no significant changes in systolic or diastolic blood pressure or in body weight in either of the groups (Table 2).

Figure 1 shows the changes in albumin excretion levels in both groups. The reduction in albumin excretion levels in the EPA group was significant both in NIDDM and IDDM patients. Combined values were also significantly reduced from 65 (30, 141) to 36 (15, 85) mg/g creatinine ($p<0.001$), while the excretion levels were unchanged in the Reference group (55 (21, 145) to 54 (17, 174) mg/g creatinine, combined values). There were no significant changes in blood chemistry, including urea nitrogen, creatinine and lipid levels during the study in either of the groups (Table 3). There also were no significant changes in fasting blood sugar and HbA_{1c} levels (Table 4).

Combined values of hematocrit were decreased slightly but significantly in the EPA group ($p<0.02$, Table 4). However, there were no significant changes in hematocrit in the Reference group. Although whole blood viscosity at 375/sec was significantly reduced in the EPA group

TABLE 2

Changes in Plasma Fatty Acid Composition, Blood Pressure and Body Weight[a]

Type of DM	EPA mol% in plasma		Systolic BP[b] (mmHg)		Diastolic BP (mmHg)		Body Weight (kg)	
	Before	After	Before	After	Before	After	Before	After
				(EPA group)				
IDDM n=5	2.7 (1.7)[c]	5.7* (2.0)	128 (33)	126 (22)	75 (15)	74 (7)	53.3 (3.8)	54.2 (3.5)
NIDDM n=11	4.5 (2.4)	8.2** (3.3)	142 (16)	145 (12)	77 (6)	84 (9)	58.6 (9.6)	58.1 (9.3)
Total n=16	4.0 (2.4)	7.5** (3.1)	137 (19)	139 (18)	77 (10)	81 (10)	57.0 (8.6)	56.9 (8.1)
				(Reference group)				
IDDN n=4	2.9 (1.3)	2.6 (1.1)	112 (12)	124 (25)	70 (10)	78 (14)	52.6 (9.9)	51.5 (9.5)
NIDDM n=6	4.0 (2.1)	4.0 (2.8)	130 (10)	132 (17)	75 (8)	78 (13)	56.7 (5.3)	55.5 (5.7)
Total n=10	3.5 (1.8)	3.4 (2.3)	122 (16)	129 (22)	72 (9)	78 (14)	55.1 (7.7)	53.9 (7.7)

[a]Comparison was made within the same group.

[b]Blood pressure.

[c]SD is shown in parentheses. Because there were no significant differences in any measured item between IDDM and NIDDM patients in either group (an unpaired t-test), combined values are also shown in the row of "Total".

*$p<0.05$; **$p<0.001$.

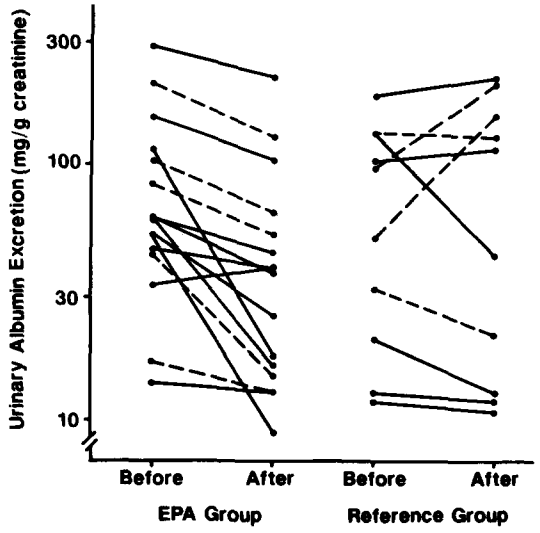

FIG. 1. Changes in albumin excretion levels in EPA and Reference groups. Urinary albumin levels were measured before and after the study (6 mon) in each group. In the EPA group albumin levels were significantly reduced after EPA administration both in IDDM patients (●——●, n=5, 68 to 39 mg/g creatinine (geometric means), $p<0.02$) and in NIDDM patients (●—●, n=11, 65 to 34 mg/g creatinine, $p<0.01$). Changes in combined values (n=16) of both IDDM and NIDDM patients were 65 to 36 mg/g creatinine, $p<0.001$. In the Reference group no significant changes were observed either in IDDM patients (● - - ●, n=4, 71 to 98 mg/g creatinine) or in NIDDM patients (●—●, n=6, 46 to 35 mg/g creatinine). Combined values (n=10) did not change significantly either (55 to 54 mg/g creatinine).

after the study, when normalized to the initial hematocrit values (data not shown), this difference disappeared (Table 4).

DISCUSSION

Since Prickett *et al.* (13) reported the beneficial effects of fish oil on experimental nephritis of NZB × NZW F_1 mice, several papers have appeared reporting effects of fish oil on experimental nephritis and renal insufficiency (14-21). Beneficial effects of fish oil on immunoglobulin A (IgA) nephropathy (22) and hemodialysis patients (12) were also reported from our laboratory. In our preliminary experiments with streptozotocin-induced diabetes in rats, EPA ethyl ester was shown to significantly reduce proteinuria (23). In the present study, we tried to answer whether the same beneficial effects on proteinuria by EPA ethyl ester could be applied to human diabetics, and showed that albumin levels were reduced by EPA administration. This finding is consistent with many of the experimental kidney diseases in rats (23).

It is not clear how EPA would reduce microalbuminuria. However, some drugs are known to reduce proteinuria of diabetic patients, as described in the introduction, and EPA shares some of these drugs' characteristics. Among them are anti-aggregatory effects and reduction in thromboxane production (24), which are favorable for microcirculation of the kidney. Fish oil administration is reported to reduce blood pressure and vascular reactivity to angiotensin II and norepinephrine

TABLE 3

Changes in Urea Nitrogen, Creatinine, Total Cholesterol and Triglycerides in Serum (mg/dl)[a]

Type of DM	Urea nitrogen		Creatinine		Total cholesterol		Triglycerides[b]	
	Before	After	Before	After	Before	After	Before	After
				(EPA group)				
IDDM	21.7 (3.2)[c]	19.3 (2.2)	0.88 (0.15)	0.95 (0.15)	185 (53)	171 (40)	106 (60,188)	89 (58,138)
NIDDM	17.4 (4.5)	14.8 (4.5)	0.90 (0.37)	0.77 (0.12)	185 (52)	179 (37)	104 (55,188)	97 (63,150)
Total	18.7 (4.6)	16.2 (4.5)	0.89 (0.32)	0.82 (0.16)	185 (52)	176 (38)	105 (56,195)	94 (61,153)
				(Reference group)				
IDDN	22.8 (7.5)	19.9 (7.4)	1.08 (0.29)	1.18 (0.19)	217 (27)	195 (14)	110 (76,160)	108 (70,169)
NIDDM	20.1 (6.8)	19.0 (3.8)	1.02 (0.13)	1.00 (0.10)	197 (40)	197 (40)	68 (52,87)	81 (56,116)
Total	21.1 (7.2)	19.4 (5.6)	1.04 (0.21)	1.07 (0.17)	205 (37)	196 (32)	83 (55,126)	91 (60,138)

[a] Comparison was made within the same group.

[b] Values were log-transformed and are expressed as mean (−SD, +SD).

[c] SD is shown in parentheses. Because there were no significant differences in any measured item between IDDM and NIDDM, combined values are also shown in the row of "Total". No significant differences were observed between before and after the study in any item in either group.

TABLE 4

Changes in Blood Sugar, Blood Viscosity and Hematocrit[a]

Type of DM	Fasting blood sugar (mg/dl)		HbA_{1c} (%)		Whole blood viscosity at 375/sec (cP)		Hematocrit (%)	
	Before	After	Before	After	Before	After	Before	After
				(EPA group)				
IDDM	175 (30)[b] *	125† (14)	7.1 (1.6)	7.1 (1.5)	4.08 (0.96)	4.06 (0.48)	42.5 (3.9)	41.4 (4.8)
NIDDM	136 (21) *	142 (17)	5.8 (1.3)	5.6 (1.4)	3.93 (0.51)	3.80 (0.30)	42.7 (2.2)	40.8* (3.7)
Total	—	—	6.2 (1.5)	6.0 (1.6)	3.97 (0.70)	3.89 (0.40)	42.6 (2.8)	41.0** (3.9)
				(Reference group)				
IDDN	138 (35)	133 (44)	6.3 (1.7)	5.5 (0.8)	3.98 (0.28)	4.07 (0.30)	41.8 (3.0)	42.5 (2.5)
NIDDM	148 (15)	134 (22)	6.8 (1.2)	6.7 (0.6)	4.09 (0.51)	4.05 (0.49)	43.7 (1.7)	44.0 (2.8)
Total	144 (25)	134 (33)	6.6 (1.5)	6.2 (0.9)	4.05 (0.43)	4.06 (0.43)	42.9 (2.5)	43.4 (2.8)

[a]Comparison was made within the same group.

[b]SD is shown in parentheses. Because there were no significant differences between IDDM and NIDDM patients in any measured item except for fasting blood sugar values, combined values are also shown in the row of "Total". There was a significant difference in fasting blood sugar values between IDDM and NIDDM before the study.

†$p=0.06$; *$p<0.05$; **$p<0.02$.

(25). Hence, in a stressful situation, EPA may sufficiently protect renal blood flow. The membrane potential of glomerular podocytes is important for preventing albuminuria because of electrical repulsion. We speculate that introduction of EPA into podocyte cell membranes may result in an increase in membrane potential resulting from changes in micro-environment to prevent albuminuria. Indeed, in our experiments with streptozotocin-induced diabetes in rats (23), EPA levels in total phospholipids of the whole kidney after 4 weeks on an EPA-rich diet increased markedly (up to 5.4%) compared to those of control rats on an EPA-free diet (0.1%).

The major impurities contained in the present EPA ethyl ester preparation seem to have nothing to do with the effect on albuminuria, because 18:4n-3 fatty acid was not detected in the total fatty acid fraction of plasma, and arachidonic acid levels did not change after EPA administration (data not shown).

Blood rheology is one of the important factors which affect the renal vasculature. Because the hematocrit of the blood in the glomerulus is very high due to ultrafiltration, a further increase in blood viscosity must be very harmful to the glomerulus. Kamada *et al.* (26) observed an increment in erythrocyte membrane fluidity by dietary supplementation with fish oil in diabetics. In the present study, we could not find any beneficial effects of EPA on whole blood viscosity if standardized to the initial values of hematocrit. Although we did not measure red blood cell (RBC) filterability, it might have been increased by EPA administration as had been shown in healthy volunteers (27) or in some patients including diabetics (11) and renal allograft recipients (28). If it were the case in the present study, more filterable RBCs might have facilitated the recovering of disturbed glomeruli by reducing shear stress.

The only adverse effect that we could find in the EPA group was a significant reduction in hematocrit ($\Delta=-1.6\%$). Since fish oil administration prolongs bleeding time (29), there might have been minor gastroenteral bleeding in the EPA group, although no overt bleeding was observed.

Haines *et al.* (30) reported that fish oil supplements (15 g MaxEPA per day) for 3 to 6 weeks did not reduce albuminuria in IDDM patients. However, the duration of fish oil administration seems to be too short to affect albumin excretion rates, and besides, the effects of MaxEPA may be different from those of 90% pure EPA ethyl ester.

Glauber *et al.* (31) reported that dietary supplementation with 18 g MaxEPA significantly increased fasting glucose levels of nontreated type II diabetics in one month. In our study there was no deterioration with regard to blood sugar levels in one month of EPA administration (data not shown) or in 6 mon of the administration (Table 3). The main difference between their study and ours was that the subjects used in the present study were diabetics under appropriate treatment, while theirs were not. The dose of n-3 fatty acids in their study (~5 g) was three times more than ours, which might be a cause of the discrepancy. Again, direct comparison between MaxEPA and EPA ethyl ester may be difficult.

In conclusion EPA ethyl ester administration reduces microalbuminuria in diabetic patients and may retard the appearance of diabetic nephropathy. Consequently, further studies seem to be justified. However, reduction in hematocrit in our present study and elevation in blood

sugar levels in others urge that studies administering EPA-containing materials to diabetics should be done only with caution.

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On the Effect of Peroxisomal β-Oxidation and Carnitine Palmitoyltransferase Activity by Eicosapentaenoic Acid in Liver and Heart from Rats

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Repeated administration of highly purified eicosapentaenoic acid (as ethyl ester) resulted in a decrease in plasma triglycerides and high density lipoprotein (HDL) cholesterol. This was accompanied by a stimulation in the activities of carnitine palmitoyltransferase, fatty acyl-CoA oxidase and peroxisomal β-oxidation in the liver. The results suggest that the triglyceride-lowering effect observed with eicosapentaenoic acid may be due to a reduced supply of fatty acids for hepatic triglyceride synthesis because of increased fatty acid oxidation. Eicosapentaenoic acid feeding marginally affected the triglyceride content of heart and mitochondrial and peroxisomal enzyme activities.

Lipids 25, 546–548 (1990).

Marine oils are rich in polyunsaturated fatty acids of the n-3 family, especially eicosapentaenoic acid (EPA, 20:5) and decosahexaenoic acid (22:6). Dietary intake of these fatty acids reduces plasma triglycerides in human hyper- and normolipidemic subjects and in experimental animals (1–7).

The mechanism by which EPA reduces plasma triglycerides may include increased removal and/or production of lipoprotein particles (7–10). Production of very low density lipoproteins by the liver may be reduced by interference with the esterification (8) as well as fatty acid oxidation, i.e., mitochondrial and peroxisomal oxidation of fatty acids. It was therefore of particular interest to investigate whether stimulation of fatty acyl-CoA oxidase and carnitine palmitoyltransferase activities, key enzymes in peroxisomal and mitochondrial oxidation of fatty acids, might contribute to the triglyceride-lowering effect observed.

The present data support the concept that fish oil fatty acids act by partitioning free fatty acids from triglyceride synthesis toward fatty acid oxidation. Whether this effect contributes to the hypotriglyceridemic effect should be considered.

MATERIALS AND METHODS

Chemicals. Ethyl eicosapentaenoic acid (purity 92%) was obtained from Norsk Hydro AS, Research Centre, Porsgrunn, Norway. Palmitic acid (PMA) was from Sigma Chemical Co., St. Louis, MO. All other chemicals were obtained from common commercial sources and were of reagent grade.

Animals and treatments. Male Wistar rats from Möllegaard Breeding Laboratory, Ejby, Denmark, weighing 180–200 g, were housed individually in metal wire cages in a room maintained at 12 hr light-dark cycles and 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. EPA and palmitic acid were suspended in 0.5% sodium carboxymethyl cellulose (CMS) and 0.5% tocopherol. In the dose-response experiments, the individual agents were administered by gastric intubation in a volume of 1 mL once a day for 5 days and the animals were killed at the start of the sixth day after 12 hr of starvation. The animals were separately treated from low to high dose levels with the fatty acids (EPA and PMA) and the doses were: 250, 600 and 1,300 mg/day/kg body weight. The control animal groups received only CMS and 0.5% tocopherol. All animals had free access to water and food. The food composition was as earlier described (11), containing 55% carbohydrate (w/w), 25% protein and 2.1% fat (w/w) and all necessary minerals and vitamins.

The body weights were recorded daily. At the end of the experiments, the rats were fasted and weighed. Under light halothane anesthesia, cardiac puncture was performed to obtain blood samples, and the livers and hearts were removed and immediately chilled on ice and weighed. Serum was prepared from the blood samples by centrifugating the clotted whole blood at 1000 × *g* for 10 min.

Analytical methods. The tissues 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 plus postnuclear fraction was used as the total homogenate (12).

Protein was assayed by Bio-Rad protein assay kit (Bio-Rad, Richmond, CA).

The enzymatic activities of palmitoyl-CoA dependent dehydrogenase (usually termed peroxisomal β-oxidation; ref. 13), fatty acyl-CoA oxidase (13), and carnitine palmitoyltransferase with no malonyl-CoA present (13) were determined as described.

Lipid analyses were carried out using the monotest cholesterol enzymatic kit (Boehringer, Mannheim, Germany) and the Biopak Triglyceride enzymatic kit (Biotrol, Paris, France).

Presentation of results. Data are presented as mean ± SD. Three animals in each experimental group and six controls were used. Data were statistically analyzed by Student's t-test.

RESULTS

All animals treated with EPA and palmitic acid at various doses gained weight at the same rate as controls. Food consumption was similar in each experimental group irrespective of the dietary regimen, indicating that appetite was not affected and that the acids were well tolerated. Rats treated with saturated and polyunsaturated fatty acid appeared healthy and looked and behaved normal.

Serum and tissue lipids. The hypotriglyceridemic and hypocholesterolemic effects of EPA were investigated in

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Abbreviations: EPA, eicosapentaenoic acid; PMA, palmitic acid.

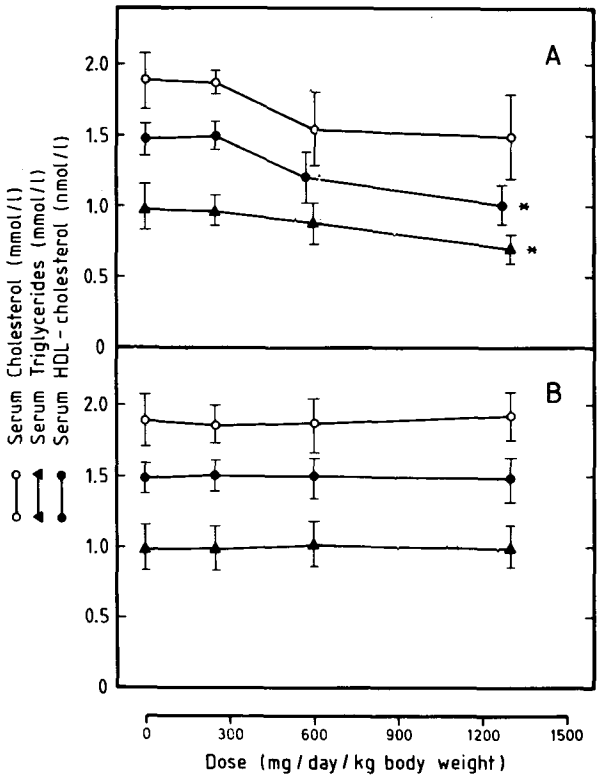

FIG. 1. Dose-dependent changes of serum cholesterol (O—O), HDL-cholesterol (● — ●) and triglycerides (▲—▲) in animals treated with EPA (A) and palmitic acid (B).

rats. Figure 1A shows that at the highest dose of EPA, a 30–35% decrease in serum triglycerides and a 20–25% decrease in HDL cholesterol occurred. The concentration of serum cholesterol tended to decrease as a function of dose. However, the reduction was not significant (Fig. 1A). In keeping with previous observations (14), plasma triglycerides and cholesterol were not significantly lowered after administration of palmitic acid (Fig. 1B).

No hepatomegaly resulted after EPA and palmitic acid feeding and no significant changes of the hepatic protein content were observed (Table 1). Repeated administration of the polyunsaturated and saturated acids only marginally affected the concentration of triglycerides and cholesterol in liver (Table 1) and heart (data not shown).

Carnitine palmitoyltransferase activity. The activity of carnitine palmitoyltransferase in total liver homogenates was found to increase in a dose-dependent manner. No changes in the activity of carnitine palmitoyltransferase were found in the total heart homogenates (Fig. 2).

Peroxisomal activities. The activities of fatty acyl-CoA oxidase (Fig. 3) and peroxisomal β-oxidation were significantly increased after administration of EPA (Table 1). Repeated administration of palmitic acid, however, marginally affected these peroxisomal activities in the liver. No changes in the activity of fatty acyl-CoA oxidase (Fig. 3) and peroxisomal β-oxidation (data not shown) were observed in heart homogenates of EPA-fed animals.

DISCUSSION

Recently we have shown that the potency of selected compounds as proliferators of peroxisomes depends on their accessibility for β-oxidation (15). Experimental data strongly suggest that the minimal structural requirement for peroxisome proliferation may be a carboxylic acid group linked to a hydrophobic backbone with poor susceptibility to β-oxidation (15). The stimulation of peroxisomal β-oxidation with eicosapentaenoic acid (Fig. 3), but not with palmitic acid, conforms to the previously defined requirement for initiation of peroxisomal proliferation. Production of liver peroxisomes accompanied by stimulated peroxisomal β-oxidation is observed after feeding certain high fat diets, especially diets rich in C_{20}–C_{22} fatty acids which are relatively poorly oxidized by the mitochondrial

TABLE 1

Dose-Dependent Changes of Liver Weight, Liver Lipids and Hepatic Enzyme Activities in Rats Treated with EPA and Palmitic Acids for 5 Days[a]

Parameters	Compound	Dose (mg/day/kg body weight) 0	250	600	1300
Liver g/g body weight	EPA	34.8 ± 0.10	3.58 ± 0.08	3.51 ± 0.12	3.75 ± 0.20
	PMA	3.46 ± 0.08	3.52 ± 0.05	3.51 ± 0.10	3.45 ± 0.10
Protein mg/g liver	EPA	148.2 ± 4.1	154.2 ± 6.4	149.6 ± 4.2	150.2 ± 6.1
	PMA	151.6 ± 4.6	152.1 ± 8.6	150.8 ± 3.6	148.6 ± 4.2
Triglycerides μmol/g liver	EPA	4.5 ± 0.5	5.1 ± 1.0	4.7 ± 0.9	3.9 ± 0.2
	PMA	4.6 ± 0.3	4.8 ± 0.6	5.2 ± 0.6	5.0 ± 0.6
Cholesterol μmol/g liver	EPA	11.7 ± 1.2	11.9 ± 1.1	12.0 ± 0.2	12.2 ± 0.7
	PMA	12.1 ± 0.6	12.4 ± 0.7	12.0 ± 1.0	11.8 ± 0.6
Peroxisomal β-oxidation nmol/g liver	EPA	500 ± 23	485 ± 83	550 ± 30	*675 ± 55
	PMA	480 ± 50	490 ± 70	510 ± 20	505 ± 40
Fatty acyl-CoA oxidase μmol/g liver	EPA	923 ± 30	984 ± 23	1003 ± 121	*1215 ± 61
	PMA	910 ± 40	950 ± 36	920 ± 40	890 ± 20
Carnitine palmitoyltransferase nmol/g liver	EPA	1050 ± 50	1045 ± 40	*1220 ± 50	*1480 ± 30
	PMA	1060 ± 40	1055 ± 30	1080 ± 80	1020 ± 50

[a]The tabulated values are means ± SD of six control animals and three rats in each treatment group. *P < 0.05.

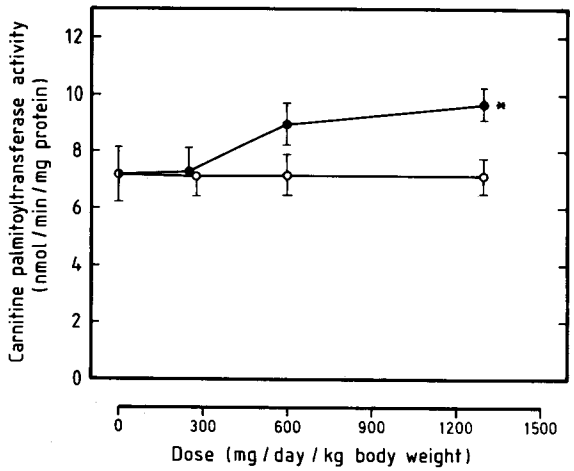

FIG. 2. Dose-dependent changes in the activity of carnitine palmitoyltransferase in liver (● — ●) and heart (O—O) after EPA administration for 5 days. *P < 0.05.

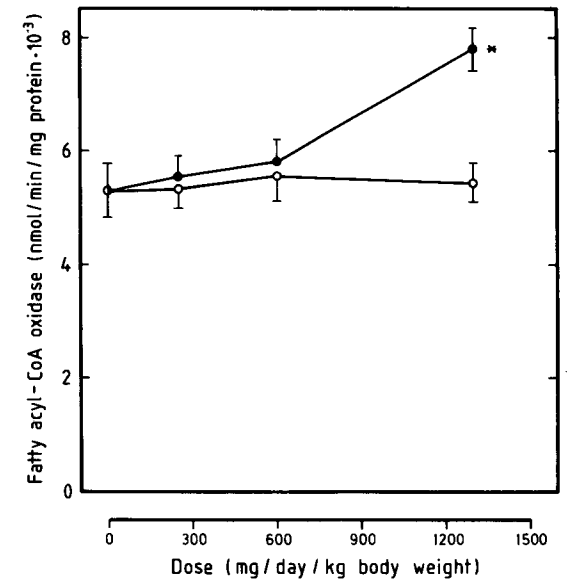

FIG. 3. Dose-dependent changes in the activity of fatty acyl-CoA oxidase in liver (● — ●) and heart (O—O) after EPA administration for 5 days. *P < 0.05.

β-oxidation system. The present study shows for the first time that administration of pure EPA stimulates peroxisomal β-oxidation. It is possible that induction of β-oxidation by a high fat diet (13) is due to its content of eicosapentaenoic acid, i.e. fatty acids which are poorly oxidized by mitochondria and which seem to be dependent on peroxisomes for efficient chain shortening (16). Increased peroxisomal β-oxidation appears not to be attributable to essential fatty acid deficiency (17) or the amount of 22:1 fatty acids or *trans*-fatty acids in the fish oils.

Formation of enlarged mitochondria (17) and increased activity of carnitine palmitoyltransferase (13) have been found in rats fed a diet containing a high level of fish oil. Wong and co-workers (5) have observed increased ketogenesis of dietary fish oil. In addition, the activity of carnitine palmitoyltransferase in hepatic mitochondria isolated from rats fed n-3 lipids was very resistant to inhibition by the negative effector malonyl-CoA. Thus, one hypothesis for hepatic reduction of triacylglycerol is that polyunsaturated fatty acids alter the hepatic partitioning of free fatty acids, i.e. by channeling them away from triacylglycerol synthesis and toward fatty acid oxidation, especially peroxisomal β-oxidation (5). Altogether, the results indicate that the lowering of plasma triglycerides by fish oil reflects diminished lipogenesis, increased fatty acid oxidation (possibly in peroxisomes), and diminished secretion of triglycerides by the liver. Drevon and co-workers (4,8), however, have reported that almost no increase in production of acid-soluble material could be detected in the presence of eicosapentaenoic acid during short-term incubations.

The present study confirms earlier observations (1-7) that treatment with EPA decreases plasma levels of triglycerides (Fig. 1). Furthermore, EPA was shown to stimulate the activity of carnitine palmitoyltransferase (Fig. 2). This was accompanied by a tendency to reduce the liver content of triglycerides (Table 1). Assuming that carnitine palmitoyltransferase activity runs in parallel with mitochondrial fatty acid oxidation (13), the results suggest that the triglyceride-lowering effect observed with pure EPA might be due to increased fatty acid oxidation, i.e. both mitochondrial and peroxisomal oxidation of fatty acids accompanied by a reduction in the availability of the substrates (fatty acids) for triglyceride biosynthesis.

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Intestinal Absorption of Retinol and Retinyl Palmitate in the Rat. Effects of Tetrahydrolipstatin

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The aim of the present study was to characterize the intestinal absorption of retinol and retinyl palmitate in thoracic duct and bile duct fistulated rats and to investigate the effect of a simultaneously administered lipase inhibitor, tetrahydrolipstatin (THL). Absorption was determined as lymphatic recovery over a 24-hr period, including an initial 12-hr continuous intraduodenal infusion of either [11,12-^{3}H]retinol or [11,12-^{3}H]retinyl palmitate given in emulsified glyceryl trioleate or in mixed micellar solution of monoolein and oleic acid. From micellar dispersion, labeled retinol and retinyl palmitate were recovered in the lymph to 50–60% and both to the same extent. Administered in emulsified form, labeled retinol from fed retinyl palmitate was recovered to 47%, but retinol from fed retinol to only 18%. THL (10^{-4} M) in the infusate had no significant effect on the recovery of ^{14}C-labeled oleic acid. The recovery of label from emulsified glyceryl tri[1-^{14}C]oleate was significantly decreased at this concentration of THL (76.5% vs 19.6% recovery). When administered in emulsified form, retinol absorption was not significantly affected by THL at 10^{-4} M, while retinyl palmitate absorption was very significantly decreased (5.0% compared to 47.8%). In the presence of THL, retinol absorption from retinyl palmitate in micellar solution was decreased (from 58% to 17%). Most of the retinol in the lymph extracts (72.2 to 91.3) was present as retinyl ester, regardless of the chemical and physical form of administration. Furthermore, THL did not induce any change in this pattern.
***Lipids 25,* 549–552 (1990).**

The intestinal absorption of nonpolar fats, such as cholesterol, is dependent on a mixed bile salt micellar system, normally generated from the hydrolysis of dietary triglycerides by the action of gastrointestinal lipases. The absorption of highly nonpolar long-chain cholesterol esters is also directly dependent on the lipolytic process as the esters must be hydrolyzed to free cholesterol prior to absorption (1).

It has recently been shown that a lipase inhibitor, tetrahydrolipstatin (THL), almost completely prevents the intestinal absorption of cholesterol from cholesteryl oleate in micellar as well as emulsified dispersions, while free cholesterol is not affected (1). In the present study we have extended these experiments to include the effect of THL on the intestinal absorption of the analogous pair retinol/retinyl palmitate.

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Abbreviations: DMSO, dimethylsulfoxide; TLC, thin-layer chromatography; THL, tetrahydrolipstatin; EDTA, ethylenediaminetetraacetic acid.

MATERIALS AND METHODS

Chemicals. [1-^{14}C]Oleic acid, [11,12-^{3}H]retinol and glyceryl tri[^{14}C]oleate were purchased from Amersham International, Buckinghamshire, U.K. [11,12-^{3}H]Retinyl palmitate was synthesized from free labeled retinol according to the method described by Baldwin and Daubert (2) as modified in our laboratory for use with light-sensitive retinol derivatives. The radiopurity was better than 92% as determined by thin-layer chromatography (TLC) radioactivity. Sodium taurocholate and sodium taurodeoxycholate were synthesized according to Norman (3). 1-Monoolein was prepared according to Mattson and Volpenheim (4). Lecithin was purified as described by Singleton *et al.* (5).

THL [(S)-1-(2S,3S)-3-hexyl-4-oxo-2-oxetanyl)methyldodecyl(S)-2-formamido-3-methyl-valerate] was obtained from F. Hoffmann La Roche Ltd. (Basel, Switzerland). All solvents were redistilled before use and all other reagents were of analytical grade.

Animals. Male Sprague-Dawley rats weighing 275–325 g were obtained from ALAB (Stockholm, Sweden), fed with a standard rat diet and kept in a room with controlled environmental temperature, humidity and lighting cycle (12 hr light/12 hr dark).

Surgery. After an overnight fast, the rats were anesthetized by inhalation of diethyl ether. The thoracic duct was cannulated according to the method described by Bollman *et al.* (6) and modified later by Lindström (7). The bile duct was cannulated allowing intact pancreatic flow to the intestinal lumen. A duodenal fistula was made inserting a silicone-tipped vinyl catheter in the duodenum through the gastric fundus. Immediately after surgery, 1 mg/kg of diazepam was given intraperitoneally.

A glucosaline solution (2.5% glucose, 0.5% NaCl and 0.05% KCl) was infused through the duodenal catheter at a rate of 3 mL/hr for 18–24 hr. The animals were allowed to drink *ad libitum* the same glucosaline solution. After this recovery period, the experimental infusates containing labelled lipids were given intraduodenally at the same rate for 12 hr. After this period, the lipid-containing infusates were substituted again by the glucosaline solution which was infused for another 12-hr period. Drinking was not allowed during this period. Lymph was collected hourly for 24 hr in tubes moistened with ethylenediaminetetraacetic acid (EDTA) solution.

This experiment has been approved by the Ethical Committee of the University of Lund.

Micellar infusates. Suitable amounts of either unlabelled or radioactive lipids were taken from organic stock solutions and placed into a round flask. The solvents were evaporated under nitrogen at room temperature and protected from light. Total bile salts were dissolved in 1/5 volume (i.e., 10 mL) of buffer (12 mM Na_2HPO_4; 8 mM NaH_2PO_4; 113 mM NaCl; 5 mM KCl; 1 mM $CaCl_2$ and 10 mM glucose, pH 6.5). This mixture was stirred with an egg-shaped magnetic bar at 500 rpm till there was no evidence of floating fat droplets. The

remaining 4/5 volume of buffer (i.e., 40 mL) was added with continuous stirring. A suitable amount of THL was taken from a stock dimethyl sulfoxide (DMSO) solution (25 mg THL/mL DMSO) and added to the whole volume to reach a final concentration of 10^{-4} M. The same amount of DMSO (100 μL) was added to the control infusates. The final concentration of DMSO in all the infusates was approximately 2.2 mg/mL. The pH of the infusate was set at 6.5 before infusion.

Infusate A: 10 mM [1-^{14}C]oleic acid; 5mM 1-monoolein; 2 μM [11,12-^{3}H]retinol; 20 mM sodium taurocholate and 5 mM sodium taurodeoxycholate in buffer solution. This was a clear solution. Slight turbidity appeared when THL 10^{-4}M was added.

Infusate B: 10 mM [1-^{14}C]oleic acid; 5 mM 1-monoolein; 2 μM [11,12-^{3}H]retinyl palmitate; 20 mM sodium taurocholate and 5 mM sodium taurodeoxycholate in buffer solution. This was a clear solution. Slight turbidity appeared when 10^{-4}M THL was added.

Emulsified infusates. Suitable amounts of either unlabelled or radioactive lipids were taken from organic stock solutions and put in 20-mL glass vials. The solvents were evaporated under nitrogen at room temperature and protected from light. Total bile salts were dissolved in 1/5 volume (i.e., 10 mL) of buffer (12 mM Na_2HPO_4; 8 mM NaH_2PO_4; 113 mM NaCl; 5 mM KCl; 1 mM $CaCl_2$ and 10 mM glucose, ph 6.5) and added to the vial. A sufficient amount (100 μL) of THL dissolved in DMSO (25 mg THL/mL DMSO) or 100 μL of DMSO (control) was also added to the vial. This mixture was sonicated 4 × 30″ (Branson sonifier). After sonication the whole was transferred to a beaker and the remaining 4/5 volume of buffer (i.e., 40 mL) was added. The mixture was sonicated again (4 × 30 ″). The final concentration of DMSO in all the emulsions was 2.2 mg/mL. In emulsions containing THL, the final concentration was always 10^{-4} M. The pH of the infusate was set at 6.5 before infusion.

Infusate C: 5mM glyceryl tri[^{14}C]oleate; 0.286 mM phosphatidylcholine; 2μM [11,12-^{3}H]retinol; 20 mM sodium taurocholate and 5 mM sodium taurodeoxycholate in buffer solution.

Infusate D: 5mM glyceryl tri[^{14}C]oleate; 0.286 mM phosphatidylcholine; 2μM [11,12-^{3}H]palmitate; 20 mM sodium taurocholate and 5 mM sodium taurodeoxycholate in buffer solution.

Micellar solutions were infused using a syringe infusion pump. Emulsions were infused with a peristaltic pump (Microperpex®, LKB, Bromma, Sweden) with continuous magnetic stirring of the reservoir flask. The infusates were protected from light during infusion.

Radioactivity determinations. Two hundred mL aliquots of the hourly lymph samples and 800 μL of distilled water were mixed with 10 mL of emulsifying scintillant (Ready Safe, Beckman, Fullerton, CA). The radioactivity was counted in a Packard Tri-Carb scintillation counter equipped with an automatic quench correction program.

After extraction of the pooled lymph samples with chloroform/methanol (2:1, v/v) and splitting of the phase with water, the relative amounts of free and esterified retinol were determined in the chloroform phase. To separate retinol and retinyl esters, aliquots of the chloroform phase were evaporated, redissolved in a small volume of chloroform and run on TLC plates (Alufolien, Merck, Darmstadt, Germany) using a solvent system consisting of light petroleum ether/diethyl ether/acetic acid (79:20:1, v/v/v).

RESULTS

Lymphatic transport of retinol and retinyl palmitate. The lymphatic output of ^{3}H-activity derived from the infused [11,12-^{3}H]retinol increased during the first 3 hr and reached a steady value 4–5 hr after the start of the intraduodenal infusion of the probe. The kinetic profile was similar regardless of the physicochemical state of the infusate (i.e., micellar solution or emulsion). Despite the similarity of the kinetic profile, the steady-state level reached was different after infusing emulsions and micellar solutions (approximately 45% and 20% of the infused dose/hr for emulsions and micellar solutions, respectively). When [11,12-^{3}H]retinyl palmitate was infused either in micellar or emulsified form, the steady-state level reached was around 50–60% for both systems. These results are depicted in Figures 1 and 2.

After the infusion of micellar solutions containing tritium-labeled retinol or retinyl palmitate, the total recoveries of ^{3}H-activity in lymph were 49.4% and 58.0% respectively. The difference was probably significant (P = 0.055). Table 1 summarizes these results.

Following the infusion of emulsions containing tritium-labeled retinol, the total percentage of ^{3}H-activity recovered in lymph was 19.1%. In contrast, the recovery of ^{3}H-activity derived from the infusion of [^{3}H]retinyl palmitate was 47.8%. The difference between these values is highly significant (P < 0.0001). Table 1 summarizes the recovery values under the different experimental conditions.

Effects of tetrahydrolipstatin on retinol and retinyl palmitate lymphatic transport. The presence of THL at a final concentration of 10^{-4}M in the micellar solution decreased to a high extent the total lymphatic output of ^{3}H-radioactivity derived from [^{3}H]retinyl palmitate

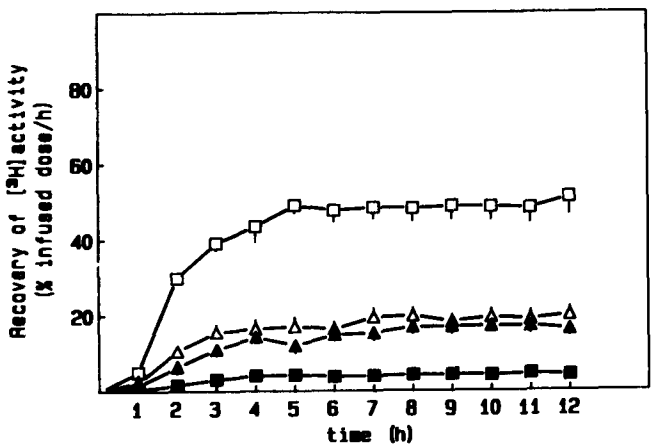

FIG. 1. Hourly recovery (% of the infused dose/hr) of ^{3}H-activity in lymph during a 12-hr infusion of emulsions containing: [^{3}H]retinol + [^{14}C]triolein + phosphatidylcholine + bile salts with (▲) or without (△) THL; [^{3}H]retinyl palmitate + [^{14}C]triolein + phosphatidylcholine + bile salts with (■) or without (□) THL. Data points represent mean ± SEM.

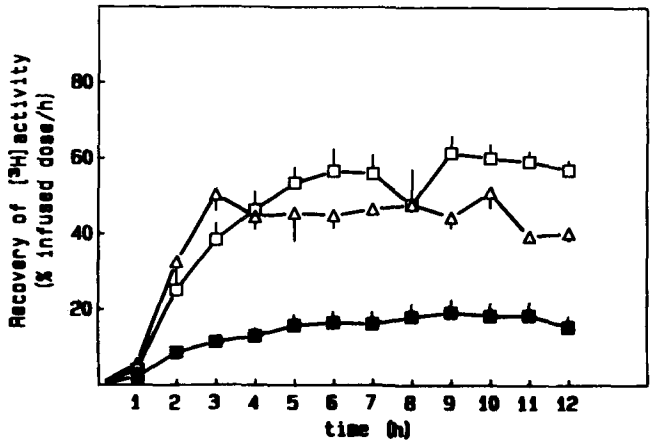

FIG. 2. Hourly % recovery (% of the infused dose/hr) of ^{3}H activity in lymph during a 12-hr infusion of micellar solutions containing: [^{3}H]retinol + [^{14}C]oleic acid + monoolein + bile salts (△); [^{3}H]retinyl palmitate + [^{14}C]oleic acid + monoolein + bile salts with (■) or without (□) THL. Data points represent mean ± SEM.

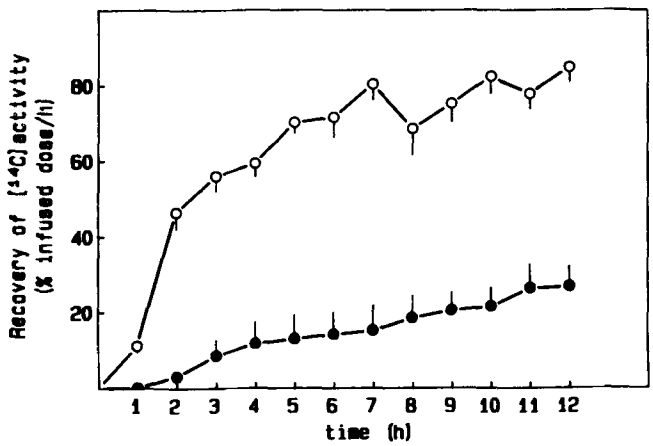

FIG. 3. Hourly % recovery of ^{14}C activity in lymph after a 12-hr infusion of emulsions containing: [^{3}H]retinol + [^{14}C]triolein + phosphatidylcholine + bile salts with (●) or without (○) THL. Data points represent mean ± SEM.

TABLE 1

Total 24-hr Lymphatic Recovery of [^{3}H]Activity from Infused [^{3}H]Retinol and [^{3}H]Retinyl Palmitate[a]

	% recovery in 24-hr lymph
Micellar infusates	
Retinol	49.4 ± 3.8[b]
Retinyl palmitate	58.0 ± 8.2
Retinyl palmitate + THL 10^{-4}M	17.4 ± 8.7[c]
Emulsified infusates	
Retinol	18.0 ± 6.0[d]
Retinol + THL 10^{-4}M	15.7 ± 2.1
Retinyl palmitate	47.8 ± 7.8[e]
Retinyl palmitate + THL 10^{-4}M	5.0 ± 2.4[f]

[a]Data are presented as mean % recovery ± standard deviation.

[b]Probably significantly different compared to micellar retinyl palmitate ($P < 0.055$).

[c]Significantly lower than micellar retinyl palmitate ($P < 0.0001$).

[d]Significantly lower than micellar retinol ($P < 0.0001$).

[e]Significantly higher than emulsified retinol ($P < 0.0001$).

[f]Significantly lower than micellar retinyl palmitate ($P < 0.0001$).

(control 58.0% recovery; 10^{-4}M THL 17.4% recovery, inhibition = 70%, $P < 0.0001$).

The presence of 10^{-4}M THL in emulsions containing [11,12-^{3}H]retinyl palmitate led to a more pronounced decrease in the total lymphatic output of ^{3}H-radioactivity (5.0% recovery vs 47.8% in controls). In this case the percentage inhibition was 90%.

The total lymphatic recovery of ^{3}H-radioactivity from emulsions containing [^{3}H]retinol was slightly lower in the presence of 10^{-4}M THL, although the difference with respect to controls was not significant (15.7% vs 18.0%, $P = 0.34$, Table 1).

The TLC analysis of the pooled lymph extracts showed that most of the ^{3}H-radioactivity (72.2 to 91.3%) is found in the retinyl palmitate fraction regardless of the chemical form of retinol infused. THL did not induce any significant difference in the distribution pattern of retinol/retinyl palmitate in the lymph extracts (77.0% to 95.6% of the radioactivity was found in the retinyl palmitate fraction).

The lymphatic output of ^{14}C-radioactivity after infusing emulsified glyceryl tri[1-^{14}C]oleate is significantly decreased (76.5% vs 19.6% recovery in controls and THL 10^{-4}M; $P = 0.0001$, 74.4% inhibition). The kinetics of this process is presented in Figure 3. THL had no effect on the incorporation of ^{14}C-radioactivity derived from micellar infusates containing ^{14}C-labeled oleic acid.

DISCUSSION

Most reports in the literature focus on the intestinal absorption of free retinol and its provitamin forms (8–11), whereas little is known about the relative bioavailability of the esterified form. Adhikari *et al.* (12) studied the absorption of [^{3}H]retinol and unlabeled retinyl palmitate after oral administration to normal and protein-deficient rats. The experimental design did not allow a comparison of the extent of absorption, as only the amount found in the intestinal contents, small intestine and muscles were accounted for. To our knowledge, the present study is the first to report the relative lymphatic transport of [^{3}H]retinol and [^{3}H]retinyl palmitate in the rat. In our experiments, the concentrations of retinol and retinyl palmitate in the infusions were adjusted to give approximately the recommended daily intake of vitamin A during the 12-hr intraduodenal infusion (13). This is particularly relevant considering the results of Hollander and Muralidhara (11) showing that the kinetics of the absorptive process for vitamin A is different when given at physiological and pharmacological levels.

In the present investigation, labeled retinol and retinyl palmitate have been infused intraduodenally in micellar and emulsified form. In the former dispersion, the fat should be in a chemical and physical form available for uptake by the intestinal mucosa, although the retinyl ester would be expected to have to undergo hydrolysis prior to absorption. In the emulsified form, micelles

should be expected to be generated by hydrolysis of the triglycerides (and the retinyl ester bond should be hydrolyzed).

If the transport to the lymph of labeled retinol infused in micellar form (49.4%) is compared to the emulsified (18.0%) form, a significant difference is seen. Such a difference was not seen in a similar study in which cholesterol was the nonpolar lipid (1). So far this difference is unexplained.

The transport of retinol to the lymph from micellar retinol and retinyl palmitate is similar, as is the transport from emulsified retinyl ester. This agrees with the findings for cholesterol and cholesteryl oleate in a previous study (1), although there is a general tendency to a higher absorption from micellar compared to emulsified probes. Such a difference may be explained by the immediate availability of the lipids in micellar form when the infusate reaches the intestine.

The presence of 10^{-4}M THL in the infusate significantly reduces the recovery of triglyceride in the lymph while it does not significantly affect the recovery of fatty acid from micellar infusates (this study and ref. 1). This difference is most likely explained by an inhibition of hydrolysis of triglycerides by THL in the lumen. This effect, however, does not seem strong enough to significantly inhibit retinol absorption.

The lymphatic transport of radioactive retinol from retinyl palmitate is significantly inhibited by THL from micellar as well as emulsified substrates indicating the importance of inhibition of the enzyme hydrolyzing retinyl palmitate. The enzyme most probably responsible for the hydrolysis of retinyl esters is the carboxyl ester lipase (cholesterol ester hydrolase) of pancreatic origin (14). This enzyme has been ascribed importance in the absorption of cholesterol and cholesteryl esters by Bhat and Brockman (15). Although THL at the concentration used in this study had no significant effect on the absorption of free cholesterol from micellar or emulsified dispersions, the inhibition of cholesteryl oleate absorption in both administration forms was much more marked (0.13% and 0.09% transport to the lymph) as compared to the absorption of retinol from retinyl palmitate (17.4 and 5.0%, respectively). A difference in the experimental design between this and the previous study is the difference in concentration of the probes in the infusates (2 μM for retinol and retinyl ester and 150 μM for cholesterol and its ester). How this difference affects the kinetics of digestion and absorption is not known.

The results of this investigation support the idea that retinyl esters have to be hydrolyzed before absorption, and that the enzyme which hydrolyzes retinyl esters is of no importance for the absorption of retinol from the intestine.

TLC analysis of the lymph extracts indicates that retinyl ester is the major form of vitamin A secreted by the enterocyte to the chyle. There was no difference in the distribution pattern of free and esterified retinol in lymph after the administration of retinol or its ester. Furthermore, THL did not induce any significant change in this pattern. This indicates that THL does not modify the reesterification process that takes place in the enterocyte. Similarly, we have previously shown that THL does not affect reesterification of cholesterol. This process, therefore, is not dependent on enzymes which can be efficiently inhibited by THL, or, alternatively, that THL, if absorbed into the enterocyte is metabolized, losing its inhibitory properties.

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Effect of Increasing the Level of ω-3 Fatty Acids on Rat Skeletal Muscle Sarcoplasmic Reticulum

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The effect of dietary supplementation with fish oil as compared to corn oil on the lipid dynamics and calcium ATPase activity of rat skeletal sarcoplasmic reticulum was examined. After four-week supplementation with fish oil, the levels of eicosapentaenoic (20:5ω3), docosapentaenoic (22:5ω3) and docosahexaenoic (22:6ω3) acids in the total lipids were 5.3, 5.5 and 28.1% of the total fatty acids, respectively. In contrast, with corn oil only 22:6 was found (8.9%). The level of these fatty acids in phosphatidylethanolamine from the membranes of animals fed fish oil was 4.2 (20:5), 5.4 (22:5) and 49.1% (22:6); and for phosphatidylcholine it was 5.4 (20:5), 4.6 (22:5) and 17.4% (22:6). Again, in corn oil fed animals, only 22:6 was found in appreciable amounts, namely 28.3% in phosphatidylethanolamine and 1.8% in phosphatidylcholine. The steady state fluorescence anisotropy of 1,6-diphenyl-1,3,5-hexatriene (DPH) was used to assess lipid order and was found to be only slightly less for membranes from animals supplemented with fish oil (0.120) as compared to those supplemented with corn oil (0.124). The calcium ATPase was found to be unaffected by supplementation consistent with the observed modest changes in lipid order as well as with suggestions that the enzyme is relatively insensitive to the level of unsaturation. It could be argued that if large increases in fatty acyl polyunsaturation in mammalian cell membranes would lead to marked alterations in bulk membrane lipid motional properties, this may not be in the interest of preserving physiological function. The complex mixture of phospholipid molecular species present in natural membranes may buffer against this by a type of passive adaptation, without the expenditure of metabolic energy, thus providing a homeoviscous environment able to optimally support membrane protein function.
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The fatty acyl composition of cell membranes can be markedly altered by dietary means, especially by changing the degree of polyunsaturation. For this reason it is important to understand the consequences of such modification in terms of membrane lipid dynamics and function. A great deal of effort has been expended in determining the consequences of dietary modifications in terms of physical parameters such as membrane lipid fluidity, a term used loosely to describe the more precise parameters of rate and order of motion. However, the effects on lipid dynamics of increasing polyunsaturation to high levels is still poorly understood (1–3).

We have been focusing on the effects of increasing the levels of 22:6 and 20:5ω3 series esterified fatty acids in cell membranes (4). There are a wide range of functional effects resulting from ω3 fatty acyl supplementation. For example, there is an increase in hyperthermic sensitivity in cultured L1210 cells when supplemented with 22:6ω3 (5), altered physiological responsiveness and decreased cyclicAMP levels in rat atria after dietary ω3 fatty acyl supplementation (6), effects on mitochondrial function by dietary or hormone mediated effects (7; also, Stubbs and Thayer, unpublished data), reduced cardiac inotropic responsiveness to the α-agonist phenylephrine in isolated perfused rat hearts after dietary ω3 fatty acyl supplementation (8), possibly contributing to the anti-ischemic effect of dietary fish oil (9).

Most tissues take up ω3 fatty acids very readily, while other tissues already have high levels, such as the brain, retina, muscle, etc. Therefore, it is important to determine the consequences in terms of membrane lipid dynamics and function of large increases in the level of the highly unsaturated C_{20} fatty acyl constituents of membrane phospholipids to see if such effects could underlie functional modifications.

The structural characteristics of sarcroplasmic reticulum (SR) Ca^{2+} ATPase have been extensively studied (see ref. 10 for review). A close association between enzyme activity and lipid motional parameters previously has been demonstrated in a number of studies (e.g., refs. 10–15). The lipid motional parameters are affected in turn by the nature of the fatty acyl chains. There have been only a few studies on the effect of elevated polyunsaturated fatty acid levels on skeletal muscle SR. In most species, SR already has appreciable levels of ω3 fatty acids, and recently Infante (16) suggested that 22:6ω3 phospholipids may be especially important for Ca^{2+} ATPase functioning. Modest changes to the fatty acid composition after safflower oil supplementation have been reported (17), but no effects on the Ca^{2+} ATPase activity were found. In a more recent study (18) using rabbits, 22:6ω3 seemed to be virtually absent, and even after fish oil dietary supplementation the values remained very low and, not surprisingly, little effect on Ca^{2+} ATPase was found. By contrast, the mouse skeletal muscle SR has high levels of ω3 fatty acids (19,20) and these rose to even higher levels after fish oil dietary supplementation. Again, no effect on the activity of the Ca^{2+} ATPase was shown, however, correlation with effects on lipid dynamics were not investigated. From other studies, using reconstitution (21), it has been suggested that while the phospholipids of SR at least need to be unsaturated, the extent of unsaturation may be less important in controlling the activity as compared to chain length.

The following study was therefore undertaken to address these questions: i) What are the consequences in terms of bulk lipid dynamics of large increases in ω3

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Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; DPH-PC, 1-acyl-2-propanoyl(DPH)-glycerophosphocholine; GLC, gas liquid chromatography; HPTLC, high performance thin-layer chromatography; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SR, sacroplasmic reticulum; TMA-DPH, trimethylammonium-DPH; fatty acids are designated as the number of carbons:number of *cis*-double bonds.

esterified fatty acids; and ii) what is the effect on SR Ca^{2+} ATPase activity?

MATERIALS AND METHODS

Animals and diets. Male Sprague Dawley rats (75–100 g) were fed with fat free chow (Diets, Inc., Bethlehem, PA) supplemented with either 50 g/kg (12 energy %) corn or menhaden oil. Details are as described elsewhere (8). 1,6-Diphenyl-1,3,5-hexatriene (DPH), trimethylammonium-DPH and 1-acyl-2-propanoyl(DPH)-glycerophosphocholine (DPH-PC) were from Molecular Probes (Eugene, OR).

Preparation of SR membranes. SR membranes from rat skeletal muscle were prepared by a method based on that of MacLennon (22). Briefly, rat rear leg white muscle was removed and cooled in ice water. The muscle was then cut into small pieces and homogenized in a Waring blender with 3 vol 0.12M NaCl and 5 mM imidazole, pH 7.4. The suspension was then centrifuged at 1600 × *g* for 10 min. The pellet was resuspended and reprocessed similarly. The supernatants were filtered through muslin and centrifuged at 10000 × *g* for 15 min. The supernatant was resuspended and then centrifuged at 44000 × *g* for 50 min. Protein was determined according to Lowry *et al.* (23). The yield of SR membrane from rat skeletal muscle is of the order of 2–4 mg protein/animal.

Determination of activity Ca^{2+} ATPase activity. Calcium ATPase activity was determined by a coupled enzyme assay (24). The assay mixture (1 mL) consisted of 50 mM HEPES buffer, pH 7.4, 5 mM $MgSO_4$, 1 mM $CaCl_2$, 100 mM phosphoenolpyruvate, 15 mM NADH, 250 mM ATP, pyruvate kinase (9 units) and lactate dehydrogenase (13 units). The calcium ATPase activity was calculated from the rate of NADH oxidation in the presence and absence of 5 μM ionophore to uncouple Ca^{2+} transport from the calcium ATPase activity.

Lipid separations and analysis. Lipids were extracted according to the method of Bligh and Dyer (25), and phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were separated using Merck high performance thin-layer chromatography (HPTLC) plates (Darmstadt, West Germany) (26). Bands were identified with the aid of standards, visualized by spraying with dichlorofluorescein, and then scraped from the plate. The fatty acids were saponified (27) and analyzed by gas liquid chromatography (GLC) on a Hewlett-Packard 5890A GLC (Hewlett Packard Co., Palo Alto, CA) using a diethylene glycol succinate column. The methyl esters were identified by use of authentic standards and based on equivalent chain length data (28).

Fluorescence measurements. Intact membranes and multilamellar vesicles were prepared and labeled with DPH, TMA-DPH or DPH-PC as previously described (29).

Fluorescence lifetime and anisotropy data were obtained using an SLM 48000 multifrequency phase-modulation fluorimeter (SLM Instruments, Urbana, IL) with excitation from a Liconix HeCd laser (Sunnyvale, CA) at 325 nm. The light was modulated by a Pockels cell for a range of frequencies from 5–150 MHz. The phase shift and demodulated emission observed through a 420 nm high-pass filter and a Glan Thomson polarizer, set at the magic angle, was analyzed using an IBM PC-XT and associated SLM software as previously described (30, 31). POPOP (lifetime 1.35 ns) was used as the lifetime reference. Steady state fluorescence anisotropy was determined as previously described (4).

RESULTS

Lipid composition. The fatty acid composition of rat SR lipids after corn and fish oil supplementation is shown in Table 1. Fish oil supplementation led to a marked increase in the level of all the ω3 fatty acids, especially 22:6. The ω3 fatty acids mainly replaced 20:4 and also 18:2, leading to a large increase in polyunsaturation but little change in the levels of saturated fatty acids. The PE contained 45% of 22:6 after fish oil supplementation compared to only 25.3% with the corn oil; for PC the levels of 22:6 were 1.71% (corn oil) and 15.13% (fish oil). The proportion of saturated and monounsaturated fatty acids in the SR were not appreciably different for the two diets. Thus, by choosing to compare fish oil and corn oil supplementation we were able to essentially compare membranes with a different degree of polyunsaturation.

Ca^{2+} ATPase activity. The Ca^{2+} dependent ATPase activity for SR from animals fed corn oil supplemented diets was essentially the same as for fish oil fed animals (Table 2). In the presence of ionomycin (5 μM), the activities increased by a factor of ~1.8–2. Thus there was no difference in the Ca^{2+} ATPase activity for the two dietary treatments.

Lipids dynamics. The effect of the fish oil supplemented diet as compared to the corn oil diet on membrane lipid dynamics in intact membranes was assessed by fluroescence spectroscopic measurements on the three DPH fluorophores—DPH, TMA-DPH and DPH-PC. Comparisons of the steady state fluorescence anisotropy values for membranes from animals receiving corn and fish oil supplemented diets are shown in Table 2. The results show that although fish oil supplementation leads to a lower anisotropy value for DPH when compared to membranes from animals that had received corn oil supplementation, the difference is barely significant. Multilamellar vesicles made from total SR lipid extracts labelled with DPH were also examined, as were vesicles made from extracted PC. No major differences in the steady state fluorescence anisotropy values were apparent, although at 8°C a difference was apparent for PC. TMA-DPH probes a region closer to the phospholipid head groups, while DPH-PC is a PC molecule with an *sn*-2 DPH positioned at a depth between that of TMA-DPH and DPH. No major differences in the steady state fluorescence anisotropies were apparent for the membranes from the two dietary treatments.

Comparison of the steady state anisotropies for DPH in intact membranes and in vesicles of extracted PC and for TMA-DPH in intact membranes for a range of temperatures is shown in Figure 1. For DPH in intact membranes a small difference was maintained over the temperature range, whereas for TMA-DPH there was little difference over the range. For PC there was little difference at ~37°C, but an increasing difference for lower temperatures.

If the fluorescence lifetime of a fluorophore is altered then this will affect the steady state fluorescence anisotropy. Therefore, it is important when comparing the steady state fluorescence anisotropies of different samples to make sure that the fluorescence lifetime is not

TABLE 1

Major Fatty Acids of Rat Sarcoplasmic Reticulum Lipids After Dietary Supplementation With Corn or Fish Oil[a]

	Total lipids		Phosphatidylcholine		Phosphatidylethanolamine	
	Corn	Fish	Corn	Fish	Corn	Fish
14:0	0.36 ± 0.03	0.66 ± 0.05	0.22 ± 0.09	0.52 ± 0.24	0.21 ± 0.30	0.21 ± 0.17
16:0	25.00 ± 0.99	26.13 ± 0.67	37.45 ± 1.36	39.70 ± 1.03	10.16 ± 0.51	10.99 ± 1.64
16:1ω7	1.08 ± 0.05	2.69 ± 0.14	0.87 ± 0.10	2.98 ± 0.30	0.29 ± 0.21	0.73 ± 0.27
18:0	14.19 ± 0.64	11.80 ± 0.23	8.02 ± 0.74	5.32 ± 0.89	26.44 ± 2.63	21.96 ± 2.33
18:1ω9	5.97 ± 0.28	6.48 ± 0.29	5.06 ± 0.30	7.69 ± 1.11	4.62 ± 0.28	3.74 ± 0.20
18:1ω7	4.42 ± 0.18	4.72 ± 0.12	6.51 ± 0.73	8.14 ± 1.05	2.00 ± 0.18	1.91 ± 0.56
18:2ω6	18.61 ± 1.15	5.96 ± 0.41	20.92 ± 1.31	6.25 ± 1.41	6.96 ± 0.49	1.88 ± 0.37
20:4ω6	20.60 ± 0.85	6.23 ± 0.74	18.84 ± 2.58	5.90 ± 0.68	20.92 ± 1.73	3.81 ± 0.51
20:5ω3	tr[b]	5.38 ± 0.89	tr	4.46 ± 0.68	tr	3.76 ± 0.52
22:5ω3	1.52 ± 0.23	5.04 ± 0.07	0.41 ± 0.07	3.90 ± 0.31	3.08 ± 1.02	6.05 ± 0.67
22:6ω3	8.19 ± 0.47	24.91 ± 1.49	1.71 ± 0.52	15.13 ± 2.72	25.31 ± 2.37	44.96 ± 1.28

[a]Results are the mean of three separate experiments ± std. dev. [b]Trace.

TABLE 2

Effect of Dietary Corn and Fish Oil Supplementation on the Calcium ATPase Activity and on the Fluorescence Anisotropies of DPH Fluorophores[a]

	Dietary treatment	
	Corn	Fish
(A) Ca^{2+} ATPase activity (I.U.):	5.9 ± 2.49[b]	5.66 ± 1.87[b]
(B) Fluorescence anisotropy		
DPH:intact membranes	0.120 ± 0.006[c]	0.124 ± 0.006[c]
lipids[d]	0.097	0.102
PC[e]	0.085	0.084
PC[f]	0.173	0.165
DPH-PC:intact membranes	0.256	0.254
TMA-DPH:intact membranes	0.254	0.252

Experimental details are given in the Materials and Methods section.
[a]All measurements were at 37°C except where stated.
[b]Mean from six pairs of animals ± std. dev., P<0.1 (paired t-test).
[c]Mean from four pairs of animals ± std. dev., P<0.8 (paired t-test).
[d]Multilamellar vesicles of extracted lipids.
[e]Multilamellar vesicles of extracted and separated phosphatidylcholine.
[f]8°C.

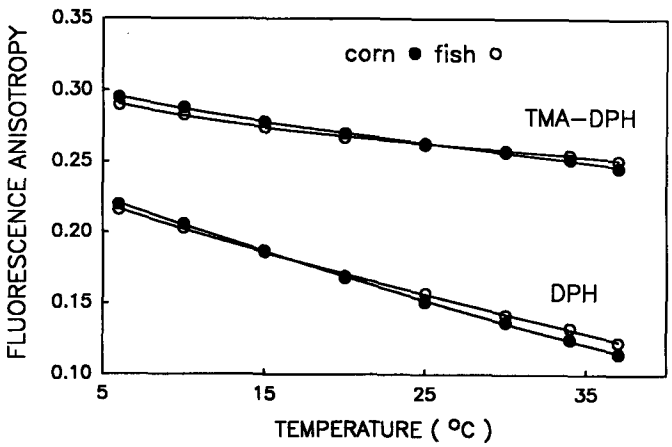

FIG. 1. Temperature dependence of the steady state fluorescence anisotropy of DPH and TMA-DPH in intact SR membranes. Details are as described in Materials and Methods.

different. The fluorescence lifetime data for DPH and TMA-DPH are shown in Table 3. Recently it has been shown that the fluorescence lifetime data can be analyzed in the form of a continuous distribution as an alternative to the discrete multiexponential analysis (for examples see refs. 32-37). Both forms are shown in Table 3. It can be seen that the major lifetime centers, pre-exponents and distributional widths (i.e., half-maximum peak height, assuming Lorentzian forms for the distribution) are the same for membranes from animals receiving corn and fish supplemented diets. Therefore, the comparison of the steady state anisotropy values as indicators of the relative (bulk) lipid motional properties are reasonable for these samples. We also determined time-resolved anisotropy parameters for TMA-DPH in intact SR membranes. The most appropriate model (using a limiting anisotropy of 0.395) was found to be a single rotational correlation time and r_∞, the values being 1.34 ns and

TABLE 3

Comparison of the Fluorescence Decay Properties of DPH and TMA-DPH in SR From Animals Fed With Corn or Fish Oil Supplementations[a]

	f_1	τ_1	w_1	f_2	τ_2	w_2	χ^2
(A) DPH							
Corn							
20°C	0.83	10.61		0.17	5.06		2.14
	0.99	9.81	5.08	0.01	1.80	0.01	1.68
37°C	0.83	9.84		0.17	1.17		1.57
	0.99	9.06	4.9	0.01	1.53	1.02	1.12
Fish							
20°C	0.84	10.53		0.16	4.92		1.47
	0.99	9.81	4.85	0.01	2.39	0.0	1.05
37°C	0.8	9.94		0.2	4.94		1.65
	0.99	9.04	4.77	0.01	1.17	0.02	1.34
(B) TMA-DPH							
Corn							
37°C	0.77	5.24		0.23	1.34		10.14
	1.00	4.40	4.36[b]				1.95
Fish							
37°C	0.78	5.20		0.22	1.28		10.71
	1.00	4.41	4.40[b]				1.78

[a]SR membranes were labeled at a DPH or TMA-DPH/lipid ratio of 1:400, and measured by phase-modulation fluorometry at frequencies between 5 and 120 MHz. The χ^2 values were calculated using errors of 0.2° and 0.002 in phase and modulation, respectively. $f_{1,2}$, Fraction of exponential or Lorentzian term; $\tau_{1,2}$, lifetime centers (ns); $w_{1,2}$, half-widths of Lorentzians (ns).

[b]Unimodal distribution.

0.259, respectively, for corn oil supplementation, and 1.31 ns and 0.257 for fish oil.

DISCUSSION

A major point we wished to address in this work was whether high enough levels of ω3 fatty acyl supplementation could be achieved by dietary means to affect the bulk physical properties and functioning of the membrane. For comparison we used a standard, fat-free, chow diet supplemented with corn oil. Corn oil was chosen since it gave a more controlled comparison than a standard diet and since the polyunsaturated fatty acids are of the ω6 type. In fact, the fatty acyl composition of the corn oil is very close to that of the standard chow diet. The main aim of this study was to obtain two membrane preparations with a substantial difference in the polyunsaturated fatty acid content. In our experience, the actual level of supplementation can be varied considerably from 5–20% with substantial levels of incorporation being achieved. The level used (12%) reflected a compromise between an over-long period of dietary treatment and the difficulty of handling diets with high fat levels.

It has been proposed that the activity of SR Ca^{2+} ATPase is insensitive to the degree of unsaturation, but rather is affected by fatty acyl chain length (21). This suggestion was made mainly on the basis of reconstitution studies which used monounsaturated lipids. The effect of reconstitution into ω3 containing phospholipids has not yet been tested. The dietary approach affords the opportunity to examine this idea using a much more unsaturated and more natural system.

There have been two previous studies on the effect of fish oil supplementation on SR membranes. In one study on rabbit SR, ω3 fatty acids were hardly present before supplementation and not at all elevated after fish oil supplementation, so that the question of the effect of large levels of ω3 supplementation could not be addressed. By contrast, in the mouse large increases in the ω3 fatty acids recently have been achieved (19,20). However, only a total lipid analysis was performed, leaving the question of the extent of incorporation into the major phospholipids and its effect on lipid dynamics. Very little effect was found in respect to Ca^{2+} ATPase activity of the SR, and although some effect on Ca^{2+} transport was noted, this was not attributed to specific effects of the unsaturated fatty acids on the Ca^{2+} ATPase itself, but was thought to be due to destabilizing of the membrane and uncoupling of the enzyme.

With rat skeletal SR, we were able to achieve marked increases of the ω3 esterified fatty acids by dietary means. PE in particular increased its 22:6 content dramatically after fish oil supplementation. In a number of studies PE has been shown to have a role in Ca^{2+} ATPase activity (38–40). The lipids in SR are asymmetrically distributed across the lipid phase (13,41,42; also see review in ref. 43) with 70% of the PE located in the cytoplasmic side of the membrane. This means that the major fatty acid at the *sn*-2 position on this side of the membrane was 22:6ω3. The protein is also asymmetrically distributed (44), but the relationship between lipid and protein assymetry remains to be established. Conformational changes are, however, known to be important in the Ca^{2+} ATPase kinetic mechanism and it has been shown recently that ATPase movement accompanying phosphorylation involves the lipid phase (41). In spite of these considerations, we were unable to demonstrate an effect on ATPase activity. This would support the idea that the SR Ca^{2+} ATPase is insensitive to unsaturation changes. Since the Ca^{2+} ATPase is sensitive to the lipid dynamics of the lipid phase, this leaves the question whether the supplementation achieved any effect on lipid dynamics.

A number of fluorophores were used in the present study to examine the lipid dynamics and its modulation by increased levels of ω3 fatty acids. The multiple double bonds in the C_{20} and C_{22} fatty acyl chains cause the chain to assume a helical configuration (45,1) and the chain is shortened to 1.8 nm. This means that the double bonds are distributed evenly along the chain. The first occurs at about 0.3 nm from the glycerol backbone and the last at about 1.5 nm. DPH-PC contains a propanoyl group, which means that the DPH spans from about 0.2–0.3 nm from the glycerol backbone to 15 nm into the bilayer (46,47). Thus, the DPH of DPH-PC and the 22:6 double bonds are located in the same region of the bilayer. The TMA-DPH probed region will be more towards the bilayer surface, but should still overlap with the double bonds. The location of free DPH is somewhat uncertain, although it would appear to be located mainly in the center of the bilayer (48). Therefore, we would conclude that by and large, the DPH fluorophores should adequately probe any effects that ω3 fatty acyl unsaturation

might have. The results showed only very modest disordering of the SR membranes brought about by increased ω3 unsaturation. It is, of course, possible that other components of the membrane would change in a manner that would compensate for the increase in ω3 unsaturation. For example, cholesterol levels could have changed, although in SR cholesterol is only a minor component, accounting for less than 5% of the lipids (49,50). Since we did not examine this in the present work, we cannot rule out the possibility. However, in most previous studies dealing with elevations of unsaturated fatty acids cholesterol levels have not been found to change. The general conclusion is that this is probably due to the already high degree of unsaturation in SR membranes, ω3 supplementation is unable to further decrease lipid order. One is therefore led to conclude that the lipid "disorder" of the SR membranes is already near a maximal value that can be attained or that the extremely complex mixture of molecular phospholipids species is able to absorb the changes in unsaturation without large effects on lipid dynamics. This could explain the lack of an effect on the Ca^{2+} ATPase activity.

In contrast to the natural membrane studies, when single species of phospholipids with differing levels of *cis*-unsaturation are examined and compared in terms of lipid order and dynamics, marked differences are apparent (4,51,52). Although the gel-to-liquid phase transition temperature in a highly unsaturated species may be similar to that in monounsaturated species (for example, the phase transitions of 16:0/18:1-PC and 16:0/22:6-PC are very similar; refs. 53,54), it is clear that bilayers of phospholipids with a 22:6ω3 fatty acyl chain are considerably less ordered than are monounsaturated species (4,55). This contrasts with the much smaller differences in lipid order and dynamics when natural membranes with marked differences in unsaturation are compared, as in the liver microsome (4) or in SR in the present study. In mammalian tissues, there does not seem to a need for a mechanism to prevent comprehensive changes in the membrane phospholipid fatty acid profiles. We suggest that in natural membranes the complex mixture of phospholipid molecular species can, by itself, act as a passive buffer to prevent the response from large changes in fatty acyl chain compositions and motional properties. The physical mechanism behind such a "passive homeoviscous adaptation" remains to be elucidated. We note, however, that micro-organizational features in membranes such as domains or specific protein-lipid interactions might fall outside the sphere of this process and are retained as a means to modulate membrane protein functioning.

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Comparison of Cell Membrane Phospholipid Fatty Acids in Five Rat Strains Fed Four Test Diets

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The fatty acid composition of phospholipids in peritoneal exudate cells and spleen cells was assessed in five rat strains fed four test diets of differing fatty acid composition. Distinctive patterns of fatty acids were seen in the total phospholipid preparations in both cell types in response to the diets which contained either olive, sunflower, linseed or fish oil. In general, similar fatty acid profiles were seen in each of the rat strains fed the same diet with the only evidence of possible genetic (strain) variation being a relative deficiency of Δ4 desaturase in Dark Agouti rats.
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Following absorption, dietary fatty acids are transported in lipoproteins and taken up by hepatic and other cells, where they may be elongated and desaturated through multiple enzymatic steps (1). Twenty-carbon fatty acids derived from dietary precursors or provided in the diet can be transferred *via* plasma lipoproteins to cells throughout the body where they act as substrates for several oxygenase enzymes. Two of these enzymes (cyclooxygenase and 5-lipoxygenase) are pivotal in the multienzyme pathways which lead to the synthesis of the prostaglandins and leukotrienes (eicosanoids), which have important mediating functions in many homeostatic and pathological processes throughout the body. Thus, the effects of treatments which involve changing the proportions of fatty acids in the diet will depend upon multiple metabolic transformations, each of which is potentially subject to varying degrees of biological variation. Under these circumstances, possible genetically determined diversity in response becomes an important issue. A search for such diversity in rat strains is important as a basis for assessing the likely generality of findings obtained in a single rat strain and may also yield a model for metabolic diversity in human populations.

Important differences between rat strains have been observed in some relevant experimental settings. For example, rat strains may differ in their clinical responses to the anti-inflammatory effects of fish-oil supplemented diets on adjuvant induced polyarthritis (2). Rat strains have also been shown to differ in the rate at which they metabolize chylomicrons following fat ingestion (3). Accordingly, the present study was undertaken to assess the extent to which rat strains may vary in their responses to modification of dietary fatty acids in relation to the composition of phospholipid fatty acids in leucocyte membranes.

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Abbreviations: AA, arachidonic acid; ALA, α-linolenic acid; DGLA, dihomoylinolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; GLC, gas-liquid chromatography; LA, linoleic acid; PBS, phosphate buffered saline; TLC, thin-layer chromatography.

METHODS

Rats. Five rat strains were studied—Dark Agouti, Ginger Hooded, Porton, Sprague Dawley and Hooded Wistar. Weanling rats were obtained from the Gilles Plains Animal Breeding Facility of the South Australian Department of Agriculture. Cohorts of 16 rats were divided into four groups providing four rats for each dietary treatment group. The rat strains were entered sequentially at 1-week intervals with all animals entering the dietary treatment period at six weeks of age. Studies with Dark Agouti and Hooded Wistar (control) rats were repeated to corroborate differences seen in the Dark Agouti rats in the initial study.

Diets. Diets were prepared from fat-free rat chow (Milne Feeds, Perth, Australia) to which the oil supplements were added to achieve a final fat content of 10% (w/w, dry weight). Four oil supplements were used—sunflower (Flora, Unilever Pty. Ltd., Adelaide, Australia), olive (F.H. Faulding & Co. Pty. Ltd., Adelaide, Australia), linseed (Diggers Trading Co., Adelaide, Australia) and fish oil (Maxepa, R.P. Scherer, Melbourne, Australia). Vitamin E was added to achieve a final concentration of 1.84 mg/g oil. The oils and other constituents were mixed with sufficient water to allow 3 × 3 × 1 cm biscuits to be prepared. These were air dried overnight at room temperature. The food was not examined for lipid oxidation, but all diets contained the same levels of vitamin E. The proportions of major fatty acids in the diets are shown in Table 1. The diets were stored at −20°C until used. The rats were fed test diets and water *ad libitum*, with food being changed each evening. The diets were continued for four weeks until induction of peritoneal exudates. All diets resulted in similar weight gains; e.g., the average weight gain over the four-week period for Porton rats were; olive, 182 g; sunflower, 167 g; linseed, 169 g; and fish, 176 g.

TABLE 1

Fatty Acid Profile of Diets[a]

	Diet			
	Olive	Sunflower	Linseed	Fish
Oleic 18:1n-9	74.0	26.6	22.1	10.0
Linoleic 18:2n-6	8.0	57.7	17.0	1.7
Arachidonic 20:4n-6	—	—	—	1.1
α-Linolenic 18:3n-3	1.0	1.6	46.8	0.8
Eicosapentaenoic 20:5n-3	—	—	—	16.5
Docosahexaenoic 22:6n-3	—	—	—	11.7

[a]Expressed as percentage of total fatty acids.

Induction of peritoneal exudates. Exudates were induced 4 hr before sacrifice by injection into the peritoneal cavity of 3% bacteriological grade peptone (Oxoid Ltd., Hampshire, U.K.) in 10 mL of normal saline. At 4 hr, rats were anaesthetized (Halothane) and blood was collected by cardiac puncture. The anaesthetized animals were sacrificed by cervical dislocation. Peritoneal exudate cells were harvested by lavage with 20 mL phosphate buffered saline (PBS). Typically, 1×10^8 leucocytes were obtained. The cells comprised 80–90% neutrophils as assessed microscopically following nuclear staining with Gentian Violet. The cell preparations were washed twice in PBS, which included one red cell lysis treatment with hypotonic saline (30 seconds) and finally resuspended in normal saline. Membrane lipids were then extracted in chloroform/methanol (2:1, v/v) containing butylated hydroxy anisole (0.005% w/w) according to the method of Bligh and Dyer (4) and stored at −20°C prior to gas-liquid chromatographic (GLC) analyses.

Preparation of spleen cells. The spleen was removed and cells were dispersed by passage through a fine wire mesh. Cells were sedimented by centrifugation. Erythrocytes were lysed by 20-second exposure to distilled water. The wash and lysis procedure was repeated once, after which cells were washed and finally resuspended in normal saline. Membrane lipids were then extracted in chloroform/methanol as described above for peritoneal exudate cells.

Separation of tissue phospholipids. Phospholipids in cell membrane extracts were separated from the total extracted lipid by thin-layer chromatography (TLC) on silica gel H plates developed in petroleum ether/diethyl ether/acetic acid (90:15:1, v/v/v). The band containing the total phospholipids remaining at the origin was scraped into the methylating solution and methylated in 1% (v/v) H_2SO_4 in methanol by heating at 70°C for 3 hr. The resulting fatty acid methyl esters were extracted and analyzed by gas chromatography.

Tissue phospholipid fatty acid analysis. Analyses of the fatty acid methyl esters of total phospholipids were performed using capillary gas chromatography (Hewlett Packard HP 5880 gas chromatograph; Hewlett Packard, Palo Alto, CA). The column was a 50-meter glass column (0.56 mm, I.D.) coated with SP2340 (Supelco Inc.) prepared by Chromolytic Technology Ltd. (Boronia, Victoria). The esters were separated using a carrier gas (helium) flow of 25 cm/sec with a temperature gradient of 120°C to 200°C at 5°C per min. The injection/split temperature was 250°C and the flame ionization detector temperature was 300°C. Fatty acid methyl esters were identified against authentic lipid standards supplied by Nuchek Prep (Elysian, MN). The flame ionization detector was calibrated so that the response obtained for each of the fatty acids between C_{14}–C_{24} was identical regardless of the number of double bonds.

Statistical analysis. Newman-Keuls multiple comparisons analysis was used to identify dietary or strain differences (5).

RESULTS

Consistency and reproducibility of findings within groups. Distinctive patterns in the percentage of fatty acids in phospholipids of cell preparations were seen in response to each of the dietary treatments in all rat strains. The effects of the diets were consistent within strains with little variability between animals; e.g., the coefficients of variation for peritoneal exudate cell linoleic acid (LA, 18:2n-6) determinations for Porton rats on the different diets were: olive, 3%; sunflower, 6%; linseed, 11%; and fish, 9%. All determinations had similar values. With one exception (detailed below), no systematic difference was seen between strains given the same dietary treatments.

n-9 Fatty acids. The oleic acid-rich olive oil diet was associated with substantially higher proportions of oleic acid (18:1n-9) in phospholipids in cell membranes than found with the other diets (Table 2). Levels of 20:3n-9 were slightly increased in the cell membranes of olive oil-fed rats as compared with the other treatment groups. However, all levels were less than 2%.

n-6 Fatty acids. There appeared to be no simple relationship between dietary LA and n-6 fatty acids in membranes of peritoneal exudate cells or spleen cells. Although by far the highest proportion of dietary linoleic acid was present in the sunflower oil diet (Table 1), the highest proportions of linoleic acid in cell membrane phospholipids were found in samples from the linseed oil-fed and sunflower oil-fed rats (Table 3). Substantially lower levels were found in olive oil- and fish oil-fed rats. However, arachidonate (AA, 20:4n-6) levels in both cell types were highest in olive oil- and sunflower oil-fed rats and lowest in linseed oil- and fish oil-fed animals. AA was a smaller proportion of the phospholipid fatty acids of spleen cells than peritoneal exudate cells in all treatment groups. Proportions of dihomoγlinolenic acid levels (DGLA, 20:3n-6) were consistently less than 2% in both cell types (data not shown).

The proportions of long chain metabolites, 22:4n-6 and 22:5n-6, were higher in phospholipids from olive oil- and sunflower oil-fed rats (Table 4) as compared with their counterparts from linseed oil- and fish oil-fed rats in which these fatty acids were all less than 1%.

In rats fed olive oil or sunflower oil diets, significantly less 22:5n-6 was found in spleen cell phospholipids of Dark Agouti rats as compared with the other rat strains (Newman-Keuls, $p < 0.05$) (Table 4). These differences were confirmed in a second experiment in which Dark Agouti and Hooded Wistar rats were given the same dietary treatments (data not shown). Less 22:5n-6 was also found in peritoneal exudate cells from Dark Agouti rats fed sunflower oil.

n-3 Fatty acids. Despite the high level of α-linolenic acid (ALA, 18:3n-3) in the diet of linseed oil-fed rats, only small amounts of ALA were found in the cell membrane phospholipids (Table 5). ALA was detected in very small amounts or was undetectable in the phospholipids from the other treatment groups.

Fish oil-fed rats had two to three times higher proportions of eicosapentaenoic acid (EPA, 20:5n-3) in cellular phospholipids compared with linseed oil-fed rats (Table 5). EPA levels were <1% or undetectable in phospholipids from olive oil- and sunflower oil-fed rats.

In both fish oil- and linseed oil-fed rats, peritoneal exudate cell phospholipids were found to have higher proportions of EPA compared with phospholipids from spleen cells. The proportion of 22:5n-3, one of the metabolites of EPA, in membrane phospholipids was greater in spleen cells than peritoneal exudate cells in all

TABLE 2

n-9 Fatty Acid Content[a]

Diet	Rat strain	Peritoneal exudate cells		Spleen cells	
		18:1	20:3	18:1	20:3
Olive oil	DA	25.1 ± 2.9	0.7 ± 0.1	20.5 ± 2.7	0.9 ± 0.1
	GH	18.9 ± 1.3	0.9 ± 0.1	16.7 ± 2.0	1.0 ± 0.0
	P	18.3 ± 0.7	1.3 ± 0.3	17.9 ± 0.8	1.3 ± 0.2
	SD	20.7 ± 0.6	1.5 ± 0.1	16.8 ± 0.1	1.7 ± 0.2
	HW	22.9 ± 1.6	0.7 ± 0.1	19.9 ± 0.7	1.0 ± 0.1
Sunflower oil	DA	13.1 ± 3.0	n.d.[b]	8.9 ± 0.4	n.d.
	GH	8.8 ± 0.4	n.d.	7.9 ± 0.5	n.d.
	P	8.2 ± 0.4	n.d.	7.8 ± 0.4	n.d.
	SD	11.1 ± 0.2	n.d.	8.0 ± 0.2	n.d.
	HW	13.2 ± 2.6	n.d.	9.7 ± 2.3	n.d.
Linseed oil	DA	12.5 ± 0.9	0.4 ± 0.1	11.0 ± 0.1	0.2 ± 0.0
	GH	11.8 ± 0.7	0.1 ± 0.1	10.5 ± 0.1	0.1 ± 0.0
	P	13.0 ± 0.9	n.d.	11.6 ± 0.5	n.d.
	SD	14.5 ± 0.8	0.1 ± 0.0	11.1 ± 0.3	0.1 ± 0.1
	HW	12.8 ± 0.3	0.3 ± 0.1	10.8 ± 0.2	0.2 ± 0.0
Fish oil	DA	12.2 ± 1.2	n.d.	10.0 ± 0.2	0.2 ± 0.0
	GH	12.4 ± 1.8	0.1 ± 0.1	9.6 ± 0.4	0.2 ± 0.0
	P	11.4 ± 1.1	0.1 ± 0.0	10.0 ± 0.2	0.2 ± 0.0
	SD	14.3 ± 1.1	0.5 ± 0.5	9.9 ± 0.6	0.2 ± 0.0
	HW	11.9 ± 0.5	n.d.	9.3 ± 0.3	0.2 ± 0.0

[a]Results are expressed as % of total phospholipid fatty acids and represent the means ± S.D. of data from four rats in each strain. DA, Dark Agouti; GH, Ginger Hooded; P; Porton; SD, Sprague Dawley; HW, Hooded Wistar.

[b]Not detectable; minimum detectable level, 0.1.

TABLE 3

n-6 Fatty Acid Content[a]

Diet	Rat strain	Peritoneal exudate cells		Spleen cells	
		18:2	20:4	18:2	20:4
Olive oil	DA	5.5 ± 0.4	20.7 ± 2.2	6.5 ± 0.2	19.3 ± 2.0
	GH	4.8 ± 1.2	26.6 ± 2.4	5.2 ± 0.8	23.4 ± 2.0
	P	4.8 ± 0.1	24.7 ± 0.3	5.3 ± 0.2	22.1 ± 0.3
	SD	4.5 ± 0.2	23.3 ± 0.4	5.5 ± 0.2	21.4 ± 0.2
	HW	6.0 ± 0.1	24.6 ± 1.9	6.5 ± 0.4	21.4 ± 0.8
Sunflower oil	DA	11.9 ± 0.4	26.4 ± 3.2	12.8 ± 0.2	23.7 ± 0.4
	GH	11.7 ± 0.5	29.2 ± 1.1	11.9 ± 0.8	24.2 ± 0.5
	P	13.2 ± 0.8	29.0 ± 1.0	13.8 ± 1.0	24.6 ± 0.8
	SD	12.1 ± 0.6	24.5 ± 0.7	12.6 ± 0.7	22.1 ± 0.4
	HW	12.8 ± 0.1	27.7 ± 1.9	12.7 ± 0.3	23.9 ± 2.0
Linseed oil	DA	19.6 ± 2.6	14.0 ± 0.9	14.5 ± 0.2	12.4 ± 0.3
	GH	12.2 ± 0.2	20.9 ± 0.9	13.6 ± 0.1	16.3 ± 0.3
	P	12.1 ± 1.3	15.3 ± 1.1	13.4 ± 0.5	12.8 ± 0.7
	SD	11.6 ± 0.4	18.7 ± 1.0	13.5 ± 0.5	14.5 ± 0.6
	HW	14.9 ± 1.5	18.2 ± 0.5	14.9 ± 0.3	13.8 ± 0.3
Fish oil	DA	4.8 ± 0.8	14.1 ± 0.5	3.7 ± 0.2	9.3 ± 0.1
	GH	5.3 ± 1.0	13.2 ± 1.6	4.2 ± 0.5	9.8 ± 0.3
	P	3.3 ± 0.3	12.7 ± 0.7	3.1 ± 0.2	9.3 ± 0.1
	SD	4.1 ± 0.3	14.0 ± 0.2	3.4 ± 0.2	9.7 ± 0.3
	HW	4.6 ± 0.1	14.5 ± 0.8	4.0 ± 0.1	10.0 ± 0.1

[a]Results are expressed as % of total phospholipid fatty acids and represent the means ± S.D. of data from four rats in each strain. Abbreviations as in Table 2.

TABLE 4

22-Carbon n-6 Fatty Acid Content[a]

Diet	Rat Strain	Peritoneal exudate cells		Spleen cells	
		22:4	22:5	22:4	22:5
Olive oil	DA	1.9 ± 0.0	n.d.[b]	1.7 ± 0.2	0.2 ± 0.1[c]
	GH	2.4 ± 0.4	0.4 ± 0.2	2.5 ± 0.1	0.6 ± 0.3
	P	1.7 ± 0.1	0.4 ± 0.1	2.1 ± 0.2	0.5 ± 0.1
	SD	1.9 ± 0.1	0.4 ± 0.0	2.3 ± 0.0	0.7 ± 0.1
	HW	1.4 ± 0.4	0.1 ± 0.1	1.9 ± 0.1	0.3 ± 0.1
Sunflower oil	DA	3.7 ± 0.5	0.3 ± 0.1	3.5 ± 0.1	0.4 ± 0.0[c]
	GH	2.8 ± 0.2	1.0 ± 0.2	3.5 ± 0.2	1.5 ± 0.2
	P	2.3 ± 0.3	0.5 ± 0.1	3.9 ± 0.2	0.7 ± 0.2
	SD	2.5 ± 0.2	0.6 ± 0.1	3.7 ± 0.1	1.0 ± 0.2
	HW	2.3 ± 0.5	0.7 ± 0.1	3.8 ± 0.3	1.2 ± 0.2

[a]Results are expressed as % of total phospholipid fatty acids and represent the means ± S.D. of data from four rats in each strain. Abbreviations as in Table 2.

[b]Not detectable, minimum detectable level, 0.1.

[c]$p < 0.05$, compared with all other strains, Newman-Keuls Analysis.

TABLE 5

n-3 Fatty Acid Content[a]

Diet	Rat strain	Peritoneal exudate cells		Spleen cells	
		18:3	20:5	18:3	20:5
Linseed oil	DA	1.6 ± 0.3	5.2 ± 0.6	1.8 ± 0.1	4.5 ± 0.2
	GH	1.1 ± 0.3	5.2 ± 0.3	1.9 ± 0.1	3.2 ± 0.2
	P	1.7 ± 0.2	5.9 ± 1.4	2.2 ± 0.4	4.1 ± 0.2
	SD	1.7 ± 0.2	4.3 ± 0.4	2.1 ± 0.1	3.6 ± 0.2
	HW	1.2 ± 0.2	5.2 ± 0.5	1.9 ± 0.1	3.8 ± 0.2
Fish oil	DA	0.5 ± 0.3	13.6 ± 0.9	n.d.[b]	10.7 ± 0.1
	GH	0.1 ± 0.1	13.5 ± 1.3	0.1 ± 0.1	9.9 ± 0.4
	P	0.5 ± 0.1	15.6 ± 0.7	n.d.	11.3 ± 0.2
	SD	n.d.	11.8 ± 1.1	n.d.	10.2 ± 0.3
	HW	0.5 ± 0.4	13.4 ± 0.6	0.1 ± 0.1	10.4 ± 0.3

[a]Results are expressed as % of total phospholipid fatty acids and represent the means ± S.D. of data from four rats in each strain. Abbreviations as in Table 2.

treatment groups (Table 6). Only trace amounts of 22:5n-3 were found in phospholipids from the olive oil- and sunflower oil-fed rats.

By far the highest proportions of docosahexaenoic acid (DHA) 22:6n-3 were found in the fish oil-fed animals. DHA constituted a higher proportion of phospholipids of spleen cells than peritoneal exudate cells in all dietary groups except the sunflower oil-fed group. In linseed oil-fed rats, DHA levels were significantly reduced in spleen cell phospholipids from Dark Agouti rats as compared with spleen cell preparations from all other strains (Newman-Keuls, $p < 0.05$) (Table 6). This was confirmed in a second experiment in which Dark Agouti and Hooded Wistar rats were given the same dietary treatments (data not shown). The DHA level in peritoneal exudate cells from linseed oil-fed Dark Agouti rats was lower than those in the other strains, but this difference was not statistically significant (Newman-Keuls, $p = 0.05$).

DISCUSSION

In the present investigations, the cell membrane fatty acid composition of the five rat strains responded in a similar manner to the different dietary treatments. A consistent exception was seen in Dark Agouti rats which, by comparison with the other strains fed similar diets, had reduced levels of long-chain fatty acids (22:5n-6 and 22:6n-3) in cellular phospholipids. DHA was reduced in Dark Agouti rats on the linseed oil diet which is rich in precursor α-linolenic acid (ALA). This reduction was not evident with the sunflower or olive oil diets, possibly due to the greatly reduced levels of dietary α-linolenic acid which may be at saturating levels for the metabolism of ALA to DHA on the linseed oil diet. The long-chain n-6 fatty acid, 22:5n-6, was reduced in Dark Agouti rats on the sunflower and olive oil diets with low levels (<1%) being present in all strains on the other diets. These

TABLE 6

22-Carbon n-3 Fatty Acid Content[a]

Diet	Rat strain	Peritoneal exudate cells		Spleen cells	
		22:5	22:6	22:5	22:6
Olive oil	DA	0.3 ± 0.1	0.8 ± 0.4	0.9 ± 0.1	1.4 ± 0.3
	GH	0.2 ± 0.2	1.1 ± 0.5	0.4 ± 0.1	1.6 ± 0.2
	P	0.1 ± 0.0	0.9 ± 0.7	0.4 ± 0.0	1.3 ± 0.1
	SD	0.1 ± 0.1	0.5 ± 0.0	0.5 ± 0.1	1.3 ± 0.1
	HW	0.1 ± 0.2	0.8 ± 0.3	0.5 ± 0.0	1.7 ± 0.1
Sunflower oil	DA	0.2 ± 0.2	0.7 ± 0.6	0.2 ± 0.0	0.3 ± 0.1
	GH	n.d.[b]	1.4 ± 0.1	0.2 ± 0.0	1.1 ± 0.1
	P	n.d.	0.2 ± 0.1	0.2 ± 0.0	0.4 ± 0.1
	SD	n.d.	1.0 ± 0.1	0.2 ± 0.1	0.8 ± 0.1
	HW	0.1 ± 0.3	0.6 ± 0.4	0.2 ± 0.1	0.8 ± 0.5
Linseed oil	DA	2.2 ± 0.3	0.2 ± 0.0	4.2 ± 0.1	0.4 ± 0.0[c]
	GH	1.7 ± 0.1	0.9 ± 0.4	3.7 ± 0.3	1.8 ± 0.2
	P	1.7 ± 0.1	0.6 ± 0.1	3.6 ± 0.1	1.3 ± 0.2
	SD	1.6 ± 0.1	1.1 ± 0.1	3.7 ± 0.2	1.3 ± 0.1
	HW	1.3 ± 0.2	0.4 ± 0.1	4.1 ± 0.3	1.2 ± 0.2
Fish oil	DA	2.4 ± 0.1	2.4 ± 0.2	4.9 ± 0.2	4.8 ± 0.2
	GH	3.2 ± 1.3	3.2 ± 0.6	4.8 ± 0.2	5.4 ± 0.3
	P	2.7 ± 0.6	3.2 ± 0.5	4.7 ± 0.4	4.8 ± 0.4
	SD	2.0 ± 0.2	2.5 ± 0.5	4.9 ± 0.1	5.0 ± 0.3
	HW	2.1 ± 0.6	2.4 ± 0.3	4.9 ± 0.2	5.8 ± 0.2

[a]Results are expressed as % of total phospholipid fatty acids and represent the means ± S.D. of data from four rats in each strain. Abbreviations as in Table 2.

[b]Not detectable, minimum detectable level, 0.1.

[c]$p < 0.05$, compared with all other strains, Newman-Keuls Analysis.

findings, which were confirmed in a subsequent experiment, suggest the possibility of a relative deficiency in Dark Agouti rats of Δ4 desaturase activity, the enzyme putatively responsible for the synthesis of these fatty acids.

Each of the four test diets was associated with distinct effects common to all of the rat strains studied. However, the unique effects of each diet on phospholipid fatty acid profiles cannot be entirely predicted from the dietary fatty acid profiles. For example, olive oil and sunflower oil have similar ALA levels, but olive oil was associated with higher proportions of the 22-carbon n-3 fatty acids compared with the sunflower oil diet, possibly due to the greater LA/ALA ratio in sunflower oil (36:1) as compared with olive oil (8:1). This latter finding may be relevant to the association of olive oil rich diets with low risk of occlusive vascular disease in humans (6), an association also seen with fish oil diets which are rich in 20- and 22-carbon n-3 fatty acids (7).

The linseed oil diet and the fish oil diet contain substantial amounts of oleic acid (linseed 22% and fish 9%) and yielded similar proportions of n-9 fatty acids in the phospholipids. Both diets were associated with substantially lower levels of arachidonic acid than were found with the olive oil and sunflower oil diets. The fish oil diet was associated with low linoleic acid levels in the phospholipid fractions as expected from the low linoleic acid content of fish oil. However, the linseed oil diet was associated with high levels of linoleic acid in the phospholipids exceeding, or equal to, those found with the sunflower oil diet despite the fact that the content of linoleic acid in the linseed oil diet was only one third of that found in the sunflower oil diet. Similar results have been reported by other workers (8,9). These findings suggest that the effect of the linseed oil diet upon AA levels may result, at least in part, from inhibition of elongase and desaturase enzymes which convert LA to AA and the 22-carbon n-6 fatty acids. Our findings in these *in vivo* studies are thus concordant with *in vitro* biochemical studies in which it has been shown that ALA competitively inhibits the metabolism of LA and its products along this pathway (10). Fish oil fatty acids have been found to be even more potent inhibitors of the Δ6 desaturase than those in the linseed oil (11). In addition, the fish oil diet may depress the AA content of phospholipids through the competitive incorporation of C_{20} and C_{22} n-3 fatty acids found in the diet at the expense of their n-6 fatty acid counterparts.

The limited strain comparisons detailed here suggest that diet may in general be a more important determinant of membrane fatty acid composition than genetic (strain) factors. Nevertheless, there is suggestive evidence in favor of a relative Δ4 desaturase deficiency in one strain, indicating that variable expression of lipid remodeling enzymes can occur.

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Liver Phosphatidylcholine Hydroperoxidation Provoked by Ethionine-Containing Choline-Deficient Diet in Mice

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It is shown that peroxidation of phosphatidylcholine (PC) is enhanced in liver of mice fed a hepatocarcinogenic choline-deficient diet containing 0.1% w/w ethionine. Mice were divided into 4 groups and fed for 4 weeks one of the following diets: choline-supplemented; choline-supplemented containing ethionine; choline-deficient; and choline-deficient containing ethionine. Phosphatidylcholine hydroperoxide (PCOOH) of liver lipids was measured by high performance liquid chromatography using a chemiluminescence detector. Mice fed a choline-deficient diet containing ethionine showed 6-fold higher PCOOH levels than the choline-supplemented control mice: the PCOOH/PC molar ratios of liver lipids were 32.3×10^{-5} and 5.6×10^{-5}, respectively. In addition to this remarkable degree of lipid peroxidation in liver of mice fed the choline-deficient diet containing ethionine, we also observed a significant liver fatty infiltration, a decrease in plasma and liver α-tocopherol, and an increase in liver injury-indicative enzyme activities. Also, marker enzymes for hepatocarcinogenesis, glucose-6-phosphatase and γ-glutamyl transpeptidase were affected. These data suggest that enhanced hydroperoxidation of phosphatidylcholine may participate in hepatocarcinogenesis provoked by choline deficiency in the presence of ethionine.
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The occurrence of hepatocellular carcinomas in rats (1-4) and mice (5-7) after long-term intake of a diet deficient in choline and methionine has revived interest in the relationship between the lack of these lipotropic factors and the formation of neoplasms. Choline is an essential precursor in the biosynthesis of choline phospholipids and of acetylcholine, and is an important source for "labile" methyl groups *in vivo*. Choline deficiency is known to affect almost all organs (8) due to the important roles played by phosphatidylcholine (PC) as constitutent of cell membranes.

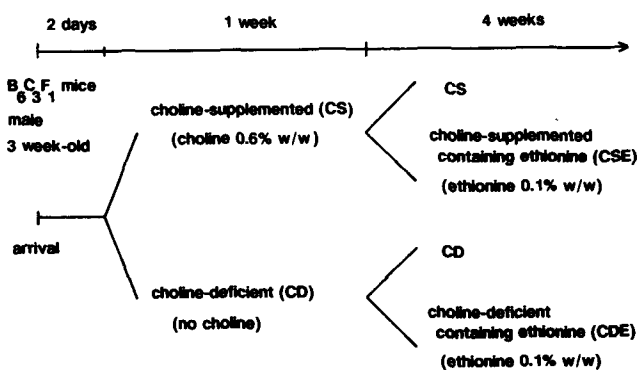
Depletion of PC should have mechanistic relevance, particularly in the liver, where a firm relationship between carcinogenesis and choline deficiency has been established (8,9). Moreover, changes in PC/phosphatidylethanolamine (PE) ratio are known to affect the structural and functional states of hepatocyte membranes (9,10). Among the mechanisms proposed for carcinogenesis, lipid peroxidation has been suspected as a possible route as conjugated dienes were increased in livers of choline-deficient rats (11,12). Nevertheless, this observation was not conclusive in confirming the participation of lipid peroxides in the carcinogenic process.

On the other hand, we have been investigating the toxic effects of dietary lipid peroxides *in vivo* (13-15) as they relate to certain diseases (16). Although it is known that liver PC is reduced during choline deficiency, no reports are available concerning the qualitative state of the remaining PC or, moreover, whether PC itself is peroxidized or not. We therefore investigated specifically the changes in PC caused by choline deficiency alone, or together with dietary ethionine. Ethionine, an analog of methionine, is also a hepatocarcinogen (17), and its carcinogenicity is promoted by choline deficiency (8,18-20).

Our results show that PC peroxidation is elevated in choline-deficient mice, and that addition of ethionine to this diet further enhances PC hydroperoxide accumulation. This suggests a possible role of lipid peroxidative damage in the process of hepatocarcinogenesis induced by dietary modifications.

MATERIALS AND METHODS

Animals and diets. Male 3-week old $B_6C_3F_1$ mice (Charles River Japan Inc., Atsugi, Japan) were acclimatized to a 12-hr light/dark cycle in a temperature controlled room at 25°C. For two days after arrival, they were fed a powdered choline-supplemented (CS) diet [Dyets Inc., Bethlehem, PA; described by Shinozuka *et al.*, (18)]. As shown in Scheme 1, the mice were then divided into 2 groups. One group received the CS diet and the other the choline-deficient (CD) diet of the same composition except for the lack of choline. After one week of feeding the CS or the CD diets, each group was divided into two subgroups, which were fed diets containing or lacking 0.1% (w/w) of D,L-ethionine (Nakarai Chemicals Co., Kyoto, Japan). Mice were fed one of these 4 diets—CS, CD, CS diet containing ethionine (CSE) or CD diet containing ethionine (CDE)—over a period of 4 weeks. Diets were stocked at 0°C to prevent autoxidation and



SCHEME 1. Dietary choline-deficiency and ethionine-feeding in mice. The scheme illustrates the present experimental approach.

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Abbreviations: CD, choline-deficient diet; CDE, CD diet containing ethionine; CL, chemiluminescence; CL-HPLC, chemiluminescence-high performance liquid chromatography; CS, choline-supplemented diet; CSE, CS diet containing ethionine; γ-GGT, gamma-glutamyl transpeptidase; G-6-Pase, glucose-6-phosphatase; GPT, glutamic-pyruvic transaminase; GOT, glutamic-oxaloacetic transaminase; PC, phosphatidylcholine; PCOOH, phosphatidylcholine hydroperoxide; PE, phosphatidylethanolamine; TBA, thiobarbituric acid; TG, triglycerides; TL, total lipids.

supplied every 2 days to assure freshness. Mice were sacrificed under light ether anesthesia. Heparinized blood was collected by heart puncture, and the livers were surgically dissected, weighed and immediately frozen for subsequent analysis.

Determination of PC and PC hydroperoxide in liver. Total lipids (TL) of liver were extracted with chloroform/methanol (2:1, v/v) from liver homogenate (40% w/w) in 0.15 M NaCl containing 0.002% butylhydroxytoluene as antioxidant (21-23). After two successive washings with the same solvent, the chloroform layer was collected and dried under reduced pressure, and the TL content was gravimetrically determined. The PC content was quantified by the Iatroscan thin-layer chromatography/flame ionization detector (TLC/FID) method on a Iatroscan TH 10 apparatus (Iatron Laboratories Inc., Tokyo, Japan) with Chromarod S-II as the stationary phase, according to the method of Hazel (24). A standard curve for L-α-PC from egg yolk (Avanti Polar Lipids, Inc., Birmingham, AL) was made under the same conditions, and a linear relation between the PC peak height (y, in cm) and the PC concentration (x, μg) was obtained: $y = 1.8282x - 4.523$.

PC hydroperoxide (PCOOH) was determined with a chemiluminescence-high performance liquid chromatography (CL-HPLC) system that consisted of a CL analyzer (OX-7, Tohoku Electronic Ind., Sendai, Japan) and a UV detector (JASCO UVIDEC-100-III, Japan Spectroscopic Co., Tokyo, Japan) as described by Miyazawa and colleagues (16,21-23). The extracted liver TL was redissolved in chloroform/methanol (2:1, v/v) and injected into the CL-HPLC using a solution of luminol and cytochrome c as the luminescent reagent (21,23). PCOOH concentration was expressed as nmoles or pmoles of hydroperoxide-O_2, based on a calibration curve obtained with photochemically peroxidized egg yolk PC (16,21-23). The standard linear equation in a log $\times$ log plot was expressed as $y = 9.6576x^{1.1217}$, where y is the integrated CL count (in counts/s) of the PCOOH chromatographic peak area, and x is the PCOOH concentration (pmoles). The curve was linear over the range of 50 to 1000 pmoles of PCOOH.

Biochemical assays. Glutamic oxaloacetic transaminase (GOT, E.C. 2.6.1.1) and glutamic pyruvic transaminase (GPT, E.C. 2.6.1.2) were assayed in plasma using the colorimetric laboratory kit, Iatrozyme TA-Lq (Iatron Laboratories Inc., Tokyo, Japan). The α-tocopherol content was measured by high-performance liquid chromatography (HPLC) (25).

In liver, glucose-6-phosphatase (G-6-Pase) activity was measured according to the method of Harper (26), thiobarbituric acid (TBA) reactive substances were assayed as described by Ohkawa *et al.* (27), and α-tocopherol was determined by HPLC (28). Gamma-glutamyltranspeptidase (γ-GGT) was measured in microsomal suspensions obtained by the standard methods described by Szasz (29). Units of enzyme were expressed as μmoles of *p*-nitroaniline/min at 37°C (30). Assays were done at similar protein concentrations in a suspension containing 50 mM Tris/HCl buffer, pH 7.4, and 1 mM EDTA. Protein was determined by the method of Hartree (31), using serum bovine albumin (Sigma Chemical Co., St. Louis, MO) as a standard.

Statistical analysis. All data were analyzed using the Student's t-test.

RESULTS

Growth and liver fat infiltration. No deaths of mice occurred during the experiments, although growth retardation occurred in mice receiving diets containing ethionine (Table 1). Growth was poor especially in CDE mice with around 14% lower weights than CS mice. No growth differences were noted between the CS and CD groups.

CDE mice had the highest liver-to-body weight ratios among all the dietary groups with CDE mice being 15% higher than CS mice (Table 1). This is most likely a consequence of both low body weight and liver fat accumulation. In fact, the livers of CD and CDE mice had a yellowish appearance when dissected, indicating fatty infiltration. This was confirmed by TL gravimetric determination (Table 1), as CD and CDE mice had more than twice the liver TL content of CS mice.

Liver PC and PCOOH. The PC content expressed *per unit weight of liver* was lower in CD and CDE mice when compared with control CS (Table 2). However, when expressed on a *per liver basis,* PC was significantly decreased in CD mice, elevated in CSE mice, and unchanged in the CDE group in comparison with the CS group. Nevertheless, the PC/PE ratios in livers of CD, CSE and CDE mice were lower than in CS mice. The proportion of PCOOH relative to nonperoxidized PC (PC/PCOOH molar ratio) was approximately 2, 4 and 6 times higher in CD, CSE and CDE mice, respectively, compared with the CS group. When PCOOH is expressed as *nmoles per g of liver,* a 5- to 6-fold increase over CS controls was observed in mice that had received ethionine, CSE and CDE. These proportions of PCOOH were in good agreement with those calculated on a per liver basis.

Effects on plasma. Glutamic-oxaloacetic transaminase (GOT) and glutamic-pyruvic transaminase (GPT) activities were significantly elevated in CDE mice, with a 6-fold increase in GPT activity found in CS mice (Table 3). In comparison with the control CS mice, plasma α-tocopherol levels were diminished in CD and CDE mice, but elevated in CSE mice.

Liver assays. Lower α-tocopherol contents were found in the liver of the CD, CSE and CDE groups compared with the control CS group (Table 4). Levels of TBA-reactive substances were 62% higher in CDE mice than in CS mice. G-6-Pase was one order of magnitude lower in CDE mice as compared to the other groups. In mice fed the CSE and CDE diets (Table 4), microsomal γ-GGT activity was approximately double that in CS mice.

TABLE 1

Effects of Dietary Choline-Deficiency and Ethionine on Mice Growth and Liver Weight After 4 Weeks

Diet	Body weight (g)	Liver/Body weight ratio (%)	Liver total lipid (mg/100mg liver)
CS	25.05 ± 1.38	4.74 ± 0.21	3.27 ± 0.09
CD	24.41 ± 1.06	4.89 ± 0.19	6.19 ± 1.72[a]
CSE	23.22 ± 0.87[a,b]	4.96 ± 0.28	4.26 ± 0.14
CDE	21.54 ± 0.99[a,b,c]	5.46 ± 0.34[a,b]	7.76 ± 0.89[a,c]

Mean ± SD for 5 to 6 mice per each group.
[a]Significantly different from CS at P<0.005.
[b]Significantly different from CD at P<0.005.
[c]Significantly different from CSE at P<0.005.

TABLE 2

PC and PCOOH Contents in Livers of Mice Fed Choline-Deficient Diet Containing Ethionine

Diet	PC (mg/liver)	PC (mg/g liver)	PC/PE	PCOOH (nmoles/liver)	PCOOH (nmoles/g liver)	PCOOH/PC (10^5 × ratio)
CS	18.5 ± 3.1	14.5 ± 2.7	1.88 ± 0.08	1.5 ± 0.5	1.2 ± 0.4	5.6 ± 0.9
CD	12.5 ± 2.3[a]	10.4 ± 1.6[a]	1.44 ± 0.12[a]	2.1 ± 0.2	1.6 ± 0.5	11.4 ± 1.6[a]
CSE	26.7 ± 3.1[a,b]	22.8 ± 2.5[a,b]	1.66 ± 0.14[a]	7.5 ± 1.4[a,b]	5.9 ± 2.0[a,b]	21.2 ± 5.9[a,b]
CDE	20.9 ± 2.2[b,c]	16.9 ± 1.6[b,c]	1.36 ± 0.09[a,c]	8.8 ± 1.2[a,b]	6.9 ± 1.7[a,b]	32.3 ± 7.9[a,b]

Mean ± SD of 5 to 6 mice per each group.
[a]Significantly different from CS mice at P<0.005.
[b]Significantly different from CD mice at P<0.005.
[c]Significantly different from CSE at P<0.005.

TABLE 3

Effects of Choline-Deficient Diet Containing Ethionine on Plasma

Diet	GOT (Karmen Units)	GPT (Karmen Units)	α-Tocopherol (μg/mL plasma)
CS	91.4 ± 16.9	26.6 ± 4.9	5.2 ± 0.2
CD	98.0 ± 8.6	25.9 ± 5.6	4.5 ± 0.4[a]
CSE	67.4 ± 12.5[b,c]	20.5 ± 1.5	6.7 ± 0.3[a]
CDE	136.4 ± 22.5[a,b,c]	120.3 ± 36.5[a,b,c]	4.8 ± 1.0[c]

Mean ± SD of 5 to 6 mice per each group.
[a]Significantly different from CS at P<0.005.
[b]Significantly different from CD at P<0.005.
[c]Significantly different from CSE at P<0.005.

DISCUSSION

It is controversial whether mice respond to lipotropic deficiency in the same way as do rats (3). Previous studies with mice under ethionine ingestion (32,33), or choline deficiency (5–7), or both (34,35) have demonstrated that these dietary conditions cause growth inhibition, fatty liver, hepatocytic necrosis, alterations in methionine utilization, and liver nodular transformation.

The results shown in Table 1 are in concordance with the previous observations in regard to growth inhibition in CSE and CDE mice and fatty livers in CD and CDE mice. It appears that the CDE group was the one most affected by the combined effects of choline deficiency and ethionine as reflected in their particularly high liver-to-body weight ratios. The TLC/FID charts showed that triglycerides (TG) were the most elevated lipid class in the livers of CD and CDE mice. This increase in TG, and thus of TL, altered the normal proportions of the other components when expressed relative to TL. Calculation of PC and PCOOH amounts on a per liver basis eliminated the apparent effect of "dilution" of TL by TG (Table 2). CD mice had a significant decrease in PC with levels about 30% lower than those for CS mice, as would be expected to result from choline deficiency. Surprising, however, was the finding that CDE mice had normal levels of PC, although they received the same CD diet as CD mice. We do not know exactly the reasons why the PC levels were not similar to those of the CD group. However, it seems likely that the fatty livers resulting from choline deficiency and ethionine have different mechanistic causes, as has been suggested previously (36). Thus, in our experiments, livers of CD mice became fatty with loss of PC, while in CDE no decreases in PC were observed, suggesting a specific action of ethionine on PC

TABLE 4

Effects of Choline-Deficient Diet Containing Ethionine on Liver Biochemistry

Diet	α-Tocopherol (μg/g liver)	TBA-reactive substances (nmoles MDA[a]/ g liver)	G-6-Pase (μmoles phosphorus/ min/g liver)	γ-GGT activity[b] (Unit[c])
CS	9.0 ± 0.9	386 ± 7	20.4 ± 3.1	2.1 (100)[d]
CD	6.0 ± 0.7[e]	423 ± 38	26.2 ± 2.7[e]	2.7 (120)
CSE	5.1 ± 1.0[e]	342 ± 17[e,f]	24.3 ± 3.7	5.0 (223)
CDE	7.2 ± 1.4	625 ± 83[e,f,g]	3.2 ± 1.5[e,f,g]	4.4 (239)

Mean ± SD of 5 to 6 mice per each group.
[a]MDA: malondialdehyde.
[b]The incubation media contained 4.4 mM of L-γ-glutamyl-p-nitroanilide as substrate and 22 mM of glycylglycine as acceptor in 50 mM ammediol buffer, pH 8.6, and the reaction was monitored at 405 nm, for 15 min at 37°C (Ref. 29).
[c]Enzyme activity in μmol of p-nitroaniline released/min at 37°C.
[d]Relative activity in %/μg protein, considering CS as 100%.
[e]Significantly different from CS at P<0.005.
[f]Significantly different from CD at P<0.005.
[g]Significantly different from CSE at P<0.005.

maintenance against the usual reduction caused by choline deficiency. It was also observed that ethionine significantly elevated PC levels when choline was supplemented (Table 2, CSE).

No similar case has been reported before. However, it is of interest to speculate on the data published by Tsuge *et al.* (37). These authors measured concomitantly both of the enzymes involved in choline metabolism in rat liver, i.e., choline dehydrogenase (E.C. 1.1.99.1) which oxidizes choline to betaine, and choline kinase (E.C. 2.7.1.32), which starts the cytidine diphosphocholine pathway for the synthesis of PC. They demonstrated that the activity of choline kinase is increased when ethionine is fed together with choline. Even though their experiments were neither carried out with mice, nor did they measure PC or employ choline deprivation, it is reasonable to expect favored biosynthesis of PC due to enhanced formation of the precursor phosphorylcholine upon ethionine supplementation.

In our study, the PC/PE ratios were lowered in all groups when compared to CS controls, especially in CD and CDE mice (Table 2). It can be deduced from observed PC levels whether these decreases were due to diminished levels of PC or to elevated PE.

The six-fold elevation of PCOOH/PC in CDE mice was followed by a four-fold elevation in the CSE group, indicating increased peroxidation due to ethionine. The molar PCOOH/PC ratios were calculated assuming that liver PC consisted only of 1,2-dipalmitoyl PC (Table 2). Recalculations for the PC molecular species that are most abundant, namely those containing stearoyl and arachidonoyl groups (38), gave similar values and the same proportionalities as presented in Table 2. The meaning of this elevation in peroxidized PC in CDE mice, which also showed the highest TBA reactive substances value (Table 4), is unknown. However, the former significant alterations were accompanied by elevated GOT and GPT activities in the plasma of CDE mice (Table 3), an indication of liver necrosis. The other biochemical indicators, normally taken as markers of rat hepatocarcinogenesis (30), were also altered in CDE, with the lowest G-6-Pase and enhanced γ-GGT activities (Table 4). The pathogenesis of hepatocellular neoplasia in mice is not well understood (39,40), and thus, it is difficult to interpret such alterations in terms of those observed in the rat (30). Nevertheless, it is noteworthy that choline could to some extent prevent these biochemical disturbances, since in CSE mice these enzymes remained essentially at normal levels (Table 4), despite the elevation in PCOOH (Table 2).

We have previously reported that choline, more than ethanolamine, efficiently decomposes lipid hydroperoxides *in vitro* (41). In this process, unesterified choline catalyzes the reduction of lipid hydroperoxides to the corresponding alcohols, products identical to those that are formed when glutathione peroxidase acts on hydroperoxides (42). Although further experiments are required to demonstrate this antioxidative action of choline also *in vivo*, the presence of peroxidized PC in mice liver during nutritional stress can be thought of as an indication of the defensive action of choline towards free radical attack.

α-Tocopherol was decreased in livers of the CD, CSE and CDE groups (Table 4), in which greater amounts of PCOOH were observed. These decreases can be interpreted as being caused by consumption of α-tocopherol in peroxidation reactions. However, differences in the absorption of this vitamin due to dietary deficiencies must also be considered as possible cause. Recent studies have also demonstrated that α-tocopherol is structurally associated with bilayer membrane phospholipids and that a direct correlation exists between the degree of unsaturation of component phospholipids and tocopherol levels (43). Changes caused by choline deficiency and/or by increased peroxidation could interfere with normal association patterns of α-tocopherol in hepatocyte membranes.

Although we have presently concentrated our attention on the peroxidation of PC, the possibility of other phospholipids and other classes of lipids being affected by similar mechanisms cannot be excluded. The major finding we report is the enhanced peroxidation of PC in mice fed with ethionine which cannot be prevented by choline supplementation. This may suggest a potential new role of lipid peroxidation in hepatocarcinogenesis caused by diet.

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METHODS

Advantages of Total Lipid Hydrogenation Prior to Lipid Class Determination on Chromarods-SIII

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The changes in detector responses of lipid classes for standards and natural lipid samples after hydrogenation were investigated with the TLC/FID Iatroscan system using Chromarods-SIII (Newman-Howells Associates, Ltd., Midwales, U.K.). Samples included lipids of human plasma and erythrocytes, egg lipids, a fish oil concentrate and triacylglycerols of sea scallop, as well as standards (mono-, di- and triacylglycerols, phosphatidylcholines, phosphatidylethanolamines, lysophosphatidylcholines, free fatty acid, and sterol). The duration of hydrogenation was increased from 45 min to 90 min for some samples to bring hydrogenation to completion. Since the detector response of a lipid class is known to depend on its relative position on the Chromarod, different solvent systems which would vary the migration rate were investigated. The increases in response after hydrogenation, given as relative percent increase (RI), were significant for all compounds examined ($p<0.05$), and ranged from 7 to 45%. Among the triacylglycerols, the fish oil and the sea scallop triacylglycerols with wide ranges of fatty acid composition had the highest RI while the other lipid classes did not differ from each other. Differences in the solvent systems did not affect the RI except for fish oil triacylglycerols. The precision of peak area measurements was usually better after hydrogenation. The combination of greater sample stability, greater response, improved resolution, and simpler choice of standards make hydrogenation a significant advance in TLC/FID quantification.
Lipids 25, **570–574 (1990).**

In recent years, a quantitative thin-layer chromatographic system based on Chromarods (Newman-Howells Associates, Ltd., Midwales, U.K.) scanned by a flame ionization detector (FID) (the Iatroscan) has gained acceptance as one of the techniques available for the analysis and quantification of a variety of involatile natural products (1–3). A number of methods have been proposed to increase the response of various lipid classes as well as for improving separation and quantification, but eliminating subfractionation effects due to the variety of fatty acids (refs. 4–6; Ohshima, T., and Ackman, R.G., unpublished data) by total hydrogenation has several attractive features.

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Abbreviations: FID, flame ionization detector; GLC, gas-liquid chromatography; LPC, lysophosphatidylcholine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; RBC, red blood cells; RI, relative increase; SM, sphingomyelin; TG, triacylglycerols; TLC/FID, thin-layer chromatography/flame ionization detection.

Hydrogenation of unsaturated triacylglycerols and their ether analogues has been used to improve the resolution during gas-liquid chromatography (GLC) and to prevent decomposition of polyunsaturated species during high temperature GLC (7). Nutter *et al.* (8) have shown that hydrogenation provided more uniform results from charring when applied to quantitative analysis of lipid classes by thin-layer chromatography with densitometry. Several publications from this laboratory discuss hydrogenation to improve separation and accuracy of lipid composition analysis by thin-layer chromatography/flame ionization detection (TLC/FID) (refs. 4–6; Ohshima, T., and Ackman, R.G., unpublished data) since sharper peaks without subfractionation could be obtained with hydrogenated lipids. Ackman and Ratnayake (5,6) have compared the response, before and after hydrogenation, of lipid standards and fish oils on Chromarods-SII (Newman-Howells Assoc., Ltd.). The recently introduced Chromarods-SIII are the only format now available and we have used them to re-evaluate the total hydrogenation process with a variety of natural lipid mixtures as well as to standards. Some aspects of solvent system polarity and catalyst carryover have also been investigated.

MATERIALS AND METHODS

Platinum oxide (Adams' catalyst, Fluka AG, Buchs, Switzerland) was purchased from Caledon Laboratories Ltd. (Georgetown, Ontario, Canada). Authentic standards of tripalmitolein, 1-monopalmitolein, 1,3-diolein, cholesterol, arachidonic acid, palmityl palmitate, octadecanol, and egg source lysophosphatidylcholine (LPC) were purchased from Serdary Research Laboratory Inc. (London, Ontario, Canada). Egg lipids were also freshly isolated using the method of Bligh and Dyer (9). The egg lipid triacylglycerols were separated by conventional thin-layer chromatography. Phosphatidylethanolamine (PE) and phosphatidylcholine (PC) were separated from the egg lipids using the procedure of Lea *et al.* (10). Lipids were extracted from human erythrocytes using the procedure of Rose and Oklander (11), while the method of Folch *et al.* (12) was used for human plasma. Highly refined fish oil in capsules was supplied by the CPL Company, Karlshamns AB (Karlshamn, Sweden). Lipids of the sea scallop *Placopecten magellanicus* were a gift from Mr. G. E. Napolitano. All solvents were reagent grade and distilled in glass before use. Standards were checked for purity by TLC/FID. Fatty acid compositions of lipid classes were determined by GLC analysis carried out on a Perkin-Elmer Model 8420 (Perkin-Elmer, Norwalk, CT) equipped with a FID and digital integrator. Methyl esters were analyzed using a SUPELCOWAX-10 column (30 m × 0.32 mm i.d., phase thickness 0.25 μm; Supelco,

Bellefonte, PA). The GLC analysis was temperature programmed from 195°C to 240°C at 3°C per min after an initial 8 min at 195°C, and was held at 240°C for 10 min. Other parameters were: split ratio, 1:32; helium (carrier gas) flow rate, 1.2 mL/min; and injection port temperature, 250°C.

Hydrogenation was done over platinum oxide catalyst. Chloroform, methanol, and a combination of methanol and hexane (3:2, v/v) were used as solvents, depending upon the lipid classes being hydrogenated. The solution containing the dispersed catalyst was stirred vigorously by means of a Teflon-coated magnetic bar while hydrogen was passed over the reaction surface.

A wax ester, hexadecyl hexadecanoate (palmityl palmitate), was used as an internal standard in all cases except in the analysis of plasma, where octadecanol was used. Volumetric solutions of the lipids and internal standard were mixed in the flask and exactly one-half was withdrawn and made up to a standard solution with chloroform/methanol (2:1, v/v) for use as reference (solution A). After addition of more solvent, hydrogenation was carried out at room temperature for 45 min in the case of standards, although for most of the natural lipid mixtures the period was extended to 60–90 min to ensure total hydrogenation. All samples were checked for complete hydrogenation by GLC of the fatty acid methyl esters. The flask and the catalyst were washed repeatedly with solvent to ensure complete transfer of lipids to a centrifuge tube. The solution was centrifuged (1800 rpm × 10 min) to remove the catalyst. The total solution was concentrated and then made up to a suitable volume with chloroform/methanol (2:1, v/v; solution B) to match the concentration of solution A. Chloroform/methanol (5:1, v/v) has also been suggested (Ohshima, T., and Ackman, R.G., unpublished data). Whichever solvent system is chosen, homogeneity of the solution must be ensured before spotting, as the solubility of hydrogenated lipids is usually markedly lower than that of the original unhydrogenated material.

Drummond disposable Microcaps (1 μL) were used to apply the solutions to Chromarods-SIII (Newman-Howells Assoc., Ltd.). Since the response of a lipid class is known to depend on its relative position on the Chromarod (13), different solvent systems which vary the R_f were chosen. These included: A) hexane/diethyl ether/formic acid (97:3:1, v/v/v); B) hexane/chloroform (85:15, v/v) with the chloroform containing 5% isopropanol and 0.5% formic acid; C) hexane/diethyl ether/formic acid (90:10:1, v/v/v); and D) chloroform/methanol/water (70:30:3.5, v/v/v). Solvent systems A and B were used as developing solvents for neutral lipids while system D was used for phospholipids. For diacylglycerol, an initial solvent focusing step (13) with system D for 5 min was followed by complete development with system A. For analysis of erythrocyte lipids, an initial development for 25 min with system D was followed by a 40-min development with system C. For plasma lipids with octadecanol as internal standard, the first development was with system C. The rods were then partially scanned, leaving the phospholipids intact at the origin. The phospholipids were then developed using system D. For calculations, the area of the phospholipids in the second scan were related to the area of the octadecanol in the first scan (14). The Chromarods-SIII (Newman-Howells Assoc., Ltd.) were scanned in an Iatroscan Mark III. The Iatroscan was run under the following conditions: Hydrogen flow, 160 mL/min; air flow, 2100 mL/min; scan-speed, 4.17 mm/second; chart speed, 16 cm/min. A Spectra-Physics SP4200 integrator (Spectra-Physics, St. Albans, U.K.) was used for recording and area integration.

Five Chromarods (Newman-Howells Assoc., Ltd.) were spotted with solution A and five with solution B, ideally containing 4–5 μg of sample. The internal standard mass and area counts for the sample class and internal standard were used to calculate the mass of sample for each of the ten Chromarods, and results were averaged for unhydrogenated and hydrogenated samples. The relative increase in response was calculated by the ratio of average sample mass before and after hydrogenation. The analysis was repeated at least five times using three separate sets of rods, each set being used at least once and the other two analyses taking place on sets randomly selected without replacement. The relative increase in response is reported as the mean of these determinations with standard error of the mean in parentheses. One-way and two-way analysis of variance were done using the 5% level of significance (15). The protected least significant difference test was used to compare the means.

RESULTS AND DISCUSSION

Table 1 gives the types of lipid class analyzed, their sources, fatty acid compositions, and relative increase in response (RI), expressed as percentage increase between hydrogenated and unhydrogenated samples in the different solvents used. There were significant increases in the response for the hydrogenated lipid samples in all cases. Two way analysis of variance with lipid class and solvent factors (A *vs* B) indicated that class was highly significant ($p<0.05$) and that the increase in RI was different between classes. Solvent was also apparently a significant factor, but this was found to be due to large increases for the sea scallop and fish oil triacylglycerols (TG) in particular. For example, the refined fish oil and sea scallop TG had fatty acid compositions ranging from C_{12} to C_{22} with high concentrations of polyenes. As expected, owing to the multiplicity of molecules possible (16), these TG had the highest increases in response. The peak shape also visibly improved after hydrogenation, as shown for fish oil concentrate (Fig. 1B). The same TG sample in solvent system B had a significantly lower RI. This was probably due to the fact that the peak shape was better even before hydrogenation in system B as compared to system A (Fig. 1C). Moreover, after hydrogenation the peak width reduction at peak half-height was significantly less in solvent system B than in system A ($p<0.05$). The other TG classes—egg, tripalmitolein, and plasma—had similar RI of about 10%, all three being lower than the fish oil. Although the RI were higher for all TG in solvent system A as compared to system B, the differences were not found to be significant except for the fish oil. Although plasma TG had a range of fatty acids much greater than the standard tripalmitolein, the RI were not different. While tripalmitolein in an earlier study with Chromarods-SII (5,6) did not show an increase in response after hydrogenation, in the present study the RI was significantly greater than 1.0.

METHODS

TABLE 1

Sources, Fatty Acid Composition, and Percentage Increases in Response After Hydrogenation of Lipid Classes

Lipid class	source	% Fatty acid composition, subtotalled, by type[a]				Percent response increase[b] solvent systems used[c]			
		sat-	mono-	di-	poly-	A	B	C	D
Triacylglycerols	Tripalmitolein	nd	100	nd	nd	9(1)	7(1)	—	—
	Egg	36	51	10	3	11(1)	9(1)	—	—
	Fish oil	29	28	3	40	45(3)	33(1)	—	—
	Sea scallop	26	17	3	54	43(4)	35(3)	—	—
	Plasma[d]	30	50	14	6	—	—	9(5)	—
Diacylglycerol	Diolein	nd	100	nd	nd	8(.01)[f]	10(2)	—	—
Monoacylglycerol	Monopalmitolein	nd	100	nd	nd	9(1)	7(1)	—	—
Fatty acids	Arachidonic	nd	nd	nd	100	14(3)	12(3)	—	—
	Plasma[d]	45	44	2	9	—	—	8(0.4)	—
Steryl ester	Plasma[d]	14	24	46	16	—	—	9(3)	—
Sterols	Cholesterol	—	—	—	—	10(1)	7(0.4)	—	—
	Plasma[d]	—	—	—	—	—	—	5(1)	—
	RBC[d,e]	—	—	—	—	—	—	7(0.4)[f]	—
Phosphatidylcholines	Egg	49	34	9	8	—	—	—	12(1)
	Plasma[d]	45	13	18	24	—	—	—	14(1)
	RBC[d,e]	49	20	15	16	—	—	—	12(4)
Phosphatidylethanolamine	Egg	46	23	12	19	—	—	—	11(3)
Lysophosphatidylcholine	Egg	91	7	2	—	—	—	—	11(2)

[a] Sat, saturated; mono, monoenes; di, dienes; poly, polyenes; and nd, not detected.

[b] Values represent % increase in response with S.E.M. in parentheses (n = 5–8).

[c] Solvent A, hexane/diethyl ether/formic acid (97:3:1, v/v/v); Solvent B, hexane/chloroform (85:15, v/v), the chloroform containing 5% isopropanol and 0.5% formic acid; Solvent C, hexane/diethyl ether/formic acid (90:10:1, v/v/v); and Solvent D, chloroform/methanol/water (70:30:3.5, v/v/v).

[d] Analyzed as native mixtures.

[e] RBC, red blood cells (erythrocytes).

[f] Refer to text for solvent system.

Arachidonic acid showed a RI that was greater than previously reported. The peak tailing was greater in solvent system B compared to A, probably due to the lower content of formic acid in B. The free fatty acid fraction from plasma had a greater range of acids than the arachidonic acid standard, and thus might have been expected to give a larger RI, but this was not the case. The peak shapes and TG-FFA resolution, however, improved after hydrogenation (Fig. 2B).

For hydrogenated diolein, peak splitting had been previously observed in solvent system A. The suggested cause of the splitting was acid catalyzed isomerization (5,6). An alternative explanation may be found in the nonuniformity of the distribution of the diacylglycerol around the Chromarod at the time of application. This phenomenon may be more pronounced in the hydrogenated diacylglycerol than the unhydrogenated form due to the markedly lower solubility of the former. Moreover, since the evaporating solvent will be chilled, solubility will be further reduced. By solvent focusing (13) in polar solvent system D for 5 min, the splitting of the peak was eliminated. In addition, the actual degree of isomerization during hydrogenation was found to be negligible by using boric acid impregnated Chromarods (17). It should be noted that 1,2- and 1,3-diacylglycerols were separated equally as well on the Chromarods-SIII without boric acid impregnation in solvent system B as had been reported earlier (17) with boric acid.

Standard cholesterol, and sterols of plasma and erythrocytes, showed similar RI. The steryl ester of plasma, in spite of its complex fatty acid composition which can lead to subfractionation on planar silica gel TLC (18), showed only a moderate increase in response. In the solvent system used, the steryl ester migrated very close to the solvent front. The use of a low polarity solvent system could lead to its subfractionation on the Chromarods as well (4).

The PC from the three natural sources and egg PE showed a similar RI. The PE from blood has not been reported because this lipid class was present in only trace quantities in the blood sample analyzed. The lysophosphatidylcholines from egg showed a RI that was much lower, 1.11 ± 0.04, than the 1.20 (from a different source) reported previously (5,6).

The precision of peak areas was usually better for the five rods in a frame containing hydrogenated lipids than it was for the five rods containing unhydrogenated samples. For one hundred analyses the average coefficient of variation for hydrogenated lipids was 5.0%, while for unhydrogenated lipids it was 5.9%.

Care was taken to ensure that no bias was introduced by repeatedly using five particular rods for the unhydrogenated samples. Three different sets of rods that displayed intraset variation in absolute response (ca. 12% coefficient of variation) were used. Although interset variation was low, it might be responsible for some of the SEM of the determinations, but allows greater confidence in predicting response from different sets of rods. Selecting three sets among many that give closer agreement may bias the results and would certainly prove expensive,

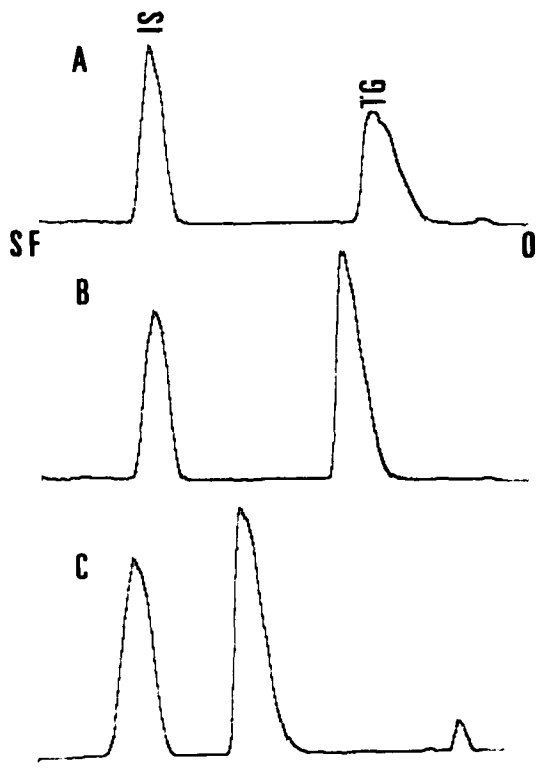

FIG. 1. Iatroscan TLC/FID chromatograms showing the effect of hydrogenation on peak shape of fish oil triacylglycerols on Chromarod-SIII. A, unhydrogenated; B, hydrogenated [developed in solvent system A (hexane/diethyl ether/formic acid, 97:3:1, v/v/v]; C, unhydrogenated [developed in solvent system B (hexane/chloroform, 85:15, v/v), the chloroform containing 5% isopropanol and 0.5% formic acid]. IS, internal standard, wax ester; TG, triacylglycerols; O, origin; and SF, solvent front. (See text for discussion of the small "oxidized lipid" peak.)

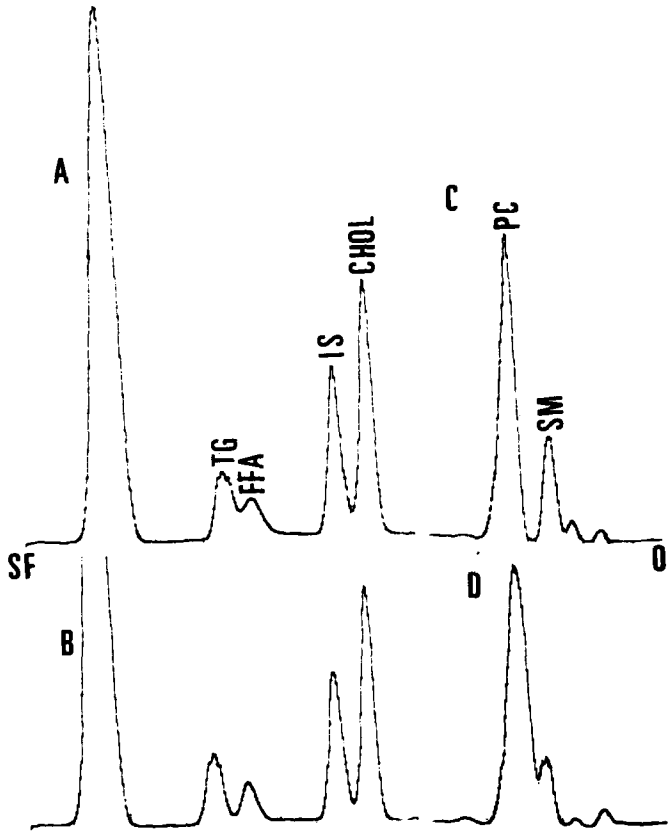

FIG. 2. Iatroscan TLC/FID chromatograms showing the effect of hydrogenation on the separation of human plasma lipids on Chromarods-SIII [in solvent system D (hexane/diethyl ether/formic acid, 90:10:1, v/v/v)], partial scan followed by complete redevelopment for analysis of polar lipids [in solvent system C (chloroform/methanol/water, 70:30:3.5, v/v/v)]. A, neutral lipids, unhydrogenated; B, neutral lipids, hydrogenated; C, polar lipids, unhydrogenated; D, polar lipids, hydrogenated; SE, steryl ester; FFA, free fatty acid; IS, internal standard, fatty alcohol; SM, sphingomyelin; PC, phosphatidylcholine; TG, triacylglycerols; O, origin; and SF, solvent front.

and using one set of rods alone would likely produce data with lower standard errors but may not be applicable to a typical analysis.

The reasons for the differences in the data in Table 1 and those reported by Ackman and Ratnayake (5,6) may be due to a number of factors. The data reported by Ackman and Ratnayake (5,6) were obtained by using the older Chromarods-SII (Newman-Howell Assoc., Ltd.). These have been discontinued and replaced by Chromarods-SIII. The Chromarods-SIII have a much more uniform machine-made coating of silica gel (5 μm mesh). The RI could also vary with operating parameters such as load level, solvent system used, etc. Although the RI may vary, what is important is that there is a definite increase in response after hydrogenation for all lipid classes tested.

In the case of lipid extracted from plasma, an improvement in separation after hydrogenation can be seen (Fig. 2B). In most complex lipid samples, a better separation may be observed after hydrogenation; this has been pointed out previously (refs. 5,6; Ohshima, T., and Ackman, R.G., unpublished data).

One of the less helpful results obtained was that the resolution between PC and sphingomyelin (SM) decreased after hydrogenation, as shown in Figures 2C and 2D. This could be corrected using a less polar solvent system (not shown). Modified solvent systems may thus sometimes be necessary for optimal separation for hydrogenated samples compared to unhydrogenated ones.

The choice of solvent to be used in hydrogenation should also be considered carefully. Chloroform seems to be an ideal choice for lipid samples or standards when no appreciable quantity of phospholipids is present. Because such lipid mixtures occur rarely, methanol often can serve as suitable solvent. There was a tendency for the catalyst to remain suspended when chloroform was used as the solvent and when phospholipids were present in the sample. This was found in all samples containing phospholipids, although at this stage it is difficult to correlate these findings. It was observed that when the hydrogenated sample in chloroform was left undisturbed in the volumetric flask, there was a tendency for the platinum black metal to float at the surface. The solution below could be carefully removed, but with small quantitites of samples being handled, this could lead to sample loss. To avoid this problem, methanol was used as the solvent, but only if the total lipid was soluble in it. In all cases care had to be taken during recovery to prevent preferential loss of lipid classes because the solubility of the saturated lipid samples was markedly less than that of the

unsaturated precursor. A mixture of methanol and hexane was used for samples having phospholipids and neutral lipid when the lipid was not soluble in methanol alone. The samples were checked by TLC/FID to be certain that no artifacts were formed from the use of methanol as solvent. In a previous study (5,6), it was pointed out that hydrogenation in methanol could theoretically lead to the formation of methyl esters, but this was not found to be the case.

Advantages of hydrogenation other than an important and useful increase in response include a greater stability of the lipid mixture and better resolution among lipid classes, advantages that have already been discussed (refs. 5,6; Ohshima, T., and Ackman, R.G., unpublished data). The refined fish oil, when tested for purity immediately after opening the capsule, showed no oxidation material. In later runs, despite careful handling and nitrogen flushing, oxidized material was observed at the origin in the original but not in the hydrogenated fish oil samples (Figs. 1A and 1C). The instability of highly unsaturated fatty acids is a problem in other types of lipid analyses, for example, in high-performance liquid chromatography (19). Hydrogenation simplifies the choice, cost, and stability of the standards that must be used.

Fraser *et al.* (20) hydrogenated a modified fish oil (Marinol) and came to the conclusion that markedly different degrees of unsaturation do not affect lipid quantitation by the Iatroscan, although a careful examination of their data suggests that for the same weight of lipid there is an increase in peak area after hydrogenation.

Our work confirms that the degree of unsaturation, apart from the range of fatty acid composition, is indeed one of the factors affecting quantitation. All chemically "pure" unsaturated standards, including cholesterol, showed a RI of about 10%. It may be hypothesized that this represents less breakup of the saturated fatty acid (or cholesterol) by radiant heat in the periphery of the flame; more material then enters the flame core to produce ions. The subfractionation so obvious in the marine triacylglycerols can be eliminated by the hydrogenation, concentrating the material and thus producing a major additional increment in response. Lipids such as the egg and plasma PC evidently do not subfractionate appreciably (Fig. 2) and the increment from this source is minimal (Table 1).

Impregnation of Chromarods (Newman-Howells Assoc., Ltd.) with copper (II) sulfate is also known to increase the response of lipid classes in the TLC/FID system (refs. 21-23; Iatron Laboratories Inc., personal communications). A combination of hydrogenation followed by analysis on copper (II) sulfate impregnated Chromarods could provide further benefit in sensitivity. Preliminary results suggest that this is indeed the case. With our current advance in the state of the art, the combination of improved sample stability, greater response, simpler choice of standards, and improved resolution mark the use of hydrogenation as a significant advancement in the art of TLC/FID quantitation.

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COMMUNICATIONS

Serum Apolipoproteins B and A-I and Naturally Occurring Fatty Liver in Dairy Cows

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Serum lipids and apolipoprotein (apo) B and A-I concentrations were determined in 164 dairy cows which had undergone liver biopsy in early lactation. The animals were divided into groups according to fatty liver severity on the basis of hepatic triglyceride content. The serum free fatty acid (FFA) concentration was higher in cows that developed fatty livers than in normal cows, and it correlated highly with liver triglycerides. Serum total cholesterol and triglyceride levels did not correlate with hepatic triglycerides. Both apo B and apo A-I levels were significantly decreased in fatty liver cows. In particular, apo B levels showed a strongly negative correlation with liver triglycerides. The present results suggest that hepatic apolipoprotein synthesis is impeded in fatty liver cows.
Lipids 25, 575–577 (1990).

Fatty liver (liver steatosis) arises in humans and animals in response to various toxic, nutritional or hormonal effects. This has been reported as a physiological or pathological situation resulting from natural or induced excessive fat mobilization (1,2). In two common diseases occurring in ruminants—"fatty liver" in dairy cows and "pregnancy toxaemia" in sheep—fat mobilization results in the development of liver steatosis (3,4). The ruminant fatty liver is of interest for the comparative study of the relationship between free fatty acid (FFA) uptake, triglyceride synthesis and triglyceride secretion, since endogenous fatty acid synthesis does not take place in the ruminant liver (5). On the other hand, the supply of blood lipids from intestine is limited in ruminants, unlike in non-ruminant animals. This is due to the particularities of ruminant nutrition and nutrient digestion (6).

During the first month of lactation, high yielding dairy cows show increased mobilization of body fat, which is caused by their negative energy balance resulting from high milk production (3). A rise in serum FFA results in an increased supply of FFA to the liver and thus in enhanced triglyceride synthesis and accumulation. Normally, the level of liver triglyceride increases in the 2–3 weeks following calving, and then decreases progressively (3). However, in many cases fatty liver develops and has a significant effect on liver structure and function (3,7). At this time some modifications in the blood lipoprotein profile have been reported in fatty liver cows (8–10). Recent studies carried out on goat hepatocytes (11) and on sheep *in vivo* (12,13), have shown that there is limited triglyceride-rich lipoprotein secretion by the liver in ruminants as compared to non-ruminants. Consequently, ruminants may be susceptible to fatty infiltration of the liver during periods of high fatty acid uptake. One may hypothesize that hepatic steatosis in cows may involve a decreased hepatic lipoprotein triglyceride output which is accompanied by an increased FFA uptake by the liver (14).

Apolipoprotein synthesis may be rate-limiting for very low density lipoprotein (VLDL) assembly and secretion, and probably determines the maximal capacity of secretion of triglycerides by the liver (15). It has been reported that humans affected by liver diseases frequently show an abnormal apolipoprotein profile (1,16). Thus, apolipoprotein profiling has been used in an attempt to follow normal and deranged lipid transport (16). Hence, the objective of this study was to examine the relationship between fatty liver and apo B and apo A-I serum concentrations. We recently proposed a quantitative assay for these apolipoproteins in bovines (17,18). This study was carried out on cows which had undergone liver biopsies, because only liver samples can give reliable information as to the extent of fatty liver in living animals.

MATERIALS AND METHODS

Animals and sampling. In this study, 164 Holstein × Friesian multiparous cows from commercial dairy herds (average milk yield of 6.200 kg) fed a complete diet based on maize silage were used. The cows were apparently healthy and without depressed milk production. Blood samples were taken 5–21 days after calving from the jugular vein in the morning, and 2–4 hr after milking and feeding, and serum was prepared by low speed centrifugation. Liver samples were taken from each cow by percutaneous needle biopsy under local anaesthesia immediately after the blood sampling. Collected samples were stored at −20°C until analysis.

Serum and liver analysis. FFA (19), triglycerides (20) and total cholesterol (21) concentrations in the serum were determined by enzymatic procedures. A single radial immunodiffusion assay was developed to determine the apo B and apo A-I serum concentrations using anti-apo B or anti-apo A-I monospecific rabbit antiserum (17,18). All assays were carried out in triplicate. Liver triglyceride was assayed in the lipid extract obtained from the biopsy sample as described previously (10). Results are expressed in milligrams of triglyceride per gram of wet tissue weight.

Calculations and statistical analysis. The animals were divided into experimental groups according to the triglyceride content of the fatty liver. Cows with less than 50 mg of triglyceride per gram of wet liver weight were considered to be in the normal group, those with 50–100 mg in the moderate fatty liver group, and those with more than 100 mg were considered as severely fatty in agreement

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Abbreviations: ANOVA, analysis of variance; apo, apolipoprotein; FFA, free fatty acids; VLDL, very low density lipoproteins.

with the classification proposed by Gaal *et al.* (22). Values are given as means ± SEM, and data were analyzed by Student's t-test or analysis of variance (ANOVA) with minimum significant difference. Differences were considered statistically significant at $P < 0.05$. Analyses of correlation were performed with Statistical Analysis System Software (SAS Institute Inc., Cary, NC).

RESULTS

The average serum concentrations for lipids, apo B and apo A-I in the cows studied are shown in Table 1. The serum FFA were higher in both moderate and severe fatty liver cows than in normal cows. Serum cholesterol and triglyceride concentrations were not significantly different between the groups. The concentrations of apo B were significantly different in all groups, with the lowest values in the severe fatty liver cows. A similar trend was observed for the concentration of apo A-I, but the differences became significant only between the normal and severe fatty liver groups. Correlation coefficients between liver triglyceride content and serum concentration of lipid and apolipoprotein, calculated by combining all data, demonstrated that the FFA presented a positive and the apolipoproteins a negative correlation with liver triglycerides. In particular, the liver triglycerides and serum FFA ($y = 17.03 + 57.78x$; $r = 0.43$), and serum apo B and liver triglycerides ($y = 13.39 - 0.02x$; $r = 0.34$) did correlate highly ($P < 0.001$).

DISCUSSION

Differences exist in the serum lipids and lipoproteins of ruminant and non-ruminant animals in relation to their nutritional and metabolic peculiarities (23,24). Bovines, in particular, have low serum triglyceride and apo B levels and high cholesterol and apo A-I levels. In the dairy cow, the lactation stage has a significant effect on serum lipid and lipoprotein concentrations which relates to milk production (23,25). The early lactation post-partum period is of particular interest. During this period, initial lactation and body fat mobilization lead to significant changes in lipid and lipoprotein metabolism in the dairy cow (25,26). The fatty liver that is formed during this period seems to enhance the changes in lipid and lipoprotein profiles that occur in early lactation. In the present study, high levels of FFA were observed in fatty liver cows, which is in agreement with previous results (3,10). On the other hand, we found that fatty liver is related to a reduction in serum concentrations of apo B and apo A-I, even though the effect on serum triglycerides and cholesterol is not consistent.

Recent studies in our laboratory have shown that severe fatty liver is associated with a decrease and an alteration in the distribution of apo A-I-containing lipoproteins (10). The possible origin of these modifications has already been discussed (10) and it could plausibly be explained as the result of reduced secretion of VLDL by the liver. These observations are consistent with the results of the present study, so that fatty liver appears to affect apo B levels even more markedly than those of apo A-I (28 *vs* 13% decrease, respectively, when control and severe fatty liver cows were compared). However, the physiological level of apo B in bovines is low, which is consistent with the serum lipid and lipoprotein patterns (17). Since the secretion of VLDL is apparently reduced, whereas the serum concentration of triglyceride, and presumably therefore the pool size, is not reduced, it seems possible that the fractional removal rate of VLDL also could have been affected. The observed increase in the triglyceride/apo B ratio suggests the presence of larger triglyceride-rich lipoproteins in the fatty liver cows. Like non-ruminant species in nutritional conditions that stimulate liver lipogenesis (15), it is probable that these cows have lipoprotein particles with a greater load of triglycerides.

Since triglyceride synthesis may not be limiting in the production of VLDL particles, the availability of other lipid constituents and/or apolipoproteins may be involved in the development of fatty liver. It has been shown (7,27) that liver steatosis in cows is accompanied by several changes in hepatic structure including increased cell volume, compression of hepatic sinusoids, mitochondrial damage, and a decreased volume of rough endoplasmic

TABLE 1

Concentration of Some Serum Constituents of Cows Classified as "Normal" or "Fatty Liver" Based on Liver Triglyceride Determination

	Normal cows	Fatty liver cows		
		Moderate	Severe	r
	(n = 102)	(n = 40)	(n = 22)	(n = 164)
Liver				
Triglycerides[a]	<50	50–100	>100	—
(mg/g wet weight)	(20 ± 1)	(68 ± 2)	(180 ± 15)	
Serum				
Free fatty acids (mM)	0.50 ± 0.03[b]	0.75 ± 0.08[c]	0.95 ± 0.12[c]	0.43[f]
Triglycerides (mg/100 mL)	13.9 ± 0.4[b]	14.7 ± 0.5[b]	13.4 ± 0.9[b]	−0.02
Cholesterol (mg/100 mL)	117.5 ± 4.3[b]	110.2 ± 4.9[b]	108.3 ± 8.8[b]	−0.11
Apo B (mg/100 mL)	13.3 ± 0.4[b]	11.2 ± 0.4[c]	9.6 ± 0.4[d]	−0.34[f]
Apo A-I (mg/100 mL)	132.4 ± 2.8[b]	129.6 ± 4.6[b,c]	119.3 ± 5.5[c]	−0.15[e]

[a]The basis of classification.

[b–d]Values in a row with different superscript are significantly different ($P < 0.05$); Means ± standard error of (n) samples.

r = correlation coefficient; [e]$P < 0.05$; [f]$P < 0.001$.

reticulum. At the same time, as structural changes take place, serum concentrations of albumin are decreased suggesting a failure in liver protein synthesis (3). The negative relationship between liver fat content and serum apo B concentration is of interest since secretion of VLDL is totally dependent upon apo B (28). Impaired synthesis of apo B may result in its decreased availability for lipoprotein formation and, in turn, to enhanced triglyceride accumulation in the liver. In recent experiments carried out in our laboratory we have shown that the early lactation period in dairy cows is characterized by a drop in apo B serum concentration (29). At the same time, the level of liver apo B mRNA was reported to be lower when compared to other stages of lactation (30). Thus, it may be suggested that failure in apo B synthesis is directly involved in fatty liver development.

The molcular events in lipoprotein formation and apolipoprotein synthesis in ruminants remain unknown. Further studies on molecular aspects of liver apolipoprotein gene expression and its control might be helpful for a better understanding of the origin of fatty liver in these animals.

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Inevitable Generation of Primary Alcohols During Reduction of Oxidized Lipids with Sodium Borohydride

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This report deals with the fluorometric determination of fatty alcohols generated by the reduction of the ester linkage of lipids with $NaBH_4$, and with the limitations of the reduction method for assaying oxidized lipids. Optimum conditions for the fluorometric analysis of primary and secondary alcohols using 1-anthroyl nitrile were obtained. After reduction with $NaBH_4$ in MeOH or in MeOH/benzene (8:2, v/v), the formation of 1-hexadecanol from a variety of palmitic acid esters was measured fluorometrically by reverse-phase high-performance liquid chromatography (HPLC): From glycerides and methyl palmitate, 1–3% (w/w) 1-hexadecanol was produced and a trace was produced from cholesteryl palmitate (10 min, 21°C). 1-Hexadecanol was never generated from palmitic acid. Although considerable improvement occurred with the choice of the solvent for the $NaBH_4$ reduction, the generation of primary alcohols from ester lipids usually seems inevitable.
Lipids 25, 578–580 (1990).

Procedures used for the structural analysis of oxidized lipids include the reduction with KI (1,2), $SnCl_2$ (3–5), triphenylphosphine (6–8), $NaBH_4$ (9–12), and $LiAlH_4$ (13,14), as well as catalytic hydrogenation (15,16). $NaBH_4$ reduction is one of the most widely used methods since it is simple and quantitative, and the reaction is relatively free of by-products. With this method, fatty acid hydroperoxides and ketones are reduced to secondary alcohols, and fatty aldehydes, scission products of hydroperoxides, are reduced to primary alcohols. After derivatization or directly, the alcohols can be identified and determined by high-performance liquid chromatography (HPLC), gas-liquid chromatography (GLC), gas chromatography-mass spectrometry (GC-MS) and similar techniques.

In the course of our work on low oxidation levels in lipids, the need arose for $NaBH_4$ reduction of very small amounts of oxidized lipids present in large amounts of unoxidized lipids. We consistently observed a significant amount of long-chain primary alcohols in addition to secondary alcohols derived from oxidized fatty acid residues in the reduction mixture. This generation of primary alcohols implies possible reduction of the ester linkage of the unoxidized lipids.

We describe here a fluorometric assay of primary alcohols generated by reduction of fatty acid esters with $NaBH_4$ and the limitations of this method for the measurement of oxidized lipids.

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Abbreviations: HPLC, high-performance liquid chromatography; GLC, gas-liquid chromatography; GC-MS, gas chromatography-mass spectrometry, 16:0-OH, 1-hexadecanol; 18:0-OH, 1-octadecanol; Me(OH)18:0, methyl 12-hydroxy-octadecanoate; Me(OH)18:2, methyl 9- or 13-hydroxy-octadecadienoate; 18:2-OH, 1-octadecadienol; PTFE, polytetrafluoroethylene.

MATERIALS AND METHODS

Chemicals. Reagents for the fluorometric analysis included 1-anthroyl nitrile which was purchased from Wako Pure Chemical Ind. (Osaka, Japan), and quinuclidine purchased from Ishizu Pharmaceutical Co. (Osaka, Japan). Other chemicals used were purchased from the following sources: $NaBH_4$ from Wako Pure Chemical Ind. (Osaka, Japan); 1-hexadecanol (16:0-OH, TCI-GR), 1-octadecanol (18:0-OH, TCI-GR), glyceryl tripalmitate (TCI-EP), glyceryl 1,3-dipalmitate (TCI-EP), methyl palmitate (TCI-EP) and methyl linoleate (TCI-EP) from Tokyo Kasei Kogyo Co. (Tokyo, Japan); methyl 12-hydroxy-octadecanoate (Me(OH)18:0, ca. 99%), cholesteryl palmitate (ca. 99%), dipalmitoyl DL-α-phosphatidylcholine (ca. 99%) and linolenic acid (ca. 95%) from Sigma Chemical Co. (St. Louis, MO); palmitic acid (ca. 99%) from Kishida Chemical Co. (Osaka, Japan).

$NaBH_4$ reduction. To 5 mg of fatty acid ester dissolved in 1.5 mL of solvent, 10 mg of $NaBH_4$ was added, and the mixture was shaken gently for the prescribed time. To the reaction mixture was added 200 μL of 1N HCl, 1.5 mL of deionized water, and 3 mL of $CHCl_3$, and when necessary, 18:0-OH (internal standard). The preparation was shaken, centrifuged, and the lower layer was washed with 1 mL of deionized water. The organic layer was transferred to another test tube, and the solvent was evaporated to dryness under reduced pressure using a centrifugal concentrator (Taiyo S.C. Co., Tokyo, Japan).

Preparation of anthroyl derivatives. To a portion of $NaBH_4$-reduced residue or hydroxyl compounds in a polytetrafluoroethylene (PTFE) screw-capped reaction tube were added 100 μL of anthroyl nitrile reagent (8 mg/mL CH_3CN) and 50 μL of quinuclidine reagent (2.4 mg/mL CH_3CN). The vial was tightly closed and shaken gently for 40 min at 60°C. After cooling, the reaction was stopped by adding 50 μL of MeOH. Five μL of resulting solution was analyzed by HPLC.

HPLC. Separation and determination of the anthroyl derivatives was accomplished using an HPLC system with a fluorescence detector MODEL 420 (excitation filter 360 nm, emission filter 440 nm, Waters Assoc. Inc., St. Louis, MO) and a Model C-R5A data processor (Shimadzu Co., Kyoto, Japan). An analytical column, Finepak SIL C18 (4.6 mm i.d. × 250 mm, Japan Spectroscopic Co., Tokyo, Japan), was used and elution was carried out isocratically, using MeOH/water (93:7 or 95:5, v/v) at a flow rate of 2.0 mL/min.

RESULTS AND DISCUSSION

Fluorometric assay conditions. For the fluorometric analysis of the fatty alcohols derived from fatty acid esters, we used 1-anthroyl nitrile which was developed for derivatization of hydroxy steroids (17,18) and for prostaglandin assays in insects (19,20). With Me(OH)18:0 and 16:0-OH as standards, optimum assay conditions, reaction time, temperature and concentration of the

TABLE 1

Generation of 1-Hexadecanol by Reduction of Palmitic Acid Esters with $NaBH_4$

Substrate	Temp. (°C)	Time 10 min (%)	Time 30 min (%)	Time 60 min (%) w/w	Solvent
Methyl palmitate	0	0.62	0.68	0.73	MeOH
	21	1.10	1.23	1.22	MeOH
Glyceryl tripalmitate	21	1.23	1.37	1.46	MeOH/benzene[a] (8:2, v/v)
Glyceryl 1,3-dipalmitate	21	2.95	3.84	3.94	MeOH/benzene[a] (8:2, v/v)
Dipalmitoyl DL-α-phosphatidylcholine	21	1.47	1.85	1.97	MeOH
Cholesteryl palmitate	21	trace	0.03	0.03	MeOH/benzene[a] (8:2, v/v)
Palmitic acid	21	nil	nil	nil	MeOH

[a]Glyceryl tripalmitate, glyceryl 1,3-dipalmitate and cholesteryl palmitate are difficult to dissolve in MeOH; hence MeOH/benzene (8:2, v/v) was used as solvent.

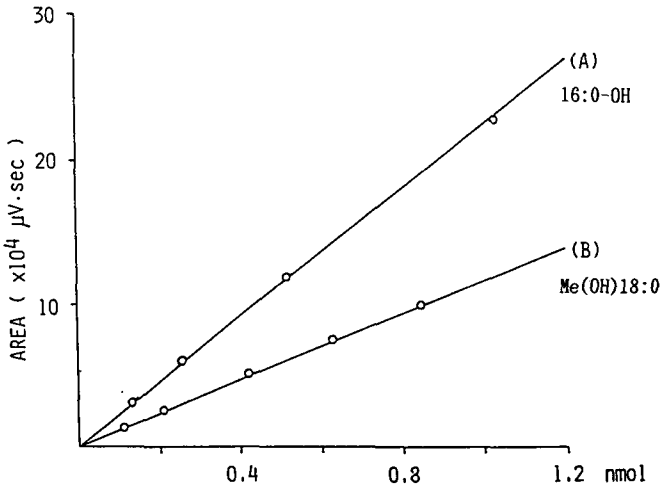

FIG. 1. Standard curves for the anthroyl derivatives of the primary and the secondary alcohols. Each data point represents the mean of two injections at each concentration. (A) 1-hexadecanol (16:0-OH), (B) methyl-12-hydroxy-octadecanoate (Me(OH)18:0).

reagents were examined. As described in Materials and Methods, a much higher concentration (5.3 mg/mL) of anthroyl nitrile in the reaction mixture than the 0.1% reported in the literature (19,20) was essential, especially for the quantitative determination of secondary alcohol. Other conditions determined were the same as those described (19,20). Calibration curves for the alcohols were obtained by measuring the peak areas of 5 μL injections on the reverse-phase column (Finepak SIL C18). They were linear at least in the range of 0–15 nmol tested; some are shown in Figure 1. The different slopes of the calibration curves between those for primary and secondary alcohols reflect the reactivity of the alcohols: the extent of conversion to anthroyl derivatives examined by GLC was almost quantitative for the primary alcohol and 50–60% for the secondary alcohol. The detection limit for the primary alcohol was 1 ng per injection. When a state-of-the-art, more sensitive fluorescence detector was used, more than 100 times the sensitivity was obtained.

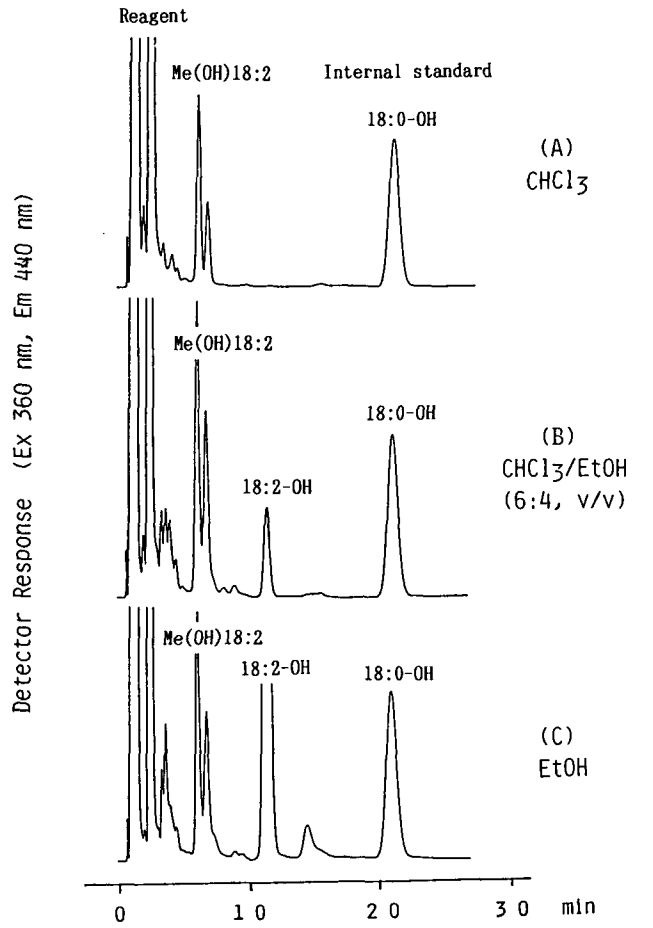

FIG. 2. HPLC of anthroyl derivatives prepared from oxidized methyl linoleate. Autoxidized methyl linoleate (peroxide value 30, measured iodometrically) was reduced with $NaBH_4$ for 40 min at 21°C in (A) $CHCl_3$, (B) $CHCl_3$/EtOH (6:4, v/v), and (C) EtOH. Aliquots of the fluorescent derivatives, a 50-μg portion of the oxidized linoleate and a 319-ng portion of 1-octadecanol (18:0-OH, internal standard), were subjected to HPLC after derivatization with 1-anthroyl nitrile, and eluted with MeOH/water (95:5 v/v), at 2.0 mL/min. 18:2-OH and Me(OH)18:2 are anthroyl derivatives of 1-octadecadienol and geometrical isomers of methyl 9- or 13-hydroxy-octadecadienoate, respectively. Other conditions are described in Materials and Methods.

Generation of fatty alcohols by reduction with $NaBH_4$. After reduction with $NaBH_4$ in MeOH or in MeOH/benzene (8:2, v/v), the amounts of 16:0-OH (palmityl alcohol) generated from a variety of palmitic acid esters were determined fluorometrically by reverse-phase HPLC. As summarized in Table 1, 1–3% (w/w) 16:0-OH was generated from glycerides and methyl palmitate, and a trace from cholesteryl palmitate (10 min, 21°C). Formation of 16:0-OH from palmitic acid was not detected. These results suggest that unless the reduction is done with care, especially for the slightly oxidized lipids, the analytical data obtained are confusing and difficult to interpret.

Autoxidized methyl linoleate (peroxide value 30 measured iodometrically) was reduced with $NaBH_4$ for 40 min at 21°C in solvents such as EtOH, $CHCl_3$, and their mixtures, MeOH, tetrahydrofuran, tetrahydrofuran/EtOH mixtures, and ethyl acetate. After derivatization with 1-anthroyl nitrile, the fluorescent derivatives were analyzed by reverse-phase HPLC. Typical examples are shown in Figure 2. In EtOH as in MeOH, the methyl linoleate hydroperoxides produced were completely reduced to secondary alcohols, geometrical isomers of methyl 9- or 13-hydroxy-octadecadienoate (Me18:2-OH, eluted at 6–7 min, as in Figure 2C). However, larger amounts of the primary alcohol, 1-octadecadienol (18:2-OH, eluted at 11.4 min) and the doubly reduced products, 9- or 13-hydroxy-octadecadienols (eluted at 3–4 min) were noted. Small peaks at 9 and 14 min corresponded to alcohols derived from methyl oleate due to impurities present. In $CHCl_3$ (Fig. 2A), as in tetrahydrofuran, reduction of the peroxides was incomplete, though little 18:2-OH was detected. In a $CHCl_3$/EtOH mixture (Fig. 2B), as in a tetrahydrofuran/EtOH mixture and in ethyl acetate, maximum production of Me18:2-OH was obtained; however, 18:2-OH was still generated. Using methyl linolenate hydroperoxides prepared enzymatically (9,21), the solvent effect of the $NaBH_4$ reduction was further examined under the conditions described above (40 min, 21°C). With $CHCl_3$ as solvent, only 3% of the hydroperoxides were reduced to the corresponding secondary alcohols, and with MeOH the hydroperoxides were reduced almost quantitatively (97%). Since the reduction rate for the ester linkage is smaller than that for the hydroperoxides, and because the rate can be controlled by the solvent used, solvents should be optimized to obtain a complete reduction of the hydroperoxides along with a minimum reduction of the ester linkage. Use of $CHCl_3$/EtOH mixtures and tetrahydrofuran/EtOH mixtures led to a considerable improvement in minimizing reduction of the ester linkage. However, the generation of primary alcohols from ester lipids generally seems inevitable. Therefore, complementary use of other reagents, such as $SnCl_2$ or triphenylphosphine, is advisable, especially when low lipid oxidation levels are to be determined *in vitro* or *in vivo*.

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Role of Cholesterol in the Microsomal Membrane[1]

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It is generally assumed that cholesterol is the public enemy of health. However, in many respects cholesterol is a crucial molecule that serves essential functions in humans. Cholesterol is the substrate for the biosynthesis of bile acids and of steroidal hormones, including gluco- and mineral-corticoids, sex hormones and progesterone. In addition, 7-dehydrocholesterol is a precursor of vitamin D_3. Cholesterol is also an important component of biological membranes. Membranes are generally thought to consist of phospholipid bilayers into which membrane proteins are embedded. Yet, cholesterol molecules are present in most animal membrane structures. Due to its amphipathic nature bearing an OH-group and a hydrocarbon skeleton with rigid rings and a branched chain of eight carbons, cholesterol is perfectly suited to mesh with lipid bilayers.

The cholesterol molecule has evolved from the lanosterol structure by demethylation of the α face thus creating a surface that is more suitable to serve its function in membranes (Fig. 1). In some membranes, the cholesterol/phospholipid ratio is very high. It is 0.83 in liver plasma membranes (1) and 0.90 in erythrocyte membranes (2). In rat liver microsomes (3) it is 0.30, but it can be elevated to 0.61 by administering cholesterol to rats for 21 days at a level of 1% of the diet. Therefore, the liver microsomal membrane is a convenient model to study the effect of cholesterol at different concentrations on the composition, physical properties and enzyme kinetics of membranes.

An increase in the cholesterol concentration in a lipid bilayer evokes a condensing effect at temperatures higher than the transition temperature of the bilayer. This causes a decrease in the rotational diffusion of the hydrophobic fatty acid chains of phospholipids and a decrease in the translational diffusion.

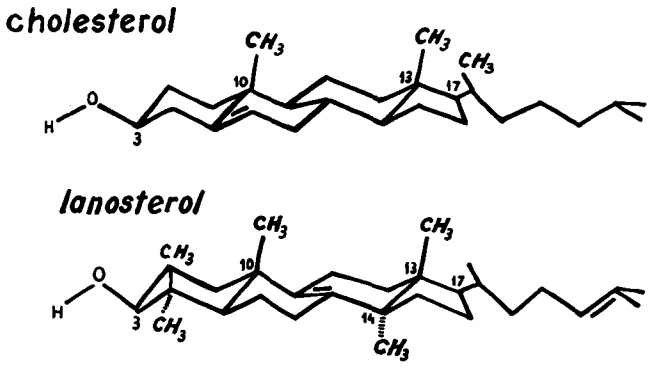

FIG. 1. Structure of cholesterol and lanosterol.

We measured rotational diffusions in liver microsomal membranes by determining the stationary fluorescence anisotropy (r_s) of microsomes labeled with diphenylhexatriene (DPH) and we observed an increase in r_s that depended on the amount of cholesterol that had been incorporated (4) (Fig. 2). The relationship is

$$r_s = \frac{I_\parallel - I_\perp}{I_\parallel + 2I_\perp}$$

where $I_\parallel$ and $I_\perp$ are the fluorescence intensities parallel and perpendicular to the plane of polarization of the excitation beam. The result indicated that with increasing cholesterol levels, a decrease in the rate and range of rotational diffusion occurred.

Lateral diffusion was investigated by labeling the microsomes with pyrene and by measuring the quotient of the fluorescence intensities of the excimer *vs* the monomer formed. This quotient is dependent on the proportion of pyrene and on the collisions of the molecules that depend on lateral diffusion. We observed a decrease in the slopes due to cholesterol incorporation which is indicative of a decrease in lateral diffusion (Fig. 2).

EFFECT OF CHOLESTEROL ON URIDINE DIPHOSPHATE (UDP)-GLUCURONYLTRANSFERASE

With above results in hand, we studied the effect of increased cholesterol levels in the diet on the lipid

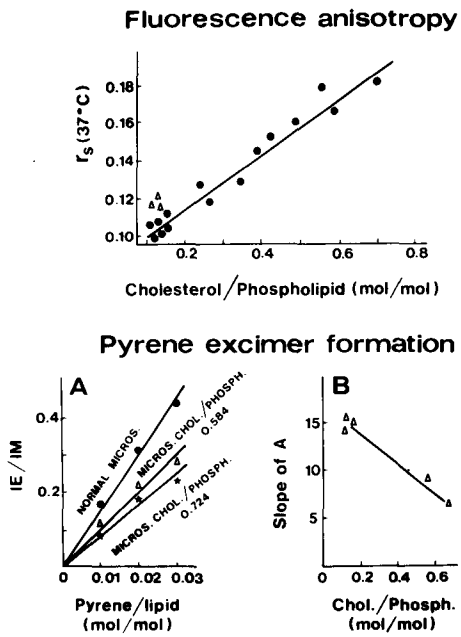

FIG. 2. Effect of cholesterol on rotational and translational diffusion in microsomes. Rotational diffusion was measured by the fluorescence anisotropy of diphenylhexatriene-labeled microsomes. Membranes (approx. 40 μg of lipid/mL) were labeled with $2.5.10^{-7}$M diphenylhexatriene. In the pyrene excimer formation, Ie/Im was obtained from the fluorescence intensities at 472 nm and 392 nm of microsomes labeled with pyrene at different cholesterol concentrations.

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Abbreviations: DPH, diphenylhexatriene; ESR, electron spin resonance; PC, phosphatidylcholine; PE, phosphatidylethanolamine; UDP, uridine diphosphate.

composition of liver microsomes in guinea pigs. We chose the UDP-glucuronyltransferase as enzyme because it is membrane-bound and its activity is known to be modified by the lipid environment.

Administration of cholesterol at 3% of the diet for twenty days increased the cholesterol/phospholipid and phosphatidylcholine/phosphatidylethanolamine molar ratios of guinea pig liver microsomes, but it did not modify, in general, the other lipids. Also, the fatty acid composition essentially was maintained (5) (Table 1). The changes that were observed were accompanied by a decrease in the fluidity of the bulk lipids of the membrane.

A significant effect resulting from these membrane changes was exerted on UDP-glucuronyltransferase activity. This enzyme is bound to the liver microsomal membrane and catalyzes the transfer of the glucuronyl group from the UDP.GA to *p*-nitrophenol. The reaction proceeds in a random-ordered sequence in which two substrates are involved. Therefore, 4 K_m values were measured (Fig. 3). It was found (Table 2) that cholesterol incorporation into the membrane decreased the 4 K_m values and increased the maximal velocity of the reaction, indicating a change in the affinity of the enzyme for the substrates and an increase in enzyme activity. However, the changes produced were more fundamental as the Hill coefficient of the reaction was increased from 0.40 to 0.68. The Hill coefficient is commonly used to measure the cooperativity of a reaction, with a coefficient <1 indicating a non-Michaelian reaction and negative cooperativity. Cholesterol incorporation into the membrane promoted a reaction change from non-Michaelian to Michaelian that correlated with a decrease in bulk membrane fluidity.

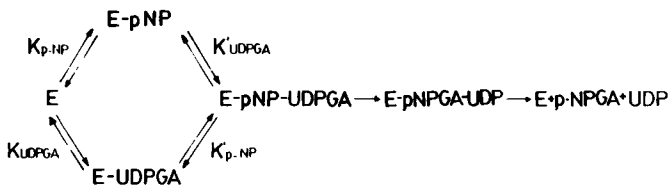

FIG. 3. Postulated reaction mechanism for UDP-glucuronyl transferase.

TABLE 1

Effect of Dietary Cholesterol (3%) on Lipid Composition of Guinea Pig Liver Microsomes (% weight)[a]

	Control	+ Cholesterol
Cholesterol	13.4 ± 0.4	28.7 ± 2.3
Cholesteryl esters	2.8 ± 0.1	4.6 ± 0.8
Triacylglycerol	3.6 ± 0.2	3.9 ± 0.9
Phosphatidylcholine	47.8 ± 1.8	41.5 ± 1.9
Phosphatidylethanolamine	25.3 ± 0.4	13.7 ± 0.5
Phosphatidylinositol	7.1 ± 0.1	7.6 ± 0.6
Double bonds/sat. acid	2.97 ± 0.03	3.04 ± 0.04
Cholesterol/Pcholine mol/mol	0.31 ± 0.10	0.46 ± 0.08
Pcholine/Pethanolamine mol/mol	1.75	2.78

[a]Lipids were separated and determined by thin-layer chromatography. Results are the mean of five animals analyzed separately ± S.E.

TABLE 2

Effect of Dietary Cholesterol on the r_s of Microsomal Membrane and Kinetic Constants of UDP-Glucuronyltransferase[a]

	Normal	+ Cholesterol
r_s	0.112 ± 0.004	0.131 ± 0.003
K_{UDPGA}(mM)	12.3 ± 0.4	8.1 ± 0.3
K'_{UDPGA}(mM)	10.6 ± 0.2	7.2 ± 0.1
$K_{p.NP}$(mM)	0.12 ± 0.03	0.07 ± 0.01
$K'_{p.NP}$(mM)	0.10 ± 0.01	0.06 ± 0.01
Vmax (nmol/min/mg prot)	10.5 ± 1.1	15.9 ± 1.3
Hill coefficient	0.40 ± 0.02	0.68 ± 0.03

[a]Fluorescence anisotropies (r_s) of diphenylhexatriene were measured at 37°C. Results are the mean of five animals analyzed separately ± S.E. K_{pNP} and K_{UDPG} correspond to the dissociation constants for the binary complex and K'_{pNP} and K'_{UDPGA} correspond to the dissociation constants for the ternary complex as shown in Figure 3.

TABLE 3

Effect of *in vitro* Change of Guinea Pig Microsomal Cholesterol Content on the Fluidity of the Membrane and Kinetic Properties of UDP-Glucuronyltransferase[a]

	−Cholesterol	Normal	+Cholesterol
Chol/Phosph	0.270	0.368	0.513
r_s	0.102	0.112	0.154
K_{UDPGA}(mM)	13.1	12.0	8.9
K'_{UDPGA}(mM)	10.9	10.3	6.2
Kp.NP (mM)	0.20	0.17	0.10
K'p.NP (mM)	0.15	0.12	0.08
Vmax (nmol/min/mg prot)	8.7	10.1	14.3
Hill coefficient	0.38	0.47	0.74

[a]Results are the mean of five animals analyzed separately ± S.E.

In vivo incorporation of cholesterol caused not only an increase in cholesterol/phospholipid ratio and viscosity of the microsomal membranes, but also a change in the phosphatidylcholine/phosphatidylethanolamine ratio. To determine whether or not this latter change could evoke the observed effect on the UDP-glucuronyltransferase, a similar change in cholesterol content of the microsomes was brought about by *in vitro* incorporation. *In vitro* incorporation or deprivation of cholesterol in microsomes was accomplished by use of a cytosolic cholesterol transfer protein in the presence of cholesterol-enriched or cholesterol-deprived liposomes. Similar results were obtained upon cholesterol incorporation or depletion of the microsomal membrane which decreased or increased, respectively, the fluidity of the bulk bilayer lipids which, in turn, affected the kinetics of the UDP-glucuronyltransferase reaction (Table 3) (6).

Zakim and colleagues (7) using a delipidated and purified UDP-glucuronyltransferase reconstituted the enzyme with lecithins in the liquid-crystalline state or in the gel phase and observed that the reaction was non-Michaelian with lipids in the gel phase, but Michaelian with lipids in the liquid-crystalline phase. These results,

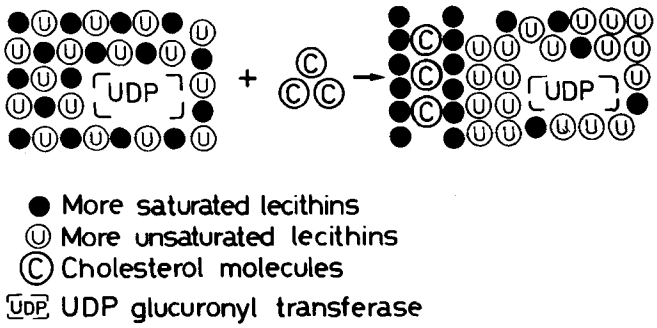

FIG. 4. Possible selective effect of cholesterol on phospholipid distribution in lipid bilayers.

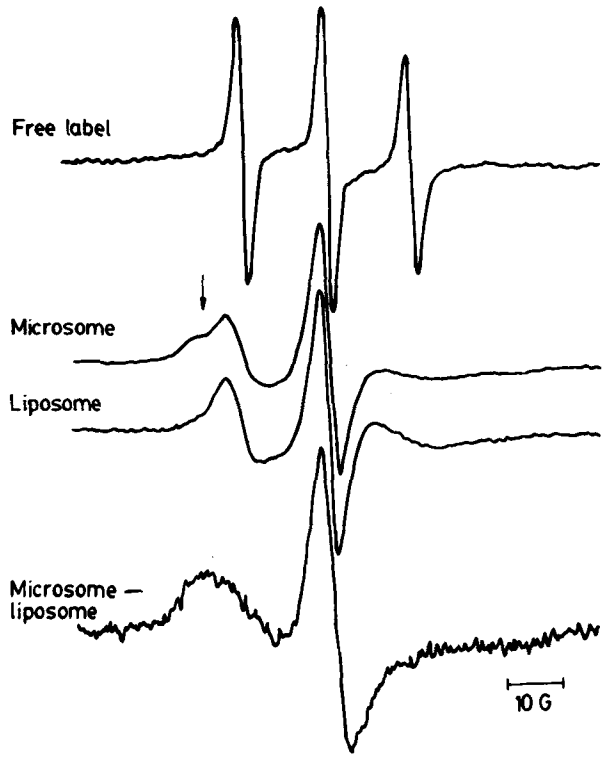

FIG. 5. ESR spectra of 3-doxyl-androstan-17-ol free and incorporated in guinea pig microsomes and liposomes. Arrow points to the shoulder produced by immobilized lipids.

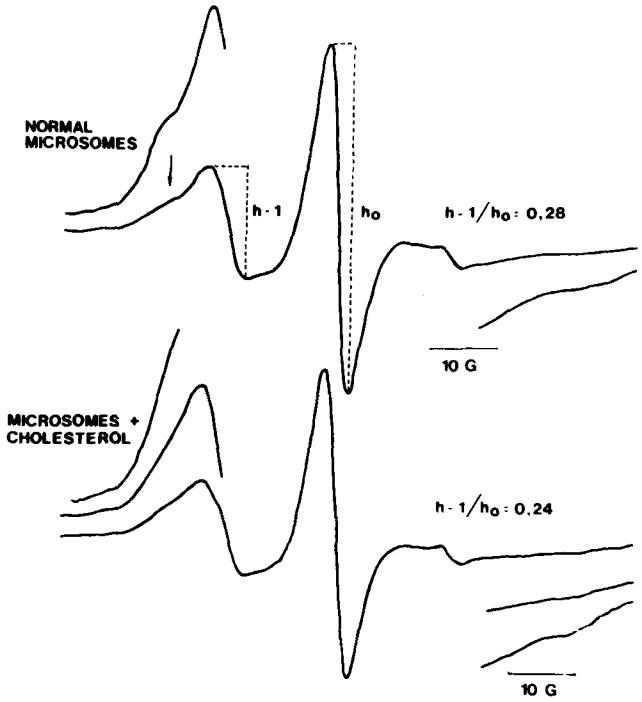

FIG. 6. Effect of cholesterol on ESR spectra of 3-doxyl-androstan-17-ol in guinea pig microsomes. Arrow points to the shoulder produced by immobilized lipids.

when compared with ours, indicate that one must differentiate between the bulk lipids of the membrane and the lipids bound in the enzyme domain.

It is entirely possible that in our experiments cholesterol incorporation into the membrane, while increasing the r_S and the order of the bulk lipids, would produce a fluidizing effect on the bound lipids. This effect could be produced by cholesterol if it would exert selectivity for specific phospholipids with lesser fluidity. It could, for example, associate preferentially with saturated lecithins, leaving behind unsaturated molecules that would remain in the enzyme domain (Fig. 4). In this respect, Melchior and Scautto (8), as well as Jain (9) and Presti *et al.* (10) have shown that cholesterol incorporation into a lipid membrane causes separation of cholesterol-phospholipid domains from pure phospholipid domains. Moreover, Gruyer and Bloch (11) have found that cholesterol increases the microviscosity of saturated phosphatidylcholine vesicles than the viscosity of unsaturated vesicles.

Additional information can be obtained by electron spin resonance (ESR). We labeled both liver microsomes and liposomes prepared from microsomal lipids with 3-doxyl-androstan-17-ol and studied the ESR spectra. The spectrum of the free label shows three peaks and indicates substantial mobility. Doxyl-androstan-17-ol incorporation into the microsomes decreased mobility as judged by the main spectrum, but gave rise to a small shoulder that corresponded to the lipids surrounding the microsomal proteins. This shoulder did not appear when the probe was incorporated in the pure microsomal lipids. By subtraction of the spectra, it was possible to obtain the spectrum of the protein-bound lipids (Fig. 5). Addition of cholesterol to the labeled microsomes resulted in a general decrease in the mobility of the main spectrum representative of the bulk lipids and caused a decrease in h_{-1}/h_0 ratio. However, it also caused the disappearance of the shoulder of the immobilized lipids (Fig. 6).

The interaction of cholesterol in microsomes and its effect on the UDP-glucuronyltransferase reaction may be representative of what generally happens in biological membranes. We suggest that cholesterol incorporation into a membrane, due to the specific affinity of cholesterol for selected lipids, can alter the composition of the bulk lipid phase and modify the microenvironment and properties of specific membrane proteins.

EFFECT ON FATTY ACID DESATURASES

The fatty acid desaturases are integral enzymes of the microsomal membranes which have been shown to be

responsive to cholesterol. There are three important desaturases: the Δ6, Δ5 and Δ9 desaturases that catalyze the formation of double bonds in Δ6, Δ5 and Δ9 position of fatty acids. All require the presence of the microsomal electron transport system that consists of the integral proteins NADH cytochrome b_5 reductase and cytochrome b_5 that transports electrons from NADH to the desaturase. All three components of the system are embedded in the microsomal membrane and, therefore, may be affected by the fluidity of the lipid bilayer.

In these experiments, we used rat liver because the fatty acid composition of guinea pig liver was not altered by the cholesterol diet. In rat, 1% dietary cholesterol administered for 21 days resulted in a significant change in the proportion of unsaturated acids in liver microsomes (3). After 21 days of cholesterol administration, the cholesterol/phospholipid molar ratio of the rat liver microsomes was increased from 0.30 to 0.61, and the bulk lipid fluidity, (as expressed by the fluorescence anisotropy of DPH) was markedly decreased (the r_s increased) (Fig. 7). The proportion of oleic acid was increased, whereas the proportions of arachidonic, eicosapentaenoic (20:5n-3) and docosahexaenoic acids (22:6n-3) were markedly decreased (Fig. 8). The effect was reversible (12) as depletion of cholesterol reversed the trends.

The changes in unsaturated fatty acid composition undoubtedly indicate a change in Δ9, Δ6 and Δ5 desaturase activities (Fig. 7). In fact, when the desaturase activity was measured, we found that cholesterol incorporation into the membrane increased Δ9 desaturase activity and increased Δ6 and Δ5 desaturases. The changes were reversible. Thus, *in vivo* cholesterol incorporation into rat liver microsomes decreases fluidity but increases phosphatidylcholine/phosphatidylethanolamine (PC/PE) ratios resulting in an increase in the desaturation of stearic acid to oleic acid and a decreased conversion of linoleic acid to arachidonic acid and of α-linolenic acid to eicosapentaenoic and docosahexaenoic acids.

Using liposomes enriched with cholesterol in the presence of a cytosolic cholesterol transfer protein, we produced *in vitro* incorporation of cholesterol into the microsomal membrane. Under these conditions (Fig. 9) we observed a decrease in membrane fluidity and an increase not only in the Δ9 desaturase activity, but also in Δ5 and Δ6 desaturase activities (4). The PC/PE ratio was not changed. Therefore, the same viscotropic effect was apparently produced *in vitro* by cholesterol incorporation on all three desaturases. We also observed that when we fluidized the microsomes by *in vitro* n-butyl or isoamyl alcohol treatment, a decrease in Δ9 and in Δ6 desaturase activity was brought about (13).

In the *in vivo* experiment, cholesterol incorporation increased not only the viscosity of the membrane but also the PC/PE molar ratio. Therefore, it is probable that this phospholipid change causes a decrease in the activities of Δ6 and Δ5 desaturases, which would overlap with the viscotropic effect. In order to test this, we fed rats for 21 days with a diet containing 5% of a β-sitosterol-campesterol mixture (14). This diet resulted in an increase in phytosterol in the membrane and a decrease in the fluidity of the microsomes. However, the PC/PE ratio was not changed. When the activities of the desaturases were measured, we found that the three desaturases were activated, as was observed in the *in vitro* experiments

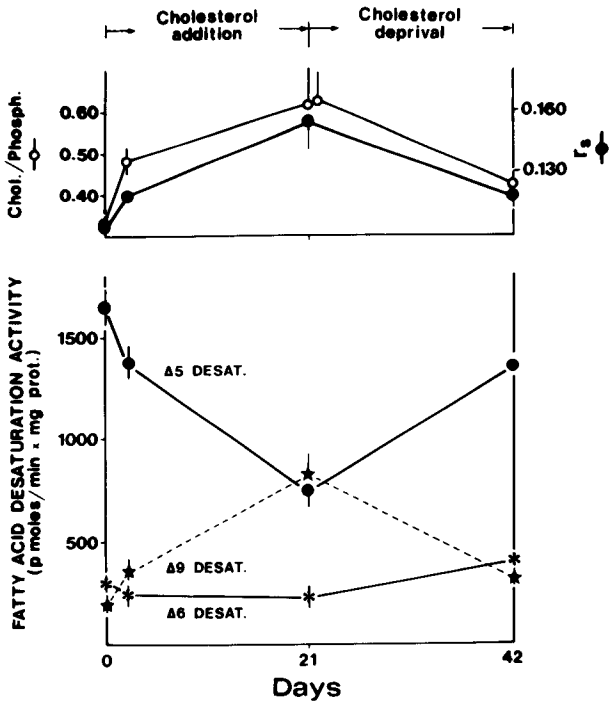

FIG. 7. Effect of dietary cholesterol on the cholesterol/phospholipid ratio, r_s and Δ9, Δ6 and Δ5 desaturase activities of rat liver microsomes. Δ6 Desaturation was measured by the microsomal [1-^{14}C]linoleic acid conversion to γ-linolenic acid; Δ5 desaturation was measured by the conversion of [1-^{14}C]20:3n-6 to arachidonic acid and Δ9 desaturation was measured by the conversion of [1-^{14}C]-palmitic acid to palmitoleic acid.

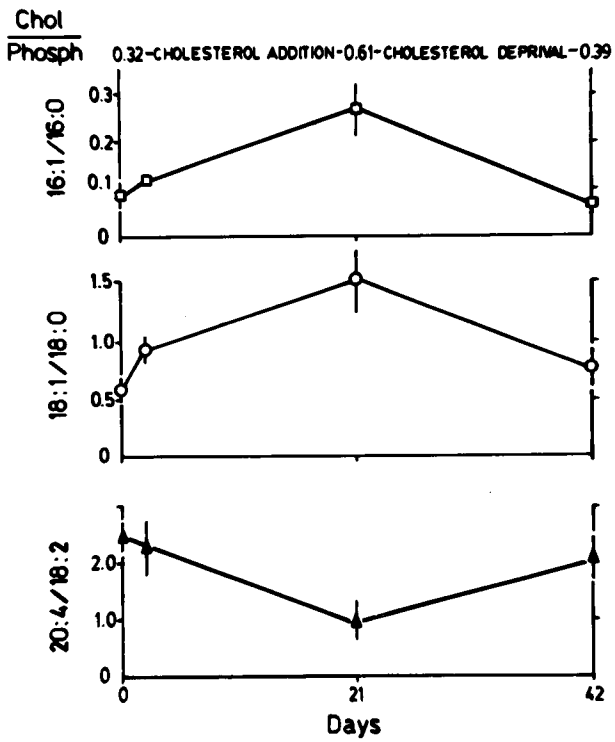

FIG. 8. Effect of dietary cholesterol on the fatty acid composition of rat liver microsomes.

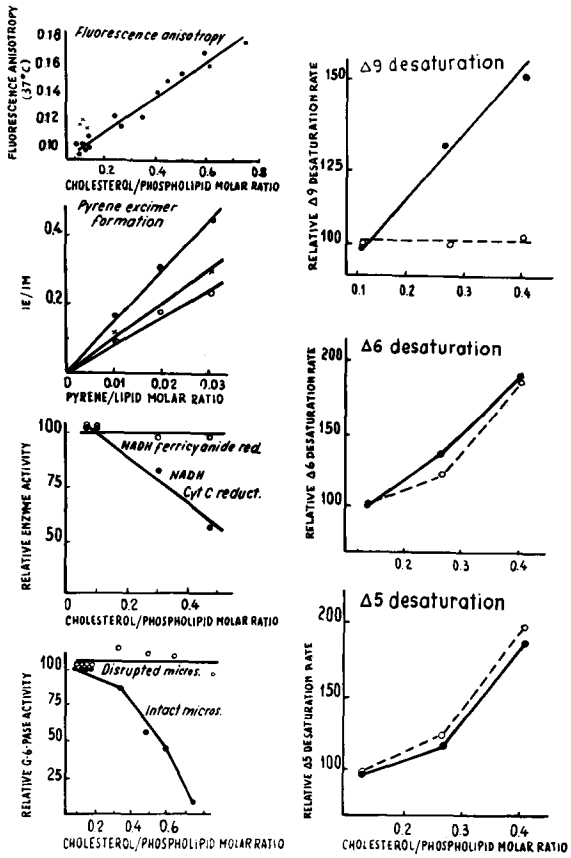

FIG. 9. Effect of cholesterol enrichment of microsomes *in vitro* on fluorescence anisotropy and pyrene excimer formation, electron transport system of microsomes, Δ9, Δ6 and Δ5 desaturation of fatty acids and glucose-6-Pase. Pyrene excimer formation: Cholesterol/phospholipid was equal to 0.120 mol/mol (●—●); 0.584 mol/mol (X—X); and 0.724 mol/mol (O—O). The fatty acid desaturases were tested at 2 μM (●—●) and 66 μM (O—O) concentration of substrate (4).

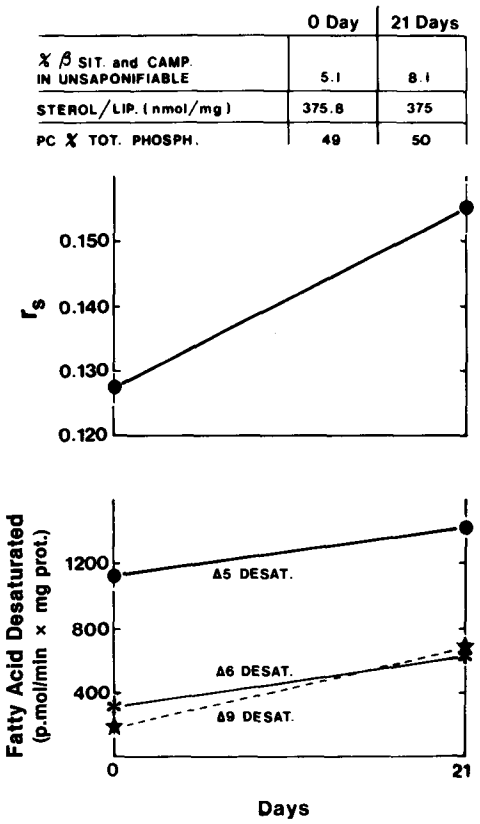

FIG. 10. Effect of 5% dietary campesterol (40%), β-sitosterol (60%) during 21 days on lipid composition, fluorescence anisotropy and Δ9, Δ6 and Δ5 desaturase activity of rat liver microsomes.

(Fig. 10). Therefore, the viscotropic effect was shown only in those instances in which the PC/PE ratio was not increased. When the cholesterol diet was used, just the opposite was observed. Therefore, the activities of Δ5 and Δ6 desaturases would not be regulated by a viscotropic change *in vivo*, as are the Δ9 desaturase, except in the case when the PC/PE ratio was not changed.

Activation of the Δ9, Δ6 and Δ5 desaturases by the phytosterol diet modified the fatty acid composition of liver microsomes as shown by an increase of the ratios of 16:1/16:0 and 18:1/18:0, as well as by an increase in 20:4n-6, 20:5n-3, 22:6n-3 and in the ratio of 20:4n-6 plus 22:4n-6/18:2.

The effects of cholesterol on both the UDP-glucuronyltransferase and fatty acid desaturases of liver microsomes illustrates that diet can cause chemical as well as physical membrane changes that result in specific modifications of membrane-bound enzymes. Some of these changes are due to viscotropic alterations, but others involve changes in the microenvironment of the membrane-bound enzyme.

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Changes in Murine Tissue Concentrations of Dolichol and Dolichol Derivatives Associated with Age

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The concentrations of the three major cellular forms of dolichol (free, esterified and phosphorylated) were determined in murine liver, kidney and heart. The tissue levels of these forms of dolichol were studied in detail as a function of age. Changes in the activities of dolichyl phosphate phosphatase and dolichol kinase were also determined. In liver, the concentration of unesterified dolichol, fatty acyl dolichol and dolichyl phosphate increased markedly over a period of 6 to 25 months (four-fold, 5.5-fold and nine-fold, respectively). In kidney only, free dolichol and phosphorylated dolichol increased (approximately four-fold in each case). However, this tissue consistently showed the highest concentrations of all forms of dolichol as compared to liver and heart. In heart, both free and esterified dolichol concentrations increased (approximately 3.25-fold in each case); dolichyl phosphate levels were not determined in this tissue. In all tissues studied, the activity of the dolichyl phosphate phosphatase enzyme was considerably higher than that of the dolichol kinase enzyme. In liver, there was no evidence to suggest that either enzyme was critical in determining the relative concentrations of dolichol and dolichyl phosphate. Evidence for such a role for the kinase in the kidney was stronger. Treatment of aging mice with meclofenoxate, a drug that is reported to cause dissolution of lipofuscin, failed to prevent accumulation of dolichol and dolichyl phosphate with age. These observations suggest that not all accumulated dolichol is associated with lipofuscin. Meclofenoxate treatment had no consistent effect on the activities of the enzymes studied.
Lipids 25, 586–593 (1990).

The accumulation of lipofuscin is arguably the single most discernible cytological change that occurs in the aging cell. The formation of the lipopigment is a continuous process that commences early in life and increases with age (1). Although the presence of this pigment within aging cells has long been recognized (2), its nature remains poorly understood. It is thought to contain an autofluorescent, conjugated Schiff base that may be formed by the *in vivo* peroxidation of polyunsaturated lipids. The aldehyde product of this reaction may subsequently complex with the amino groups of proteins (3). There is strong evidence that the pigment is associated with lysosomes (4,5).

As well as its chronological accumulation, lipofuscin is reported to build up within neurons of sufferers of a number of neurodegenerative disorders. Amongst these disorders are Batten Disease (6,7) and Alzheimers' Disease (8–10).

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Abbreviations: DP, dolichyl phosphate; DTT, dithiothreitol; ED, esterified (fatty acyl) dolichol; FD, free (unesterified) dolichol; HPLC, high performance liquid chromatography; i.p., intraperitoneal; NMR, nuclear magnetic resonance; TD, total dolichol; TLC, thin-layer chromatography.

The pathogenicity of lipofuscin in both the aging cell and in disease conditions is unproven. Rats fed vitamin E-deficient diets are reported to show a decrease in learning and memory functions along with an increase in lipopigment accumulation (11); while mice receiving the drug centrophenoxine show an increase in cognitive functions with a decrease in neuronal lipofuscin (12).

Age-associated increases in dolichols were first observed in human brain (13) and have since been reported in both neural and non-neural tissues of rats (14–18), mice (19) and dogs (20). More recent evidence suggests that the active metabolite of dolichol (dolichyl phosphate) might also accumulate with age (15).

Dolichol, like lipofuscin, is reported to accumulate in both Batten Disease (14,21–23) and Alzheimers' Disease (14,24,25). Analysis of lipofuscin isolated from tissues of Batten Disease patients suggests that the pigment might contain significant amounts of dolichols that exist in the free form (13,14,24,26,27).

An association between dolichol and lipofuscin is strengthened by several observations. Lipofuscin is reported to exhibit a similar nuclear magnetic resonance (NMR) spectrum to pure dolichol (28), the induction of lipopigment granules in rat brain is accompanied by increases in brain dolichols (29,30) and, finally, tissues thought to be rich in lipofuscin such as testis and kidney (31,32) also appear to be rich in dolichol (18,19).

The drug meclofenoxate (dimethylaminoethyl *p*-chlorophenoxyacetate) has been shown to improve cognitive function in old mice (12) and in the elderly (33,34). Such improvements are accompanied by a reduction in lipofuscin accumulation in both neural (35–39) and non-neural tissues (40). Similar reductions also have been reported in cultured cells (41). Meclofenoxate may therefore offer a convenient, but hitherto unused, method to explore the relationship between lipofuscin and dolichol.

The following study is an attempt to measure the changes in concentrations of all three major forms of dolichol (free, acylated and phosphorylated) in the liver, kidney and heart of aging mice. Two of the potentially most important enzymes involved in dolichol metabolism, i.e., dolichyl phosphate phosphatase and the CTP-dependent dolichol kinase, were also studied as a function of age. The effect of lipofuscin dissolution by meclofenoxate on these parameters was noted.

MATERIALS AND METHODS

Materials. Dolichol was a gift from Kuraray Co. (Okayama, Japan), as was [1-^{14}C]dolichol (50 mCi/mL). Standard dolichol and dolichyl phosphate were purchased from Sigma Chemical Co., Ltd. (Poole, Dorset, U.K.). Meclofenoxate was a gift from Anphar Rolland Laboratories, France.

[^{3}H]Dolichol was prepared by the reduction of dolichal with sodium boro-[^{3}H]hydride (5–10 Ci/mmole, Amersham International Ltd., Buckingham, U.K.) (42,43). Labelled and unlabelled dolichyl phosphate were prepared

by the method of Danilov and Chojnacki (44), with the exception that the Sephadex LH-20 desalting step was replaced by washing the $CHCl_3$ extract with 3 × 2.0 mL volumes of water to effect the removal of ammonium acetate. All other reagents, unless otherwise stated, were of analytical grade.

To minimize sample loss due to interaction with glass surfaces, all glassware was first treated with a solution of dimethyldichlorosilane (Repelcoat; BDH Chemicals Ltd., Dorset, U.K.) in accordance with the manufacturer's instructions.

Animals and treatment. A colony of 220 mice (strain MF1) was established in the animal unit of the University Hospital Medical School, Nottingham. All mice were maintained on identical, commercially prepared mouse chow and reared under identical conditions. The colony was divided into six subcolonies (SC1–SC6), each consisting of 40 individuals (20 male and 20 female), with the exception of SC1 which consisted of 20 individuals (10 male and 10 female). All members of a subcolony were born within two days of each other and were considered to be of identical age.

Members of a single subcolony were weighed and sacrificed (cervical dislocation) at predetermined ages ranging from 6 to 29 months. For 12 weeks prior to sacrifice 50% of the subcolony received daily intraperitoneal (i.p.) injections of meclofenoxate (80 mg/Kg of body weight in a final volume of 0.1 mL physiological saline). The remaining half of the subcolony received identical daily injections of physiological saline.

On sacrifice, heart, liver and kidney were removed, trimmed of excess fat, rinsed in distilled water and blotted dry. Tissues were immediately stored in sealed plastic bags at −80°C until assayed. Assays were always performed within one month of sacrifice, usually within a few days. Preliminary experiments indicated that dolichol, dolichyl phosphate and the related enzymes were stable under these conditions for up to three months.

Tissue preparation. Frozen tissue was allowed to thaw at room temperature before being "rough chopped" with scissors and homogenized at 4°C in a 0.25 M sucrose-Tris buffer (pH 7.5). Protein was determined by the method of Lowry *et al.* (45).

Assay for dolichyl phosphate phosphatase activity. The activity of dolichyl phosphate phosphatase enzyme was determined by the conversion of [^{3}H]dolichyl phosphate to [^{3}H]dolichol (46,47).

The standard reaction mixture contained 150 μM [^{3}H]dolichyl phosphate (110,000 DPM), 200 mM Tris-HCl (pH 7.5), 10 mM dithiothreitol (DTT) and 0.5% w/v Triton X-100 in a final volume of 400 μL. The reaction was started by the addition of 350 μg of homogenate protein. Tubes were incubated at 37°C for 30 min in a shaking water bath (40 strokes/min).

The reaction was terminated by the addition of 800 μL of $CHCl_3/CH_3OH$ (2:1, v/v) which also extracted the dolichol and dolichyl phosphate. After centrifugation at 2000 rpm for 20 min, the lower $CHCl_3$ layer was retained while the upper CH_3OH/H_2O was re-extracted by the addition of 2 × 400 μL of $CHCl_3$. $CHCl_3$ layers were combined and washed with 3 × 400 μL of CH_3OH/H_2O (1:1, v/v) before being taken to dryness under a stream of nitrogen.

Assay for dolichol kinase activity. Dolichol kinase activity in tissue homogenates was assessed by measuring the enzymatic conversion of [^{3}H]dolichol to [^{3}H]-dolichyl phosphate (48).

The standard incubation mixture contained 5 μM [^{3}H]dolichol (400,000 DPM), 200 mM Tris-HCl (pH 7.5), 60 mM $MgCl_2$, 15 mM CTP, 6.25 mM NaF, 0.2 mM *p*-chloromercuriphenyl sulphonic acid (*p*-CMBS) and 0.5% (w/v) Triton X-100 in a final volume of 200 μL. Incubation conditions and the extraction of labelled substrate were identical to the methodology described for the assay of dolichyl phosphate phosphatase.

Assay for radioactivity. [^{3}H]Dolichol and [^{3}H]dolichyl phosphate were separated by thin-layer chromatography (TLC) on SG 81 silica-impregnated paper (Whatmans Ltd., U.K.) that had been pre-treated according to the method of Steiner and Lester (49).

Dried $CHCl_3$ extracts from enzyme assays were redissolved in approximately 100 μL of $CHCl_3$ and spotted along a 15 mm line on a 200 mm × 200 mm TLC sheet. Standard dolichol and dolichyl phosphate (5 μg of each) were spotted alongside of the "test" lanes. TLC plates were developed in $CHCl_3/CH_3OH/H_2O$/acetic acid (75:20:2.5:2.5, by vol) the solvent front being allowed to run 120 mm from the origin. Authentic dolichol and dolichyl phosphate were visualized by exposure to iodine vapor. "Test" lanes were protected from iodine by a glass sheet. Bands corresponding to standard dolichol (Rf= 0.92) and dolichyl phosphate (Rf=0.40) were removed and cut into small strips. Radioactivity was assessed in a scintillation counter after the addition of 10.0 mL of Scintillator 199™ (Fisons Scientific Apparatus Ltd., Leichester, U.K.).

The activity of dolichyl phosphate phosphatase was expressed as μg product formed per mg protein per min and that of dolichol kinase as ng of product per mg protein per min.

Tissue extraction of dolichol and dolichyl phosphate. Crude tissue homogenates were extracted using a modification of the method described by Folch *et al.* (50).

For each 5.0 mL volume of homogenate (1.0 g tissue), 5.0 mL of CH_3OH was added and the mixture homogenized with 10 strokes of a motor driven Potter-Elvehjem homogenizer (medium speed setting). To allow correction of losses during extraction and isolation procedures, 1.5 ng of [1-^{14}C]dolichol and [1-^{14}C]dolichyl phosphate (110,000 DPM of each) were added. This was followed by $CHCl_3$ at the ratio of 5.0 mL per 5.0 mL of original tissue homogenate. The mixture was homogenized as previously described.

After an extraction period of 60 min at room temperature, centrifugation at 1500 rpm for 15 min gave three distinct layers. The lower $CHCl_3$ layer was removed and retained, while the upper CH_3OH/H_2O was washed with 2 × 5.0 mL volumes of $CHCl_3$. Finally the tissue pellet at the $CHCl_3/CH_3OH/H_2O$ interface was left to re-extract for 30 min in 6.0 mL of $CHCl_3/CH_3OH/H_2O$ (1:1:0.3, by vol). After centrifugation, the $CHCl_3$ layer was retained while the CH_3OH/H_2O layer was washed with 2 × 6.0 mL volumes of $CHCl_3/CH_3OH/H_2O$ (1:1:0.3, by vol).

Combined $CHCl_3$ layers from the extraction procedures were washed with 3 × 6.0-mL volumes of CH_3OH/H_2O (1:1, v/v) before being taken to dryness under a stream of nitrogen. Recovery of labelled standards at this

time was routinely in excess of 95% for dolichol and 92% for dolichyl phosphate.

Separation of dolichol and dolichyl phosphate. Dolichol and dolichyl phosphate were routinely separated by chromatography on 3.0 mL columns of DEAE cellulose (Sigma Chemical Company) equilibrated with $CHCl_3$/CH_3OH (2:1, v/v). Dry tissue extracts were re-dissolved in 0.5 mL of $CHCl_3$/CH_3OH (2:1, v/v) and applied to the column. A dolichol rich fraction was eluted with 10.0 mL of $CHCl_3$/CH_3OH (2:1, v/v), which contained 95% of labelled standard, while a dolichyl phosphate rich fraction was eluted with 20.0 mL of $CHCl_3$/CH_3OH (2:1, v/v) containing 0.25 M ammonium acetate. The recovery of dolichyl phosphate was routinely in excess of 92% (estimated by labelled standard), and the recovery of other dolichyl phosphate derivatives was assumed to be of the same order.

The dolichol-rich fraction was divided into two equal aliquots, one for the assessment of free dolichol and the other for assessment of total dolichol (free and ester forms). Both aliquots were taken to dryness under nitrogen.

The dolichyl phosphate-rich fraction from the ion exchange column was washed with 3 × 3.0 mL volumes of water to remove ammonium acetate and taken to dryness under nitrogen.

Saponification. The dry aliquot for total dolichol assessment was redissolved in 3.0 mL of cyclohexane. The saponification step involved a 45 min boiling period in a sealed tube in the presence of 1.0 mL of pyrogallol (1% in CH_3OH) and 2.0 mL of KOH (60%) in CH_3CH_2OH/H_2O (1:1, v/v). Dolichol was extracted from the mixture by the addition of 3.0 mL of freshly redistilled diethyl ether and 2.0 mL of water. After vortex mixing and centrifugation (1700 rpm for 5 min), the top ether layer was removed and retained. The remaining tube contents were re-extracted with a 3.0-mL volume of diethyl ether. Combined ether extracts were taken to dryness under nitrogen.

Sample purification. Dry aliquots for determination of total, free and phosphorylated dolichol were redissolved in 0.5 mL of propanol/methanol (20:80, v/v) and applied to Sep-pak RP C_{18} cartridges (Waters Associates, U.K.). Elution of the cartridges with propanol/methanol (20:80, v/v) (8.0 mL) removed polar contaminants from the sample while reduction in the polarity of the eluent to 80:20 (v/v) propanol/methanol (16.0 mL) permitted elution of a dolichol-rich fraction. For the elution of dolichyl phosphate, 0.1% ortho-phosphoric acid was added to the mobile phase.

Dolichol- and dolichyl phosphate-rich fractions (85% and 70% recovery, respectively, of labelled standard) from Sep-pak were taken to dryness under nitrogen and stored at −80°C for high performance liquid chromatography (HPLC) analysis.

High performance liquid chromatography. HPLC quantitation of dolichol and dolichyl phosphate was performed on an Ultrasphere ODS RP C_{18} column (5 μm; 4.6 mm × 250 mm) fitted with a precolumn of identical packing material (5 μm; 4.6 mm × 40 mm) (Beckman Ltd., U.K.). Column temperature was maintained at 24°C by a jacket and controller (Conair Churchill). Detection was at 210 nm on a Kratos SF 773 UV detector coupled to a SP 4270 integrator (both of Spectra Physics Ltd., Hertshire, U.K.). A flow rate of 1.0 mL/min was achieved using a single SP 8700 isocratic pump (also of Spectra Physics Ltd.).

Dry samples for HPLC were redissolved in 100 μL of HPLC mobile phase, and 10 μL was injected onto the column via a Reodyne 7125 injection system. The standard mobile phase for the elution of dolichol and dolichyl phosphate was propan-2-ol/methanol/hexane (40:40:20, by vol) (all HPLC grade) containing 0.1% ortho-phosphoric acid (19).

Dolichol/dolichyl phosphate, 17-21, were recovered after each HPLC run and assayed for radioactivity to assess the overall recovery of labelled standards (usually 60-80% for dolichol and 40-50% for dolichyl phosphate). The peak areas of sample dolichol/dolichyl phosphate (isoprenologs 18-20) were calculated by "valley to valley" integration and the concentration in the sample calculated from double log plots of standard dolichol (1-28 μg/10 μL) and dolichyl phosphate (0.05-12.35 μg/10 μL).

Dolichol and dolichyl phosphate concentrations were expressed as μg/g wet weight of tissue.

RESULTS

Figure 1 shows the body weight and liver weight of animals sacrificed at different ages. It demonstrates a steady rise in body weight until 20 months of age, followed by a small fall by 29 months (12% of maximum for males, 5% for females). Throughout this period males were heavier than females, as expected. Mean liver weights fluctuated only very slightly, showing a weak peak at 20 months. Treatment with meclofenoxate for 12 weeks prior to sacrifice caused no marked difference in total body weight or liver weight. Body weight of females older than 11 months was slightly lower than in controls. The weights of kidneys and hearts (data not shown) varied very little during this period.

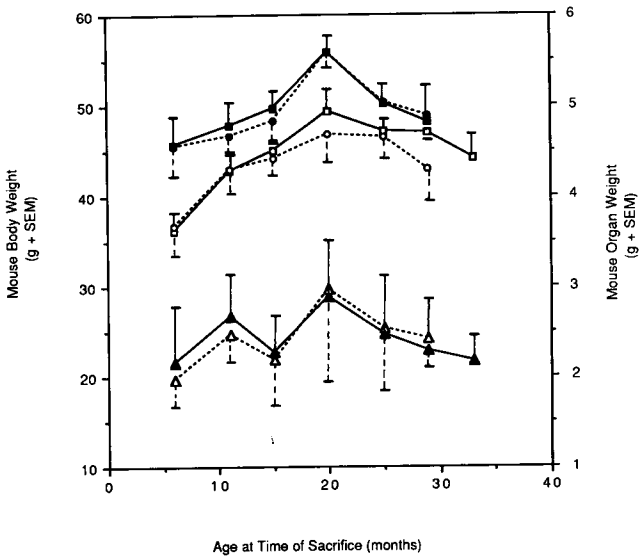


FIG. 1. Mean body weight and liver weight of male and female mice at the time of sacrifice. Body weight: —■—, control males; -●-, meclofenoxate males; —□—, control females; -○-, meclofenoxate females; liver weight: —▲—, control mice; and -△-, meclofenoxate mice.

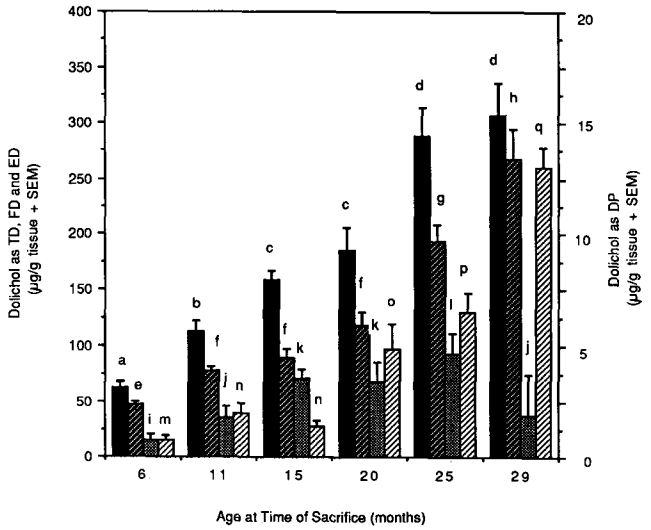
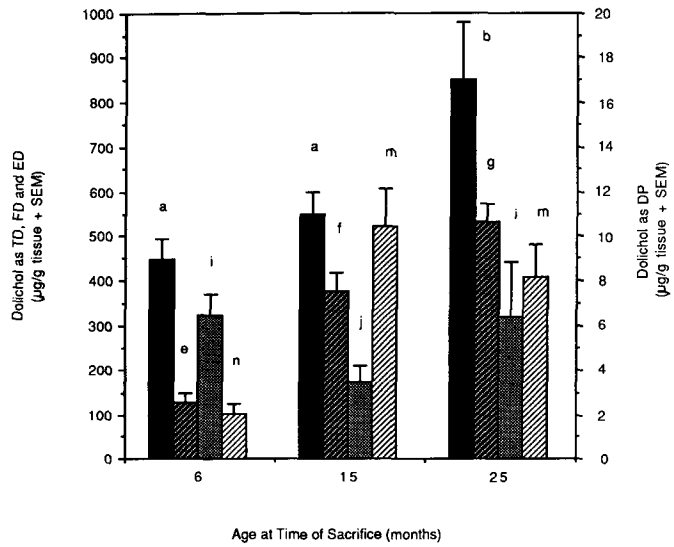


FIG. 2. Variation with age of the concentration of total dolichol (TD), of free (unesterified) dolichol (FD), of esterified (fatty acyl) dolichol (ED) and of dolichyl phosphate (DP) in liver of control mice. The standard error of the mean of three determinations on the liver of each of 3–8 mice at each time point is given. Statistically significant differences ($P < 0.05$) between the mean values are indicated by different letters, the series a to d, e to h, i to l and m to q relating respectively to total dolichol (solid bar), free dolichol (white diagonal lined bar), fatty acyl dolichol (grey bar) and dolichyl phosphate (black diagonal lined bar). Thus the same letter against two mean values indicates that differences between them are not statistically significant.



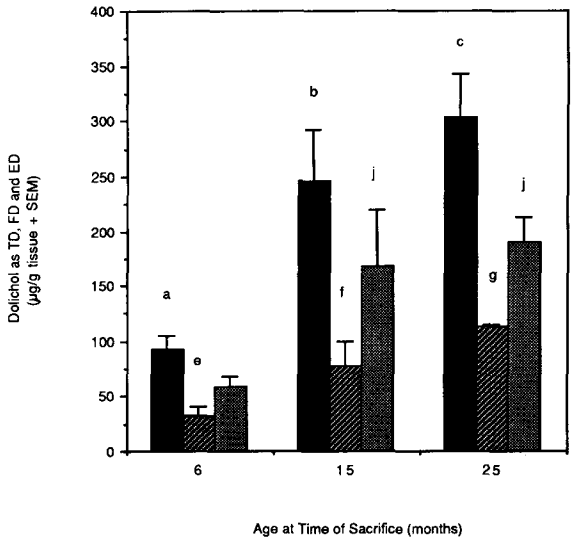


FIG. 3. Variation with age of the concentration of dolichol and its derivatives in kidney (a) and heart (b) of control mice. Details are as given in Figure 2.

At all ages, no statistically significant differences (Student *t*-test, $p=0.05$) between male and female mice with respect to the concentrations of free, esterified and phosphorylated dolichol and enzyme activity were observed. Meclofenoxate did not preferentially influence dolichol concentrations or enzyme activity of one sex (data not shown).

The concentration in liver of unesterified (free) dolichol and its fatty acid ester at regular time points during the life of mice up to 29 months is shown in Figure 2. A steady increase in total and free dolichol of five- to six-fold occurred during this period. A similar increase in the concentration of fatty acid ester of dolichol occurred up to the age of 25 months, after which a fall was apparent. Figures 3a and 3b demonstrate similar large increases in the free and total dolichol of kidney and heart over the 6 to 25 month period, with the proportion of ester varying. Kidney was the richest source of both free dolichol and its fatty acyl derivative at all time points studied. Generally, the proportion of the fatty acid ester was highest in heart and lowest in liver.

Changes in concentration of dolichyl phosphate in liver and kidney are summarized in Figures 2 and 3a, respectively. Heart tissue yielded insufficient dolichyl phosphate (less than 0.1 μg/g tissue) for a reliable assay. The results showed a 15-fold increase in hepatic dolichyl phosphate, most of this occurring between 15 and 29 months of age. In kidney, a five-fold increase was observed between 6 and 15 months of age and the concentration remained equally high over the next 10 months. Kidney was a richer source of dolichyl phosphate than was liver at 6, 15 and 25 months.

The activity of dolichyl phosphate phosphatase was assayed and the results are reported in Figure 4. Although the activity in liver fell slightly in early adult life, it then rose to reach the highest level at 29 months, being approximately double that observed at 6 months. In kidney at 6 months, the activity was about five times that in liver of the same age but it then changed very little over the next 19 months. A moderate increase in activity occurred in heart such that, although at six months it was just over half of that in liver, by 25 months both tissues showed very similar activities.

Dolichol kinase activity (Fig. 5) in kidney peaked at 15 months of age, and at this stage was much higher than in liver and heart. Differences between the tissues were less marked at 6 and 25 months.

The concentrations of dolichol and fatty acyl dolichol in livers of animals treated with meclofenoxate for 12 weeks prior to sacrifice are recorded in Figure 6.

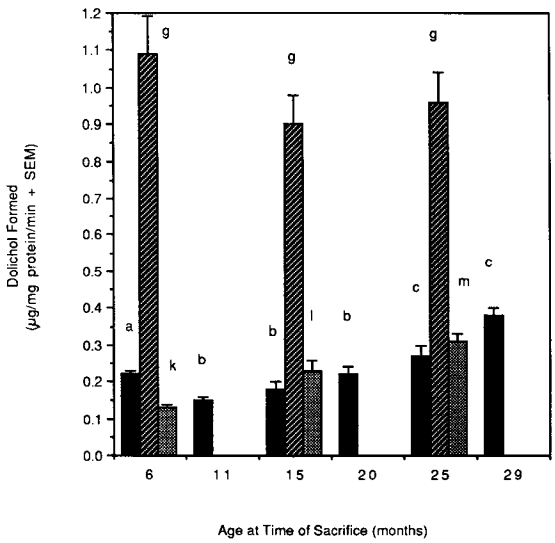


FIG. 4. Variation with age of the activity of dolichyl phosphate phosphatase in liver, kidney and heart of control mice. Statistically significant differences ($P < 0.05$) between the mean values are indicated by different letters, the series a to f for liver (solid bar), g to j for kidney (black diagonal lined bar) and k to m for heart (grey bar). Thus the same letter against two mean values indicates that the differences between them are not statistically significant.

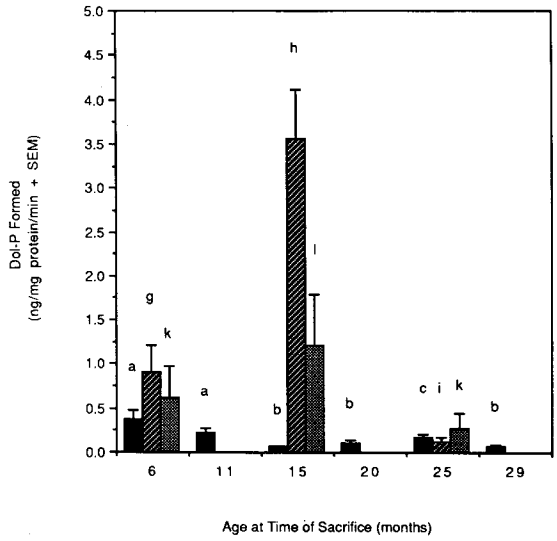


FIG. 5. Variation with age of the activity of dolichol kinase in liver, kidney and heart of control mice. Statistical data are as detailed in Figure 4.

Comparisons with Figure 2 reveal that in the livers of young adult mice (six-months-old), the treatment caused a marked increased (2.5-fold) in the concentration of both free and esterified dolichol. Over the next 14 months, the differences became less, having almost disappeared at 20 and 25 months of age. However, an increase seen at 29 months in untreated animals was prevented by treatment with meclofenoxate. The increase in the amount of fatty acyl dolichol was maintained until the 25th month, varying between 1.2 and 2.5 times the control figure, but then fell and became lower than the control figure.

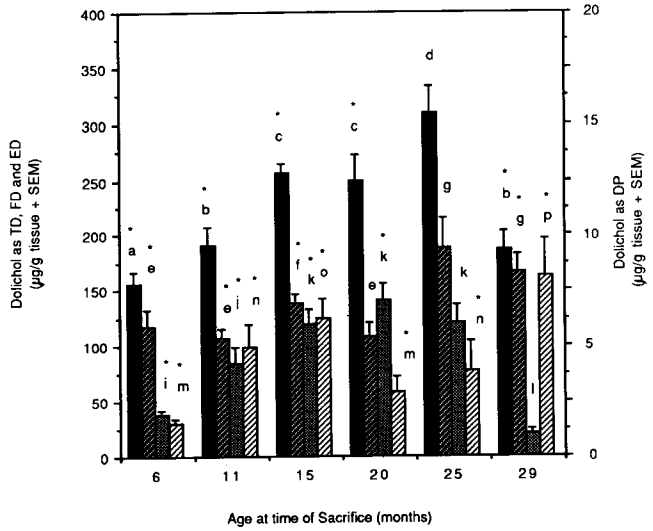


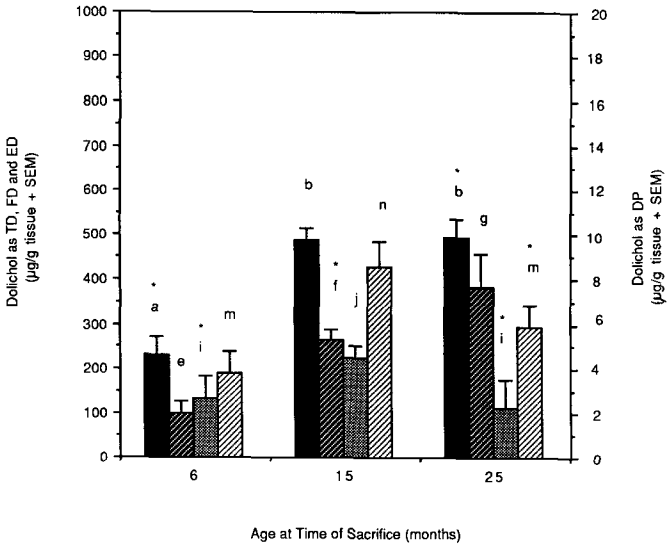
FIG. 6. Variation with age of the concentration of dolichol and its derivatives in liver of mice treated with meclofenoxate. Details are as given in Figure 2. The presence of an asterisk (*) denotes a significantly different value ($P < 0.05$) from controls (i.e., a comparison with Figure 2.)

In kidney (Fig. 7a), meclofenoxate caused a moderate fall in dolichol concentrations in older mice (compare with Fig. 3a), but its effect on fatty acyl dolichol was much more dramatic, causing a decrease to less than a half of that at both 6 and 25 months. In heart (Fig. 7b), the most significant effect of meclofenoxate treatment was at 15 months, when both free and esterified dolichol were markedly lower than in controls (Fig. 3b). Meclofenoxate caused a smaller response to aging of dolichyl phosphate levels in liver (Fig. 6) at 20 months and beyond, but an increase was not prevented. Similarly, in kidney (Fig. 7a), the increases were much less marked.

With regard to dolichyl phosphate phosphatase in liver, meclofenoxate stimulated an increase in activity at all ages, with that at 25 months being particularly marked (almost three-fold, see Fig. 8). In kidney and heart, differences caused by the treatment were small, becoming negligible in older mice. Although meclofenoxate reduced the size of the peak of kidney dolichol kinase activity at 15 months, by 25 months its effect was stimulatory. Figure 9 shows that the treatment caused a fall in kinase activity in heart, especially in younger mice, in contrast to liver, where after six months meclofenoxate consistently stimulated a small increase in dolichol kinase activity.

DISCUSSION

The finding that kidney is the richest source of dolichol in mice is in keeping with the observations of Pullarkat *et al.* (19) and is in contrast to rat and human tissues in which liver yields the most (51–54). Mouse kidney is also a richer source of lipofuscin than liver and heart (31,32,53,54). The actual concentration of total dolichol in heart and liver of six-month-old mice was very close (within 2%) to that reported by Pullarkat *et al.* (19) for a different strain of mice whereas in kidney the yield was 33% higher. Comparisons of older mice suggest that



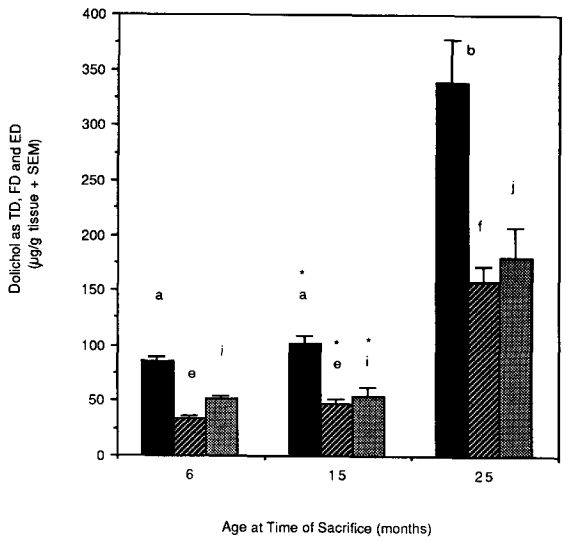


FIG. 7. Variation with age of the activity of dolichyl phosphate phosphatiase in liver, kidney and heart of mice treated with meclofenoxate. Statistical data are as detailed in Figure 4. The presence of an asterisk (*) denotes a significantly different value (P <0.05) from controls (i.e., a comparison with Figure 4.)

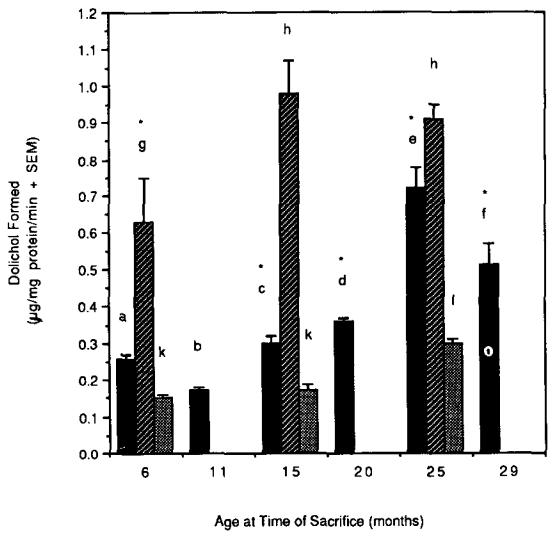


FIG. 8. Variation with age of the activity of dolichyl phosphate phosphatase in liver, kidney and heart of mice treated with meclofenoxate. Statistical data are as detailed in Figure 4. The presence of an asterisk (*) denotes a significantly different avlue (P <0.05) from controls (i.e., a comparison with Figure 4.)

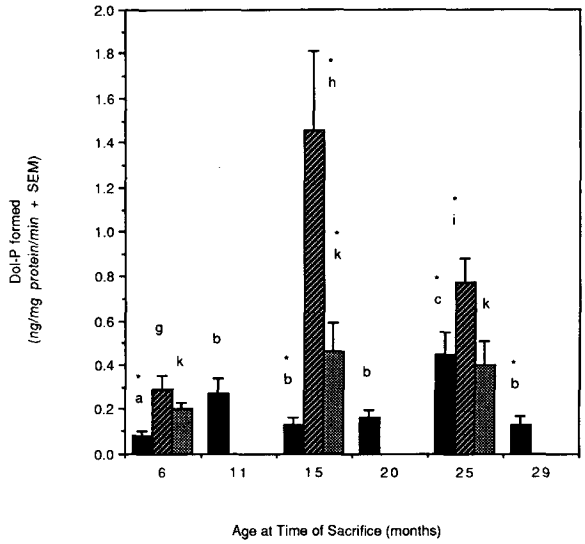


FIG. 9. Variation with age of the activity of dolichol kinase in liver, kidney and heart of mice treated with meclofenoxate. Statistical data are as detailed in Figure 4. The presence of an asterisk (*) denotes a significantly different value (P<0.05) from controls (i.e., a comparison with Fig. 5.)

Pullarkat's strain, while showing significant increases in concentration of dolichol with age, responded less markedly, especially in kidney and liver, than did the Nottingham strain. A more recent report by Crick and Rip (55) support our observations of increasing dolichol concentrations in murine tissues with age. However, the authors also report a decrease in the concentration of liver dolichyl phosphate in mice aged between 1 and 24 months. The reason for this difference between the two studies is not clear, but it may be due, at least in part, to the different methodology employed and/or biological variation between the strains of mice studied.

There are no published results against which to compare directly the assays of fatty acyl dolichol reported for mice in Figures 2, 3a and 3b. The proportion of ester varies markedly from species to species for any single tissue and from tissue to tissue in any single species (54,56-60). Figure 2 shows that in liver the fraction of dolichol that is esterified remains fairly constant at about 65% of the total dolichol over the 25 months studied. Kidney started with an equally high level at six months, but then remained at a concentration lower than the liver over the next 19 months.

Sakakihara and Volpe (16) observed that in rat brain the fraction of dolichol that was esterified remained fairly constant during development and aging. This relationship is similar to that of cholesterol and its fatty acid esters, which led the authors to suggest that dolichyl ester may be a convenient storage form of dolichol. It has been

reported (57) that in rat liver the concentration of free dolichol remains fairly constant, but that of ester increases during early stages of growth (weanling to 5 months of age). All such observations suggest that at all stages of development and aging, most tissues contain a substantial fraction of their dolichol in the fatty acyl form.

The proportion of dolichol recovered as phosphate was routinly less than 4% in all three murine tissues studied. Most authors using similar extraction procedures report a low figure, e.g., in rat liver 2%, 4% and 10% (51,61) and in human liver 4% (53). It has been argued by Keller *et al.* (62) that these figures are low due to inadequate extraction procedures. By direct saponification of tissue followed by ether extraction, Keller's group reports higher concentrations of dolichyl phosphate in rat tissues. Indeed, they suggest that in several tissues the concentration exceeds that of non-phosphorylated dolichol. Preliminary attempts by the authors to repeat the extraction procedure of Keller *et al.* (62) on murine tissues failed to detect higher concentrations of dolichyl phosphate than are shown in Figures 2 and 3a.

The concentration of dolichyl phosphate in rat tissues during the first few months of life is reported to remain fairly constant (15,20). Keller's group also reports (20) that in two normal English setters, aged 13 and 45 months, the concentration of dolichyl phosphate was essentially the same. On the other hand, the results reported in Figures 2 and 3a, which represent the first systematic investigation of dolichyl phosphate concentrations in a group of aging animals demonstrate very clearly marked increases in both liver and kidney as aging proceeds.

The enzyme studies (Figs. 4 and 5) show a much higher dolichyl phosphate phosphatase than dolichol kinase activity at all ages and in all three tissues studied. The range of values in murine liver 0.2–0.38 μg product/mg protein/min) is intermediate between values reported for rat liver by Boscoboinik *et al.* (63) and Rip *et al.* (47), i.e., 0.16 and 1.70 μg product/mg protein/min, respectively. In heart, a moderate increase in phosphatase activity between 6 and 25 months echoes the increased dolichol content observed over the same period. However, the 1.7-fold increase in hepatic phosphatase between 6 and 29 months, although substantial, is considerably less than the change in concentration of dolichol over the same period. Similarly, in kidney the increase with age in the concentration of dolichol is not matched by a corresponding increase in phosphatase activity. The liver and kidney results are consistent with the view expressed by Ekström *et al.* (64,65) that the formation of dolichol may not be solely by dephosphorylation of dolichyl phosphate. However, further information on the rate of degradation of dolichol and on its rate of *de novo* synthesis in these tissues is essential if this view is to be established further.

Comparison of Figures 2 and 5 shows little or no correlation of changes in the activity of hepatic dolichyl kinase with changes in the concentration of its product dolichyl phosphate or its substrate dolichol. This may be partly the result of the location of at least some of the putative substrate and product in separate subcellular pools that are not available to the enzyme. It may also suggest that the concentrations of dolichol and dolichyl phosphate are also strongly influenced by the rate of their *de novo* synthesis and/or catabolism. Whatever the reasons, it is clear that the results in this paper provide no support for the view that the activities of dolichol kinase and dolichyl phosphate phosphatase dictate the tissue concentrations of dolichol and dolichyl phosphate in mouse liver.

In kidney, the kinase is much more active than in liver, in keeping with the higher concentration of dolichyl phosphate in the former tissue. Also, the large increase in activity at 15 months as compared with that at 6 months correlates well with a large peak in the concentration of dolichyl phosphate at the later age. However, the big drop in enzyme activity at 25 months of age is accompanied by a relatively small fall in the concentration of dolichyl phosphate. Thus, although the evidence from kidney is more consistent with the kinase having a critical role in the control of dolichyl phosphate levels, it is clearly not the whole story.

There was no correlation between murine heart dolichol content and the activity of the kinase in this tissue. This, coupled with the failure to detect dolichyl phosphate, leaves the importance of the role of this enzyme in this tissue obscure.

The effect of meclofenoxate treatment on liver dolichol was biphasic. In mice before 20 months of age, it resulted in elevated levels with respect to controls, but in older mice (29 months of age) it brought levels of dolichol and dolichyl ester below those of controls. This latter phase was seen in kidney at all times studied, as well as in heart at 15 months of age, and is consistent with an association of dolichol and its ester with lipofuscin. The meclofenoxate treatment adopted is reported to be sufficient to cause dissolution of most of the lipofuscin present (38,39). However, appreciable quantities of dolichol still accumulate in the presence of meclofenoxate. This suggests that not all of the accumulated dolichol in these tissues is associated with lipofuscin.

The response of dolichyl phosphate concentrations in liver and kidney to meclofenoxate treatment was similar in showing an increase (over controls) in younger mice but attenuating the age-related increase observed in older control mice. Whether or not this fall in very old mice results from (or in) the corresponding latter fall in dolichol, or if it has any important consequences for the rate and extent of protein N-glycosylation is uncertain and requires further investigation.

Neither dolichyl phosphate phosphatase nor dolichol kinase showed a consistent response to meclofenoxate treatment. Correlations of these responses with changes in dolichol and dolichyl phosphate concentrations were poor.

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Caecal and Colonic Uptake of both Linoleic Acid and Cholesterol in Rats Following Intestinal Resection

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Caecal and colonic uptake of both linoleic acid and cholesterol were studied in rats after distal small bowel resection (DSBR). The results showed that the surgical operation increased the caecal and colonic uptake of linoleic acid. Supplementation with linolenic acid inhibited caecal and colonic uptake of linoleic acid. Experiments carried out in the presence of rotenone and ouabain suggest that facilitated diffusion is the predominant mechanism of caecal and colonic linoleic acid absorption, at least at low concentrations. An increase in caecal and colonic uptake of cholesterol was observed after the surgical operation. The study showed that facilitated diffusion seems to be the mechanism of linoleic acid absorption in the caecum and colon, and that both organ growth and changes in transport function of the epithelial cells of caecum and colon appear to be involved in the adaptive response of the bowel to intestinal resection.
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The long-term outcome of massive small bowel resection depends mainly on the adaptive capacity of the remnant intestine. Both, morphological and functional adaptations of the remaining small intestine have been extensively studied (1). However, few studies have examined the effect of distal small bowel resection (DSBR) on the adaptive processes in the large bowel.

Linoleic acid is the most common essential fatty acid in the mammalian diet. Dietary deficiency of linoleic acid or its intestinal malabsorption can result in a wide variety of disorders (2). However, despite the biological importance of linoleic acid, its mode of absorption by the large intestine has not been studied previously. On the other hand, it is well known that DSBR affects cholesterol metabolism, including hepatic cholesterol synthesis (3,4).

The aim of the current study was to investigate the effect of DSBR on cholesterol and linoleic acid transport in the large intestine (caecum and colon). The mechanism of intestinal absorption of linoleic acid in the large intestine was evaluated.

MATERIALS AND METHODS

Chemicals. [^{14}C]Linoleic acid and [^{14}C]cholesterol (Amersham, UK) with specific activities of 50-60 mCi/mmol were used as tracers. [^{3}H]Inulin (Amersham, UK) with a specific activity of 1-5 mCi/mmol was used as an extracellular water marker. Cholesterol and linoleic acid (99% of purity) and all other chemicals were from Sigma Chemical Co. (St. Louis, MO).

Animals. Male Wistar rats weighing about 300 g were maintained on a standard pellet diet with free access to tap water. The rats were randomly assigned to one of three groups: sham-operated, 50% and 75% DSBR. The rats were anaesthetized with intraperitoneal sodium pentobarbital (4,5 mg/100g body wt) after a 24-hr fast period. Laparotomy was performed, and rats assigned for DSBR underwent either 50% or 75% DSBR, as described by Murillo *et al.* (5). Briefly, the blood vessels of the resected intestinal segment were tied and sectioned, but the blood supply and innervation of the remaining intestine were carefully maintained. Intestinal continuity was reestablished by end-to-end anatomosis with Mersilene 3/0 thread. In another group, sham operation was performed, and the intestine was cut and reanastomosed without resection. In each instance, continuity of the gut was restored by end-to-end anastomosis. Six weeks after the surgical operation, animals were starved overnight (with access to water only) and killed by stunning and cervical dislocation. Both control and experimental groups were treated in the same manner to prevent effects that could mask differences between groups.

Tissue preparation. After the animals were killed, the abdomen was opened, caecum and colon were rapidly removed, gently rinsed free of intestinal contents with ice-cold saline (0.9% NaCl) solution and their weights (caecum and colon) and lengths (colon) recorded.

Intestinal sacs weighing about 100 mg were tied off and kept in cold saline solution until used. No fluid was placed in the serosal compartment. The intestinal sacs were preincubated for 10 min at 37°C in gassed Ringer's solution at pH 6.5 for linoleic acid or pH 7.4 for cholesterol (see below), to allow for equilibration at this temperature. In order to get intestinal tissue depleted of cellular adenosine triphosphate (ATP), in a separate experiment sacs were preincubated in the presence of 80 μM rotenone (metabolic inhibitor) and 20 μM ouabain (Na^+,K^+-ATPase inhibitor) for 15 min at 37°C (6). Sacs were then transferred to the incubation medium. Both, preincubation and incubation solutions were mixed at identical stirring rates of 750 revolutions/min in order to achieve low effective resistance of the intestinal unstirred water layer.

Preparation of incubation solution. Lipids were dissolved in 10 mM NaTC-Ringer's solution which contained (in mM): 140 NaCl; 10 KH_2PO_4; 2.4 K_2HPO_4 and 1.2 $MgCl_2$ instead of 1.2 $CaCl_2$ to avoid fatty acid soap formation.

Linoleic acid micellar solutions were prepared as previously described (7). Briefly, an appropriate amount of both ^{14}C-labeled and unlabeled linoleic acid were dissolved in a small volume of ethanol. Ten mM NaTC-Ringer solution at pH 6.5 [to obtain maximum absorption (7,9)], previously gassed with 95% O_2 5% CO_2, was added to give a final linoleic acid concentration of 0.05 mM. The micellar solution was prepared by ultrasonic irradiation for 10 min. If necessary, the pH was readjusted to 6.5. On separate occasions, linolenic acid was added to the linoleic acid solution at a concentration of 3 mM.

The technique used to prepare the cholesterol micellar solutions has been published previously (8). An

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Abbreviations: DSBR, distal small bowel resection; NaTC, sodium taurocholate.

appropriate amount of both ^{14}C-labeled and unlabeled cholesterol was dissolved in an exact volume of chloroform/methanol (2:1, v/v). The chloroform/methanol phase was evaporated and the Ringer's solution (pH 7.4) previously gassed was added. The micellar solution was prepared by ultrasonic irradiation for 10 min. The solution was then further diluted by the addition of Ringer's solution with NaTC 10 mM and traces of [^{14}C]cholesterol to give a final cholesterol concentration of 0.05 and 0.2 mM.

A trace amount of [^{3}H]inulin as a radiolabeled volume marker was also added.

Linoleic acid and cholesterol uptake. After preincubation, sacs were transferred to the incubation medium containing [^{3}H]inulin and either [^{14}C]linoleic acid or [^{14}C]cholesterol. Following incubation of intestinal sacs in labeled solutions for 6 min at 37°C, sacs were removed and quickly rinsed with 1 mM NaTC in order to remove some of the adherent incubation solution off the sac's surface. Sacs were then gently blotted on filter paper, weighed and dried overnight at 60°C. This temperature did not lead to any loss of linoleic acid. Dry sacs were weighed and saponified with 0.75 N NaOH. Scintillation fluid was added and radioactivity was determined.

Expression of results. The rate of uptake of solutes was calculated after correcting the total tissue ^{14}C radioactivity for the mass of the probe molecule present in the adherent mucosal fluid, and these rates were expressed as the pmoles of substrate taken up into the mucosal per mg wet intestinal tissue per 6 min incubation.

Values obtained are reported as the mean ± S.E. of results observed for a minimum of five separate experiments. Each experiment is a pool of two large intestine (caecum or colon) from two different rats.

The effect of DSBR on the intestinal uptake of substrate was examined by analysis of variance (ANOVA) procedures. The unpaired student's t-test was used to test the significance of the difference between the means for sham-operated and resected rats.

RESULTS

Animal characteristics. Postoperative mortality was 10% and 20% after 50% and 75% DSBR, respectively. Deaths occurred within the first 5 post-operative days and were attributed to the surgery. Initial body weights in each group of animals were the same. At the time of study, 6 wk after the surgical operation, mean body weights were significantly lower in both 50% and 75% resected rats, this decrease being higher after 75% than after 50% DSBR. Caecal tissue wet weights were significantly increased after both 50% and 75% DSBR, the increase being related to the extent of intestine removed. However, colonic tissue mass, expressed as mg/cm, only significantly increased after the massive resection (75%) (Table 1).

Uptake of linoleic acid and effect of linolenic acid. The absorption of 0.05 mM linoleic acid was studied in caecum and colon after DSBR. The results show that the surgical operation increased the caecal and colonic uptake of linoleic acid, the increase not being relative to the extent of the intestine removed (Fig. 1). Since the intestinal uptake of linoleic acid in jejunum appears to be mediated by a facilitated diffusion mechanism, and inhibited by linolenic acid (7,9), we also studied the effect of linolenic acid (3 mM) on caecal and colonic uptake of linoleic acid (0.05 mM). Results in Figure 1 indicate that the addition of linolenic acid inhibited the caecal and colonic uptake of linoleic acid in the three groups of animals, the rate of the inhibition being similar between sham and resected animals.

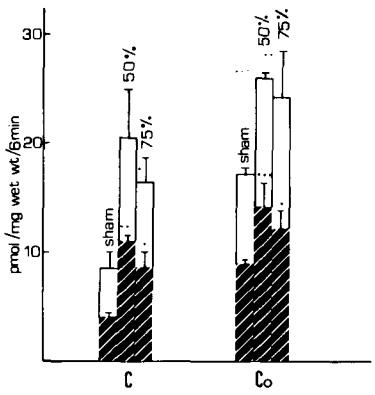

FIG. 1. Effect of DSBR on caecal (C) and colonic (Co) uptake of linoleic acid (0.05mM). Influence of linolenic acid (3mM) (white diagonal lines). Results are given as means ±S.E. of five separate experiments. Each experiment was done on 2 pooled large intestines (caecum or colon) from two rats. *p<0.05, **p<0.01, ***p<0.005, ****p<0.001 50% or 75% resected animals compared with corresponding sham animals.

TABLE 1

Effect of DSBR on Body Weight and Intestinal Tissue[a]

	Sham	50%	75%
Body weight (g)			
At entry	306 ± 2	306 ± 7	302 ± 8
At study	387 ± 13	354 ± 10*	249 ± 12**,[b]
Intestinal tissue			
Caecum (g)	1.91 ± 0.12	2.90 ± 0.26***	4.20 ± 0.32***,[b]
Colon (mg/cm)	0.093 ± 0.006	0.112 ± 0.033	0.166 ± 0.028*

[a]Results are given as means ±S.E. of 10 animals in each group. *p<0.05, **p<0.01, ***p<0.005, ***p<0.001. 50% or 75% resected animals compared with sham animals.

[b]p<0.001. 75% compared with 50% resected animals.

Linoleic acid uptake by ATP-depleted intestinal tissue. Uptake of 0.05 mM linoleic acid was investigated in caecal and colonic intestine of control rats depleted of ATP by 15-min preincubation in the presence of rotenone and ouabain, in order to explore the possible energetic requirements of linoleic acid intestinal uptake in these segments (caecum and colon). Neither in the caecum nor in the colon, does the addition of these compounds change the rate of linoleic acid uptake from its rate of uptake under basal conditions (Table 2).

Uptake of cholesterol. The effect of DSBR on the uptake of cholesterol in the caecum and colon is shown in Figure 2. The surgical operation increased both caecal (Fig. 2A) and colonic (Fig. 2B) uptake of cholesterol measured at 0.05 and 0.2 mM. The observed increase in the caecum was higher after 75% than after 50% DSBR. However, in the colon, the observed enhancement in cholesterol uptake was not related to the extent of intestine removed at any concentration measured.

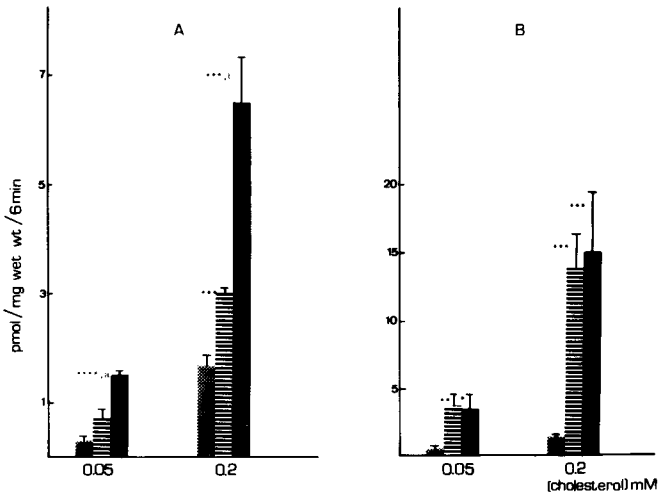

FIG. 2. Effect of DSBR on caecal (A) and colonic (B) uptake of cholesterol. Results are given as means ±S.E. of five separate experiments. Each experiment was done on 2 pooled large intestines (caecum or colon). *p<0.05, **p<0.01, ***p<0.005, ****p<0.001 50% (horizontal lined bar) or 75% (solid bar) resected animals compared with sham animals (shaded bar). [a]p<0.05, [b]p<0.01 75% compared with 50% resected animals.

DISCUSSION

The caecal and colonic uptake of linoleic acid was studied *in vitro* in sham-operated and resected animals. The results show that the uptake of linoleic acid increased in the caecum and colon after DSBR, neither enhancements being related to the extent of intestine resected (Fig. 1).

It has been previously reported that the uptake of linoleic acid by small intestine is by a facilitated diffusion mechanism which could be carrier mediated (7,9). Thus, the addition of other long-chain unsaturated fatty acids (such as oleic, linolenic or arachidonic acid) to the solution containing low concentrations of linoleic acid inhibited linoleic acid absorption in small intestine by competing for a common carrier which may be responsible for the absorption of the compounds.

In order to explore the mechanism responsible for linoleic acid uptake in the large intestine, linolenic acid was added to the linoleic acid micellar solution. The results showed that the addition of 3 mM linolenic acid inhibited the caecal and colonic uptake of linoleic acid in the three groups of animals (Fig. 1), the rate of inhibition being very similar between sham-operated and resected animals in both caecum (46% and 48% for 50% and 75% DSBR, respectively, vs 52% for sham-operated rats) and colon (47% and 48% for 50% and 75% DSBR, respectively vs 48% for sham-operated animals). These findings suggest (7,9) that both the caecal and colonic uptake of linoleic acid is mediated by either active transport or facilitated diffusion.

In order to differentiate both mechanisms, experiments were performed in the caecum and colon of sham rats by preincubating the tissue for 15 min with rotenone and ouabain that deplete the intestinal cells of ATP. The results showed that addition of these agents to the preincubation medium did not change the uptake of linoleic acid either in the caecum or colon, when compared to absorption under basal conditions (Table 2). These results may indicate that at low luminal concentrations, the absorption of linoleic acid in the caecum and colon does not require metabolic energy, and suggest that linoleic acid might be absorbed by a facilitated, carrier-mediated diffusion mechanism. However, the possibility that the observed decrese in caecal and colonic linoleic acid uptake in all groups of rats after the addition of linolenic acid could also be caused by an enlargement of the micellar size could not be ruled out. Thus, an expansion in the size of the micelles would decrease the micellar rate of diffusion towards the absorptive surface of the enterocytes (10).

The study of caecal and colonic uptake of cholesterol shows that cholesterol uptake increased in both caecum (Fig. 2A) and colon (Fig. 2B) of resected rats compared with that found in sham-operated rats, in both concentrations used (0.05 and 0.2mM). The observed increase in the caecum was higher after 75% than after 50% DSBR. However, in the colon, the observed enhancement in cholesterol uptake was not related to the extent of intestine removed at any of the concentrations. The results observed in the colon after surgical operation are not in agreement with previous reports that did not find significant changes in the uptake of cholesterol in the colon following intestinal resection (11). However, as far as we are aware, the caecal absorption of cholesterol as a result of DSBR has not been previously studied.

TABLE 2

Effect of Rotenone and Ouabain on Linoleic Acid Uptake[a]

	Linoleic acid	Linoleic acid plus rotenone/ouabain
Uptake rate[b]		
Caecum	5.6 ± 1.12	4.85 ± 0.96
Colon	16.84 ± 0.7	14.7 ± 0.8

[a]Results are given as means ±S.E. of five separate experiments. Each experiment is a pool of two large intestine (caecum or colon) from two different rats.

[b]Uptake rate expressed as pmoles/mg wet intestinal tissue/6 min.

In agreement with previous reports (1,12), the current results show that when substantial parts of the small intestine are resected (75%), the changes of mass parameters were much larger in the caecum than in the colon. Thus, after 50% DSBR significant increase in intestinal growth was limited to caecum (Table 1). Light microscopy studies (13) revealed that the mucosa took part in the growth response, and that the mucosal growth was due to hyperplasia.

The increased caecal and colonic uptake of linoleic acid and cholesterol observed after DSBR could not be simply due to morphological changes that occur after the surgical operation, since the results are expressed as unit wet intestinal tissue. Furthermore, 50% DSBR increased colonic uptake of linoleic acid and cholesterol without significant concomitant increase in intestinal growth. Therefore, DSBR, in addition to inducing a compensatory intestinal growth, stimulates the transport in epithelial cells of caecum and colon.

Several causes could contribute to the explanation. The increase in both linoleic acid and cholesterol uptake in caecum and colon after DSBR can be explained in different ways. It has been suggested that the rate of cholesterol esterification with fatty acids within the mucosal cell is limiting the rate of uptake of both substrates (14). However, we have previously demonstrated that the rate of esterification by acyl-CoA:cholesterol acyltransferase is not modified in both caecum and colon after the surgical operation (Molina, M.T., Vázquez, C.M., and Ruiz-Gutierrez, V., unpublished data). On the other hand, changes in both the effective resistance of the unstirred water layer and the permeability properties of the caecum and colonic membrane itself could occur after DSBR and modify the caecal and colonic uptake of lipids.

In conclusion, the study shows that i) facilitated diffusion seems to be the mechanism of linoleic acid absorption in caecum and colon, at least at low concentrations and ii) both, organ growth and changes in transport in epithelial cells appear to be involved in the adaptive response of the large bowel to intestinal resection. This suggests that, in patients with DSBR, the loss of essential fatty acids and cholesterol might be partially compensated by increased absorption in the large intestine.

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Accumulation of Eicosapentaenoic Acid in Plasma Phospholipids of Subjects Fed Canola Oil

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The metabolism of α-linolenic acid from canola oil was studied in eight normolipidemic men. The 42-day study was divided into three periods: a 6-day pre-experimental and two 18-day experimental. Approximately 75% of the dietary fat (28% of total energy) was provided by a mixture of fats during the pre-experimental period and either canola oil (CO) or sunflower oil (SO) during the experimental periods. The CO and SO diets were fed in a cross-over design. The ratios of linoleic to linolenic acid were 2.6:1 and 73.9:1 in the CO and SO diets, respectively. Dietary fat source had an effect on plasma phospholipid fatty acids: 18:1n-9, 18:3n-3 and 20:5n-3 were higher ($p < 0.05$), and 18:2n-6 was lower in the phosphatidylcholine fraction; 18:1n-9 was higher and 20:4n-6 lower in the phosphatidylethanolamine fraction; and 18:1n-9 and 20:5n-3 were higher and 20:4n-6 and 22:6n-3 were lower in the alkenylacyl ethanolamine phospholipid fraction on the CO diet as compared to the SO diet. Consumption of the canola oil diet resulted in higher n-3 fatty acid levels and lower n-6 fatty acid levels in plasma phospholipids than consumption of the sunflower oil diet.
Lipids 25, 598–601 (1990).

The recognition of thrombogenesis as an important process in coronary heart disease has led to investigation of the effects of dietary fatty acids on lipid metabolism and prostanoid synthesis. Fatty acids of the n-3 family have received considerable attention in this regard. Evidence of an inverse relationship between the level of dietary eicosapentaenoic acid (EPA) and platelet function is accumulating (1–4). This effect appears to be associated with significant increases in the level of EPA and a corresponding decrease in arachidonic acid (AA) in platelet phospholipids (3). Although humans possess the capacity to convert α-linolenic acid (LNA) to EPA, they do not appear to do so to any great extent (5); hence, there is controversy surrounding the relative importance of dietary LNA to platelet function (4). Nevertheless, consumption of LNA has resulted in increased EPA content of plasma (5–7) and platelet phospholipids (6–8). Similarly, the level of dietary LNA has been inversely related to human platelet aggregation (8,9). However, many of the studies on the metabolism of LNA have been confounded by methodology and have not taken into account dietary concentrations of pertinent fatty acids. In addition, fatty acid composition often is reported for total plasma or platelet phospholipid as opposed to individual phospholipid species. Fatty acid composition varies among different phospholipid species and the concentrations of the different species vary among tissues (10). Thus, it is possible that subtle but potentially important changes are masked when phospholipid species are not analyzed separately.

Canola oil is characterized by a high oleic acid content and a relatively low linoleic acid to linolenic acid (LA/LNA) ratio. The objective of the present study was to compare the effect of dietary canola oil with dietary sunflower oil—which has a relatively high LA content and a relatively high LA/LNA ratio—on the fatty acid composition of plasma phospholipids in humans.

EXPERIMENTAL PROCEDURES

Subjects and diets. The present study was part of a larger study reported in detail elsewhere (11). Briefly, the 42-day metabolic study was divided into three periods: a 6-day pre-experimental period and two 18-day experimental periods. All subjects (eight normolipidemic men) received a mixed fat diet during the pre-experimental period and either a canola oil (CO) diet or a sunflower oil (SO) diet during the experimental periods. Half of the subjects received the CO diet during the first experimental period and the SO diet during the second experimental period (CAN-SUN group) while the other half received the SO diet during the first experimental period and the CO diet during the second experimental period (SUN-CAN group).

A two-day menu cycle, made up of conventional foods, provided approximately 3000 Kcals/day. Protein, fat and carbohydrate provided 14.5, 36 and 49.5% of total energy, respectively. Approximately 75% of the dietary fat (28% of total energy) came from added fats. The fatty acid composition of the diets and the ratio of LA to LNA are presented in Table 1.

TABLE 1

Fatty Acid Composition of Mixed Fat, Canola Oil and Sunflower Oil Diets

	Fatty acid level (% of total fatty acids)		
Fatty acid[a]	Mixed fat[b]	Canola oil	Sunflower oil
14:0	3.6	1.2	1.2
16:0	21.9	8.6	10.6
16:1	2.4	0.9	0.8
18:0	11.2	3.2	5.8
18:1	37.7	53.8	19.5
18:2	18.4	20.8	59.1
18:3	1.2	7.9	0.8
20:0	0.6	0.8	0.5
20:1	0.5	1.3	0.3
22:0	0.2	0.3	0.8
22:1	0.3	—	—
18:2/18:3	15/1	2.6/1	74/1

[a] Carbon number:number of double bonds.

[b] Added fat included beef tallow, lard, corn oil, vegetable shortening and butter.

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Abbreviations: AA, arachidonic acid; CAN-SUN, canola-sunflower group; CO, canola oil; EPA, eicosapentaenoic acid; LA, linoleic acid; LNA, α-linolenic acid; OA, oleic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PPE, alkenylacyl ethanolamine phospholipid; PPP, platelet poor plasma; SO, sunflower oil; SUN-CAN, sunflower-canola group; TLC, thin-layer chromatography.

Plasma phospholipid analysis. Blood samples were taken after a 12-hr fast on days 7, 25 and 43. Platelets were removed from the samples by centrifuging at 1400 × *g* for 15 min. The platelet poor plasma (PPP) was removed and stored at −10°C. Lipids were extracted from PPP using the method of Folch *et al.* (12). Phospholipid species were separated using 2-directional thin-layer chromatography (TLC) (13) on pre-coated plates (Silica Gel 60, E. Merck, Terochem Laboratories Ltd., Edmonton, Canada). Following the first separation, alkenylacyl ethanolamine phospholipids (PPE) were hydrolyzed with HCl fumes (13). The developed plates were dipped in a dichlorofluorescein solution, exposed to ammonia fumes and viewed under UV light. The phosphatidylcholine (PC), phosphatidylethanolamine (PE) and PPE spots were scraped from the plate into tubes and transmethylated using H_2SO_4/methanol (14). The methyl esters were separated on a capillary column, 30 m × 0.25 mm id coated with DB-225 (J&W Scientific, Folsom, CA) using a Perkin Elmer gas chromatograph (model 8500, Perkin Elmer, Norwalk, CT). Injector, detector and column temperatures were 225°C, 225°C and 205°C, respectively. The carrier gas was helium, at a linear velocity of 25 cm/second. The methyl ester peaks were identified using known standards (Nu-Chek Prep., Elysian, MN). The levels of fatty acids in the PPE fraction were corrected for the amounts of contaminants in blank TLC plates in spots having retention times equivalent to palmitic acid and stearic acid.

Statistical analysis. The data were subjected to a modified analysis of variance of a two-way classification for a completely randomized block design, using diet type as a co-variate. Group means were compared by the Bonferroni procedure. Both of these analyses were performed using a SAS computer program (1984, 1986, SAS Institute Inc., Cary, NC).

RESULTS

Plasma PC exhibited the most dramatic alterations in fatty acid composition with changes in dietary fat source (Table 2). When the data for both groups of subjects (CAN-SUN and SUN-CAN) were combined, consumption of the canola diet was associated with higher mean levels of OA, LNA and EPA ($p < 0.0001$) and lower levels of stearic acid ($p < 0.004$) and LA ($p < 0.0001$) than consumption of the sunflower diet. The effect of the dietary fat source on plasma PC fatty acid patterns also was evident when mean levels of fatty acids within diet groups were compared (Table 2). For the CAN-SUN group, the mean levels of OA, LNA and EPA were significantly higher while mean levels of stearic acid and LA were significantly lower following the canola diet than following the sunflower diet. Similarly, mean levels of OA, LNA and EPA were significantly lower while levels of LA were significantly higher following the sunflower diet than following the canola diet for the SUN-CAN group. For the CAN-SUN group, levels of OA and LNA were higher ($p < 0.05$) following the canola diet than the mixed fat diet, while no differences in fatty acid composition were found between the mixed fat and sunflower diet in the SUN-CAN group.

The plasma PE fraction (Table 3) appeared to be less sensitive to changes in dietary fat source and there was

TABLE 2

Fatty Acid Composition of Plasma Phosphatidylcholine Following Each Diet Period

Group	Fatty acid composition (%)[a] Day 7	Day 25	Day 43	p[b]
CAN-SUN				
16:0	30.5 ± 1.5[c]	28.4 ± 2.2[c,d]	24.5 ± 1.4[d]	.49
18:0	13.2 ± 0.6[c,d]	12.7 ± 1.1[c]	15.1 ± 1.2[d]	.004
18:1	13.4 ± 0.3[c]	16.8 ± 1.3[d]	8.8 ± 0.8[e]	.0001
18:2	29.1 ± 3.1[c]	28.2 ± 3.2[c]	35.8 ± 4.2[d]	.0001
18:3	0.2 ± 0.1[c]	0.7 ± 0.4[d]	0.2 ± 0.2[c]	.0001
20:4	7.7 ± 1.0[c]	6.7 ± 2.3[c]	8.8 ± 3.5[c]	.13
20:5	0.4 ± 0.1[c,d]	0.8 ± 0.4[c]	0.1 ± 0.0[d]	.0001
22:5	0.6 ± 0.1[c]	0.7 ± 0.3[c]	0.6 ± 0.2[c]	.10
22:6	1.9 ± 0.3[c]	1.8 ± 0.5[c]	2.4 ± 0.9[c]	.41
SUN-CAN				
16:0	27.0 ± 1.9[c]	28.4 ± 5.5[c]	26.6 ± 1.4[c]	
18:0	12.8 ± 1.1[c]	13.4 ± 1.3[c]	12.6 ± 1.3[c]	
18:1	14.0 ± 1.8[c,d]	10.8 ± 1.4[c]	17.6 ± 1.9[d]	
18:2	28.8 ± 3.9[c,d]	32.9 ± 5.3[c]	27.0 ± 4.9[d]	
18:3	0.2 ± 0.1[c]	0.1 ± 0.0[c]	0.6 ± 0.1[d]	
20:4	9.7 ± 2.9[c]	8.4 ± 3.3[c]	8.3 ± 3.1[c]	
20:5	0.4 ± 0.2[c,d]	0.2 ± 0.2[c]	0.9 ± 0.6[d]	
22:5	1.0 ± 0.2[c]	0.7 ± 0.3[c]	0.9 ± 0.3[c]	
22:6	1.9 ± 0.7[c]	1.8 ± 0.9[c]	1.6 ± 0.5[c]	

[a]Values are expressed as Means ± S.D.

[b]p values for diet comparisons (i.e., canola vs sunflower oil; n=8).

[c,d,e]Values in rows with different letters differ at $p < 0.05$.

TABLE 3

Fatty Acid Composition of Plasma Phosphatidylethanolamine Following Each Diet Period

Group	Fatty acid composition (%)[a] Day 7	Day 25	Day 43	p[b]
CAN-SUN				
16:0	17.0 ± 2.0[c]	14.9 ± 3.0[c]	14.3 ± 2.5[c]	.78
18:0	21.9 ± 2.7[c]	23.4 ± 4.2[c]	25.1 ± 2.4[c]	.01
18:1	14.1 ± 0.8[c]	20.6 ± 1.9[d]	13.3 ± 2.3[c]	.0001
18:2	22.0 ± 7.8[c]	18.1 ± 2.4[c]	22.3 ± 3.4[c]	.19
18:3	0.3 ± 0.2[c]	0.8 ± 0.1[c]	0.7 ± 0.6[c]	.40
20:4	14.3 ± 2.2[c]	11.6 ± 2.8[c]	13.4 ± 0.6[c]	.045
20:5	1.0 ± 1.3[c]	0.6 ± 0.2[c]	0.1 ± 0.1[c]	.066
22:5	1.6 ± 1.2[c]	1.1 ± 0.5[c]	2.0 ± 1.6[c]	.43
22:6	4.2 ± 3.2[c]	4.0 ± 2.5[c]	4.2 ± 2.2[c]	.15
SUN-CAN				
16:0	25.8 ± 4.0[c]	16.7 ± 2.9[d]	17.0 ± 1.1[d]	
18:0	21.0 ± 3.6[c]	25.4 ± 3.1[c]	20.8 ± 2.2[c]	
18:1	14.0 ± 1.9[c]	13.1 ± 2.5[c]	19.6 ± 3.0[d]	
18:2	15.8 ± 5.6[c]	17.0 ± 3.3[c]	16.3 ± 5.9[c]	
18:3	1.0 ± 0.3[c]	1.0 ± 0.3[c]	1.2 ± 0.2[c]	
20:4	11.6 ± 4.1[c]	15.6 ± 4.2[d]	12.8 ± 3.6[c,d]	
20:5	0.5 ± 0.4[c]	0.1 ± 0.1[c]	0.8 ± 0.4[c]	
22:5	1.4 ± 0.4[c]	1.4 ± 0.2[c]	1.7 ± 0.5[c]	
22:6	3.5 ± 1.8[c]	4.2 ± 1.1[c]	3.5 ± 1.0[c]	

[a]Values are expressed as Means ± S.D.

[b]p values for diet comparisons (i.e., canola vs sunflower oil; n=8).

[c,d]Values in rows with different letters differ at $p < 0.05$.

more inter-subject variation than for the PC fraction. When the data for the experimental groups were combined, consumption of the canola diet was associated with higher mean levels of OA ($p < 0.0001$) and EPA ($p < 0.07$) and lower levels of stearic acid ($p < 0.01$) and AA ($p < 0.045$) than the sunflower diet. However, there was no difference in LNA level between the diets.

The comparison of mean fatty acid levels within diet groups also suggested that there were relatively small changes in PE fatty acid composition related to dietary fat source. Higher levels of OA were observed following the canola diet for both diet groups. Palmitic acid levels were lower following the experimental diets than following the mixed fat diet, and the AA level was higher following the sunflower diet than following the mixed fat diet for the SUN-CAN group.

Changes in the fatty acid composition of the PPE fraction (Table 4) in response to dietary fat source were similar to those observed for PE. Consumption of the canola diet was associated with higher mean levels of OA ($p < 0.014$) and EPA ($p < 0.012$) and lower levels of DHA ($p < 0.026$) as compared to the sunflower diet. The lower level of DHA on the canola diet than on the sunflower diet was due primarily to the low level following the canola diet for the SUN-CAN group. As with the PE fraction, there was no difference in LNA levels due to fat source.

DISCUSSION

In the present study, the fatty acid composition of plasma phospholipid fractions showed important differences in response to dietary fat source. Compositional changes in the PC fraction in particular reflected the dietary fats. The canola diet contained less palmitic acid, stearic acid and LA, and more OA and LNA than the sunflower diet (Table 1). The plasma PC fraction also contained less stearic acid and LA, and more OA, LNA and EPA following the canola diet than following the sunflower diet. Furthermore, the magnitude of these composition changes reflected the relative differences in the fatty acid composition of the test diets, which suggests similar relative rates of incorporation of these fatty acids into PC.

The magnitude of the changes in fatty acid composition in the plasma PE and PPE fractions, relative to the changes in dietary fatty acid composition, was less than for the PC fraction, suggesting lower relative rates or differential rates of incorporation of fatty acids into these fractions. Differential rates of incorporation of LNA into PC and PE have been reported in the rat (15) and the human (16). Adam *et al.* (16) found that the incorporation of LNA and its higher homologs into the PC fraction of high and low density lipoproteins (HDL and LDL) reflected the dietary levels of LNA. By contrast, there was no apparent incorporation of LNA into the PE fraction of HDL regardless of dietary treatment.

The fatty acid composition of platelet phospholipid fractions also responded to changes in dietary fat source (17). The PPE fraction in platelets, however, was more responsive to changes in dietary fat source than the PC fraction. In addition, higher levels of EPA were observed in both the PPE and the PC fractions in platelets than in plasma following consumption of the canola diet. The docosapentaenoic acid level also was higher in the PPE fraction in platelets following the canola diet.

The incorporation of LNA and EPA into the phospholipid fractions, in absolute amounts, in response to dietary canola oil was modest. However, the levels were consistent with those observed by other investigators (5,6,16,18). The EPA content of plasma PC of subjects given 20 mL of linseed oil (6.5 g LNA) daily for two weeks increased from 1.3 to 2.7% for omnivores and from 0.3 to 1.0% for vegetarians. Although total fat and LNA intakes were similar for the two groups, the intake of LA by the vegetarians was four times that of the omnivores (28 vs 7 g/day). In the present study, mean EPA levels were significantly higher following the canola diet than following the sunflower diet (PC, 0.8 vs 0.2%; PE, 0.7 vs 0.1%; and PPE, 3.0 vs 1.0%, respectively). The canola diet provided approximately 9.5 g of LNA and 25 g of LA daily as compared to approximately 1 g of LNA and 71 g of LA for the sunflower diet. It is generally accepted that LNA competes successfully with LA in the desaturation and elongation pathway (19). Although there were significantly lower levels of LA in the PC and PE fractions, and of AA in the PE fraction following consumption of the canola diet than following the sunflower diet, these results may simply reflect the difference in LA content of the two diets. Other investigators (18,20) have reported a decrease in plasma phospholipid AA as compared to pre-experimental levels following the consumption of linseed oil supplements. However, the fatty acid composition of the diets were not reported.

The present study demonstrated that a dietary source of LNA, namely canola oil, resulted in significant increases in the levels of EPA in plasma phospholipids. The level and pattern of incorporation of LNA and its metabolites into plasma phospholipids, however, differed from those observed in platelet phospholipids (17).

TABLE 4

Plasma Alkenylacyl Ethanolamine Phospholipid Fatty Acid Composition Following Each Diet Period

Group	Fatty acid composition (%)[a]			
	Day 7	Day 25	Day 43	p[b]
CAN-SUN				
18:1	14.1 ± 6.6[c]	13.8 ± 2.9[c]	9.0 ± 2.3[c]	.014
18:2	31.1 ± 7.6[c]	23.1 ± 8.6[c]	29.2 ± 7.1[c]	.57
18:3	1.4 ± 1.1[c]	0.6 ± 0.2[c]	0.8 ± 0.7[c]	.46
20:4	30.0 ± 8.9[c]	36.5 ± 7.7[c]	35.9 ± 2.2[c]	.076
20:5	1.1 ± 0.6[c]	3.4 ± 1.8[c]	0.7 ± 0.2[c]	.012
22:5	4.4 ± 1.4[c]	5.0 ± 1.0[c]	3.8 ± 0.7[c]	.60
22:6	9.8 ± 5.5[c]	12.4 ± 2.8[c]	12.2 ± 1.9[c]	.026
SUN-CAN				
18:1	12.3 ± 3.2[c]	8.8 ± 5.3[c]	13.5 ± 1.8[c]	
18:2	22.8 ± 8.3[c]	20.8 ± 7.5[c]	31.6 ± 17.7[c]	
18:3	1.4 ± 0.2[c]	1.2 ± 0.6[c]	1.8 ± 1.5[c]	
20:4	37.8 ± 8.0[c]	40.5 ± 11.0[c]	26.3 ± 13.6[d]	
20:5	1.6 ± 0.5[c]	1.3 ± 0.8[c]	2.6 ± 2.2[c]	
22:5	5.4 ± 1.6[c]	5.5 ± 0.5[c]	5.1 ± 2.8[c]	
22:6	9.1 ± 2.5[c]	13.2 ± 1.9[d]	7.0 ± 2.8[c]	

[a]Values are expressed as Means ± S.D.

[b]p Values for diet comparisons (i.e., canola vs sunflower oil; n=8).

[c,d]Values in rows with different letters differ at $p < 0.05$.

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Occurrence of n-5 Monounsaturated Fatty Acids in Jujube Pulp Lipids

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The pulp lipids of jujube *(Zizyphus jujuba* var. *inermis)* fruit have been shown by chromatographic, spectrometric and chemical analyses to contain a series of *cis*-monoenoic fatty acids with n-5 unsaturation as major acyl moieties. The total concentration of these n-5 fatty acids, such as 14:1n-5, 16:1n-5 and 18:1n-5, ranged from 22 to 54% of total fatty acids in the pulp lipids of 11 different sources. The main component of the n-5 homologues was 16:1n-5 in all cases. Other monoenoic acids with n-7 unsaturation, namely palmitoleic (*cis*-9-hexadecenoic) acid and *cis*-vaccenic (*cis*-11-octadecenoic) acid, as well as with n-9 unsaturation, namely oleic acid, were also identified. In the seed lipids of jujube fruit, none of the n-5 monoenoic acids could be detected. Thus the jujube pulp lipids are characterized by the predominance of n-5 monoenoic acid isomers.
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Monounsaturated fatty acids with n-5 unsaturation have been recognized as rare components of common higher plants, except for the seeds of Proteaceae (1,2) which contain considerable amounts of n-5 monoenoic acids as the characteristic acyl moieties (3–6). Application of capillary gas chromatography (GC) to fatty acid analysis has made it easy to reveal the occurrence of n-5 monoenoic acids in the lipids of several plant families and species, as has been reported by Seher and Gundlach (7), Kleiman and Payne-Wahl (8), and others. In our recent papers on the occurrence of *cis*-vaccenic (*cis*-11-octadecenoic) acid in higher plant lipids, we also reported the presence of GC peaks corresponding to n-5 monoenoic acid isomers in the seed lipids of *Mallotus japonicus* (Euphorbiaceae) (9) and the pulp lipids of mango (Anacardiaceae) (10), kaki (Ebenaceae), sweet orange (Rutaceae), etc. (11). However, the proportion of these n-5 isomers relative to total fatty acids was very low (7,9–11). In screening for fatty acid positional isomers in several fruit pulps, we have found that the pulp lipids of jujube *(Zizyphus jujuba* var. *inermis)* fruit contained a series of n-5 monoenoic acid isomers as major acyl groups along with n-7 and n-9 monoenoic acid isomers.

This paper describes the identification and estimation of n-5 fatty acid isomers in jujube pulp lipids, and also reports the fatty acid profile of the fruit lipids. No paper has hitherto been published on the fatty acids of jujube pulp lipids. Jujube, a member of the family Rhamnaceae, is considered to be native to Southern Europe, Southwest Asia and China. The fresh pulp is used as table fruit. The dried fruit is an important Chinese medicine ('da zao' in China, 'taiso' in Japan); the dried pulp is used in confection as are raisins.

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Abbreviations: FAME, fatty acid methyl ester; GC, gas chromatography; GC-MS, gas chromatography-mass spectrometry; TLC, thin-layer chromatography.

MATERIALS AND METHODS

Fatty acid standards and chemicals. The sources of fatty acid standards were as follows: myristoleic (*cis*-9-tetradecenoic) acid was from NuChek-Prep (Elysian, MN); palmitoleic (*cis*-9-hexadecenoic) and *cis*-vaccenic acids were from P.L. Biochemicals Inc. (Milwaukee, WI); and oleic, linoleic and linolenic acids were from Research Laboratory of Nippon Oil and Fats Co. (Amagasaki, Japan). These acids were methylated with BF_3/methanol and purified by thin-layer chromatography (TLC) (10). All chemicals were analytical reagent grade. All organic solvents were distilled before use.

Plant materials and extraction of total lipids. Fully ripened jujube fruit samples, grown at various districts (11 locations among 7 prefectures) in Japan, were collected in October 1988. They were immediately immersed in boiling water for 5 min to inactivate enzymes (12) and then stored at −20°C until analysis. The Chinese medicine 'taiso' and a confection made from jujube fruit were purchased from local markets. These materials (5 to 15 pieces) were peeled and divided into pulp and seed parts. The pulp total lipids were obtained by chloroform/methanol extraction and purified by the method previously described (11) to remove nonlipid contaminants. The seed total lipids of fresh fruit were obtained in the same manner.

Derivatization. Fatty acid methyl esters (FAME) of the total lipids were prepared by methanolysis (0.5 N KOH/methanol) (10) and successive methylation (BF_3/methanol) (10) and purified by TLC before capillary GC analysis. The FAME were converted to their dimethyl disulfide adducts by our rapid methylthiolation procedure (10). Trimethylsilyloxy derivatives of polyunsaturated FAME were synthesized according to conventional procedures (13) with minor modifications (10).

Capillary GC and gas chromatography-mass spectrometry (GC-MS). FAME were separated on a fused silica ULBON HR-SS-10 column (50 m × 0.25 mm i.d., chemically bonded type, Shinwakako Co., Kyoto, Japan) in a Hitachi G-3000 gas chromatograph with a splitless injector and a flame ionization detector. The carrier gas was helium at a flow rate of 16.2 cm/s. The column temperature was initially 100°C for 5 min; then it was programmed at 4°C/min to 210°C with a final 20 min hold. Capillary GC-MS analysis of FAME was carried out on a Supelcowax-10 column (30 m × 0.25 mm i.d., Supelco Inc., Bellefonte, PA) in a Hewlett-Packard 5890A gas chromatograph coupled to a 5970B Mass Selective Detector at an ionization energy of 70 eV. The column temperature was kept at 120°C for 3 min, then raised at 5°C/min to 220°C and held for 22 min. The carrier gas was helium at 8 psi. Dimethyl disulfide adducts and trimethylsilyloxy derivatives of FAME were analyzed on a glass column (1 m × 3 mm i.d.) packed with 2% OV-101 on 100–120 mesh Chromosorb WHP in a Hitachi 663-30 gas chromatograph linked to a Hitachi M-80A double focusing mass spectrometer (ionization energy, 20 eV) with an M-003 minicomputer on-line system. The column

temperature was maintained at 240°C for dimethyl disulfide adducts and at 230°C for trimethylsilyloxy derivatives.

Other chromatographic, spectrometric and chemical analyses were performed under the same conditions as those given in our previous work (10).

RESULTS AND DISCUSSION

Extractable lipids accounted for 5.5 wt% of the seed part (wet basis) and 0.8 wt% of the pulp part (wet basis) from fresh jujube fruit. The fatty acid composition of the jujube seed lipids was similar to that of common vegetable seed oils with respect to the variety of fatty acids. In the jujube pulp lipids, however, an unusual profile of fatty acids was observed as shown in Figure 1.

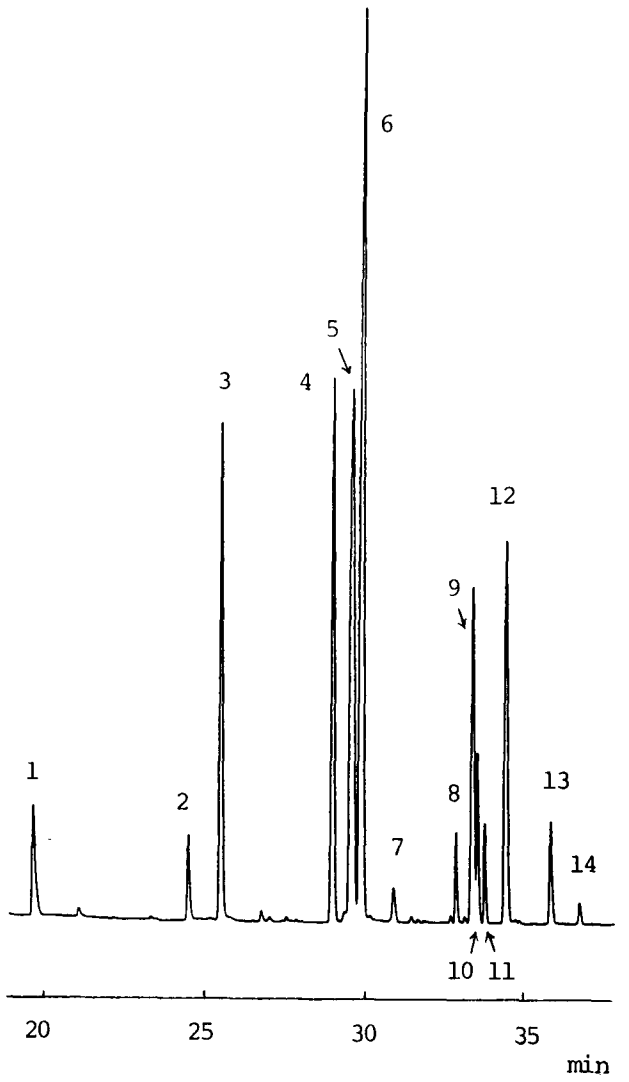

FIG. 1. Capillary gas chromatogram of fatty acid methyl esters from pulp total lipids of jujube fruit. The jujube was grown in Hyogo prefecture in October 1988. Peak identifications are detailed in the text. 1, 12:0; 2, 14:0; 3, 14:1n-5; 4, 16:0; 5, 16:1n-7; 6, 16:1n-5; 7, 17:0; 8, 18:0; 9, 18:1n-9; 10, 18:1n-7; 11, 18:1n-5; 12, 18:2(9,12); 13, 18:3(9,12,15); 14, 20:0.

Identification of isomeric monoenoates. The retention times of peaks 3, 6 and 11 in Figure 1 agreed very closely with those of n-5 *cis*-monoenoates with C_{14}, C_{16} and C_{18} chain lengths of our laboratory FAME standard mixture. The standard mixture was previously prepared from mango pulp lipids and completely identified (10). Cochromatography of commercially available authentic standards under the same GC conditions revealed that authentic methyl myristoleate eluted at the same position as peak 3; authentic methyl palmitoleate, oleate and *cis*-vaccenate coincided in elution with peaks 5, 9 and 10, respectively. We further confirmed the structures of jujube pulp fatty acids (see below) by employing more than two independent analytical techniques for the accurate qualitative and quantitative analysis of fatty acid isomers (11).

The infrared spectrum of an aliquot (5% solution in hexane) of the FAME mixture (Fig. 1) showed no absorption in the 960–970 cm^{-1} region, which indicates the absence of *trans* double bonds. The FAME mixture was then applied onto silica gel plates containing $AgNO_3$ and the plates were developed with benzene/diethyl ether (90:10, v/v) (10). No band of *trans*-monoenoate was observed. A band having the same retardation factor value as that of authentic *cis*-monoenoates was recovered and analyzed by capillary GC and GC-MS. Six major peaks coinciding with peaks 3, 5, 6, 9, 10 and 11 in Figure 1 were observed, and typical fragment ions $[M-32]^+$ and weak molecular ions resulting from electron impact ionization of methyl monoenoates were detected. The peaks were identified as *cis*-monoenoates.

Hydrogenation of an aliquot of the *cis*-monoenoate fraction in the presence of platinum black as a catalyst (10) resulted in the formation of single peaks of methyl myristate, palmitate and stearate in capillary GC. Capillary GC-MS analysis of the hydrogenated fraction further showed a series of molecular ions corresponding to the individual saturated esters and also showed fragmentation patterns indicating the absence of branched-chain fatty acid esters. These results suggested that the jujube pulp lipids contained *cis*-monoenoic acid positional isomers with straight chains only.

To confirm the double-bond position in the aliphatic chain of the *cis*-monoenoic acid isomers, an aliquot of the FAME mixture (Fig. 1) was subjected directly to the methylthiolation. The resulting dimethyl disulfide adducts were analyzed by packed-column GC-MS. Table 1 shows the summary of this mass chromatographic analysis. A set of key fragment ions due to the cleavage between the methylthio-substituted carbons (9,10) provided accurate confirmation of the original double-bond positions in the samples. Consequently, the peak assignments of the isomeric monoenoates cited in the legend of Figure 1 are correct.

The major monoenoic acid in the pulp lipids analyzed (Fig. 1) was *cis*-11-hexadecenoic acid which amounted to 25.1% of the total fatty acids. This value was slightly higher than that reported (21.4%) for the acid of *Grevillea decora* seed oil (6).

Identification of polyenoates. Two bands corresponding to authentic methyl linoleate (fraction D) and linolenate (fraction T) were recovered separately from the argentation TLC plates (see above). Capillary GC showed that the major peaks arising from fractions D and T coincided

TABLE 1

Mass Spectral Evidence for the Double-Bond Positions in Jujube Pulp Monoenoate Isomers[a]

Peak no.[b]	m/z			Molecular ion	Structure confirmed
	Key fragment ion[c]				
	A	B	C		
3	117	217	185	334	14:1n-5
5	145	217	185	362	16:1n-7
6	117	245	213	362	16:1n-5
9	173	217	185	390	18:1n-9
10	145	245	213	390	18:1n-7
11	117	273	241	390	18:1n-5

[a] Measured as dimethyl disulfide adducts. The adducts were prepared directly from a mixture of fatty acid methyl esters shown in Figure 1.

[b] Corresponding to those in Figure 1.

[c] A, $CH_3(CH_2)_xHCSCH_3$; B, $CH_3SCH(CH_2)_yCOOCH_3$; C, B-32 (CH_3OH).

Operating conditions are given in the text.

with peaks 12 and 13 in Figure 1, respectively. Hydrogenation of a combined aliquot of fractions D and T gave a major peak corresponding to that of methyl stearate, whose structure was confirmed by capillary GC-MS. Fractions D and T were then converted to trimethylsilyloxy derivatives and analyzed by packed-column GC-MS. Mass spectra of the derivatives from fractions D and T agreed quite well with those derived from authentic methyl linoleate and linolenate, respectively. Therefore, the polyenoic esters corresponding to peaks 12 and 13 in Figure 1 were attributed to methyl linoleate and linolenate. Polyenoic acid with n-5 or n-7 structures could not be detected in the jujube pulp lipids.

Hence, n-5 monoenoates do not seem to be desaturated in jujube pulp. Similarly *cis*-vaccenate does not appear to serve as precursor for polyenoic acids in jujube pulp or other fruit pulps (10,11). This is in contrast to 'cilienic (*cis*-6,11-octadecadienoic) acid' biosynthesis in the ciliate *Tetrahymena pyriformis* (14).

Fatty acid composition of pulp lipids from fresh jujube fruit and jujube fruit products. The 10 samples of FAME of fresh jujube fruit from different sources were analyzed to establish the presence of monoenoic acid isomers with n-5 unsaturation. The analytical data showed that a series of n-5 monoenoic acids with C_{14}, C_{16} and C_{18} chain lengths was present along with n-7 and n-9 monoenoic acids as the major acyl groups in the pulp lipids. We then selected the 4 representative samples shown in Table 2. Samples H-1, W-1 and G-1 were chosen because they contained the highest percentage of myristoleic acid, 16:1n-5 and 18:1n-5, respectively (for abbreviations, see the footnote of Table 2). Sample K-2 was a poor source for the total n-5 homologues (C_{14}, C_{16} and C_{18}) in the jujube fruit samples examined in the present study.

A characteristic difference in the fatty acid variety (Table 2) was observed between the pulp and seed lipids from the same source (sample K-2). In the seed lipids, none of the n-5 monoenoic acids could be detected. This suggests that the formation of n-5 monoenoates proceeds exclusively in the pulp part of jujube fruit.

TABLE 2

Fatty Acid Composition of Pulp and Seed Total Lipids of Fresh Jujube Fruit[a]

Fatty acid (wt%)	Source				
	Pulp				Seed
	H-1[b]	W-1[b]	G-1[b]	K-2[b]	K-2[b]
12:0	7.0	1.2	6.8	0.4	—
14:0	1.8	0.9	4.6	1.5	—
14:1n-5	19.5	5.6	10.2	2.7	—[c]
16:0	4.8	8.8	8.4	14.5	4.5
16:1n-7	17.2	21.7	17.5	12.1	Tr[c]
16:1n-5	33.3	33.8	19.6	16.3	—[c]
18:0	0.5	1.0	0.9	4.4	2.8
18:1n-9	2.0	5.7	1.2	9.2	46.6[c]
18:1n-7	2.6	5.8	8.4	5.6	2.6[c]
18:1n-5	1.6	3.1	4.6	2.7	—[c]
18:2(9,12)	4.1	6.8	11.2	15.6	36.5
18:3(9,12,15)	3.2	3.3	3.6	6.3	0.4
Others[d]	2.4	2.3	3.0	8.7	6.6
Sum of monoenoic isomers					
n-9 isomers	2.0	5.7	1.2	9.2	46.6
n-7 isomers	19.8	27.5	25.9	17.7	2.6
n-5 isomers	54.4	42.5	34.4	21.7	—

[a] Determined by capillary GC. Each value is the average of duplicate determinations.

[b] Abbreviations indicate source (prefecture) and sampling number of fruit. H, Hyogo; W, Wakayama; G, Gifu; K, Kagawa prefecture.

[c] Based on mass chromatography of the dimethyl disulfide adducts after capillary GC analysis.

[d] Including saturated acids (except for 12:0, 14:0, 16:0 and 18:0), minor acids (below 0.2 wt%) and unidentified acids.

Tr, trace (below 0.2 wt%).

TABLE 3

Fatty Acid Composition of Pulp Total Lipids of Jujube Products[a]

Fatty acid (wt%)	Product		
	Chinese medicine 'taiso'	Confection	
		A[b]	B[b]
12:0	4.6	8.3	0.9
14:0	4.5	5.6	1.2
14:1n-5	12.0	12.5	3.0
16:0	15.1	16.6	11.6
16:1n-7	13.6	14.8	13.4
16:1n-5	21.9	25.6	19.6
18:0	1.6	1.5	1.9
18:1n-9	6.7	2.4	9.6
18:1n-7	3.2	2.0	10.6
18:1n-5	1.5	1.2	6.7
18:2(9,12)	8.1	3.0	10.9
18:3(9,12,15)	2.2	0.5	3.3
Others[c]	5.0	6.0	7.3
Sum of monoenoic isomers			
n-9 isomers	6.7	2.4	9.6
n-7 isomers	16.8	16.8	24.0
n-5 isomers	35.4	39.3	29.3

[a] Determined by capillary GC. Each value is the average of duplicate determinations.

[b] The brands are different.

[c] Including saturated acids (except for 12:0, 14:0, 16:0 and 18:0), minor acids (below 0.2 wt%) and unidentified acids.

Table 3 shows the fatty acid compositions of the pulp lipids from jujube fruit products: the Chinese medicine 'taiso' and the confection. The presence of the n-5 monoenoic acids in these materials originating in China also supports the above findings that the n-5 monoenoic isomers are usual components of the jujube pulp lipids.

The only commercially available authentic monoenoic acid having an n-5 *cis* double bond and even number of carbon atoms is myristoleic acid. The confection made from jujube fruit (Table 3) may be useful as a source of 16:1n-5 and 18:1n-5 fatty acid standards; the confection is produced in China on a large scale and is easily available.

From the data presented in this study, it is clear that the profile of jujube pulp fatty acids is complex and clearly different from the fatty acid patterns of common vegetable seed oils and also from those of other fruit pulp lipids (10,11) on account of the predominance of n-5 monoenoic acid isomers. The biosynthetic routes of n-5 monoenoic acids in higher plants are unknown. We are trying to determine the pathways in the jujube pulp using our recently developed GC-MS method based on [2,2-$^2H_2$]-fatty acid labelling and dimethyl disulfide adduct formation, by which the pathway of *cis*-vaccenic acid biosynthesis in kaki fruit pulp has recently been confirmed (15).

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Lyso Platelet Activating Factor (LysoPAF) and Its Enantiomer. Total Synthesis and Carbon-13 NMR Spectroscopy

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Described is a reaction sequence for the total synthesis of lyso platelet activating factor (lysoPAF; 1-*O*-alkyl-*sn*-glycero-3-phosphocholine) and its enantiomer. The procedure is versatile and yields optically pure isomers of defined chain length. The synthesis is equally suited for the preparation of lysoPAF analogues and its enantiomers with unsaturation in the long aliphatic chain. First, *rac*-1(3)-*O*-alkylglycerol is prepared by alkylation of *rac*-isopropylideneglycerol with alkyl methanesulfonate followed by acid-catalyzed removal of the ketal group. The primary hydroxy group of alkylglycerol is then protected by tritylation, the secondary hydroxy group is acylated, and the protective trityl group is removed under mild acidic conditions with boric acid on silicic acid, essentially without acyl migration. Condensation of the diradylglycerol with bromoethyl dichlorophosphate in diethyl ether, hydrolysis of the resulting chloride, and nucleophilic displacement of the bromine with trimethylamine gives *rac*-1-*O*-alkyl-2-acylglycero-3-phosphocholine in good overall yield. The racemic alkylacylglycerophosphocholine is finally treated with snake venom phospholipase A_2 *(Ophiophagus hannah)* which affords 1-*O*-alkyl-*sn*-glycero-3-phosphocholine (lysoPAF) of natural configuration in optically pure form. The "unnatural" 3-*O*-alkyl-2-*O*-acyl-*sn*-glycero-1-phosphocholine enantiomer, which is not susceptible to phospholipase A_2 cleavage, gives 3-*O*-alkyl-*sn*-glycero-1-phosphocholine upon deacylation with methanolic sodium hydroxide. Homogeneity and structure of the intermediates and final products were ascertained by carbon-13 nuclear magnetic resonance spectroscopy on monomeric solutions.

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Ether phospholipids have emerged as potent mediators and modifiers of biological function. The structure of *platelet activating factor (PAF)* as 1-*O*-alkyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine (1-alkyl-2-acetyl-GPC) is now well established (1–3). PAF has been shown to evoke a multitude of biological responses ranging from platelet aggregation, systemic hypotension, and bronchoconstriction, to various circulatory, inflammatory, and allergic effects (for reviews, see refs. 4–8). *Alkyl lysophospholipids (ALP)* have also attracted attention as novel types of immunomodulators and as promising new antineoplastic agents. ALP, which are structurally related to lysoPAF, show rather selective cytostatic and cytotoxic properties, and they appear to be primarily targeted at the cell membrane (for reviews, see refs. 9 and 10).

Current intense interest in PAF and ALP, and in the metabolism, biological response, structural specificity and interaction of these agents have created a need for synthetic alkylglycerophosphocholines of defined chemical structure and in optically pure form.

Convenient semisynthetic procedures are available (11). These typically utilize the 1-*O*-alkyl-*sn*-glycerol structures present in natural products, such as bovine heart (2,3,12), porcine leukocytes (1), ratfish liver (13), or bovine erythrocytes (14). Most semisynthetically prepared alkylglycerophosphocholines are, however, heterogeneous in chain length, they usually are saturated, and they can only be obtained in natural configuration.[4]

The preparation of alkylglycerophosphocholines with defined chain-length, defined degree of unsaturation and with a defined chiral center of either configuration at glycerol C-2 requires total chemical synthesis. PAF and lysoPAF of natural configuration (*sn*-3 isomers) can be prepared by various stereoselective reaction sequences which utilize the chiral synthons *D*-mannitol (16–18), *D*-tartaric acid (19,20), or *L*-serine (18,21). The "unnatural" isomer (*sn*-1) can be prepared starting from *L*-tartaric acid (19,20), 1,2-isopropylidene-*sn*-glycerol (22), or *via* Walden inversion (18,21). However, most of these earlier procedures (16–20) involve intermediary hydrogenation steps for the removal of protecting groups and, hence, are not suited for the preparation of unsaturated alkylglycerophosphocholines. Also, as the chiral center is carried through the entire sequence, some isomerization can *a priori* not be ruled out. Both difficulties have been circumvented in the elegant synthesis described by Surles *et al.* (23) which utilizes the methoxyethoxymethyl (MEM) protecting group (24), and makes use of phospholipase A_2 (25,26) for the segregation of optical isomers late in the synthesis. More recently, Guivisdalsky and Bittman (27) chose a rather innovative approach for the synthesis of lysoPAF isomers based on stereocontrolled glycidyl arenesulfonate ring opening.

We describe here a sequence of reliable reaction steps for the total synthesis of lyso platelet activating factor and its enantiomer (see Scheme 1). Starting from commercially available racemic isopropylideneglycerol, the synthesis of the natural lysoPAF (*sn*-3 **9**) is completed in eight steps. An additional step is required to prepare the "unnatural" isomer of lysoPAF (*sn*-1 **9**). The procedure is versatile and yields optically pure enantiomers of defined chain length and degree of unsaturation. The utility of

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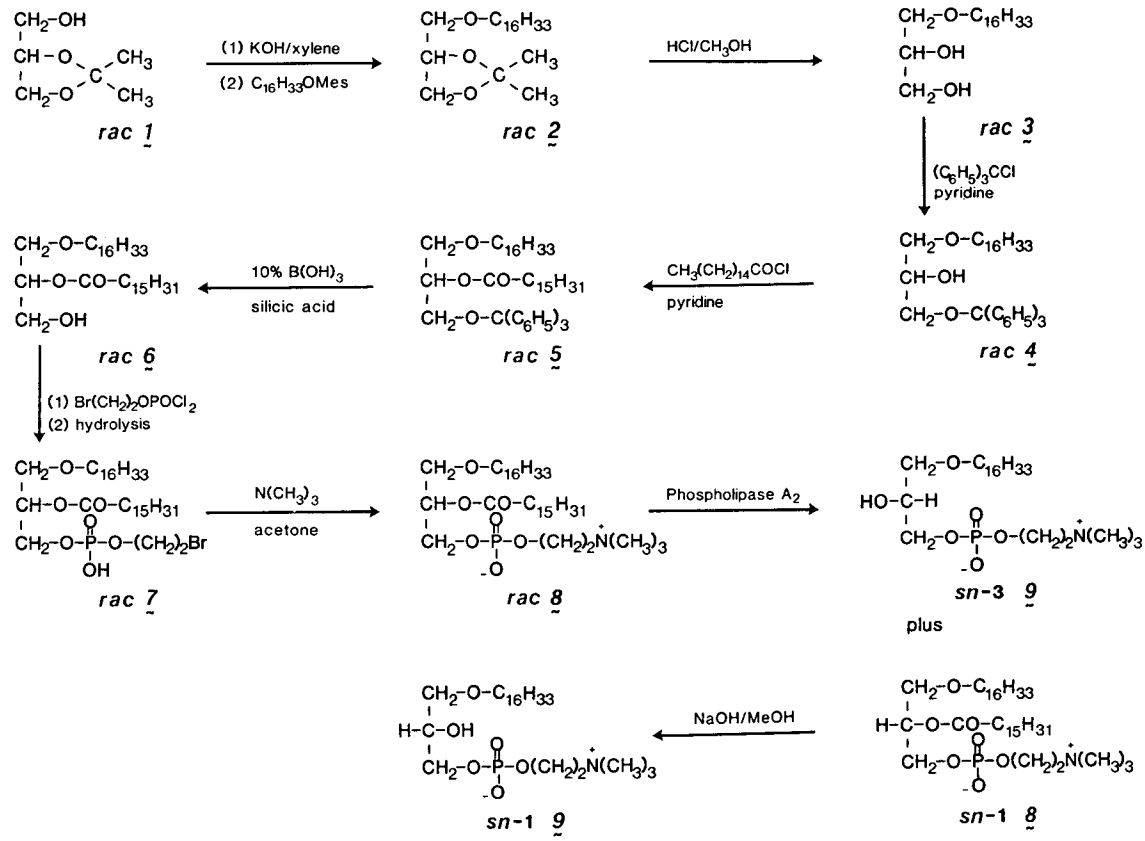
Abbreviations: GPC, glycerophosphocholine; IR, infrared spectra; lysoPAF, 1-*O*-alkyl-*sn*-glycero-3-phosphocholine; MS, mass spectra; NMR, nuclear magnetic resonance; PAF, platelet activating factor; 1-*O*-alkyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine; Ph, phenyl; TLC, thin-layer chromatography.

[4]In the present study, the configuration of glycerol derivatives is designated by "stereospecific numbering" *(sn)* (15). Natural lysoPAF is 1-*O*-alkyl-*sn*-glycero-3-phosphocholine, the *sn*-3 isomer.



SCHEME 1

the synthesis is documented for the preparation of 1-*O*-hexadecyl-*sn*-glycero-3-phosphocholine and 3-*O*-hexadecyl-*sn*-glycero-1-phosphocholine.

First, *rac*-1(3)-*O*-alkylglycerol (*rac* **3**) is prepared by alkylation of *rac*-isopropylideneglycerol (*rac* **1**) with a methanesulfonate of desired chain length and number of double bonds, followed by acid-catalyzed removal of the protective ketal group from isopropylidene glycerol ether *rac* **2**, essentially following established procedures (28). The primary hydroxy group of the resulting alkylglycerol *rac* **3** is then protected by tritylation (29), and the secondary hydroxy group of alkyl trityl glycerol ether *rac* **4** is acylated with a long-chain acyl chloride (30) to yield alkylacyltritylglycerol *rac* **5**. The reaction sequence continues with the careful removal of the trityl protective group under mild acidic conditions using activated boric acid (10%) on silicic acid (31) in dry hexane to form *rac*-1-*O*-alkyl-2-*O*-acylglycerol **6**. Under these conditions, acyl migration, and formation of 1,3-isomer, is kept to a minimum.

The phospholipid head group skeleton is then introduced immediately by base catalyzed condensation of *rac*-1,2-di-*O*-radylglycerol **6** with a three-fold excess of bromoethyl dichlorophosphate (32) under reaction conditions which have previously been optimized in our laboratory (33). Use of anhydrous diethyl ether as reaction medium, instead of chloroform or similar solvents (32), facilitates the condensation and minimizes nucleophilic substitution of both chlorines (triester formation) due to insolubility of the phosphorylation product in Et_2O (33). The phosphorylation conditions furthermore ensure preferential reaction of the primary hydroxy group of **6** rather than at the sterically hindered secondary hydroxy group of the 1,3-isomer, if such contamination would be present (33). Hydrolysis of the residual phosphochloride bond gives intermediate *rac* **7** which, upon nucleophilic displacement of the bromine with trimethylamine, produces *rac*-1-*O*-alkyl-2-acylglycero-3-phosphocholine (*rac* **8**) in good overall yield. The chemical purity of the products at each reaction step was verified by thin-layer chromatography. The structural identity of the compounds and the absence of positional isomers was ascertained by carbon-13 NMR spectroscopy (see Table 1). Spectral assignments were consistent with various lipid spectral data reported in the literature (33–36).

An essential element of our synthetic approach is the formation of optical isomers at the last stage of preparation. The possibility of racemization is thereby largely eliminated. The racemic alkylacylglycerophosphocholine (*rac* **8**) is treated with snake venom phospholipase A_2 (37) from *Ophiophagus hannah*, which cleaves only the natural *sn*-3 **8** isomer and affords 1-*O*-alkyl-*sn*-glycero-3-phosphocholine (lysoPAF) of natural configuration in optically pure form ($[\alpha]^{31}_{546}$–6.17). The carbon-13 NMR spectrum of lysoPAF (*sn*-3 **9**) is shown in Figure 1.

The "unnatural" 3-*O*-alkyl-2-*O*-acyl-*sn*-glycero-1-phosphocholine (*sn*-1 **8**), which is not susceptible to phospholipase A_2 cleavage, gives 3-*O*-alkyl-*sn*-glycero-1-phosphocholine (*sn*-1 **9**) upon deacylation with methanolic sodium hydroxide (38). The optical rotation of the "unnatural" isomer **9** was approximately equal in magnitude, but opposite in direction ($[\alpha]^{31}_{546}$+6.11) when compared to the

TABLE 1

^{13}C NMR Chemical Shifts and ^{13}C-^{31}P and ^{13}C-^{14}N Couplings of LysoPAF (9) and of the Intermediates of LysoPAF Synthesis[a]

Compound	Glycerol				Head group			C-1′ ether	Additional signals[e]
	No.	C-1	C-2[c]	C-3[b]	C-α[b]	C-β[c]	$N(CH_3)_3$[d]		
rac-2,3-Isopropylidene-glycerol	1	64.21	77.41	66.93					110.63 ($\underline{C}Me_2$); 27.87 and 26.45 ($C\underline{Me}_2$)
rac-1-*O*-Hexadecyl-2,3-isopropylidene-glycerol	2	71.91[f]	74.84	67.04				71.91[f]	109.36 ($\underline{C}Me_2$); 26.80 and 25.47 ($C\underline{Me}_2$)
rac-1-*O*-Hexadecyl-glycerol	3	70.54	71.92	64.35				72.55	
rac-1-*O*-Hexadecyl-3-*O*-tritylglycerol	4	69.91	71.67	64.70				72.09	143.94 ($\underline{C}Ph_3$); 128.71, 127.82 and 127.05 (Ph)
rac-1-*O*-Hexadecyl-2-*O*-palmitoyl-3-*O*-trityl-*rac*-glycerol	5	69.51	71.55[g]	62.70				71.68[g]	173.25 (C=O); 143.89 ($\underline{C}Ph_3$); 128.68, 127.79 and 126.99 (Ph); 34.56 ($CO\underline{C}H_2$); 25.07 ($COCH_2\underline{C}H_2$)
rac-1-*O*-Hexadecyl-2-*O*-palmitoyl-3-*O*-(2′-bromoethyl)-phosphoryl-glycerol	7	69.62	72.40 (8 Hz)	64.58 (4.9 Hz)	65.85 (4.6 Hz)	31.21 (7.4 Hz)		72.19	174.54 (C=O); 34.84 ($CO\underline{C}H_2$); 25.38 ($COCH_2\underline{C}H_2$)
rac-1-*O*-Hexadecyl-2-*O*-palmitoyl-glycero-3-phosphocholine	8	69.76	72.45 (9.1 Hz)	64.63 (4.8 Hz)	59.60 (4.6 Hz)	67.03 (7.0 Hz)[h]	54.50 (3.5 Hz)	72.22	174.51 (C=O); 34.83 ($CO\underline{C}H_2$); 25.47 ($COCH_2\underline{C}H_2$)
1-*O*-Hexadecyl-*sn*-glycero-3-phosphocholine (LysoPAF)	9	72.07[i]	70.20 (7.5 Hz)	67.92 (5.8 Hz)	59.65 (5.1 Hz)	66.89 (6.9 Hz)[h]	54.49 (3.5 Hz)	72.30[i]	

[a] Proton-decoupled 20-MHz ^{13}C NMR spectra were measured in solution at 37 ± 1°C using $CDCl_3$ for compounds 1-5 and $CDCl_3/CD_3OD/D_2O$ (50:50:15, v/v/v) for compounds 7-9 as solvent. Chemical shifts (∂) are given downfield from Me_4Si (internal standard). Chemical shifts without further specification refer to singlets. Designations of carbon atoms are those shown in Figure 1 (formula insert). Coupling constants are given in Hz.

[b] Two-bond ^{13}C-^{31}P couplings ($^2J_{CP}$, doublets) are given in parentheses.

[c] Three-bond ^{13}C-^{31}P couplings ($^3J_{CP}$, doublets) are given in parentheses.

[d] ^{13}C-^{14}N Couplings (J_{CN}, triplets) are given in parentheses.

[e] Not listed are signals due to the carbons of the aliphatic chains. With $CDCl_3$ as solvent (1-5), the aliphatic signals occur at 14.11 ppm (ω CH_3), 22.71 ± 0.02 ppm (ω-1 CH_2), 31.94 ± 0.02 ppm (ω-2 CH_2), 29.70 ± 0.01 ppm ($-(CH_2)_n-$), and 26.11 ± 0.02 ppm (C-2′ ether); with $CDCl_3/CD_3OD/D_2O$ (50:50:15, v/v/v) as solvent, the respective signals occur at 14.23 ± 0.03 ppm (ω CH_3), 23.05 ± 0.04 ppm (ω-1 CH_2), 32.34 ± 0.05 ppm (ω-2 CH_2), 30.09 ± 0.03 ppm ($-(CH_2)_n-$), and 26.47 ± 0.05 ppm (C-2′ ether).

[f] The signals for C-1 and C-1′ (C-O-C) are not resolved. The intensity of the signal at 71.91 ppm is about twice that of C-2 or C-3.

[g] Assignment to C-2 and C-1′ is tentative and may be reverse.

[h] $^3J_{CP}$ values given for choline derivatives 8 and 9 are approximate and were obtained from the multiplets after correcting for superimposed ^{13}C-^{14}N couplings (J_{CN}=3.5 Hz).

[i] Assignments of C-1 and C-1′ (C-O-C) were confirmed by ^1H-^{13}C heteronuclear shift correlation (HETCOR) experiments on a Varian Unity 300 instrument (Varian Associates, Palo Alto, CA). The methylene protons at C-1 and C-1′ were distinguishable based on the additional multiplicity observed for the C-1 methylene protons due to their nonequivalence. The proton assignments were confirmed by ^1H-^1H correlation spectroscopy (COSY).

natural isomer. This affirms the purity of both optical isomers we synthesized. LysoPAF and its enantiomer can readily be acetylated (17,18) to yield PAF and its enantiomer, respectively.

EXPERIMENTAL PROCEDURES

Racemic isopropylideneglycerol (1), trimethylamine, and triphenylchloromethane were purchased from Aldrich Chemical Co. (Milwaukee, WI). Triphenylchloromethane was recrystallized from petroleum hydrocarbon (b.p. 90–110°)/acetyl chloride (39). Palmitoyl chloride was purchased from Nu-Chek-Prep (Elysian, MN). Hexadecyl methanesulfonate was prepared from hexadecanol (Nu-Chek-Prep) and methanesulfonyl chloride (Eastman, Rochester, NY) according to established procedures (28). 2-Bromoethyl dichlorophosphate was prepared according to Hirt and Berchtold (32), as modified previously (33). Snake venom *(Ophiophagus hannah)* was purchased from Miami Serpentarium Laboratories (Miami, FL).

Solvents were purified as follows: diethyl ether was refluxed over and distilled from $LiAlH_4$, then stored over sodium wire; acetone was refluxed over and distilled from anhydrous $CaSO_4$, then stored over anhydrous Na_2SO_4; methanol was refluxed over and distilled from magnesium

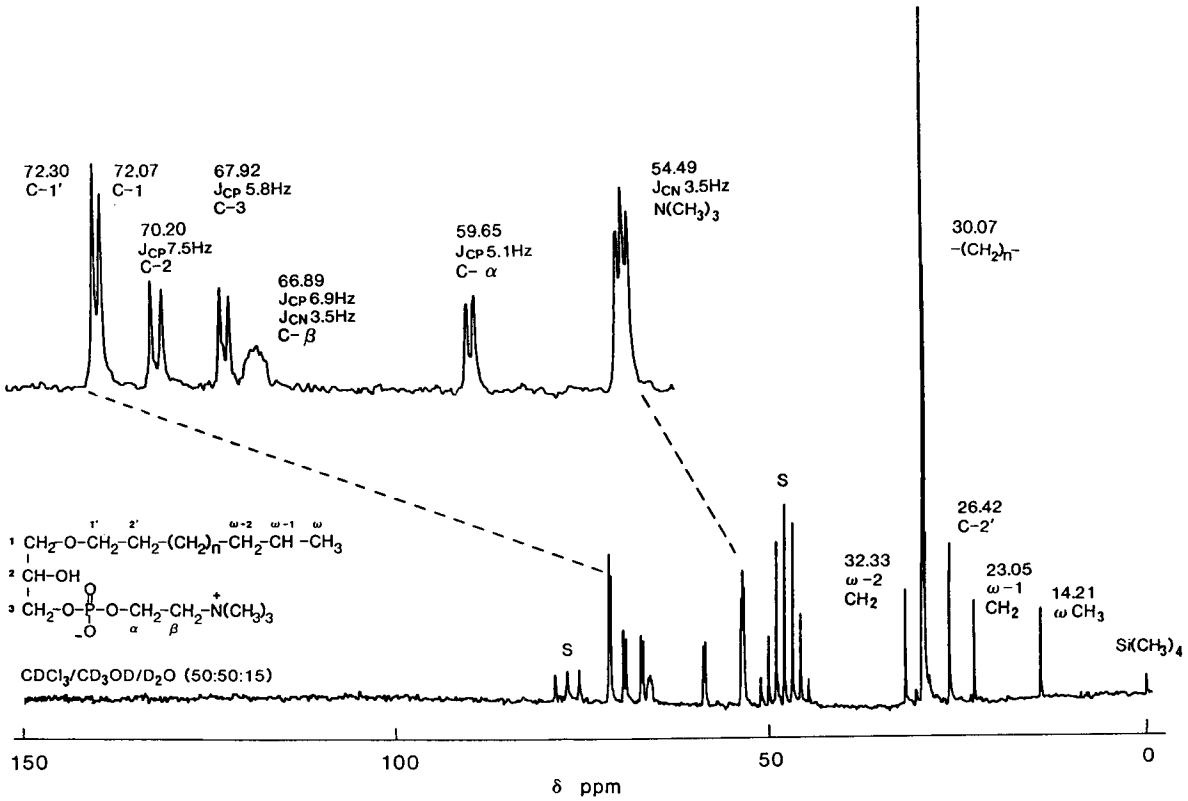


FIG. 1. Proton-decoupled 20-MHz ^{13}C NMR spectrum of 1-*O*-hexadecyl-*sn*-glycero-3-phosphocholine (lysoPAF; 9) in $CDCl_3/CD_3OD/D_2O$ (50:50:15, v/v/v) with tetramethylsilane as internal standard (0.00 ppm). S designates clusters of solvent peaks ($CDCl_3$, CD_3OD).

turnings; pyridine was refluxed over and distilled from KOH pellets; benzene, xylene and petroleum hydrocarbons were refluxed over and distilled from P_2O_5.

Analytical and preparative thin-layer chromatography (TLC) was done on layers of Silica Gel H (Merck), 0.3 and 3 mm thick, respectively. For analytical purposes, plates were developed in tanks lined with filter paper, and fractions were made visible by charring after spraying with chromic-sulfuric acid reagent (for details, see ref. 33); phosphorus-containing fractions were alternatively made visible by spraying with molybdenum reagent (40). In preparative work, 200–250 mg of sample was applied per 20 × 20-cm plate; after developing, lipid fractions were usually visible as opalescent bands, or were made visible by brief exposure to iodine vapors; bands were scraped off, and lipids were eluted. After evaporation on a rotary evaporator, lipid preparations were freeze-dried from dry benzene.

Melting points (mp) were determined on a Kofler hot stage (C. Reichert) and are uncorrected. Optical rotations were measured at the 546.1-nm mercury line using a Bendix Automatic Polarimeter 1169. Infrared spectra (IR) were recorded with a Beckman Model IR 4240 Spectrophotometer (Beckman Instruments, Fullerton, CA) using KBr pellets or neat thin films. Relative intensities of IR absorption bands are given: vs, very strong; s, strong; m, medium; and w, weak. Aliphatic CH vibrations occurring in the regions 3000–2800 cm^{-1} and 1500–1400 cm^{-1} are not individually listed. Electron impact mass spectra (MS) were recorded on a Dupont DP-102 spectrometer (Dupont, Boston, MA) at 70 eV; only significant or characteristic ions are reported. Carbon-13 NMR spectra were recorded at 20 MHz on a Varian FT-80A pulse Fourier transform instrument (Varian Associates, Palo Alto, CA) equipped with a broadband probe. Spectra were measured at 37 ± 1°C using proton noise decoupling, 8K data points and 4000 Hz spectral width. Neutral lipids were dissolved in $CDCl_3$, phospholipids were dissolved in $CDCl_3/CD_3OD/D_2O$ (50:50:15, v/v/v) to assure monomeric solutions (35,41). Spectra were measured on 10–100 mg/mL samples (10-mm o.d. tubes). Chemical shifts are expressed in ppm downfield from Me_4Si, which was used as internal standard.

rac-*1-O-Hexadecyl-2,3-isopropylideneglycerol* (2). Powdered potassium hydroxide (14 g, 0.25 mol), 320 mL of dry xylene and 5.1 g (39 mmol) of *rac*-isopropylideneglycerol (1) are refluxed for 2 hr under dry nitrogen while water is removed azeotropically. A solution of 8.3 g (26 mmol) of hexadecyl methanesulfonate (28) in dry xylene (80 mL) is added dropwise at room temperature and refluxing is continued for 4 hr. After cooling, the xylene solution is decanted, and the solid residue is washed with diethyl ether (200 mL). The combined organic phases are transferred into a separatory funnel, washed with cold water until neutral (using NaCl, if necessary, to facilitate phase separation), the combined water layers are re-extracted with Et_2O, and the ether extracts are washed with water. Removal of the solvents from the combined organic phases on a rotary evaporator and freeze-drying from benzene gives 11.0 g of crude product 2. Preparative TLC (developing solvent, hexane/Et_2O, 90:10, v/v; R_f 0.51), elution with $CHCl_3$/MeOH

(90:10, v/v), evaporation of the solvent, and freeze-drying yields 6.9 g (75%) of **2** as a colorless oil. IR (neat) 1379 (s), 1370 (s), 1350 (w), 1258 (s), 1218 (s), 1120 (vs), 1080 (vs), 1059 (vs), 975 (w), 850 (s), 792 (w), 723 (w), 660 cm^{-1} (w). MS *m/z* 356, M^+; 341, $[M - CH_3]^+$; 299, $[M + H - Me_2CO]^+$.

rac-*1*-O-*Hexadecylglycerol* (**3**). Isopropylideneglycerol ether **2** (6.9 g, 19 mmol) is refluxed for 3 hr in a solution of concentrated HCl (10 mL) in methanol (100 mL). After cooling and addition of water (200 mL), the mixture is extracted twice with a total of 400 mL of Et_2O. The combined ether extracts are washed consecutively with small amounts of cold water (until pH 5–6), 2% aqueous K_2CO_3, and cold water (until neutral), and dried over anhydrous Na_2SO_4. Filtration, evaporation of the solvent, and freeze-drying gives 5.9 g of crude product, which is recrystallized from hexane/Et_2O (70:10, v/v) to yield 5.1 g (85%) of **3** as white crystals. Hexadecylglycerol **3** is pure as judged by TLC (developing solvent, Et_2O/hexane, 95:5, v/v; R_f 0.51); mp 64–66°C (ref. 28; 65.5°C). IR (KBr) 1395 (w), 1377 (w), 1325 (m), 1255 (w), 1239 (w), 1123 (vs), 1102 (m), 1071 (m), 1059 (s), 993 (w), 936 (m), 861 (w), 718 (m), 677 cm^{-1} (m). MS *m/z* 316, M^+; 299, $[M - OH]^+$; 298, $[M - H_2O]^+$; 285, $[M - CH_2OH]^+$; 255, $[M - C_2H_5O_2]^+$; 241, $[C_{16}H_{33}O]^+$; 225, $[C_{16}H_{33}]^+$.

rac-*Hexadecyl-3*-O-*tritylglycerol* (**4**). Freshly recrystallized triphenylchloromethane (1.2 g, 4.3 mmol) is slowly added (5 min) through a powder funnel to a stirred, ice-cooled solution of hexadecylglycerol **3** (1.0 g, 3.2 mmol) in dry pyridine (6 mL), while an atmosphere of dry nitrogen is maintained. After stirring and cooling for 1 hr, the reaction mixture is kept at room temperature under N_2 for 48 hr. Dry Et_2O (100 mL) is added, and the precipitated pyridine hydrochloride is removed by suction and washed on the filter with dry Et_2O (2 × 30 mL). While cooling with ice, the filtrate is extracted consecutively with 0.5 N H_2SO_4 (until acidic), water, 2% K_2CO_3 (until basic), and water, and the organic phase is dried over anhydrous Na_2SO_4. Filtration, evaporation of the solvent, and freeze-drying leaves 1.74 g of crude **4**, which is purified by preparative TLC (developing solvent, hexane/Et_2O, 70:30, v/v; R_f 0.60). Elution with $CHCl_3$/MeOH (90:10, v/v), evaporation of the solvent, and freeze-drying yields 1.1 g (62%) of **4** as a white solid; mp 46–47°C (ref. 29; 49°C). IR (KBr) 3060 (w), 3035(w), 3025 (w), 1595 (w), 1191 (s), 1179 (s), 764 (s), 705 (vs), 645 cm^{-1} (m). MS *m/z* 558, M^+; 314, $[M - HCPh_3]^+$; 260, $[HOCPh_3]^+$; 259, $[OCPh_3]^+$; 244, $[HCPh_3]^+$.

rac-*1*-O-*Hexadecyl-2*-O-*palmitoyl-3*-O-*tritylglycerol* (**5**). Tritylated glycerol ether **4** (3.3 g, 5.9 mmol) in dry benzene (300 mL) plus dry pyridine (25 mL) is refluxed in an atmosphere of dry nitrogen for 1 hr while solvent (100 mL) is collected to remove water. The reaction mixture is cooled in an ice bath, palmitoyl chloride (5.0 g, 18 mmol) in dry benzene (75 mL) is added dropwise under stirring, and the reaction is continued first at room temperature for 30 min, then at reflux temperature for 5 hr. After standing overnight at room temperature, Et_2O (200 mL) and ice chips are added, and the mixture is transferred into a separatory funnel. After phase separation, the aqueous layer is re-extracted with Et_2O (100 mL), and the combined organic phases are washed with water until neutral. After drying over anhydrous Na_2SO_4, the solvent is removed on a rotary evaporator, and the residue is freeze-dried to give 6.5 g of crude **5**. Preparative TLC (developing solvent, hexane/Et_2O, 90:10, v/v; R_f 0.68), elution with $CHCl_3$/MeOH (90:10, v/v), evaporation of the solvent, and freeze-drying yields 4.6 g (98%) of **5** as a white solid; mp 35–36°C. IR (KBr) 3065 (w), 3055 (w), 3030 (w), 1775 (s), 1375 (w), 1175 (w), 1115 (w), 1090 (w), 775 (w), 763 (w), 745 (w), 705 cm^{-1} (s). MS *m/z* M^+ not observed; 553, $[M - CPh_3]^+$; 537, $[M - OCPh_3]^+$; 259, $[OCPh_3]^+$; 256, $[C_{15}H_{31}COOH]^+$; 243, $[CPh_3]^+$; 241, $[C_{16}H_{33}O]^+$; 239, $[C_{15}H_{31}CO]^+$.

rac-*1*-O-*Hexadecyl-2*-O-*palmitoylglycerol* (**6**). Trityl acyl glycerol ether **5** (1.5 g, 1.9 mmol) in 300 mL of dry hexane and 10%-boric acid/silicic acid (30 g, prepared according to Buchnea, ref. 31; and activated at 110°C overnight) is stirred vigorously at room temperature in an atmosphere of dry nitrogen for 2 hr. The reaction mixture is then run through filter paper, and trityl alcohol is eluted by further washing with hexane (1 L).

Alkylacylglycerol is eluted with chloroform (1 L), the solvent is removed on a rotary evaporator, and the residue is freeze-dried with benzene to leave 1.3 g of crude reaction product **6**. Thin-layer chromatography (developing solvent, hexane/Et_2O, 20:30, v/v) shows mainly 1-alkyl-2-acylglycerol (R_f 0.33), traces of 1-alkyl-3-acylglycerol (R_f 0.56), and some contamination by trityl alcohol (R_f 0.78). Crude product **6** is immediately phosphorylated to minimize isomerization.

rac-*1*-O-*Hexadecyl-2*-O-*palmitoyl-3*-O-*(2'-bromoethyl)-phosphorylglycerol* (**7**). 2-Bromoethyl dichlorophosphate (1.2 g, 5 mmol) in anhydrous Et_2O (50 mL) is placed into a three-necked flask equipped with heating mantle, magnetic stirrer, reflux condenser and drying tube, dropping funnel, and an inlet tube for dry N_2. After cooling in an ice bath, 3 mL of dry pyridine is added with stirring followed by dropwise addition of 0.92 g (1.66 mmol) of crude alkylacylglycerol **6** in 50 mL of dry Et_2O over a 30-min period at room temperature. The reaction is brought to completion by stirring for another 30 min at room temperature followed by refluxing for 4 hr. After cooling in an ice bath, 5 mL of water is added slowly and stirring is continued for 1 hr. The solvent is removed on a rotary evaporator, the residue is taken up in 100 mL of $CHCl_3$/MeOH (2:1, v/v), and the solution is extracted with 20 mL of water while the organic (lower) phase is kept at pH 5 by addition of 2N H_2SO_4. The aqueous phase is re-extracted with two portions of 50 mL of $CHCl_3$/MeOH (2:1, v/v), and the combined organic phases are evaporated. The freeze-dried residue is purified by preparative TLC (developing solvent, $CHCl_3$/MeOH/H_2O, 65:20:2, v/v/v; R_f 0.31–0.59), the product is eluted with $CHCl_3$/MeOH/H_2O (50:40:10, v/v/v), and the solvent is removed on a rotary evaporator. Freeze-drying afforded 440 mg of pure bromide **7** (two-step yield, 44%, relative to trityl compound **5**); mp 175–177°C. IR (KBr) 1735 (s), 1250 (s), 1182 (w), 1120 (s), 1084 (s), 1096 (s), 952 (w), 855 (w), 726 cm^{-1} (w).

rac-*1*-O-*Hexadecyl-2*-O-*palmitoylglycero-3-phosphocholine* (**8**). A solution of bromide **7** (333 mg, 0.45 mmol) in $CHCl_3$ is transferred into a 50-mL, thick-walled glass bulb, the solvent is removed with dry N_2, and the sample is dried *in vacuo*. Anhydrous acetone (30 mL) and 3 mL of anhydrous trimethylamine is added under N_2 while cooling the bulb in dry ice. The sealed bulb is heated in a shaking water bath *(caution: use safety glasses and*

protective shield) at 75°C for 4 hr, cooled *slowly* to dry ice temperature, and then opened carefully. The solvent is removed by a stream of nitrogen. The residue is taken up in 100 mL of $CHCl_3$/MeOH (2:1, v/v) and extracted with 20 mL of water, the organic phase is adjusted to pH 5 with 2N H_2SO_4, and the aqueous phase is separated and re-extracted with two portions of 50 mL $CHCl_3$/MeOH (2:1, v/v). The organic phases are combined, the solvent is removed on a rotary evaporator, and the residue is freeze-dried. Preparative TLC (sample applied in $CHCl_3$/MeOH, 2:1, v/v; developing solvent, $CHCl_3$/MeOH/H_2O, 65:25:4, v/v/v; R_f 0.19), elution with $CHCl_3$/MeOH/H_2O (50:40:10, v/v/v), evaporation of the solvent and freeze-drying yields 215 mg (66%) of alkylacylglycerophosphocholine 8; mp 227.5-228°C (coalescent; translucent globules at 80°C). IR (KBr) 1373 (w), 1257 (s), 1242 (s), 1170 (w), 1097 (s), 1088 (s), 1057 (s), 969 (m), 820 cm^{-1} (w).

*1-O-Hexadecyl-*sn*-glycero-3-phosphocholine* (sn-*3* 9). Alkylacylglycerophosphocholine (*rac* 8) (161 mg, 0.22 mmol) is dispersed by vortexing in 20 mL of an incubation mixture containing 20 mM Tris-HCl buffer (pH 7.5) and 10 mM $CaCl_2$. Snake venom phospholipase A_2 (5 mg powdered *Ophiophagus hannah* venom) is added, and the mixture is incubated in a shaking water bath at 37°C for 6 hr. After addition of another 5-mg portion of phospholipase A_2, hydrolysis is continued for 12 hr. The mixture is transferred into a separatory funnel with $CHCl_3$ (50 mL), MeOH (50 mL) and water (25 mL), the Bligh-Dyer (42) mixture is separated, and the upper layer is re-extracted with $CHCl_3$ (2 × 50 mL). Evaporation of the solvent and freeze-drying leaves 176 mg of a white solid. The mixture is separated into two bands (R_f 0.17 and 0.28) on 0.3-mm layers of Silica Gel H using $CHCl_3$/MeOH/H_2O (65:35:8, v/v/v) as developing solvent. Elution of the fractions with $CHCl_3$/MeOH/H_2O (50:40:10, v/v/v), evaporation of the solvent and freeze-drying gives 77 mg of alkylacylglycerophosphocholine (crude *sn*-1 8) from the upper band (R_f 0.28) and 51 mg (96% yield) of pure 1-*O*-hexadecyl-*sn*-glycero-3-phosphocholine (lysoPAF; *sn*-3 9) from the lower band (R_f 0.17); mp >240°C (coalesces and decomposes; translucent globules above 125°C). IR (KBr) 1235 (m), 1210 (s), 1095 (s) 1065 (s), 1055 (s), 972 (s), 846 cm^{-1} (m). $[\alpha]_{546}^{31}$ −6.17° (c=0.43 g/100 mL $CHCl_3$/MeOH, 1:1, v/v); ref. 27, $[\alpha]_D^{25}$ −6.09° (c=1.04 g/100 mL $CHCl_3$/MeOH, 1:1, v/v); ref. 18, $[\alpha]_D^{25}$ −6.7° (c=5 g/100 mL) for 1-*O*-octadecyl-*sn*-glycero-3-phosphocholine.

*3-O-Hexadecyl-2-O-palmitoyl-*sn*-glycero-1-phosphocholine* (sn-*1* 8). The upper fraction (crude *sn*-1 8, 77 mg) obtained in the previous reaction step is treated again with phospholipase A_2, extracted and purified by TLC, as described for the preparation of *sn*-3 9, in order to remove all of the natural isomer of 8 that is susceptible to phospholipase A_2 hydrolysis. The procedure affords 72 mg (91% yield) of pure *sn*-1 8; mp 224-224.5°C (coalescent; translucent globules at 72°C). The infrared and ^{13}C NMR spectra are essentially identical to those of *rac* 8.

*3-O-Hexadecyl-*sn*-glycero-1-phosphocholine* (sn-*1* 9). Alkylacylglycerophosphocholine *sn*-1 8 (34 mg, 0.047 mmol) in $CHCl_3$ (0.5 mL) and MeOH (0.5 mL) is stirred together with 1 mL of 0.2 N methanolic NaOH (freshly prepared and centrifuged) for 30 min at room temperature. While cooling with ice, 2 mL of water is added, and the mixture is neutralized with a few drops of concentrated acetic acid. The mixture is transferred into a separatory funnel with $CHCl_3$ (9.5 mL), MeOH (8.5 mL) and water (7 mL), the Bligh-Dyer (42) mixture is separated, and the upper layer is re-extracted with $CHCl_3$ (5 × 10 mL). Evaporation of the solvent and freeze-drying gives 44 mg of crude product which is purified on 0.3-mm layers of Silica Gel H (developing solvent, $CHCl_3$/MeOH/H_2O, 65:35:8, v/v/v). After elution with $CHCl_3$/MeOH/H_2O (50:40:10, v/v/v), evaporation of the solvent and freeze-drying, some unreacted starting material (5 mg), and 17 mg (75% yield) of pure 3-alkyl-*sn*-glycero-1-phosphocholine *sn*-1 9 is isolated from the lower TLC band (R_f 0.17); mp >240°C (coalesces and decomposes; translucent globules above 125°C). The infrared and ^{13}C NMR spectra are essentially identical to those of natural lysoPAF (*sn*-3 9). $[\alpha]_{546}^{31}$ + 6.11° (c=0.45 g/100 mL $CHCl_3$/MeOH, 1:1, v/v); ref. 27, $[\alpha]_D^{25}$ + 6.11° (c=1.05 g/100 mL $CHCl_3$/MeOH, 1:1 v/v); ref. 18, $[\alpha]_D^{25}$ + 6.7° (c=5 g/100 mL) for 3-*O*-octadecyl-*sn*-glycero-1-phosphocholine.

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Composition of Mouse Peritoneal Macrophage Phospholipid Molecular Species

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The individual molecular species composition of diacyl, alkylacyl and alkenylacyl glycerophospholipids was determined in mouse peritoneal macrophages. A marked heterogeneity in the relative composition (mol%) of macrophage ether and ester phospholipid individual species was noted. High concentrations of 16:0-20:4 were found in ether phospholipids such as alkenylacyl glycerophosphoethanolamine (GPE; 27.5 mol%) and alkylacyl glycerophosphocholine (GPC; 16.6%) as compared to mol % levels of 16:0-20:4 in diacyl GPE (5.7%) and diacyl GPC (8.1%), respectively. Interestingly, alkenylacyl GPE was highly enriched in 1-ether (16:0) relative to alkylacyl GPC. The predominant diacyl molecular species in glycerophosphoinositol (GPI) and glycerophosphoserine (GPS) were 18:0-20:4 (59.1%) and 16:0-18:1 (41.1%), respectively. It is noteworthy that the level of 18:0-20:4 was several times higher in diacyl GPI (59.1%) than in diacyl GPS (11.1%), diacyl GPE (25.7%), and diacyl GPC (3.7%). The most abundant molecular species in diacyl GPC and diacyl GPE were 16:0-18:1 (29.9%) and 18:0-20:4 (25.7%), respectively. The abundance of 20:4 in ether phospholipids, specifically 16:0-20:4 and 18:0-20:4, in alkylacyl GPC is significant in view of the role these antecedents play in the biosynthesis of platelet-activating factor (PAF) and 20:4-derived eicosanoids in stimulated macrophages. The unique molecular species composition of the peritoneal macrophage distinguishes this cell type from others.
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Macrophages play an important role in the immune system and are capable of both amplifying and suppressing immune responses (1,2). The phospholipids of murine peritoneal macrophages are highly enriched with arachidonic acid (20:4) (3). Indeed, macrophages are known to secrete large quantities of 20:4-derived eicosanoids (cyclooxygenase and lipoxygenase oxidative products) from endogenous phospholipid stores (1). Excessive production of 20:4-derived eicosanoids may exacerbate pathophysiological conditions such as asthma, arthritis and psoriasis (4,5). Therefore, an understanding of the mechanisms regulating the production of 20:4-derived eicosanoids is vital in controlling inflammation and preventing pathophysiological states.

The cascade of 20:4 release and metabolism is regulated in part by its specific phospholipid class distribution (6,7). Different classes of glycerophospholipids such as glycerophosphocholines (GPC), glycerophosphoethanolamines (GPE), glycerophosphoinositols (GPI), and glycerophosphoserines (GPS) have been proposed as sources of eicosanoid fatty acid precursors (8,9). Glycerophospholipids such as GPC and GPE can be further separated into subclasses (diacyl, alkylacyl and alkenylacyl), each differing in the covalent linkage of the aliphatic chain at the *sn*-1 position of the glycerol backbone. Recently, it has been shown that considerable amounts of GPC and GPE subclasses are present in rabbit alveolar (10) and mouse peritoneal macrophages (11). The 1-*O*-alkyl and 1-*O*-alk-1′-enyl phospholipid subclasses are unique because they contain significantly higher levels of 20:4n-6 than diacyl species in certain cell types (11–13) and could be important pools of polyunsaturated fatty acids (PUFA) in macrophages. Indeed, phospholipid molecular species may have specific biochemical functions and turnover rates (14,15). For example, platelet-activating factor (PAF, 1-*O*-alkyl-2-acetyl-glycerophosphocholine), derived primarily from 16:0-20:4 alkylacyl GPC, is a potent bioactive phospholipid with broad pro-inflammatory properties and is produced by stimulated macrophages (16,17). Therefore, in order to elucidate the mechanisms which regulate cellular eicosanoid and PAF production, it is important to characterize the molecular species of GPI and GPS classes and GPC and GPE subclasses.

Peritoneal macrophages are both biochemically and functionally distinct from alveolar and other tissue macrophages (1). This is particularly evident with regard to eicosanoid production and molecular transductional mechanisms (1,18). Although several reports have been published on the molecular species of alveolar macrophage glycerophospholipids (19,20), the molecular species composition of mouse peritoneal macrophages has not been determined. A plethora of well-documented similarities between the mouse and human macrophage (1,2) make the mouse peritoneal macrophage an excellent model system for elucidating the mechanisms regulating human macrophage metabolism and function. The purpose of the present work, therefore, was to characterize the relative composition of the various glycerophospholipid molecular species in mouse peritoneal macrophages.

MATERIALS AND METHODS

Materials. Diacyl GPC (16:0-16:0, 16:0-18:0, 16:0-18:1, 16:0-20:4, 18:0-18:0, 18:1-18:1, 18:2-18:2) used as standards were from Avanti Polar Lipids, Inc. (Birmingham, AL). Platelet-activating factor (PAF 16:0, PAF 18:0), and (18:0-20:4, 18:0-18:2) phosphatidylglycerol were from Biomol Research Labs. Inc. (Plymouth Meeting, PA). Fatty acid methyl ester standards and heptadecanoic acid were from NuChek Prep (Elysian, MN). Silica gel 60 plates and silica gel G were from E. Merck (Darmstadt, FRG). Phospholipase C (*Bacillus cereus*, Types V and XI)

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Abbreviations: ANS, 8-anilino-1-napthalenesulfonic acid ammonium salt; BHT, butylated hydroxytoluene; DMA, dimethylacetal; FAME, fatty acid methyl ester; GC, gas chromatography; GPC, glycerophosphocholine; GPE, glycerophosphoethanolamine; GPI, glycerophosphoinositol; GPS, glycerophosphoserine; HPLC, high-performance liquid chromatography; PAF, platelet-activating factor; PMN, polymorphonuclear leukocytes; PUFA, polyunsaturated fatty acids; R_f, retardation factor; RRT, relative retention time; TLC, thin-layer chromatography.

were from Sigma (St. Louis, MO). 8-Anilino-1-napthalenesulfonic acid ammonium salt (ANS) and Vitride reagent, 70% in toluene, were from Eastman Kodak (Rochester, NY). Benzoic anhydride and 4-dimethylaminopyridine were from Aldrich (Milwaukee, WI). All solvents were of high-performance liquid chromatography (HPLC) grade.

Animal and macrophage isolation. Specific pathogen-free male C57BL/6NCR mice (Frederick Cancer Research Facility, Frederick, MD) weighing 15–18 g (4–6 wk) were used. Mice were fed Purina Mouse Chow and water *ad libitum* and were injected intraperitoneally with 2 mL of 3% thioglycollate broth (Difco Labs., Detroit, MI) prepared to manufacturer's specifications, three days prior to sacrifice. Primary cultures of peritoneal macrophages were established from responsive cells as previously described (21).

Extraction and preparation of phospholipid classes. Macrophages from monolayers were extracted by the method of Folch *et al.* (22). All solvents contained 0.01% butylated hydroxytoluene (BHT) to prevent oxidation. The individual phospholipid classes were separated by thin-layer chromatography (TLC) on silica gel 60 plates using chloroform/methanol/acetic acid/water (50:37.5:3.5:2, by vol) as previously described (23). Bands were detected under ultraviolet light after spraying with 0.1% ANS. The phospholipid bands corresponding to GPI, GPS, GPC, and GPE were extracted using chloroform/methanol/water (5:5:1, v/v/v) followed by the addition of 2.25 mL chloroform and 1 mL 50 mM Tris buffer, pH 9.0 (24). The upper aqueous layer was discarded and the lower chloroform layer was removed under N_2.

Separation of phospholipid subclasses. GPC and GPE were isolated as described above and converted to benzoate derivatives following phospholipase C hydrolysis (25,26). Briefly, 0.4 mg of phospholipid was dissolved in 2 mL peroxide-free diethyl ether containing 0.005% BHT and 2 mL of 50 mM Tris-HCl buffer, pH 7.4, containing 5 mM calcium chloride and 10 units of phospholipase C *(Bacillus cereus).* The mixture was agitated in a shaking water bath for 3 hr at room temperature. The diradylglycerols were extracted 3 times with 2 mL diethyl ether. The combined extracts were passed through Pasteur pipets containing anhydrous sodium sulfate and dried under N_2. Samples were dissolved in 0.2 mL of benzene containing 10 mg benzoic anhydride and 0.1 mL of benzene containing 4 mg of 4-dimethylaminopyridine and incubated for 1 hr at room temperature (25). The reaction was stopped by placing the samples on ice, followed by the addition of 1 mL of concentrated ammonium hydroxide and 2 mL hexane (19). The diradylglycerobenzoates were extracted 3 times with hexane and backwashed once with 1 mL water. The solvent was evaporated under N_2 and the diradylglycerobenzoates were separated on silica gel G plates using benzene/hexane/diethyl ether (50:45:4, v/v/v) (25). The alkenylacyl, alkylacyl and diacylglycerobenzoates were visualized using ANS spray. The bands were scraped directly into glass tubes containing chilled ethanol, water and hexane (1:1:1, v/v/v). The mixture was vortexed, centrifuged, and the hexane layer removed and evaporated under N_2.

The diacylglycerobenzoates derived from GPI and GPS were prepared following phospholipase C hydrolysis using a modification of the methods of Colard *et al.* (27) and Nakagawa *et al.* (28). Briefly, GPI and GPS were suspended in 4 mL peroxide-free diethyl ether containing 0.005% BHT and 2 mL of 50 mM Tris-HCl buffer, pH 7.4, containing 5 mM $CaCl_2$ and 100 units of phospholipase C. The mixture was incubated in a shaking water bath for 24 hr at 25°C. The diradylglycerols were extracted as described above and diradylglycerobenzoates were prepared immediately to prevent isomerization. No evidence of positional isomerization of the diglycerides was observed following derivatization. Greater than 98% hydrolysis of GPI and GPS was obtained as verified by combined thin-layer and gas chromatographic techniques.

Characterization of individual molecular species of macrophage phospholipids. The distribution of molecular species of GPC (1,2-diacyl, 1-*O*-alkyl-2-acyl) and GPE (1,2-diacyl, 1-*O*-alk-1′-enyl-2-acyl) subclasses and GPI and GPS (1,2-diacyl) classes was analyzed as their glycerobenzoate derivatives by reversed phase HPLC (25). Separation of molecular species within each class and subclass as diradylglycerobenzoates was achieved with a Beckman System Gold Module HPLC system (Beckman Instruments, Fullerton, CA) fitted with a C-18 reverse phase pre-column and a 250 × 4.6 mm analytical column. Both columns were packed with 5 μ Ultrasphere-ODS (Altex, San Ramon, CA). Component molecular species were quantitated on-line at 230 nm using a Beckman 167 UV-visible Scanning Detector (Beckman). The diacyl, alkylacyl and alk-1′-enylacyl glycerobenzoates were separated isocratically with acetonitrile/2-propanol using ratios of 70:30, 63:37, and 65:35 (v/v), respectively. Authentic molecular species standards were prepared as described above and also analyzed by HPLC and gas chromatography (GC) for identification of the major individual molecular species. Relative retention times (RRT) of the molecular species were determined using the method of Patton *et al.* (29). The RRT was obtained by dividing the retention time of each peak by the retention time of the reference peak (16:0-18:1). A graphical relationship using log (RRT × 10) was constructed for prediction of the elution sequence of molecular species (25,29,30).

Gas chromatography. Individual molecular species of diacylglycerophospholipids (collected from HPLC as benzoates) were also identified as fatty acid methyl ester (FAME) derivatives by gas chromatography as previously described (23). Structures of alkenylacyl glycerobenzoates from plasmalogens were confirmed following conversion to their corresponding FAME and dimethyl acetals (DMA) (31,32). Briefly, alkenylacyl glycerobenzoate fractions were dissolved in 3 mL of 6% anhydrous methanolic H_2SO_4 and incubated at 80°C. After 2 hr, the reaction mixture was cooled, neutralized with excess anhydrous sodium carbonate, and extracted with 2 mL hexane and 2 mL water. Hexane extraction was repeated. The hexane phase was passed through anhydrous sodium sulfate, and the FAME and DMA were purified by TLC using toluene (32), and analyzed on a Hewlett-Packard 5890A gas chromatograph equipped with a DB-225 30 meter fused silica capillary column (i.d. 0.25 mm, J&W Scientific, Folsom, CA). The hydrogen carrier gas flow was 1.78 mL/min, split ratio was 1:66 and oven temperature was isothermally kept at 200°C. Fatty acids were identified relative to retention times of known standards. In addition, DMA derived from plasmalogens were isolated following incubation of DMA and FAME with

3 mL of 0.5 M methanolic NaOH at 80°C. After 2 hr, 3 mL of water was added and the DMA were extracted with hexane and analyzed by gas chromatography as described above. The fatty acids remained in the aqueous phase as sodium salts.

The *sn*-1 configuration of alkylacyl GPC molecular species was determined by preparing dibenzoate derivatives of the corresponding alkylglycerol (33). Briefly, isolated alkylacyl glycerobenzoates (monobenzoates) were reduced with Vitride reagent [$NaAlH_2(OCH_2CH_2OCH_3)_2$, 70% in toluene]. The lipid was dissolved in 2.5 mL of diethyl ether/toluene (4:1, v/v), 0.5 mL of Vitride reagent was added, and the mixture was incubated in a shaking water bath at 37°C for 30 min. Reactions were stopped by cooling on ice, 20% ethanol in water was added, and the product extracted twice with 5 mL diethyl ether. The extract was dissolved in chloroform/methanol (1:1, v/v) and run on a silica gel G plate using diethyl ether/acetic acid/H_2O (200:1:1, v/v/v) (33). The alkylglycerol band—retardation factor (R_f) 0.39—was scraped into a test tube containing 1 mL water and extracted twice with 2 mL diethyl ether. Dibenzoylation was achieved following evaporation and addition of 0.2 mL of benzene containing 10 mg benzoic anhydride and 0.1 mL of benzene containing 4 mg of 4-dimethylaminopyridine as described above. Following extraction, the dibenzoates were separated on silica gel G plates using hexane/diethyl ether/conc. NH_4OH (85:15:1, v/v/v) as solvents. Alkylglycerodibenzoates (R_f 0.3) were extracted from silica gel using chloroform/methanol/water (5:4:5, v/v/v). Chloroform (2 mL) extraction was repeated. The chloroform layer was removed, passed over anhydrous sodium sulfate and evaporated to dryness. The sample was redissolved in mobile phase and analyzed by HPLC using an Altex C-18 reverse phase column with detection at 228 nm. An isocratic solvent system consisting of 16% isopropanol in acetonitrile (v/v) at a flow rate of 2 mL/min was used.

RESULTS AND DISCUSSION

The data in Table 1 represent the relative retention times (RRT) of macrophage phospholipid molecular species. A graphical relationship using RRT was constructed in order to predict the elution sequence of molecular species (28–30). These RRT are consistent with previously published RRT and HPLC chromatograms (28–30,34). A representative chromatogram of the reverse phase HPLC separation of 1,2-diacyl GPC individual molecular species is shown in Figure 1. Table 2 shows the fatty acid composition of molecular species of 1,2-diacyl and 1-*O*-alkyl-2-acyl GPC of mouse peritoneal macrophages. The major molecular species in 1,2-diacyl GPC were 16:0-18:1 (29.9 mol%) and 16:0-18:2 (17.8%), whereas the predominant species in alkylacyl GPC were 16:0-18:1 (28.2%), 16:0-20:4 (16.6%) and 18:0-20:4 (11.2%). Diacyl GPC contained only 8.1% 16:0-20:4 and 3.7% 18:0-20:4. The data reported for 1,2-diacyl GPC and alkylacyl GPC molecular species are similar to values reported for rat polymorphonuclear leukocytes (PMN) (34). By comparison, Nakagawa *et al.* (19) reported higher amounts of 16:0-20:4 species in rabbit alveolar macrophage alkylacyl GPC (39%). The abundance of 1-*O*-alkyl-2-arachidonoyl GPC is noteworthy, because these molecular species are precursors to PAF (1-*O*-alkyl-2-acetyl GPC) which is known as a potent pro-inflammatory mediator secreted by stimulated macrophages (16,17). The high content of 1-radyl-2-arachidonoyl GPC molecular species in peritoneal macrophages is also of biological importance because phospholipase A_2 hydrolysis will release 20:4 which can be metabolized to a variety of functionally significant eicosanoids (1,35). It remains to be determined whether specific phospholipid molecular species provide precursors for individual classes of eicosanoids. The hydrolysis of diacyl GPC molecular species by phospholipases C and D to yield diacylglycerol and phosphatidic acid, respectively, has also been recently demonstrated (36). These GPC breakdown products can cause sustained activation of protein kinase C (36). Interestingly, the different molecular species of diacylglycerol may exert differential effects on the various isoforms of protein kinase C (37). In addition, ether-linked diradylglycerols may also be involved in activation of protein kinase C during intracellular signal transduction (38).

The molecular species composition of mouse macrophage 1,2-diacyl GPE and 1-*O*-alk-1′-enyl-2-acyl GPE

TABLE 1

Relative Retention Times of Diacylglycerobenzoate Molecular Species

Molecular species	log(RRT × 10)[a]	Molecular species	log(RRT × 10)[a]
18:2-20:5	0.486	16:0-20:3	0.867
18:2-20:4	0.647	16:0-22:4	0.890
18:1-22:6	0.687	18:0-20:4	0.920
16:1-22:5	0.647	18:0-22:5	0.944
18:2-18:2	0.715	18:1-18:1	0.975
16:0-22:6	0.715	16:0-18:1	1.000
18:1-20:4	0.769	16:0-16:0	1.009
16:0-20:4	0.791	18:0-18:2	1.009
16:0-22:5	0.813	18:0-20:3	1.024
18:1-18:2	0.843	18:0-22:4	1.121
18:0-22:6	0.843	18:0-18:1	1.138
16:0-18:2	0.867	16:0-18:0	1.171
16:1-18:1	0.867		

[a]16:0-18:1 was used as the reference time for RRT calculation. The log (RRT × 10) of other peaks was determined by dividing the retention time of each peak by the retention time of the reference peak.

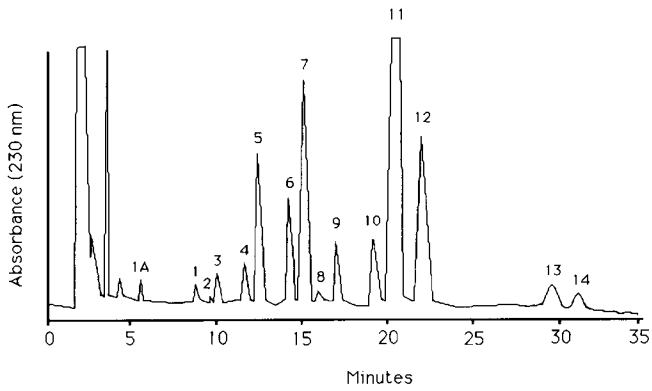

FIG. 1. HPLC separation of 1,2-diacyl GPC molecular species of mouse peritoneal macrophages. Refer to Table 2 for peak identification.

TABLE 2

Composition of Diacyl and Alkylacyl GPC Molecular Species of Mouse Peritoneal Macrophages

Peak no.[a]	Molecular species	1,2-Diacyl	1-*O*-Alkyl-2-acyl
1A	18:2-X[b]	2.8 ± 2.1	
1	18:2-20:4	0.8 ± 0.2	0.9 ± 0.3
	16:1-22:5		
2	18:1-22:6	0.4 ± 0.1	1.7 ± 0.1
3	18:2-18:2	2.0 ± 0.4	2.9 ± 1.0
	16:0-22:6		
4	18:1-20:4	2.4 ± 0.1	0.5 ± 0.0
5	16:0-20:4	8.1 ± 0.5	16.6 ± 0.3
6	18:1-18:2	7.2 ± 0.3	13.0 ± 1.2
7	16:0-18:2	17.8 ± 0.6	11.0 ± 1.7
	16:1-18:1		
	16:0-20:3		
8	16:0-22:4	0.9 ± 0.2	1.9 ± 0.9
9	18:0-20:4	3.7 ± 0.2	11.2 ± 0.4
10	18:1-18:1	6.4 ± 1.7	3.9 ± 0.0
11	16:0-18:1	29.9 ± 3.8	28.2 ± 0.7
12	16:0-16:0	12.9 ± 1.4	6.0 ± 1.9
	18:0-18:2		
13	18:0-18:1	3.6 ± 0.0	1.2 ± 0.3
14	16:0-18:0	2.5 ± 0.7	2.1 ± 0.2

[a] Peak numbers correspond to those shown in the HPLC tracings of Figure 1.

Values are mol% ± SD from two separate cell preparations of three mice each.

[b] X, unidentified fatty acid (*sn*-2 position).

TABLE 3

Composition of Diacyl and Alkenylacyl GPE Molecular Species of Mouse Peritoneal Macrophages[a]

Peak no.[b]	Molecular species	1,2-Diacyl	1-*O*-Alk-1′-enyl-2-acyl
1	18:2-20:4	0.2 ± 0.2	0.7 ± 0.0
	16:1-22:5		
2	18:1-22:6	0.7 ± 0.3	1.7 ± 0.1
3	18:2-18:2	1.9 ± 0.4	4.5 ± 0.0
	16:0-22:6		
4	18:1-20:4	2.5 ± 0.3	12.6 ± 1.6
5	16:0-20:4	5.7 ± 0.7	27.5 ± 1.1
6	16:0-22:5	3.1 ± 2.9	0.3 ± 0.3
7	18:1-18:2	7.2 ± 0.6	1.1 ± 0.3
8	16:0-18:2	5.7 ± 2.6	3.7 ± 0.2
	16:0-20:3		
9	16:0-22:4	1.7 ± 0.7	16.7 ± 0.9
10	18:0-20:4	25.7 ± 7.9	12.8 ± 3.4
11	18:0-22:5	3.2 ± 0.2	1.9 ± 0.1
12	18:1-18:1	2.5 ± 0.7	
13	16:0-18:1	13.6 ± 3.9	13.8 ± 0.9
14	16:0-16:0	11.8 ± 1.2	
	18:0-18:2		
15	18:0-20:3	2.1 ± 1.0	
16	18:0-22:4	1.7 ± 0.5	1.2 ± 0.2
17	18:0-18:1	9.4 ± 2.0	2.6 ± 0.3
18	16:0-18:0	1.9 ± 0.1	

[a] Values are mol% ± SD from two separate cell preparations of three mice each.

[b] Peak number corresponds to the HPLC elution profile of specific phospholipid-derived diradylglycerobenzoates.

(plasmalogen) is shown in Table 3. The most abundant molecular species were 18:0-20:4 (25.7%) and 16:0-20:4 (27.5%), respectively. A considerable amount of 18:0-20:4 (12.8%) was also present in alkenylacyl GPE. Consistent with other cell types (19,34), alkenylacyl GPE contained higher amounts of 16:0-20:4 (27.5%) than alkylacyl GPC (16.6%). The aliphatic chain in the *sn*-1 position of alkenylacyl GPE consisted primarily of 16:0, 18:0 and 18:1 as confirmed by DMA analysis. Furthermore, the *sn*-1 chain of alkylacyl GPC consisted mainly of 16:0, 18:0 and 18:1 as confirmed by HPLC analysis of alkylglycerodibenzoates. In both instances, 16:0 was the predominant *sn*-1 moiety (data not shown). This is consistent with the molecular species data presented in Tables 2 and 3. Previous reports on alveolar macrophages (10,19) and neutrophils (34) indicated that the *sn*-1 position of both alkylacyl GPC and alkenylacyl GPE is composed primarily of 16:0 and 18:0.

Table 4 shows the individual molecular species of diacyl GPI and GPS. The most prevalent species in GPI was 18:0-20:4 (59.1%), whereas in GPS 16:0-18:1 (41.1%) and 18:0-18:1 (19.8%) were most predominant. This contrasts with GPS 16:0-18:1 (2%) in alveolar macrophages (19). GPS also contained 11.1% 18:0-20:4 which could potentially serve as a source of 20:4 for eicosanoid biosynthesis following phospholipase A_2 hydrolysis (39). Collectively, 18:0-18:1 (19.8%) and 18:0-20:4 (11.1%) made up 30.9% of GPS species as compared to 78% in human platelets (7) and 69% in rabbit alveolar macrophages (19). Interestingly, the mol% levels of 18:0-20:4 in GPI (59.1%) were dissimilar from human platelets (71%) (7) and rabbit alveolar macrophages (39%) (19). This is significant because the mouse peritoneal macrophage is commonly

TABLE 4

Composition of Diacyl GPI and GPS Molecular Species of Mouse Peritoneal Macrophages[a]

Peak no.[b]	Molecular species	Diacyl GPI	Diacyl GPS
1	18:2-X[c]	9.3 ± 1.0	1.1 ± 0.6
2	18:1-22:6		0.8 ± 0.2
3	18:2-18:2		0.3 ± 0.2
	16:0-22:6		
4	18:1-20:4	5.4 ± 0.2	0.5 ± 0.1
5	16:0-20:4	3.7 ± 0.7	1.0 ± 0.0
6	18:1-18:2	3.9 ± 0.5	6.2 ± 0.7
	18:0-22:6		
7	16:0-18:2	3.0 ± 0.4	3.9 ± 0.4
	16:1-18:1		
	16:0-20:3		
8	16:0-22:4	1.5 ± 1.1	2.0 ± 0.2
9	18:0-20:4	59.1 ± 3.5	11.1 ± 1.2
10	18:0-22:5		2.5 ± 0.3
11	18:1-18:1	2.8 ± 0.8	3.0 ± 0.4
12	16:0-18:1	11.3 ± 1.0	41.1 ± 1.5
13	16:0-16:0		2.1 ± 1.1
	18:0-18:2		
14	18:0-22:4		1.7 ± 0.1
15	18:0-18:1	5.1 ± 2.5	19.8 ± 1.4
16	16:0-18:0		4.0 ± 0.8

[a] Values are from two separate cell preparations of three mice each.

[b] Peak number corresponds to the HPLC elution profile of specific phospholipid-derived diradylglycerobenzoates.

[c] X, unidentified fatty acid (*sn*-2 position).

used as a model system for elucidating the mechanisms regulating human macrophage metabolism and function.

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Transformations of 5-HETE by Activated Keratinocyte 15-Lipoxygenase and the Activation Mechanism

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There is convincing evidence that normal cultured human keratinocytes possess a 15-lipoxygenase activity which, however, does not appear to manifest itself without cell membrane damage. When "activated", this enzyme transforms arachidonic acid into 15-hydroxyeicosatetraenoic acid (15-HETE), and linoleic acid into 13-hydroxyoctadecadienoic acid, presumably by peroxidase action on their respective hydroperoxy intermediates. Normal but not membrane-damaged keratinocytes metabolize exogenous 5-HETE, principally by esterifying the eicosanoid intact, primarily in the triacylglycerol fraction. In the present study, membrane-damaged keratinocytes were found to transform 5-HETE to 5,15-diHETE and also to a lipoxin-like group of tetraenes. Similar, if not identical, tetraenes were produced by action of the keratinocyte enzyme on 5(S),15(S)-diHETE, which points to the role of the latter as an intermediate between 5-HETE and the tetraenes. A direction for further study of the mechanism of the "activation" step is presented.
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There is much current interest in the lipoxygenases of keratinocytes and inflammatory cells and the possible coprocessing of their products (1–3). Cultured keratinocytes offer a simplified system in which to study both coprocessing and further metabolism of eicosanoids produced by inflammatory cells. Keratinocyte 15-lipoxygenase was first described by Burrall *et al.* (4,5) and more recently has been shown to act on endogenous and exogenous substrate after "activation" by membrane damage, for example by freezing (6). Normal keratinocytes were found to rapidly esterify 5-lipoxygenase-derived exogenous 5(S)-hydroxyeicosa-6E,8Z,11Z,13E-tetraenoic acid (5-HETE) and this esterified product was stable in the living keratinocytes for up to 24 hr (6). In the present work, the focus is on the metabolism of 5-HETE by "activated" keratinocyte 15-lipoxygenase with respect to biologically active products and on the mechanism of keratinocyte 15-lipoxygenase activation.

MATERIALS AND METHODS

Cells and incubations. Keratinocyte cultures (from both human adult, and newborn donors) were obtained from Dr. Howard Green, Department of Cellular and Molecular Physiology, Harvard Medical School, Boston MA (7–9). The further handling of the cultures was exactly as previously described (6).

Buccal epithelial cells from normal donors (10) were obtained by gentle scraping with a spatula rinsed in Medium 199 with pH 7.5 40 mM HEPES buffer. All the cells recovered by this procedure were epithelial cells and approximately two-thirds retained barrier function to Trypan Blue. These cells were washed three times with 5 mL of Medium 199. After counting in a ZBI Coulter counter (Coulter Electronics, Hialeah, FL), the cells were incubated at 37°C for up to 3 hr in the presence of arachidonic acid or other eicosanoids. The reactions were interrupted by the addition of three volumes of ethanol and samples were stored at −20°C.

High performance liquid chromatography (HPLC) separations. After the addition of prostaglandin B_2 or labelled HETE as an internal standard, the samples were centrifuged and the supernatant was rotoevaporated, transferred in methanol and ultimately dissolved in mobile phase for reverse phase (RP) HPLC in a Hewlett-Packard 1090 LC (Hewlett-Packard Co., Palo Alto, CA). This was performed isocratically on Hewlett-Packard ODS-Hypersil columns, 10 or 20 cm in length × 4.6 mm at a flow rate of 0.4 mL/min. The mobile phase for the monohydroxyeicosanoids ("HETES phase") was methanol/water/acetic acid (75–80:25–20:0.1, by vol.); and for leukotrienes and tetraenes ("acetonitrile-containing phase"), water/acetonitrile/methanol/acetic acid (45:37:18:1, by vol.). Slight modifications of these conditions are given in the text. The fractions containing hydroxylated free acids were collected during the HPLC runs and were methylated with ethereal diazomethane, and rechromatographed on the same columns (delayed retention time of approximately 15 min). All the HPLC studies were run at a wavelength of 236 nm, but recall was available at 270 nm and 301 nm; *in situ* UV spectroscopy was also routinely performed. The ordinate scale is given in milliabsorption units throughout, and all chromatograms are reproduced directly from the HPLC computer plots. Retention times (R.T.) of the authentic standards are indicated in the Figure legends. Software programs for quantitation using published molar extinction coefficients (6) were employed to measure concentrations as previously described. Unlabelled eicosanoids were obtained from BioMol Research Labs (Plymouth Meeting, PA). They were pure by HPLC as free hydroxy acids and also after methylation. Fatty acids were purchased from NuChek Prep, Inc. (Elysian, MN).

Chiral separation was achieved with Baker DPG chiral columns (ionic form), 2 × 25 cm in series, under isocratic straight phase (SP) conditions with hexane/isopropanol, (100:1.5, by vol.). Under these conditions, the chiral isomers of HETEs are well separated. In order to confirm the identity of the unknown stereoisomers, mixing studies were routinely performed by addition of synthetic racemic isomers to the unknowns followed by repeat chiral phase chromatography. Racemic 15-HETE was prepared by reduction of 15-ketoeicosatetraenoic acid with sodium borohydride.

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Abbreviations: 5,15-diHETE, 5(S),15(S)-dihydroxyeicosa-6E,8Z,11Z,13E-tetraenoic acid; 5-HETE, 5(S)-hydroxyeicosa-6E,8Z,11Z,14Z-tetraenoic acid, 13-HODD, 13(S)-hydroxyoctadeca-9Z,11E-dienoic acid; HPLC, high performance liquid chromatography; LC, liquid chromatography; RP, reverse phase; R.T., retention times; SP, straight phase.

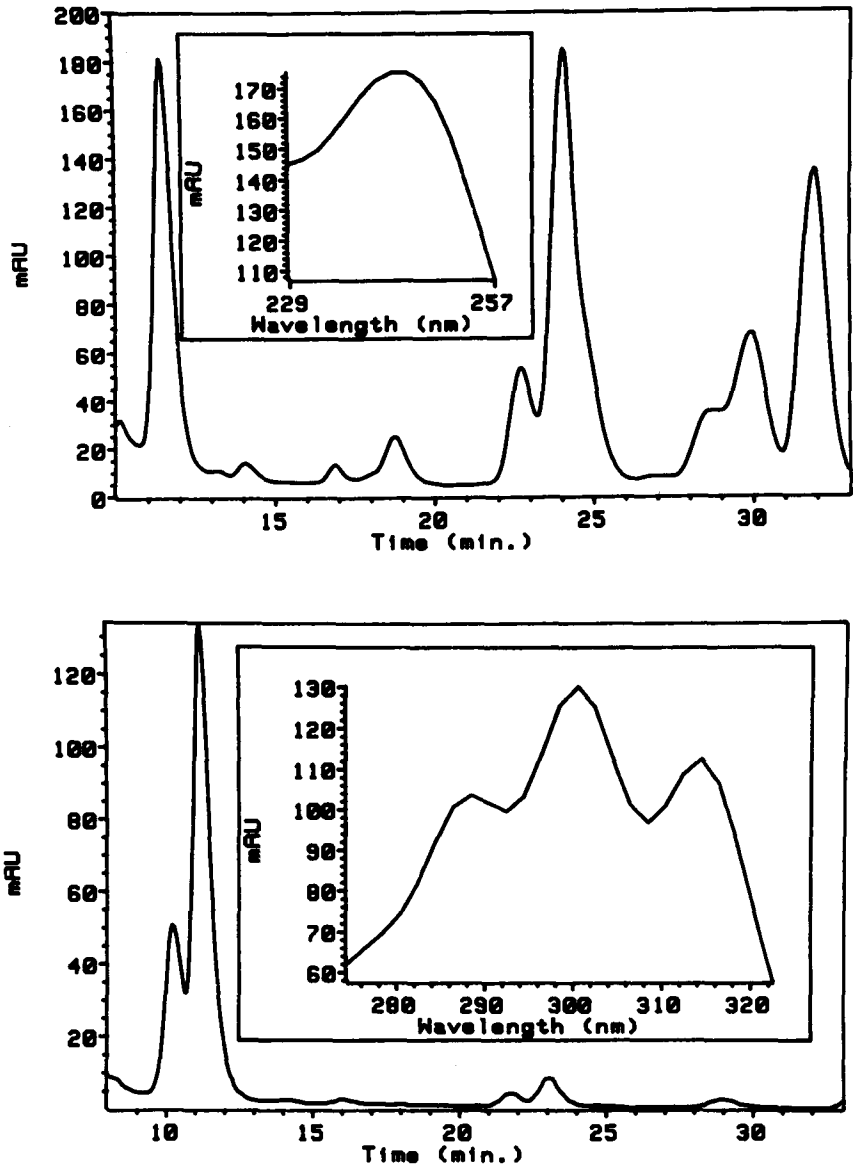

FIG. 1. A: LC (absorbance signal at 236 nm) of the products generated by the addition of 3 μg of 5-HETE to 5×10^6 frozen and thawed human keratinocytes and incubated for 3 hr at 37°C. The peak at 32 min represents residual 5-HETE, that at 24 min 15-HETE, and the new peak at 11.5 min has a λ_{max} of 243 (insert). B: The same chromatogram as in A, with the absorbance signal at 301 nm. At least two peaks are seen with the UV spectrum of a tetraene of λ_{max} 301 (insert).

RESULTS

Cultured human keratinocytes were frozen, thawed and then incubated with 5-HETE. A typical experiment is illustrated in Figure 1. Peaks corresponding to 13-HODD [13(S)-hydroxyoctadeca-9Z,11E-dienoic acid], 15-HETE, and residual exogenous 5-HETE were found in the chromatogram at 236 nm (HETES phase) as shown in Figure 1A. Chiral identification (data not shown) of the esterified mono-HETE products derived from keratinocytes showed an all (S) stereospecificity at the point of -OH insertion, consistent with the lipoxygenase origin of the products. These results are identical to the data already published for the products of cultured buccal keratinocytes (10). Another peak was observed in the 11-12 min range, which had a λ_{max} at 243. When this peak was esterified with diazomethane, its retention time was prolonged to 16 min with the same λ_{max} (data not shown). These peaks correspond to 5,15-diHETE [5(S),15(S)-dihydroxyeicosa-6E,8Z,11Z,13E-tetraenoic acid] and 5,15-diHETE methyl ester, respectively. The same liquid chromatography (LC) run is shown in Figure 1B except that the absorbance at 301 nm instead of at 236 nm is plotted. Two partially separated peaks were observed both with a λ_{max} of 301 nm and shoulders at 288 and 315 nm, which are characteristic of tetraenes. These peaks were more clearly separated on the acetonitrile-containing phase (see below).

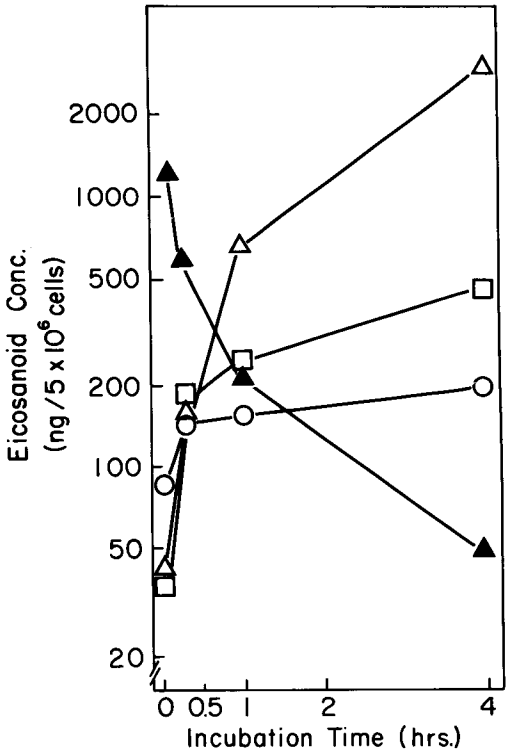

FIG. 2. Time course of production and disappearance of eicosanoids following the addition of 2.5 μg of 5-HETE to (5×10^6) frozen and thawed human keratinocytes. Closed triangles (▲), residual exogenous 5-HETE; open triangles (△), 15-HETE; open squares (□), tetraenes; open circles (○), 5,15-diHETE.

The kinetics of appearance of these peaks, as well as the disappearance rate of exogenous 5-HETE, are illustrated in Figure 2. These data show that by 5 min, substantial metabolic conversion to 5,15-diHETE had occurred. Formation of the tetraenes continued to increase for at least 4 hr. The production of 15-HETE from endogenous arachidonic acid (6) and the rapid fall in exogenous 5-HETE ($t_{1/2} < 10$ min) are also illustrated. 5-HETE did not stimulate the production of 15-HETE by keratinocytes.

Figure 3 shows an example of chromatography of the tetraenes (absorbance at 301 nm) with acetonitrile-containing phase in which there is a better separation. Rechromatography of the compound identified as 5,15-diHETE on the acetonitrile phase (absorbance at 236 nm) along with the synthetic standard also confirm the identification (Fig. 3, insert and UV spectrum).

To test the possibility that the tetraene is a metabolic product of the 5,15-diHETE, as opposed to having been produced through a separate pathway from 5-HETE, synthetic 5,15-diHETE was added to keratinocytes previously frozen and thawed. These experiments were carried out both at 3°C and at 37°C, and the contrasting results in Figure 4 show that the two major peaks change their relative abundance at the higher temperature with the preponderance of the less polar tetraene at 37°C. The tetraene at 38 min has a λ_{max} at 302 nm and that at 44 min at 300 nm. This difference, although small, was reproducible and not due to solvent effects since the elution conditions were isocratic. Other minor tetraene components were also observed. The probable significance of

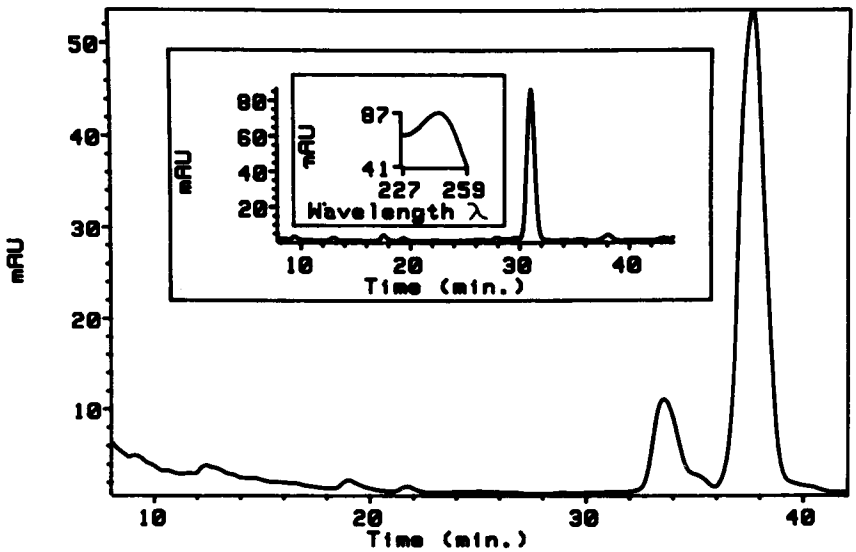

FIG. 3. LC of the products of incubation of 5-HETE with (5×10^6) frozen and thawed normal keratinocytes incubated for 1 hr at 37°C and run on the acetonitrile-containing phase. The main chromatogram shows the absorbance signal at 301 nm (tetraene peaks) and the large insert shows the same chromatogram with its signal at 236 nm together with the small insert showing a peak at 31 min and its UV spectrum with λ_{max} of 243. This latter peak and spectrum correspond exactly to those 5,15-diHETE.

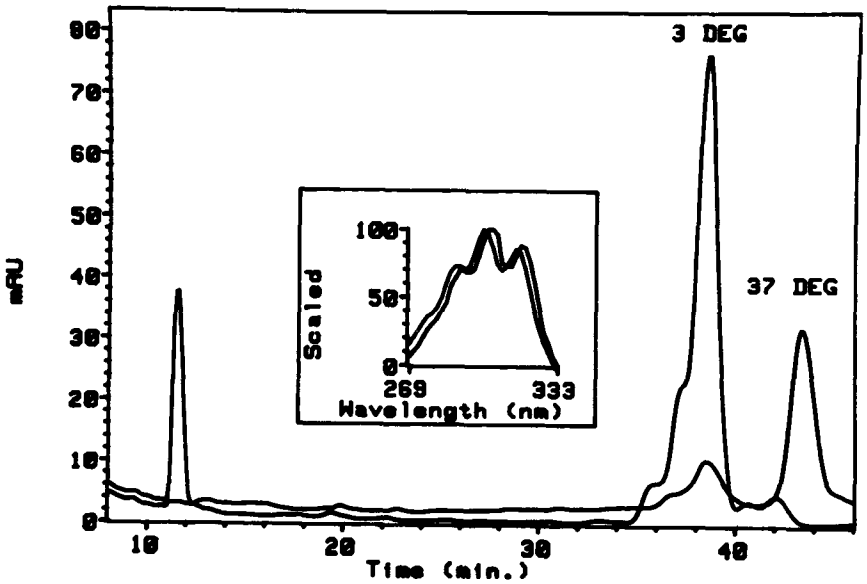

FIG. 4. Two chromatograms superimposed, both with the absorbance signal at 301 nm, representing the products of incubation of 2.5 μg of 5,15-diHETE with (5×10^6) frozen and thawed keratinocytes. The incubation temperatures were 3°C and at 37°C in separate experiments. The major peaks show a slightly different λ_{max} of the tetraene spectra as well as a shift in retention time. The minor peaks also exhibited tetraene characteristics.

TABLE 1

Keratinocyte Lipoxygenase Activities[a]

Characteristic	Cultured cells	Buccal cells *ex vivo*
Apparent activation requirement	Membrane damage	None
Acts on endogenous arachidonic acid	Yes	No
Esterification of mono-HETE	Yes	No
15-Lipoxygenase activity	Yes	Yes
12-Lipoxygenase activity	None	Yes
Chirality at point of -OH insertion	All (S)	All (S)

[a]Collation of data primarily from references 10 and 6.

this change is that a *cis-trans* isomerization occurred at the higher temperature, resulting in a change in both polarity and λ_{max}. The tetraenes produced exhibited virtually the same retention times whether the reaction was interrupted with acetic acid, ethanol or methanol, suggesting that the unexpectedly non-polar character of the tetraenes was not due to the formation of ethyl ether or methyl ether trapping products of the organic solvent used to interrupt the incubations.

The data presented thus far illustrate the metabolic fate of 5-HETE when incubated with keratinocytes which had been frozen and thawed. These data contrast with the situation in which 5-HETE is added to healthy keratinocytes in which case the principal product is esterified 5-HETE (6). This leads to the fundamental biological question of the factors involved in the generation of the "activated" state of keratinocyte 15-lipoxygenase. A comparison of lipoxygenase characteristics has been made between cultured keratinocytes from buccal origin (10) with keratinocytes from buccal cells taken *ex vivo* (Table 1). It can be seen that membrane damage appeared to be required for activation of the lipoxygenase of the cultured cells, but not for the lipoxygenases of the *ex vivo* cells, and that the enzyme could act on endogenous substrate in the case of cultured cells but not those *ex vivo*. Most significantly, the cultured cells were capable of esterification of mono-HETEs, whereas the buccal cells *ex vivo* were not. These differences are analogous to those between cultured healthy skin keratinocytes and those which had been membrane-damaged. Terminally differentiated buccal cells may lose their esterification mechanisms prior to their loss of 15-lipoxygenase activity. From these considerations the possibility presented itself that "activation" of 15-lipoxygenase may, in some way, be associated with inactivation of the enzyme system(s) which are responsible for fatty acid esterification.

A series of experiments was performed in which a very high concentration of fatty acid was presented to normal keratinocytes to see whether, under these circumstances, the requirement of membrane damage for activation might be absent. In order to minimize the risk of damage of the cells by the high concentration of arachidonic acid itself which might then lead to a confusing activation of 15-lipoxygenase, a slightly different approach was used.

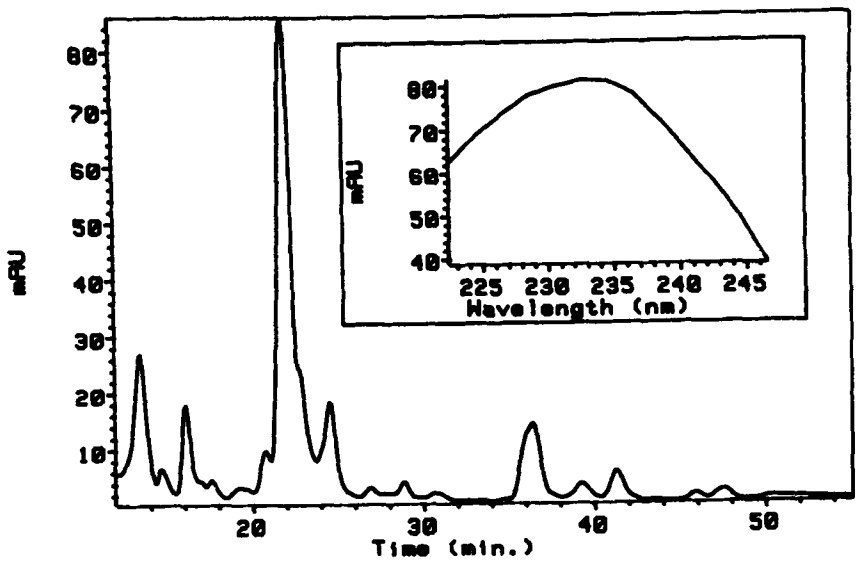

FIG. 5. LC (absorbance signal at 236 nm) of normal keratinocytes which were incubated for 3 hr with 700 μM linoleic acid. The principal peak at 22 min and its spectrum (insert) with λ_{max} at 234 indicate that the product is 13-hydroxyoctadecadienoic acid (760 ng).

Since damage to keratinocytes has been found to lead to the production of 15-HETE and 13-HODD from endogenous substrates in a ratio of 8-10:1 (6), linoleic instead of arachidonic acid was added in high concentrations (700 μM) to the keratinocytes to look for lipoxygenase product generation (Fig. 5). This experiment on normal, non-frozen keratinocytes revealed 13-HODD but no demonstrable 15-HETE. One possible explanation is that the capacity to esterify linoleic acid was swamped, leaving substrate available to an apparently activated 15-lipoxygenase.

DISCUSSION

Neutrophils and monocyte 5-lipoxygenase activation lead to 5-HETE production which may be especially marked when LTA_4 hydrolase is damaged or overwhelmed. Transcellular transfer of 5-HETE may have a regulatory role or possess other significant biological effects (11,12). This eicosanoid may be further metabolized to other products with biological activities. The 15-lipoxygenase of cultured human keratinocytes does not respond to such physiological agonists as PMA and bradykinin. In the present studies, as documented by retention times and UV spectra in three different chromatograph systems, the 15-lipoxygenase of keratinocytes, when activated, transforms 5-HETE to 5,15-diHETE. More surprising was the observation that a lipoxinoid group of tetraenes was also produced from 5-HETE and the same group of compounds was observed after incubation of keratinocytes with 5,15-diHETE. Tetraenes have also been shown to be made from 5,15-HETE by reticulocyte 15-lipoxygenase (13,14). The present results indicate that the 5,15-diHETE is the direct precursor of the tetraene and that the resulting eicosanoid has a 5(S),15(S)-dihydroxy structure, plus another structural modification which had resulted in conjugation of all four double bonds. Because of limitations in amounts and stability of these products, further characterization of these tetraenes has not yet been possible, except to rule out the presence of lipoxins A and B, which have retention times between 15 and 17 min under the conditions of the experiments. Previously, tetraenes of the lipoxin class have been produced as a result of 5-lipoxygenase acting upon 15-HETE or 15-HPETE (15,16). In this present situation, the roles are reversed. The fact that the tetraenes can be produced from a 5,15-diHETE intermediate suggests that an epoxide intermediate is not involved. The mechanism of tetraene formation under these present circumstances is not yet clear. In addition, the polarity of the tetraenes on LC approximated that of diHETEs rather than triHETEs, implying that a third hydroxyl group if present must not be free. The functional effects of this local tetraene production must remain speculative until the exact structures are characterized but the reported biological effects of lipoxins include the possibility for immune regulation (17). In addition, 5,15-diHETE has been reported to have significant biological effects (18).

In attempting to understand the difference in metabolism of 5-HETE by intact *vs* damaged cells, the critical question arises of operationally-defined "activation" of the lipoxygenase. Since different products and biological properties may result, the mechanism of "activation" becomes central. Observations found in this work and in others cited above point to the possibility that the key factor in the apparent "activation" of 15-lipoxygenase may be the interruption of fatty acid esterification pathways and therefore that there is no true activation step *per se*. The "overwhelming substrate" experiment illustrated in Figure 5 could be consistent with, but certainly does not establish the truth, of this

postulated mechanism. Multiple lines of evidence will be required to assess this possible mechanism. Whether this speculation ultimately proves to be true of keratinocytes and, if so, whether this action could represent a general cellular lipoxygenase activation mechanism remains to be shown.

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Molecular Species of 1-*O*-Alk-1′-enyl-2-acyl-, 1-*O*-Alkyl-2-acyl- and 1,2-Diacyl Glycerophospholipids in Japanese Oyster *Crassostrea gigas* (Thunberg)

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Molecular species of 1-*O*-alk-1′-enyl-2-acyl-, 1-*O*-alkyl-2-acyl-, and 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine (EPL) and *sn*-glycero-3-phosphocholine (CPL) of Japanese oyster *Crassostrea gigas* were analyzed by selected-ion monitoring gas chromatography/mass spectrometry using electron impact ionization. The characteristic fragment ions, $[RCH{=}CH + 56]^+$ due to the alkenyl residue in the *sn*-1 position and $[RCO + 74]^+$ due to the acyl residue in the *sn*-2 position of alkenylacylglycerols, $[R + 130]^+$ due to the alkyl residue in the *sn*-1 position and $[RCO + 74]^+$ due to the acyl residue in the *sn*-2 position of alkylacylglycerols, $[RCO + 74]^+$ due to the acyl residues in the *sn*-1 and/or *sn*-2 positions of diacylglycerols, and $[M-57]^+$ being indicative of the corresponding molecular weight, were used for structural assignments.

For alkenylacyl EPL and CPL, 19 and 16 molecular species were determined, respectively. Two molecular species, 18:0alkenyl-22:6n-3 and 18:0-alkenyl-22:2-non-methylene interrupted diene (NMID), amounted to 53.2% and 47.9%, respectively. The alkylacyl EPL and CPL consisted of 16 and 20 molecular species, respectively, and the prominent components were 18:0alkyl-22:2NMID, 20:1alkyl-20:1n-11 (27.4%) and 20:1alkyl-20:2NMID (16.3%) in the former, and 16:0alkyl-20:5n-3 (23.0%) and 16:0alkyl-22:6n-3 (21.6%) in the latter. For the diacyl EPL and CPL, 14 and 51 molecular species were determined, respectively. The major molecular species were 18:0-20:5n-3 (37.4%), 16:0-20:5n-3 (14.2%) and 18:1n-7-22:2NMID (13.2%) in the former, and 16:0-20:5n-3 (33.4%) and 16:0-22:6n-3 (22.3%) in the latter. It was found that there were significant differences in the molecular species between the alkylacyl and diacyl EPL and the alkylacyl and diacyl CPL; the number of molecular species was larger in CPL than in EPL, while the number of total carbons and double bonds of the major molecular species were larger in the EPL than in the CPL. Alkenylacyl EPL were similar to alkenylacyl CPL in molecular species composition.

Lipids 25, 624-632 (1990).

Naturally occurring glycerophospholipids (GPL) consist of complex mixtures of molecular species including 1-*O*-alk-1′-enyl-2-acyl (alkenylacyl), 1-*O*-alkyl-2-acyl (alkylacyl) and 1,2-diacyl (diacyl) glycerol analogues. Since molecular species composition affects the function of cell membranes (1,2), it is important to obtain detailed information on the molecular species of GPL. Analyses of molecular species of GPL have been carried out successfully by several workers (3); Takahashi *et al.* (4-6) studied the molecular species of diacyl choline glycesophospholipids (CPL) in various fishes by analyzing their acetylated diacylglycerol derivatives using reversed-phase high-performance liquid chromatography (HPLC). The molecular species of diacylglycerols derived from diacyl ethanolamine glycerophospholipids (EPL) and CPL in cod and skipjack muscles were investigated by Ohshima *et al.* (7-9) in regard to fatty acid combinations. More recently, Bell (10) quantified the molecular species of diacyl EPL, CPL and inositol glycerophospholipids (IPL) from the ripe roes of cod *Gadus morhua* by reversed-phase HPLC. Myher and Kuksis (11) quantified molecular species of the diacyl CPL of egg yolk and rat liver and the alkenylacyl and the diacyl EPL of rat heart and kidney (12) by gas-liquid chromatography (GLC) on a polar capillary column. In these studies, the peak assignments were made by comparing the retention times with those of authentic standards. However, authentic standards are not available for the more complicated structures of naturally occurring lipids. Therefore, it is necessary to obtain additional information which reflects the structure of peak components separated by GLC and/or HPLC. In order to confirm the peak assignments in GLC, Ohshima *et al.* (13-15) successfully used selected-ion monitoring (SIM) mass spectrometry (MS) to analyze the molecular species of triacylglycerols of certain vegetable oils, and diacyl and alkenylacyl GPL in the muscle lipids of bonito *Euthynnus pelamis*. Although it is well known that alkenylacyl-, alkylacyl- and diacylglycerophosphoethanolamine and glycerophosphocholine are widely distributed in the organs of various marine organisms, limited information has been available concerning the molecular species of the phospholipids in marine invertebrates. Recently, we reported detailed compositions of fatty chains in the *sn*-1 and *sn*-2 positions of alkenylacyl, alkylacyl and diacyl glycerophospholipids of the Japanese oyster (16). Our data suggested that unusual molecular species were present because 22:2NMID (non-methylene interrupted diene) fatty acids were prominent while 18:1alkenyl chains were not found. In the present study, we determined the molecular species of alkenylacyl, alkylacyl and diacyl EPL and CPL in the total wet tissue of the Japanese oyster using gas chromatography (GC) on a polar open-tubular column coupled with selected-ion monitoring mass spectrometry (MS).

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Abbreviations: Alkenylacyl, 1-*O*-alk-1′-enyl-2-acyl -; alkylacyl, 1-*O*-alkyl-2-acyl-; CPL, choline glycerophospholipids; diacyl, 1,2-diacyl-; EPL, ethanolamine glycerophospholipids; FID, flame ionization detector; GC, gas chromatography; GC/MS, gas chromatography/mass spectrometry; GPL, glycerophospholipids; HPLC, high performance liquid chromatography; IPL, inositol glycerophospholipids; NMID, non-methylene interrupted diene; PUFA, polyunsaturated fatty acids; SIM, selected-ion monitoring; *tert*-BDMS, *tert*-butyldimethylsilyl; TLC, thin-layer chromatography.

MATERIALS AND METHODS

Samples. Japanese oysters *Crassostrea gigas*, cultured commercially in Hiroshima Bay, Japan, were harvested in March, 1988. The oysters were transported to the factory, where the total wet tissue was removed, washed, frozen with a spiral-freezer at −50°C, and ice-glazed by dipping into water for 1 min. The samples were put into a pouch of laminated ethylene-vinyl acetate copolymer (20 cm × 15 cm, 60 μm in thickness), and the pouch was heat-sealed. Samples were stored for one day at −20°C, transported to the laboratory on dry ice, and used immediately. Duplicate samples (groups 1 and 2, involving 10–11 specimens each) were used.

Lipid extraction and fractionation. Frozen samples were thawed, minced, and extracted with chloroform/methanol according to the Bligh and Dyer procedure (17). The ethanolamine and choline glycerophospholipid subclasses were separated from each other by column chromatography on silicic acid (2.5 cm i.d. × 50 cm; Silica Gel 60, 70–230 mesh; E. Merck, Darmstadt, Germany) according to the method of Hanahan *et al.* (18); the lipids were eluted by stepwise application of chloroform/methanol mixtures of different ratios (4:1, 3:2 and 1:4, v/v) as eluents.

Lipid derivatization. The alkenylacylglycerol, alkylacylglycerol and diacylglycerol *tert*-butyldimethylsilyl (*tert*-BDMS) ether derivatives were obtained from the EPL and CPL as described previously (14). Briefly, an aliquot of a chloroform solution of the EPL or CPL was subjected to hydrolysis with phospholipase C (from *Bacillus cereus*, Boehringer Mannheim, Germany). The resulting 1,2-diradyl-glycerols (19) were recovered by preparative thin-layer chromatography (TLC) on precoated Silica Gel G plates (0.25 mm in thickness, E. Merck) using a mixture of petroleum ether/diethyl ether/acetic acid (85:15:1, v/v/v) as developing solvent. The diradylglycerols extracted from the Silica bands were converted to *tert*-BDMS ether derivatives, by heating with *tert*-butyldimethylchlorosilane/imidazol reagent (Gaskuro Kogyo Inc., Tokyo, Japan) at 80°C for 1 hr (20). The alkenylacylglycerol, alkylacylglycerol and diacylglycerol *tert*-BDMS derivatives were separated by preparative TLC on Silica Gel G plate, using toluene as mobile phase (21).

GLC and SIM-GC/MS of alkenylacylglycerol, alkylacylglycerol and diacylglycerol tert*-BDMS ether derivatives.* Gas-liquid chromatography (GLC) of the alkenylacylglycerol, alkylacylglycerol and diacylglycerol *tert*-BDMS ethers was carried out on a Shimadzu GC 12A instrument (Shimadzu Seisakusho, Kyoto, Japan) equipped with a flame ionization detector (FID) and a Shimadzu movable solventless injector. A MP65HT (65% methylphenylsilicone) fused silica open-tubular column (0.25 mm i.d. × 25 m, Quadrex, New Haven, CT) was used. The injection port was kept at 370°C. The column oven temperature was held at 250°C for 1 min and then programmed to 350°C at a rate of 5°C/

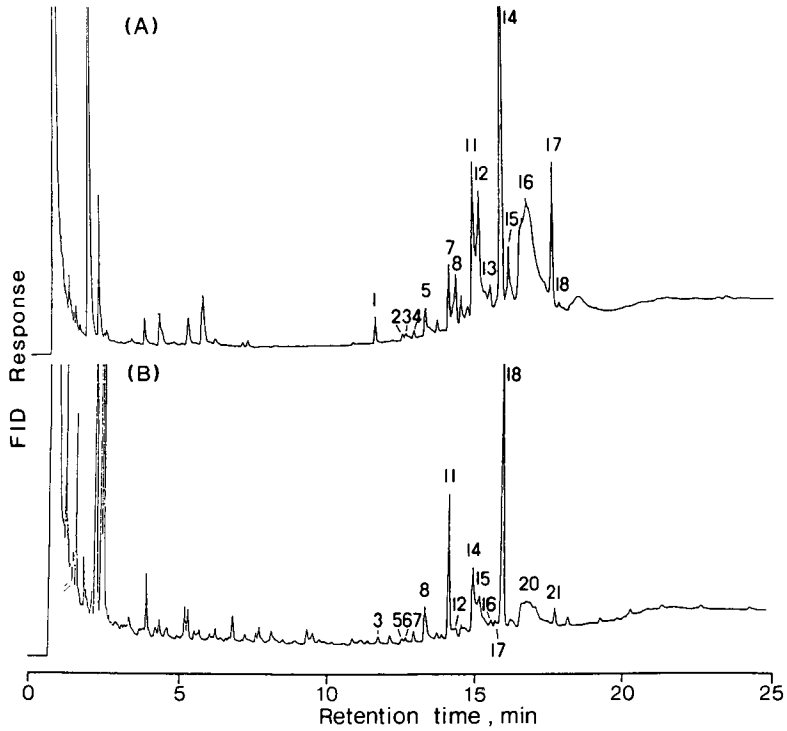

FIG. 1. FID gas liquid chromatograms of alkenylacylglycerol *tert*-BDMS ether derivatives from EPL (A) and CPL (B) of Japanese oyster. Molecular species assigned to the peak components are shown in Tables 1 and 2. Column: 65% methylphenylsilicone fused silica open-tubular capillary column, 25 m × 0.25 mm i.d.; column temp., 250°C to 350°C (program rate, 5°C/min); injection temp., 370°C; detector, FID; and carrier gas, He (2.0 Kg/cm²).

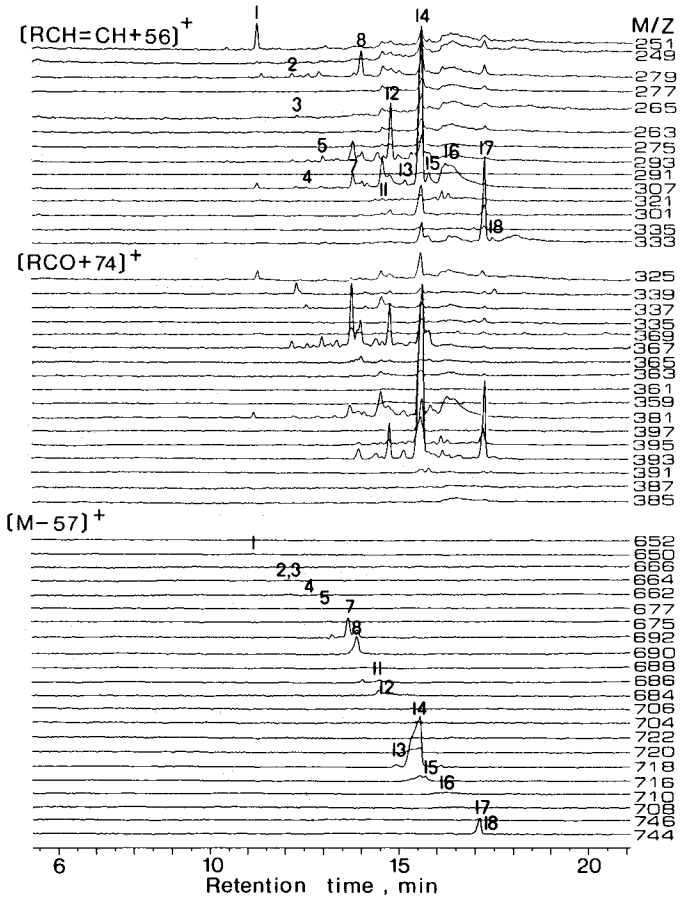

FIG. 2. Selected-ion monitoring profiles of alkenylacylglycerol *tert*-BDMS ether derivatives from EPL of Japanese oyster. GC: Column, 65% methylphenylsilicone fused silica open-tubular capillary column, 15 m × 0.25 mm i.d.; column temp., 250°C to 350°C (program rate, 5°C/min); injection temp., 370°C; and carrier gas, He (0.2 Kg/cm²). MS: EI mode; 70 eV ionization energy; 3 KV accelerating voltage; ion source temp., 310°C.

min. Helium was used as carrier gas at an inlet pressure of 2.0 Kg/cm². Usually, 1-2 μL of a 0.02% hexane solution of a sample was injected.

Selected-ion monitoring gas chromatography/mass spectrometry of the alkenylacylglycerol, alkylacylglycerol and diacylglycerol *tert*-BDMS ethers was performed on a Shimadzu QP1000 quadrupole mass spectrometer equipped with an electron impact ionization source to which the outlet of a MP65HT open-tubular column (0.25 mm i.d. × 15 m) was directly connected. The temperatures of the injection port and column oven were similar to those used for GLC as mentioned above, while the helium inlet pressure was 0.2 Kg/cm². The mass spectrometer was operated of 70eV ionization energy and 3KV accelerating voltage. The temperature of the ion source was set at 310°C.

Peak assignments of the alkenylacylglycerol, alkylacylglycerol and diacylglycerol *tert*-BDMS ethers were made based on the characteristic fragment ions as reported by Satouchi and Saito (20), i.e., $[RCH=CH + 56]^+$ for alkenyl chains, $[R + 130]^+$ for alkyl chains, and $[RCO + 74]^+$ for acyl chains. The molecular weight of the molecular species was assigned based on the ion $[M - 57]^+$.

RESULTS AND DISCUSSION

Molecular species compositions of alkenylacyl EPL and CPL. The alkenylacylglycerol *tert*-BDMS ether derivatives of EPL gave 19 prominent peaks in FID-GLC (Fig. 1). The molecular species of the alkenylacylglycerol *tert*-BDMS ethers were determined by monitoring the peaks of the characteristic fragment ions, i.e., $[RCH=CH + 56]^+$ for the alkenyl moiety, $[RCO + 74]^+$ for the acyl moiety, and $[M - 57]^+$ being indicative of the molecular weight at the same retention time on the SIM profile (13). A typical SIM profile is shown in Figure 2. Peak assignments were made as follows: when monitored at *m/z* 744, an ion peak appeared after a retention time of 17 min, indicating that the component has 42 carbons and 3 double bonds in the alkenyl and acyl moieties. Similarly, when monitored at *m/z* 333, an intense ion peak appeared at the same retention time as that of *m/z* 744. Since the *m/z* 333 is due to an $[RCH=CH + 56]^+$ ion, the alkenyl moiety of the component of peak No. 17 is a 20:1alkenyl chain. Subsequently, when monitored at *m/z* 393 for the $[RCO + 74]^+$ ion, an ion peak appeared at the same retention time. This showed that the acyl moiety of the component of peak No. 17 was 22:2NMID. Consequently, the peak No. 17 clearly corresponds to alkenylacyl EPL having 20:1alkenyl-22:2NMID as respective moieties. The elution orders of the peak components in FID-GLC should be the same as those in GC/MS, since open-tubular columns with the same liquid phase were used in both chromatographic separations. Therefore, those peaks having the same number marked on both chromatograms are ascribed to the same component.

The components of the other peaks shown in Figure 1A were analyzed for their alkenyl and acyl moieties in the same manner mentioned above, and the combinations are summarized in Table 1. Major molecular species were those consisting of 18:0alkenyl-22:6n-3 and 18:0alkenyl-22:2NMID, accounting for 31.6% and 21.6%, respectively. The molecular species of the alkenylacyl CPL, analyzed in the same manner, are shown in Table 2. Major molecular species of alkenylacyl CPL were the same as those of the EPL homologues—18:0alkenyl-22:6n-3 and 18:0alkenyl-22:2NMID.

Ohshima *et al.* (15) studied the molecular species compositions of the glycerophospholipids in the bonito white muscle, and reported that the molecular species consisting of 16:0alkenyl-22:6n-3 accounted for 57% of the total alkenylacyl glycerophospholipids. The molecular species compositions of the alkenylacyl EPL of the oyster analyzed in the present study were markedly different from those of the bonito white muscle; the number of total alkenyl and acyl carbons of the alkenylacyl EPL of the oyster was larger and the degree of unsaturation in the alkenylacyl moiety was higher.

Molecular species of alkylacyl EPL and CPL. The FID gas chromatograms of the alkylacylglycerol *tert*-BDMS ether derivatives of the alkylacyl EPL and CPL are shown in Figures 3A and 3B, respectively. The characteristic ions, $[R + 130]^+$, $[RCO + 74]^+$ and $[M - 57]^+$ were selected for assignment of the peak components on the gas chromatograms. The results ob-

TABLE 1

Molecular Species Compositions of Alkenylacyl Ethanolamine Glycerophospholipids of Japanese Oyster

Peak No.	TC:DB[a]	Alkenyl	—	Acyl	Area %[b]
1	35:1	18:0alkenyl	—	17:1n-8	0.7 ± 0.0
2	36:1	16:0alkenyl	—	20:1n-11	0.2 ± 0.0
3	36:1	18:0alkenyl	—	18:1n-9	0.2 ± 0.0
4	36:2	18:0alkenyl	—	18:2n-6	0.2 ± 0.0
5	37:1	17:0alkenyl	—	20:1n-11	1.2 ± 0.3
7	38:1	18:0alkenyl	—	20:1n-11	2.0 ± 0.1
8	38:2	16:0alkenyl	—	22:2NMID[c]	2.7 ± 0.2
	38:2	18:0alkenyl	—	20:2NMID[d]	
11	38:3	20:1alkenyl	—	18:2n-6	7.4 ± 0.3
12	38:5	18:0alkenyl	—	20:5n-3[c]	8.2 ± 0.3
	39:2	17:0alkenyl	—	22:2NMID[e]	
13	40:2	18:0alkenyl	—	22:2NMID[c]	21.6 ± 0.3
14	40:2	20:1alkenyl	—	20:1n-11	
15	40:3	18:0alkenyl	—	22:3	5.7 ± 0.3
16	40:6	18:0alkenyl	—	22:6n-3[c]	
	40:6	20:1alkenyl	—	20:5n-3	31.6 ± 0.1
	41:2	19:0alkenyl	—	22:2NMID	
17	42:3	20:1alkenyl	—	22:2NMID	6.3 ± 0.1
18	42:3	20:1alkenyl	—	22:2NMID	1.2 ± 0.1
Others[f]					10.7

[a]TC, the number of total carbons; DB, the number of double bonds.
[b]The data are presented as the mean ± standard deviation of six (two groups × three) determinations.
[c]Represents the main component.
[d,e]Each of the NMIDs has two isomers.
[f]Sum of the unknown peak components.

TABLE 2

Molecular Species Compositions of Alkenylacyl Choline Glycerophospholipids of Japanese Oyster

Peak No.	TC:DB[a]	Alkenyl	—	Acyl	Area %[b]
3	35:1	17:0alkenyl	—	18:1n-9	1.1 ± 0.0
5	36:1	16:0alkenyl	—	20:1n-11	1.4 ± 0.1
6	36:1	18:0alkenyl	—	18:1n-9[c]	
7	36:2	18:0alkenyl	—	18:2n-6	1.3 ± 0.1
8	37:2	17:0alkenyl	—	20:2NMID[d]	5.4 ± 0.1
11	38:2	18:0alkenyl	—	20:2NMID[c]	8.4 ± 0.4
12	38:2	16:0alkenyl	—	22:2NMID[e]	
14	38:5	18:0alkenyl	—	20:5n-3[c]	22.5 ± 0.5
15	39:2	17:0alkenyl	—	22:2NMID	
16	40:2	18:0alkenyl	—	22:2NMID[c]	
17	40:2	18:0alkenyl	—	22:2NMID	22.7 ± 0.7
18	40:2	20:1alkenyl	—	20:1n-11	
20	40:6	18:0alkenyl	—	22:6n-3[c]	
	40:6	20:1alkenyl	—	20:5n-3	25.2 ± 0.9
	41:2	19:0alkenyl	—	22:2NMID	
21	42:3	20:1alkenyl	—	22:2NMID	2.5 ± 0.3
Others[f]					9.5

[a]TC, the number of total carbons; DB, the number of double bonds.
[b]The data are presented as the mean ± standard deviation of six (two groups × three) determinations.
[c]Represents the main component.
[d,e]Each of the NMIDs has two isomers.
[f]Sum of the unknown peak components.

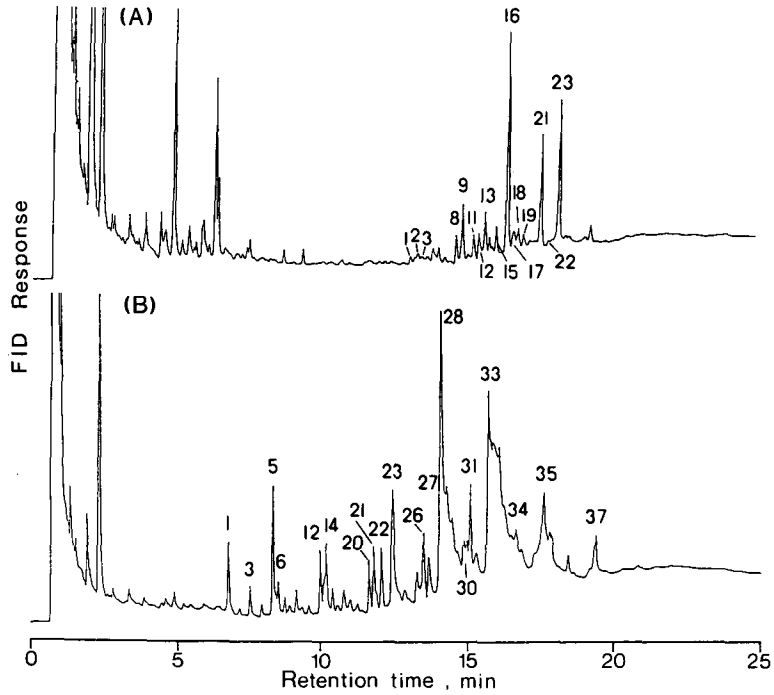

FIG. 3. FID gas liquid chromatograms of alkylacylglycerol *tert*-BDMS ether derivatives from EPL (A) and CPL (B) of Japanese oyster. Molecular species assigned to the peak components are shown in Table 3 and 4. Conditions are the same as in Figure 1.

TABLE 3

Molecular Species Compositions of Alkylacyl Ethanolamine Glycerophospholipids of Japanese Oyster

Peak No.	TC:DB[a]	Alkyl	—	Acyl	Area %[b]
1	36:0	18:0alkyl	—	18:0	0.8 ± 0.1
2	36:1	18:0alkyl	—	18:1n-9	1.1 ± 0.3
3	36:2	16:0alkyl	—	18:2n-6[c]	0.9 ± 0.3
	36:2	14:0alkyl	—	22:2NMID	
8	38:1	18:0alkyl	—	20:1n-11	3.0 ± 0.3
9	38:2	16:0alkyl	—	22:2NMID[d]	7.0 ± 0.3
11	38:5	18:0alkyl	—	20:5n-3	3.1 ± 0.4
12	38:6	16:0alkyl	—	22:6n-3[c]	8.9 ± 0.2
	39:2	17:0alkyl	—	22:2NMID	
15	40:2	18:0alkyl	—	22:2NMID[c]	
16	40:2	20:1alkyl	—	20:1n-11	27.4 ± 0.4
	40:2	18:0alkyl	—	22:2NMID	
17	40:3	18:1alkyl	—	22:2NMID	6.4 ± 0.5
18	40:3	18:1alkyl	—	22:2NMID	
19	40:6	18:0alkyl	—	22:6n-3	1.9 ± 0.8
21	41:2				9.9 ± 1.2
23	42:3	20:1alkyl		22:2NMID	16.3 ± 0.5
Others[e]					13.3

[a]TC, the number of total carbons; DB, the number of double bonds.
[b]The data are presented as the mean ± standard deviation of six (two groups × three) determinations.
[c]Represents the main component.
[d]Each of the NMIDs has two isomers.
[e]Sum of the unknown peak components.

TABLE 4

Molecular Species Compositions of Alkylacyl Choline Glycerophospholipids of Japanese Oyster

Peak No.	TC:DB[a]	Alkyl	—	Acyl	Area %[b]
1	28:0	14:0alkyl	—	14:0	2.2 ± 0.0
3	29:0	14:0alkyl	—	15:0	0.9 ± 0.1
5	30:0	14:0alkyl	—	16:0	4.9 ± 0.1
6	30:1	14:0alkyl	—	16:1n-7	1.1 ± 0.1
12	32:0	16:0alkyl	—	16:0	2.1 ± 0.1
14	32:1	16:0alkyl	—	16:1n-7	3.4 ± 0.1
20	34:0	18:0alkyl	—	16:0[c]	1.0 ± 0.0
	34:0	17:0alkyl	—	17:0	
21	34:1	16:0alkyl	—	18:1n-9	1.7 ± 0.0
22	34:2	16:0alkyl	—	18:2n-6	2.0 ± 0.1
23	34:4	14:0alkyl	—	20:4n-6	5.0 ± 0.3
26	36:2	14:0alkyl	—	22:2NMID[d]	2.6 ± 0.2
27	36:3	16:0alkyl	—	20:3	2.0 ± 0.1
28	36:5	16:0alkyl	—	20:5n-3	23.0 ± 0.6
30	38:1	18:0alkyl	—	20:1n-11	1.3 ± 0.2
31	38:2	16:0alkyl	—	22:2NMID	3.2 ± 0.2
33	38:6	16:0alkyl	—	22:6n-3	21.6 ± 0.6
34	40:2	18:0alkyl	—	22:2NMID	2.2 ± 0.4
35	40:6	18:0alkyl	—	22:6n-3	5.9 ± 1.0
37	42:3	20:1alkyl	—	22:2NMID	1.3 ± 0.5
Others[e]					12.6

[a]TC, the number of total carbons; DB, the number of double bonds.
[b]The data are presented as the mean ± standard deviation of six (two groups × three) determinations.
[c]Represents the main component.
[d]Each of the NMIDs has two isomers.
[e]Sum of the unknown peak components.

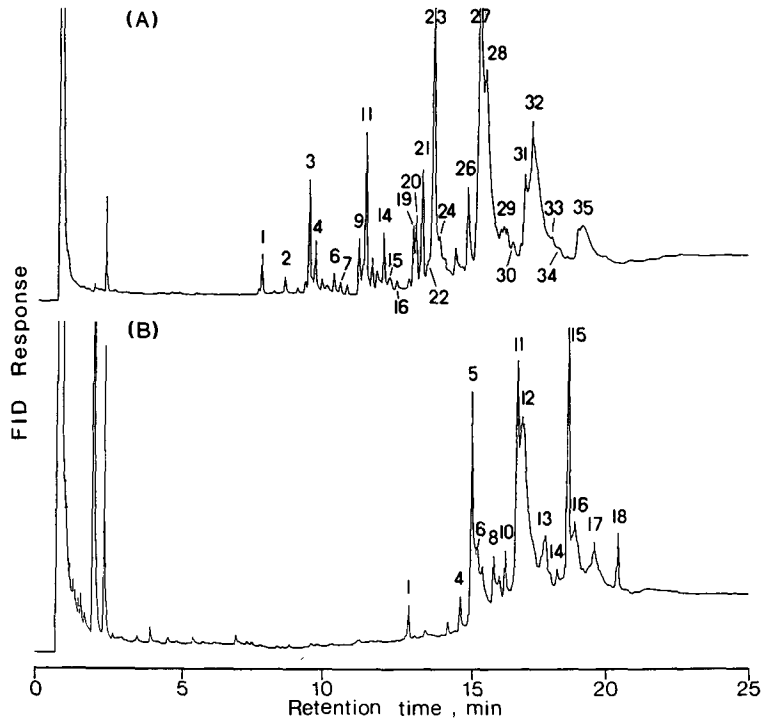

FIG. 4. The FID gas liquid chromatograms of diacylglycerol *tert*-BDMS ether derivatives from CPL (A) and EPL (B) of Japanese oyster. Molecular species assigned to the peak components are shown in Tables 5 and 6. Conditions are the same as in Figure 1.

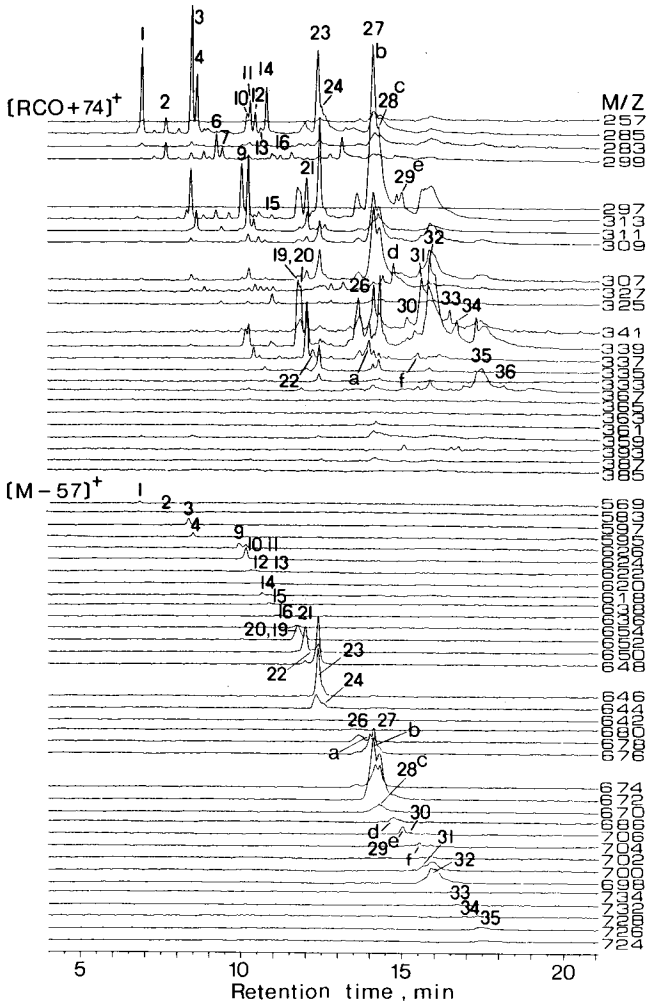

FIG. 5. Selected-ion monitoring profiles of diacylglycerol *tert*-BDMS ether derivatives from CPL of Japanese oyster. Peaks a to f are as in Table 5. GC/MS conditions are the same as in Figure 2.

tained are summarized in Tables 3 and 4. As shown in Table 3, major molecular species of the alkylacyl EPL were 20:1alkyl-20:1n-11 and 18:0alkyl-22:2NMID (27.4%), 20:1alkyl-22:2NMID (16.3%) and 16:0alkyl-22:6n-3 (8.9%). The major molecular species of alkylacyl CPL (Table 4) were those having 16:0alkyl-20:5n-3 and 16:0alkyl-22:6n-3, accounting for 23.0% and 21.6%, respectively. The number of molecular species was larger in alkylacyl CPL (20 molecular species) than in alkylacyl-EPL (16 molecular species). The composition of the molecular species showed marked differences in the alkylacyl moiety; the alkylacyl CPL were mainly composed of shorter carbon chains than in the alkylacyl EPL.

Data obtained in this study indicate that alkenylacyl EPL and alkylacyl CPL are good sources of n-3 fatty acids, such as 20:5n-3 and 22:6n-3. Similar locations of n-3 fatty acids in ether phospholipids were found in bonito (22), rainbow trout (23) and certain marine fishes (5,6). In contrast, alkenylacyl EPL (24–29) and alkylacyl CPL (30) of mammalian molecular species contain large proportions of n-6 fatty acids such as 20:4n-6. It is clear from a comparison with mammalian molecular species distributions that the ether phospholipids of oyster are markedly different. No detailed information on the molecular species of alkylacyl EPL and CPL of marine invertebrates has been published previously.

Molecular species compositions of diacyl CPL and EPL. The FID gas chromatograms of the diacylglycerol *tert*-BDMS ether derivatives of the diacyl-CPL and EPL are shown in Figures 4A and 4B, respectively. A SIM-profile of the *tert*-BDMS ethers of diacyl CPL is shown in Figure 5. Fifty-one molecular species were identified by monitoring the selected fragment ions $[RCO + 74]^+$ and $[M - 57]^+$ at the same retention time on the SIM-profile. Major molecular species of the diacyl CPL were those consisting of 16:0-20:5n-3 (33.4%) and 18:1n-7-20:5n-3 (22.3%), as shown in Table 5.

The analyses of molecular species of the diacyl EPL were done in the same manner, and the results are summarized in Table 6. Major molecular species of diacyl EPL were those having 18:0-20:5n-3 (37.4%), 16:0-20:5n-3 (14.2%) and 18:1n-7-20:2NMID (13.2%). Consequently, the number of total carbons in the acyl moieties was larger in the molecular species of diacyl EPL than in those of the diacyl CPL. The number of molecular species detected in the diacyl CPL was almost three times that in diacyl EPL.

Takahashi *et al.* (6) studied the molecular species of diacyl CPL from muscle of several species of fishes and demonstrated that the most prominent molecular species of diacyl CPL were those having 16:0-22:6. The molecular species of 16:0-20:5 was prominent in the cases of brown sole *Limanda ferruginea* and sand flounder *Linanda punctatissima* muscle. The molecular species constituted from polyunsaturated fatty acids (PUFA) alone such as 22:6-22:6, 20:5-22:6 and 20:5-20:5, occurred in significant amounts in these fishes. Ohshima *et al.* (7–9) established that the most prominent molecular species of the diacyl CPL of cod *Gadus morhua* and skipjack *Euthynnus pelamis* muscles was that consisting of 16:0-22:6, but that the molecular species consisting of 18:0-22:6 was the most abundant in diacyl EPL of cod *Gadus morhua.* Recently, it was reported that the most prominent molecular species of the diacylglycerophospholipids in the bonito white muscle was 16:0-22:6n-3. The molecular species consisting of PUFA alone, such as 22:6n-3-22:6n-3 and 20:5n-3-22:6n-3, were also present in large quantities in the diacylglycerophospholipids (14). The sum of the major molecular species, 16:0-20:5, 18:1-20:5, 16:0-22:6 and 18:1-22:6, amounted to 61.8% and 67.2% of EPL and CPL, respectively, in the ripe roes of cod (10). Small amounts of the molecular species having PUFAs at the *sn*-1 as well as the *sn*-2 position were found in cod roe. In the present study, however, no molecular species having PUFA alone in the acyl moieties were detected in the glycerophospholipids of Japanese oyster. From our results, it is clear that the diacyl-EPL in the Japanese oyster contained larger proportions of molecular species having higher carbon numbers than those of the diacyl CPL and that the number of the molecular species in diacyl CPL is larger than that in diacyl EPL.

TABLE 5

Molecular Species Compositions of Diacyl Choline Glycerophospholipids of Japanese Oyster

Peak No.	TC:DB[a]	Acyl	—	Acyl	Area %[b]
1	28:0	14:0	—	14:0	0.5 ± 0.1
2	29:0	14:0	—	15:0	0.2 ± 0.0
3	30:0	14:0	—	16:0	1.9 ± 0.3
4	30:1	14:0	—	16:1n-7	0.8 ± 0.2
6	31:0	15:0	—	16:0	0.4 ± 0.1
7	31:1	15:0	—	16:1n-7	0.3 ± 0.0
9	32:0	16:0	—	16:0	1.0 ± 0.3
10	32:1	16:0	—	16:1n-7[c]	3.4 ± 0.4
11	32:1	14:0	—	18:1n-9	
	32:1	14:0	—	18:1n-7	
12	32:2	14:0	—	18:2n-6	0.6 ± 0.1
13	32:2	14:0	—	18:2n-4	0.5 ± 0.1
14	32:3	14:0	—	18:3[c]	0.9 ± 0.2
	32:4	14:0	—	18:4n-3	
15	33:1	15:0	—	18:1n-7	0.4 ± 0.0
	33:1	16:0	—	17:1n-8[c]	
16	33:2	15:0	—	18:2n-6	0.2 ± 0.0
19	34:1	16:0	—	18:1n-9[c]	2.4 ± 0.1
20	34:1	16:0	—	18:1n-7	
21	34:2	16:1n-7	—	18:1n-7	3.3 ± 0.2
	34:2	16:0	—	18:2n-6[c]	
22	34:2	16:0	—	18:2n-4	
23	34:3	16:0	—	18:3	9.9 ± 0.1
	34:4	16:0	—	18:4n-3	
	34:4	14:0	—	20:4n-6	
	34:5	14:0	—	20:5n-3[c]	
24	34:5	16:1n-7	—	18:4n-3	
26	36:2	18:1	—	18:1n-7[c]	3.2 ± 0.1
	36:2	18:0	—	18:2n-6	
27	36:3[a,b]	18:1n-9	—	18:2n-4	33.4 ± 0.6
	36:4[b,c]	16:0	—	20:4n-6	
	36:4[b,c]	18:0	—	18:4n-3	
	36:4[b,c]	18:1n-7	—	18:3	
	36:5[b,c]	16:0	—	20:5n-3[c]	
28	36:6[c]	14:0	—	22:6n-3	
	36:6[c]	14:1	—	22:5n-3	
29	37:5[d]	17:0	—	22:5n-3[c]	1.7 ± 0.1
	38:2[e]	16:0	—	22:2NMID[d]	
30	38:2	18:1n-7	—	20:1n-7	1.5 ± 0.3
31	38:3[f]	18:2n-6	—	20:1n-7	22.3 ± 1.1
	38:4	16:2	—	22:2NMID	
	38:5	18:0	—	20:5n-3	
32	38:6	18:1n-7	—	20:5n-3	
	38:6	16:0	—	22:6n-3[c]	
	38:7	16:1n-7	—	22:6n-3	
33	40:2	18:0	—	22:2NMID	1.5 ± 0.3
34	40:3	18:1n-7	—	22:2NMID	0.8 ± 0.2
35	40:6	18:1n-7	—	22:5n-3[c]	6.8 ± 1.3
	40:6	20:1n-7	—	20:5n-3	
	40:7	18:1n-7	—	22:6n-3	
36	42:3	20:1n-7	—	22:2NMID	0.5 ± 0.3
Others[e]					1.6

[a]TC, the number of total carbons; DB, the number of double bonds.
[b]The data are presented as the mean ± standard deviation of six (two groups × three) determinations.
[c]Represents the main component.
[d]Each of the NMIDs has two isomers.
[e]Sum of the unknown peak components.

The alkenylacyl EPL and CPL were rich in the molecular species having long acyl carbon chains, compared to the alkylacyl and diacyl EPL, and CPL subclasses, although the number of molecular species of alkenylacyl EPL and CPL were the smallest of all subclasses of EPL and CPL. Twenty and 51 molecular species were found in the alkylacyl and diacyl CPL, respectively. These numbers are relatively large when compared with those of the corresponding EPL subclasses. The alkenylacyl EPL and CPL in oyster were

TABLE 6

Molecular Species Compositions of Diacyl Ethanolamine Glycerophospholipids of Japanese Oyster

Peak No.	TC:DB[a]	Acyl	—	Acyl	Area %[b]
1	34:1	16:0	—	18:1n-7	0.8 ± 0.1
4	36:4	16:0	—	20:4n-6	1.3 ± 0.2
5	36:5	16:0	—	20:5n-3[c]	14.2 ± 0.7
6	36:5	16:1n-7	—	20:4n-6	
8	37:5	17:0	—	20:5n-3	3.8 ± 0.1
10	38:4	18:0	—	20:4n-6	3.4 ± 0.1
11	38:5	18:0	—	20:5n-3[c]	37.4 ± 0.8
12	38:6	18:1n-7	—	20:5n-3	
13	40:2	18:0	—	22:2NMID[d]	5.7 ± 0.2
14	40:3	18:1n-7	—	22:2NMID[c]	13.2 ± 0.4
15	40:3	20:1n-7	—	20:2NMID	
16	40:6	20:1n-9	—	20:5n-3	7.2 ± 0.4
17	40:7	18:1n-7	—	22:6n-3	4.3 ± 1.6
18	42:4	20:2NMID	—	22:2NMID	1.7 ± 0.5
Others[e]					7.0

[a]TC, the number of total carbons; DB, the number of double bonds.
[b]The data are presented as the mean ± standard deviation of six (two groups × three) determinations.
[c]Represents the main component.
[d]Each of the NMIDs has two isomers.
[e]Sum of the unknown peak components.

richer in molecular species having higher acyl carbon numbers of NMIDs than those mostly having methylene-interrupted dienes in bonito. Molecular species containing PUFA only were not found in Japanese oyster diacyl CPL and EPL, an important difference when compared to bonito white muscle. There may be evolutionary or merely functional differences accounting for this difference.

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Effects of Free Fatty Acids on the Binding of Steroid Hormones to Bovine Serum Albumin

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The effects of binding of free fatty acids (FFA) to bovine serum albumin (BSA) on steroid hormone binding to BSA were examined. The FFA studied included myristic, palmitic, stearic, oleic and linoleic acids. The binding coefficient K was estimated from the changes in the equilibrium partition coefficient between the aqueous and the hexane phase caused by the addition to BSA to the aqueous phase. A noticeable effect of FFA binding (molar ratio FFA/BSA, 2:1) on the affinities of α-estradiol, ethynylestradiol and dehydroisoandrosterone to BSA was not observed: however, the affinities of progesterone, androsterone and testosterone were distinctly enhanced by FFA binding. Furthermore, the elution profiles of gel filtration chromatography clearly showed that progesterone and testosterone are easily liberated from the hormone/BSA complexes and that stronger binding of these hormones to BSA is caused by binding of FFA to BSA. The affinity of ethynylestradiol to BSA is stronger than that of progesterone and testosterone and is not affected by palmitic acid binding to BSA.
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Albumin and lipoprotein in serum combine to form hydrophilic lipoprotein complexes, which play an important role in the transport and metabolism of hydrophobic lipids, such as triglycerides, cholesterol and cholesterol esters (1). Steroid hormones in serum exist and are transported not only in the free (unbound) form, but also bound to β-globulin and albumin (2). This is of physiological significance, because albumin or globulin by itself cannot permeate cell membranes. It has been assumed that the concentration of the free substance determines the uptake rate. Recently, the albumin-mediated hepatic uptake of free fatty acids (3), bile acids (4) and rose bengal (5) has been reported, but the kinetics are not sufficiently clear. Albumin in serum exists in a form to which free fatty acids (FFA) (6) and many other substances are bound. It was reported that crystalline serum albumin contains 2–3 moles of FFA per mole and that the affinity of steroid hormone to albumin is increased by the removal of FFA (7). The effect of FFA binding on affinities of bovine serum albumin (BSA) for steroid hormones was investigated in detail in the present study.

EXPERIMENTAL

Chemicals. Androsterone, dehydroisoandrosterone and testosterone were purchased from Wako Pure Chemicals Ind. Ltd. (Osaka, Japan); ethynylestradiol and progesterone were from Sigma Chemical Co. (St. Louis, MO); α-estradiol was from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan). BSA was from Sigma (A-8022, purity 96–99%), and so were myristic acid (14:0), palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1) and linoleic acid (18:2).

Measurements of steroid hormone, BSA and FFA. Steroid hormones in the samples were measured by high-performance liquid chromatography (HPLC) (Shimadzu Ltd., Kyoto, Japan, LC-4A) and quantified with ultraviolet (UV) photometric and fluorescence detectors. α-Estradiol and ethynylestradiol were measured by the fluorescence (Ex 285 nm, Em 305 nm), progesterone and testosterone were measured by UV at 240 nm, and androsterone and dehydroisoandrosterone were measured by UV at 295 nm. HPLC was done with a column of LiChrospher 100 RP-18 (4 × 250 mm). The column was eluted with a mobile phase composed of acetronitrile/water (65:35, v/v) at a flow rate of 0.8 mL/min. BSA concentrations were measured by absorbance at 280 nm with a UV photometer (Nihon Bunkō Ltd., Tokyo, Japan, UVIDEC-650). Free fatty acids (FFA) were methylated with diazomethane and quantified by gas chromatography (Shimadzu Ltd., GC-14A).

Procedures. BSA obtained from commercial sources contains varying amounts of bound FFA. It has been reported that bound FFA per mole of BSA can range from less than 0.1 mole to more than 2 moles (6); the maximum amount of bound FFA can be as high as 6–13 moles per mole of BSA (8). The FFA bound to BSA used in this experiment was 0.01–0.04 mole per mole. Myristic acid, palmitic acid, stearic acid, oleic acid and linoleic acid are the main free fatty acids bound to human albumin *in vivo* (9,10) and were also detected as the FFA bound to BSA (11). These fatty acids were chosen for the present experiments. In man, the FFA/albumin molar ratio rarely exceeds 4 and, in most cases, it is between 0.5 and 2.0 (12,13). Thus, BSA with 0.2 or 2 moles FFA per mole was tested.

The experiments on the effect of FFA binding on the affinities of BSA for steroid hormones were carried out as follows: 50 mL of a 3.5% BSA (2.65×10^{-5} M) solution dissolved in a 1/15 M phosphate-buffer solution (0.012 M KH_2PO_4 and 0.055 M Na_2HPO_4; pH adjusted to 7.4) was placed into a 100-mL Erlenmeyer flask with a stopper, and 0.2 mL of an FFA solution dissolved in methanol at a concentration of 2.65×10^{-4} or 2.65×10^{-5} M/mL (molar ratio of FFA to BSA was 2 or 0.2) was added. The mixture was stirred vigorously for 6 hr. FFA added to the solution was at first suspended in the aqueous phase but dissolved gradually in the presence of BSA. Then 10 mL of steroid hormone dissolved in hexane (200 μg/mL; except in the cases of α-estradiol, 5.9 μg/mL, and ethynylestradiol, 32.3 μg/mL), was added to the solution, and the aque-

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Abbreviations: BSA, bovine serum albumin; FFA, free fatty acids; HPLC, high-performance liquid chromatography; Fr, fraction.

ous phase was stirred at 25°C. After 15 hr, an aliquot of the hexane phase was withdrawn and analyzed. The sample was dried, and the residue was dissolved in acetonitrile to measure the hormone concentration by HPLC. It was confirmed by preliminary testing that the partition of the hormones between the aqueous and the hexane phase reaches equilibrium within 15 hr.

Separately, similar measurements were made with a phosphate-buffer solution containing no BSA, and a calibration curve for the hormone concentration in the hexane phase versus that in the aqueous solution was prepared. By using the calibration curve, the amount of hormone bound to BSA in the aqueous phase was estimated from that measured in the presence of BSA.

RESULTS AND DISCUSSION

Effect of FFA on affinities of steroid hormones to BSA. In the present investigation, the binding of hormone to BSA was not directly measured but was estimated by partition experiments. A hexane solution containing a definite amount of hormone was added to a phosphate-buffer solution with or without albumin. After transfer of hormone from the hexane to the aqueous phase, the partition equilibrium for the hormone between both solution phases was established. The amount of hormone remaining in the hexane phase was measured and the amount of hormone in the aqueous phase was calculated. In the presence of BSA, the total concentration of hormone in the aqueous phase increased when compared to that in the absence of BSA, owing to the binding of hormone to BSA.

The hormone exists in both the free form and the BSA-bound form. If the partition coefficient of free hormone between both the phases is not affected by the presence of BSA, the amount of hormone in the free form and the BSA-bound form can be obtained from these partition experiments. The binding coefficient K is defined as the concentration ratio of BSA-bound form versus free form of the hormone in the aqueous phase,

$$K=[S]_b/[S]_f \quad [1]$$

where $[S]_b$ and $[S]_f$ are the concentrations of hormones of the BSA-bound form and the free form in the water phase, respectively. When the hormone concentrations in the hexane and the water phase (a phosphate-buffer solution containing no BSA) are denoted by $[S]_h^0$ and $[S]_w^0$, respectively, and the hormone concentrations in the hexane and the aqueous phase containing BSA are denoted by $[S]_h$ and $[S]_w$, respectively, the partition coefficients of the hormone between the hexane and the water phase in the absence and the presence of BSA are represented by

$$P^0=[S]_w^0/[S]_h^0 \quad [2]$$

$$P=[S]_w/[S]_h \quad [3]$$

respectively. The total concentration of steroid hormone in the aqueous phase is the sum of those of the BSA-bound form and the free form:

$$[S]_w=[S]_b+[S]_f \quad [4]$$

The partition coefficient of the hormone between the two phases in the absence of BSA is not affected by the concentration of hormone under the present experimental conditions and, therefore, the relationship

$$[S]_f/[S]_h=P^0 \quad [5]$$

holds. The binding coefficient K is then determined from the equation:

$$K=(P/P^0)-1 \quad [6]$$

Table 1 shows the experimental results of the hormone concentration in the hexane phase when equilibrium partition is attained between the hexane and the aqueous phase in the presence of BSA containing 0.2 or 2 moles per mole of free fatty acid. The FFA used in the experiments were not detected in the hexane phase, and the FFA in the aqueous phase are supposed to exist not in the free form but in the BSA-bound form, because the FFA are only slightly soluble in aqueous medium. As is shown in Table 1, the concentration in the hexane phase is markedly lowered for some hormones compared to the control (3.5% BSA

TABLE 1

Binding of Steroid Hormones to FFA-Free BSA and FFA/BSA Complexes

	Initial hormone concentration in hexane (μg/mL)	Remaining amount in hexane phase[a] (%)									
		BSA solution (FFA free)	Myristic		Palmitic		Stearic		Oleic		Linoleic	
			0.2[b]	2[b]	0.2	2	0.2	2	0.2	2	0.2	2
α-Estradiol	5.9	0.35	0.34	0.33	0.35	0.33	0.34	0.33	—	—	—	—
Ethynylestradiol	32.3	0.30	0.30	0.29	0.30	0.29	0.30	0.30	—	—	—	—
Progesterone	200	74	73	63	72	64	73	62	73	60	73	60
Androsterone	200	28	28	23	26	21	27	23	27	20	27	22
Dehydroisoandrosterone	200	10	10	10	9.9	9.5	10	9.5	10	9.5	10	9.5
Testosterone	200	6.2	5.9	4.2	5.7	4.3	5.5	3.5	5.8	3.7	5.5	3.8

[a]Remaining amounts in the hexane phase at partition equilibrium between 50 mL of a 3.5% BSA solution and 10 mL of hexane.
[b]Moles of bound-FFA per mole of BSA.

TABLE 2

Partition Coefficient and Binding Coefficient of Steroid Hormones in the Presence of FFA (Palmitic Acid)

	Partition coefficient[a] $P=[S]_w/[S]_h$				Binding coefficient[b] $K=[S]_b/[S]_f$		
		Moles of bound FFA[c] per mole of BSA			Moles of bound FFA[c] per mole of BSA		
	Phosphate buffer (without BSA)	0	0.2	2	0	0.2	2
α-Estradiol	0.91	56.9	56.9	60.4	61.5	61.5	65.4
Ethynylestradiol	0.51	66.5	66.5	68.8	129	129	134
Progesterone	0.0041	0.070	0.078	0.11	16.1	18.0	25.8
Androsterone	0.017	0.51	0.57	0.75	29.0	32.5	43.1
Dehydroisoandrosterone	0.050	1.8	1.8	1.9	35.0	35.0	37.0
Testosterone	0.27	3.0	3.3	4.5	10.1	11.2	15.7

[a]Partition coefficients ($P=[S]_w/[S]_h$) were measured between 50 mL of a 3.5% BSA solution and 10 mL of hexane; $[S]_w$, total concentrations of hormone in the water phase; $[S]_h$, concentrations in the hexane phase.
[b]Binding coefficients ($K=[S]_b/[S]_f$) were calculated by using equation 6; $[S]_b$, concentrations of BSA-bound form in the water phase; $[S]_f$, concentrations of free form in the water phase.
[c]Values shown for palmitic acid as FFA.

solution containing no FFA) in the case of BSA containing of 2 moles per mole of FFA.

The concentration in the hexane phase was lowered by ca. 16% for progesterone, 23% for androsterone, and 37% for testosterone. On the other hand, the effect of FFA binding on the affinity of BSA for hormones was hardly observed for α-estradiol, ethynylestradiol and dehydroisoandrosterone. The partition coefficients P of the hormones between the hexane and the aqueous phase were determined and the binding coefficients K were estimated according to equation 6. The results for palmitic acid are shown in Table 2. The binding coefficients of progesterone, androsterone and testosterone increased by ca. 10% in the case of 0.2 mole of FFA binding, and by 50–60% for 2 moles of FFA binding. A detectable difference in the hormone concentration was not observed upon addition of FFA in the partition experiment in the absence of BSA.

Moreover, it was confirmed that the equilibrium concentration of progesterone and testosterone in the hexane phase, in the case where FFA was added initially, was almost at the same level as that in the case where FFA was added after equilibrium partition of the hormone between the two phases had occurred. This result shows that the partition of hormone between the hexane and the aqueous phase, and therefore the binding of hormone to BSA, reaches the same equilibrium regardless of the operational procedures. The reproducibility of the results is high.

Analysis of the FFA-BSA-steroid hormone complexes. A gel filtration chromatography method was used to obtain more detailed information about the type of binding of hormones to BSA and the effect of FFA on the affinity of hormone binding to BSA. The total amounts of FFA and steroid hormone in both the free and BSA-bound forms in the aqueous solution were analyzed as follows. FFA was extracted with a mixture of isopropyl alcohol/isooctane/1N H_2SO_4 (40:10:1, v/v/v) according to the method of Chen (6) and measured by gas chromatography after methylation. The hormone was extracted into benzene and the extract was dried and dissolved in acetonitrile for HPLC analysis. The results of these measurements are summarized in Table 3. Recoveries for progesterone, testosterone, ethynylestradiol and FFA were 90–95% of the amounts initially added to the system.

An aliquot (1 mL) of the aqueous phase at equilibrium partition between the aqueous and the hexane phase was loaded on a Sephadex G-50 column (15 mm × 250 mm) and eluted with 1/15 M phosphate-buffer solution to determine the binding form. The results for progesterone, testosterone and ethynylestradiol are shown in Figures 1, 2 and 3, respectively. The contents of BSA, FFA and hormones were assayed on every fraction (2.1 mL each). The void volume was at Fraction (Fr) No. 4 under our experimental conditions.

As shown in Figure 1, in the absence of BSA, the amount of progesterone transferred into the aqueous phase was small, and progesterone in free form elutes in Fr 19–25 in gel filtration. When BSA is added to the solution, progesterone elutes in a broad range (Fr 4–25), while BSA elutes mainly near the void volume (Fr 4–8). As seen in the figure, elution of progesterone occurs earlier upon addition of BSA and the total amount eluted is larger than without BSA added. It is considered that progesterone bound to BSA is liberated gradually from BSA, owing to the lowering of the concentration of the free form in gel filtration. When a fatty acid such as myristic acid, palmitic acid or stearic acid is bound to BSA, elution is greatly affected and a large amount of progesterone is detected in Fr 4–8. The elution pattern of progesterone after Fr 13 is similar to that of BSA without bound FFA. The increasing amount of progesterone eluted in Fr 4–12 when compared to BSA without bound FFA, clearly indicates that progesterone interacts more strongly with BSA when the BSA contains bound FFA.

The chromatographic elution patterns of BSA and FFA overlap which shows that the affinity of BSA for FFA is stronger than that of the hormone for BSA.

Similar results were obtained for testosterone (Fig. 2). Testosterone in the free form elutes between Fr 14–26 with a maximum occurring in Fr 17. Addition of BSA accelerates testosterone elution. The chromatographic patterns of testosterone and BSA suggest that

TABLE 3

Recoveries of Hormones and FFA from the Hexane and the Water Phase After Equilibrium Partition Between a 3.5% BSA Solution (50 mL) and Hexane (10 mL)

		Hexane phase (%)[a]		Aqueous phase (%)[a]	
		Hormone	FFA	Hormone	FFA
Progesterone	with myristic acid	64	<1	30	88
	with palmitic acid	64	<1	31	91
	with stearic acid	62	<1	32	92
	without FFA	74	—	20	—
Testosterone	with palmitic acid	4.3	<1	94	91
	without FFA	6.2	—	92	—
Ethynylestradiol	with palmitic acid	0.29	<1	95	92
	without FFA	0.30	—	95	—

[a]The amounts added are taken as 100, and relative values are recorded.

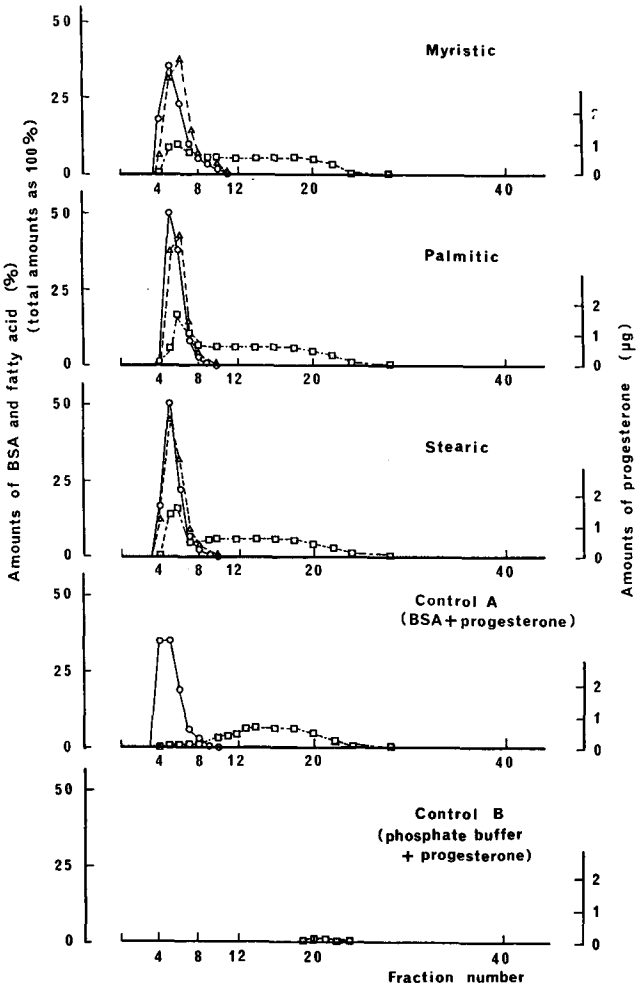

FIG. 1. Gel filtration chromatogram of the albumin-fatty acid-progesterone complex. Ten mL of progesterone dissolved in hexane (200 μg/mL) was added to 50 mL of a 3.5% BSA solution dissolved in a 1/15 M phosphate buffer solution in the absence or presence of FFA (2 moles of FFA per mole of BSA), and the mixture was stirred at 25°C for 15 hr. A defined amount (1 mL) of the aqueous phase at the equilibrium partition was loaded on a Sephadex G-50 column (15 mm × 250 mm) and eluted with 1/15 M phosphate buffer solution. The amounts of BSA (○), FFA (△) and progesterone (□) were assayed in every fraction (each 2.1 mL).

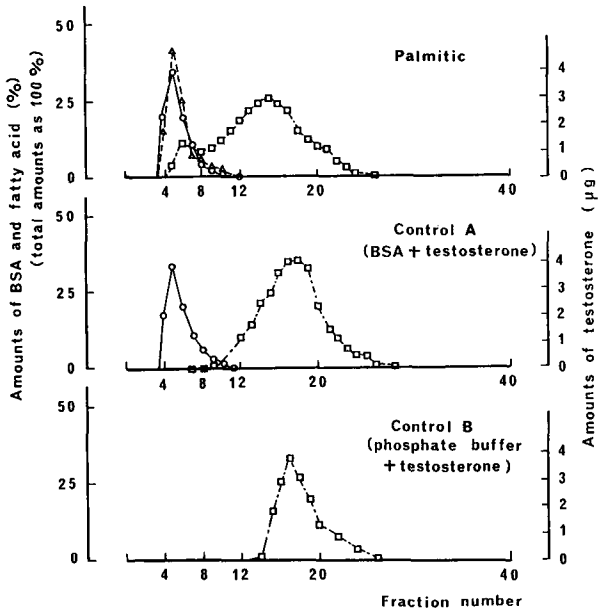

FIG. 2. Gel filtration chromatogram of the albumin-palmitic acid-testosterone complex. Ten mL of testosterone dissolved in hexane (200 μg/mL) was added to 50 mL of a 3.5% BSA solution; other experimental conditions are the same as Figure 1. The amounts of BSA (○), FFA (△) and testosterone (□) eluted from a Sephadex G-50 column were assayed in each fraction (2.1 mL).

testosterone bound to BSA is liberated during the process of gel filtration. When palmitic acid is bound to BSA, the amount of testosterone transferred into the aqueous phase is not as large as in the case of BSA without added fatty acid (Table 3), but the amount of testosterone eluted in Fr 4-11 increases remarkedly as it did in the case of progesterone. The testosterone eluted in these fractions is thought to interact with BSA because of palmitic acid binding to BSA.

As shown in Table 2, the binding coefficient K of hormone to BSA is increased by binding of FFA to BSA. The results of the gel filtration chromatography clearly show that binding of progesterone and testosterone is enhanced by binding of FFA to BSA. This finding is in contrast to a previous report (7), in which the affinity of steroid hormone binding to albumin was

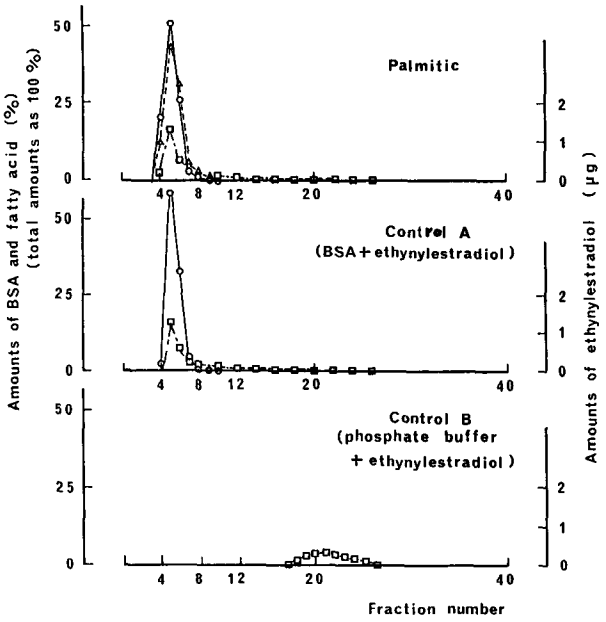

FIG. 3. Gel filtration chromatogram of the albumin-palmitic acid-ethynylestradiol complex. Ten mL of ethynylestradiol dissolved in hexane (32.3 μg/mL) was added to 50 mL of a 3.5% BSA solution; other experimental conditions are the same as in Figure 1. The amounts of BSA (○), FFA (△) and ethynylestradiol (□) eluted from a Sephadex G-50 column were assayed in each fraction (2.1 mL).

increased by FFA removal and decreased to initial levels upon addition of FFA. We cannot further comment on this apparent discrepancy, because detailed experimental conditions were not given.

On the other hand, the chromatographic elution pattern of ethynylestradiol, which is shown in Figure 3, is different from that of progesterone and testosterone. Ethynylestradiol in the free form elutes in Fr 17-26. When BSA is present, a large amount of ethynylestradiol is eluted in Fr 4-8 together with BSA, and only a small amount of ethynylestradiol is eluted in Fr 9-26. When palmitic acid is bound to BSA, the elution pattern of the hormone is essentially the same as in the case where BSA does not contain added fatty acid. It is clear that the liberation of ethynylestradiol from BSA is very slow compared to that of progesterone and testosterone. These results show that the binding of ethynylestradiol to BSA is much stronger, and that the binding of palmitic acid to BSA hardly affects the affinity of ethynylestradiol binding to BSA.

The partition coefficient of a compound between 1-octanol/water is commonly used as an index of hydrophobicity. Close correlations between the association constants with BSA and the partition coefficients were observed for phenothiazine derivatives (14), disopyramide derivatives (15) and sulfonylureas (16). In a previous paper, we reported that the -NHCOO- group of carbamate pesticides participates in the binding to BSA, and a logarithmic inverse correlation was observed between the water/1-octanol partition coefficient and the binding coefficient of these pesticides (17). The binding of FFA to albumin is thought to be caused by the electrostatic attraction of the carboxyl group of FFA to protein cationic sites, together with hydrophobic interactions between the FFA hydrocarbon chain and nonpolar side chains of the protein (18). The binding sites of BSA for FFA consist of six high-energy sites and a large number of weaker sites (8).

Carboxyl and hydroxyl groups of the steroid hormone molecules are supposed to participate in the binding to BSA. A carbonyl group exists at the C-3 position in the skeleton structure of the gestagen (e.g. progesterone), and a hydroxyl group exists at the C-3 position of the estrogens (e.g. ethynylestradiol and α-estradiol). The androgens (e.g. testosterone, androsterone and dehydroisoandrosterone) do not have such a functional group in the C-3 position (19), but these hormones are all substituted at both C-3 and C-17 positions by carboxyl or hydroxyl groups. The observed order of the water/1-octanol partition coefficients of these six hormones is as follows: progesterone (partition coefficient 1×10^{-4}) < androsterone (2×10^{-4}) < dehydroandrosterone (4.6×10^{-4}) < testosterone (6.2×10^{-4}) < α-estradiol (42×10^{-4}) < ethynylestradiol (76×10^{-4}). For progesterone, androsterone and testosterone, that have lower partition coefficients (except for dehydroisoandrosterone), the affinities of binding to BSA are enhanced by binding to FFA to BSA.

When the hormones are classified by the type and number of their functional groups, the partition coefficient in the water/1-octanol system is lowest for progesterone, which has two carboxyl groups, highest for α-estradiol and ethynylestradiol, which have two hydroxyl groups and intermediate for testosterone, androsterone and dehydroisoandrosterone, which have a carboxyl and a hydroxyl group. On the other hand, the binding coefficient K is higher for α-estradiol and ethynylestradiol, which has a higher water/1-octanol partition coefficient, and it is lower for progesterone, that has a lower partition coefficient. Thus, an inverse correlation was not observed between the binding coefficient and the partition coefficient.

We cannot derive a definite conclusion from the present investigation about the nature of binding of the hormones to albumin, or the mechanism by which binding of FFA to albumin enhances the affinities of hormone binding to albumin. Hormones are produced in a specific secretory organ and transported to the target organs through the bloodstream. The binding of hormones to carrier proteins, such as albumin in blood, affects their transport, storage and biological action. It is clear from the present *in vitro* investigation that the affinities of albumin for some steroid hormones are affected by binding of FFA to albumin, but the biological significance of this effect is not clear at this point.

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Erythrocyte Membrane Lipid Alterations in Undernourished Cerebral Palsied Children During High Intakes of a Soy Oil-Based Enteral Formula

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Five undernourished children with severe cerebral palsy (CP) were tube-fed sufficient volumes of Isocal™ to allow rapid weight gain. Isocal™ provided, on average, 88% of their daily energy intake for at least 25 days. The purpose of our study was to correct the undernutrition and to analyze the major erythrocyte phospholipids before and after feeding periods for possible feeding and disease-related differences. The fatty acid profiles of erythrocyte membranes from CP children were compared with those from 12 healthy children and with the fatty acid composition of the formula. There were no clinical or biochemical indications of essential fatty acid deficiency. The feeding of a soy oil-based formula increased the proportions of 18:2n-6 in the phospholipids. The increases occurred predominantly in phosphatidylcholine followed by phosphatidylethanolamine. Despite such large dietary intakes of soy oil, no changes were observed in the phospholipid concentrations of 20:4n-6, 18:3n-3, 20:5n-3, or in the C_{22}n-6 and C_{22}n-3 fatty acids. These findings are consistent with an inhibition of the Δ6 desaturase by high dietary linoleate.
Lipids 25, 639–645 (1990).

Children with severe quadriplegic cerebral palsy (CP) frequently suffer from undernutrition. Factors contributing to the development of the undernutrition include oral motor deficits which reduce feeding efficiency often to less than 10% of normal (1,2) and seizures and abnormal movements which increase energy requirements (3). Cerebral palsy is a descriptive term for a collection of nonprogressive neurological disorders of central origin identified in early life that are not the result of a recognized cerebral malformation (4). The undernutrition in children with severe CP has been treated using various feeding protocols involving nasogastric, gastrostomy or jejunostomy feeding (3,5–7). For rapid refeeding, we have found the nasogastric route to be effective; the long term solution in many of these children, however, requires a gastrostomy or jejunostomy with associated antireflux surgery. Although tube feeding usually does not appear to eliminate entirely the growth retardation accompanying severe CP (6,7), it does lead to weight gain, healing of pressure sores, improved circulation, and general improvements in spasticity and well-being (8). Long-term tube feeding is necessary in some of these children, and we, therefore, need to study the effects of different dietary formulations.

In this communication we describe the refeeding of five children with severe CP who were admitted to the hospital specifically for nutritional rehabilitation. The children were refed with a commonly used commercial enteral formula. Commercial enteral formulae have been used as both dietary supplements and exclusive nutrient sources for these patients. Such formulae usually have as their predominant lipid source corn or soy oil and thus contain very large proportions of polyunsaturated fatty acids (PUFA)—and the major PUFA therein, linoleic acid (18:2n-6). In this study, Isocal™ (Mead Johnson, Evansville, IN), a soy oil-based formula containing added medium-chain triglycerides (MCT, which provide 19% of lipid energy, or 7% of total energy), was fed to the undernourished CP children. Beyond describing the feeding protocol itself, we report the lipid composition of erythrocyte membranes before and after refeeding. To our knowledge, membrane lipid composition has not been assessed in patients during renourishment with a commonly used noninfant enteral formula. Infant formulae are inappropriate for rapid catch-up growth due to their low protein and mineral contents.

Erythrocytes were chosen for membrane lipid analysis because they provide a very good indicator of dietary fat effects (9,10) and because they are readily and noninvasively obtained. Diet-induced changes in red blood cell membrane fatty acids have also been shown to parallel changes in neural membrane fatty acids in experimental animals (11). As human mature erythrocytes are incapable of synthesizing phospholipids *de novo* and of altering acyl chain length and unsaturation (12), the mechanisms involved in modifying phospholipid fatty acids are restricted to the replacement of entire membrane phospholipid molecules and the deacylation of membrane phospholipids to lysophospholipids whose acylation subsequently can be regulated (13–15).

MATERIALS AND METHODS

Patients. The patients were seen at the Children's Hospital of Eastern Ontario. The 12 control children (mean age of 5.3 yr; 6 females, 6 males) were those having routine blood tests two weeks prior to minor surgery. Informed consent was obtained from parents or guardians and the study was approved by the hospital's ethics committee.

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Abbreviations: ANOVA, analysis of variance; ATPase, adenosine triphosphatase; CP, cerebral palsy; EFA, essential fatty acid; FID, flame ionization detector; MCT, medium-chain triglycerides; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PUFA, polyunsaturated fatty acid; RBC, red blood cell; SP, sphingomyelin.

The children with CP were among those admitted for nutritional assessment and treatment. By the Johns Hopkins' criteria for the severity of involvement, each of the five children was quadriplegic and would fall into class 4 which is characterized by no useful motor activity and by dependency for all needs (16). Four of the five children received low to moderate doses of anticonvulsants. None of the medications used is known to affect lipid absorption or lipid metabolism. To identify undernutrition, conventional stature-based anthropometric standards cannot be used as these children usually have skeletal abnormalities. Therefore, on the basis that decreased subcutaneous fat stores are indicative of reduced energy reserves, triceps skinfold thicknesses less than the fifth percentile (HANES I %iles) (17) combined with the failure to gain weight over the previous year were used. Anthropometric methods included several standard circumferential and skinfold measurements (Lange calipers, Cambridge Scientific Institute; Cambridge, MD) and are reported here as the mean of triplicate determinations.

Clinical feeding protocol. The essential points of the feeding protocol are as follows: Initial Phase: In this phase which typically lasts 2–4 days, the goal is the re-establishment of normal metabolism without inducing growth. Growth is equated for practical purposes with weight gain, in the absence of sodium and water retention. Second Phase: During this phase, energy intake is gradually increased to the maximum tolerated and continued until weight gain ceases or the high energy feeding is no longer tolerated. Since the volume tolerated becomes the determinant which limits total nutrient intake, a formula of relatively high energy density such as Isocal™ (Mead Johnson, Evansville, IN) is appropriate. Continuous pump-assisted nasogastric feeding was used throughout the protocol. A patient's intolerance of feeds, as indicated by vomiting, bloating or no further gain in weight, indicated that intake should be returned to maintenance levels and phase 3 of the protocol commenced. In general, total daily energy intakes from the formula range between 230–364 kJ/kg (55–87 kcal/kg) in the first phase and between 343–627 kJ/kg (82–150 kcal/kg) in the second phase. Third Phase: In this phase the patient gradually returns to normal feeding. After returning to maintenance levels of intake, tube feeding is gradually reduced while normal oral feeding is gradually re-introduced. The tube is removed when normal oral feeding is re-established.

Erythrocyte membrane isolation. Whole blood (ca. 3 mL) was collected from each of 5 CP children before and after feeding Isocal™. Whole-blood samples were also collected from 12 healthy control children and were pooled into 4 groups of 3 samples. The blood was collected in Na-heparinized glass tubes, and the erythrocyte membranes were isolated according to Burton *et al.* (18). The packed membranes along with approximately 0.5 mL 1.25 mM Na phosphate buffer were frozen under N_2.

Extraction and analysis of lipids. Total lipids were extracted from erythrocyte membranes according to Kates (19) and stored in 3 mL of chloroform/methanol (2:1, v/v). The total lipids were quantitated by the Iatroscan method (20,21). Three aliquots of 1.0 μL from each of 2 samples together with 3 different amounts of a standard (methyl heptadecanoate) of known concentration were spotted separately and equidistant along each $CuSO_4$-treated Chromarod, about 1.3 cm apart. The Chromarods were not developed but placed directly into the Iatroscan and analyzed. The flame ionization detector (FID) response was proportional to the total amount of lipid present, and a comparison of the FID response to that of the standard permitted quantitation of total lipids in the sample.

Once the concentration of the total lipids in each sample was known, the samples were evaporated to dryness under a stream of N_2 and an exact volume of chloroform/methanol (2:1, v/v) was added to give a lipid concentration of 20 μg/μL, appropriate for further Iatroscan analysis of lipid classes. Neutral and polar lipid classes were resolved and quantitated using the Iatroscan method described previously (20, 21).

Aliquots containing 3 to 4 mg total lipids were then separated into their lipid classes by 3-directional thin-layer chromatography according to Kramer *et al.* (22). The major phospholipids, phosphatidylcholine (PC), phosphatidylethanolamine (PE), sphingomyelin (SP) and combined phosphatidylserine and phosphatidylinositol (PS/PI) were transesterified with anhydrous HCl/methanol (5% by wt) (23) and analyzed by gas-liquid chromatography (24). The methyl esters were analyzed using a flexible fused silica column (30 m × 0.25 mm i.d.) bonded with 0.25 μm Supelcowax 10. The fatty acid methyl esters were identified by comparison with authentic standards (NuCheck Prep, Elysian, MN).

Statistical analyses. For comparison of the 5 pre- and post-treatment values, a paired t-test was used. Separate analyses were done to compare the pre- and post-treatment values with the control values. Because each of the control values was obtained from a pooled sample of healthy children, the variances of these data were expected to be one-third of the patients with CP. Consequently, in comparing the controls with either the pre- or post-treatment values a weighted analysis of variance (ANOVA) was done with the weights in the ratio of 1:3. To confirm the need for the weighted ANOVA, an F-test was used to compare the variances of the pre-/post-treatment data with those of the control patients. However, when the findings from the weighted ANOVA were compared with those obtained from a regular ANOVA, the results were similar. Since the weighted ANOVA is methodologically more accurate, the results from this test are reported here.

RESULTS AND DISCUSSION

The children, referred to as patients 1–5, are described in Tables 1 and 2. On average, the patients received 88% of their daily energy intake from the formula for a period of at least 25 days. No clinical evidence of essential fatty acid (EFA) or of trace element deficiencies was observed in any of the patients. Anthropometric data with reference to standards are shown in Table

2; the data provide a rough indication of the responses to the refeeding protocol. The feeding periods described in Table 1 are total feeding periods while some periods in Table 2 are shorter to facilitate the comparisons of responses to feeding. Body weights with reference to standards of weight-for-age are included only to provide a general idea of the abnormality and variability of anthropometrics in this population.

The fatty acid composition of Isocal™, based on the total area response of the flame ionization detector, was as follows: 6:0, 0.2%; 8:0, 8.9%; 10:0, 5.0%; 12:0, 0.1%; 14:0, 0.2%; 16:0, 9.7%; 16:1n-9, 0.1%; 18:0, 3.5%; 18:1n-9, 18.5%; 18:2n-6, 45.6%; 18:3n-3, 6.7%; 20:0, 0.3%; 20:1n-9, 0.5%; and 22:0, 0.3%. This formula supplies 16.9% and 2.5% of its total energy content in the form of 18:2n-6 and 18:3n-3, respectively; the amounts are more than adequate to meet the EFA requirements of these patients. For infants the estimated minimal daily requirement for 18:2n-6 is < 1%–4% of total energy intake (27–29), while for growing children and adults an intake of < 1% is sufficient to prevent EFA deficiency (27,29,30). The estimated minimal daily requirement for 18:3n-3 in children, based on one study involving a six-year-old girl, was 0.54% of total energy (31). For immobile adults, the minimal requirement was estimated at 0.2% when the intake of long-chain n-3 fatty acids was 0.08% of energy (30). Bjerve *et al.* (32) showed that the minimal daily requirement for 18:3n-3 was higher when the intake of long-chain n-3 fatty acids was extremely low. The estimated optimal ratio of n-6 to n-3 intake is 6:1 (33,34). The ratio in the formula used in this study is 9.6:1, and the n-6 and n-3 fatty acids are provided almost exclusively by 18:2n-6 and 18:3n-3, respectively.

Lipid subclass changes. The erythrocyte membrane percentage compositions (i.e., % of total membrane lipid) of cholesterol (35.3%), cholesterol esters (0.6%), triglyceride (0.1%), free fatty acids (0.4%), PC (17.9%), PE (17.3%), combined PS/PI (3.6%), and SP (19.1%) did not change during refeeding. Moreover, they were not significantly different than corresponding values of erythrocyte membranes isolated from the healthy children studied (results not shown).

The erythrocyte membrane fatty acid profiles of selected phospholipids are shown in Tables 3 and 4. Pre- and post-feeding results are compared to each other and to results obtained in the 12 healthy control children.

Fatty acid changes. The fatty acid composition of the major erythrocyte membrane phospholipids is shown in Table 3. There were significant differences between results of CP patients and controls, and of pre- and post-feeding with Isocal™. The relative concentration of saturates (16:0 and 18:0) was generally higher in CP patients prior to feeding than in controls. Feeding

TABLE 1

Patient Description

Patient	Age (years)	Sex	Feeding period (days)	Energy intake as Isocal™ (%)
1	17	M	26	85
2	15	M	25	86
3	2	F	79	100
4	2	F	99	82
5	12	M	26	85

TABLE 2

Anthropometric Profiles Before and During Clinical Refeeding

Patient	Feeding period (days)	TSF[a] (cm)	TSF %ile[b]	BSF[c] (cm)	MAMC[d] (cm)	BWt (kg)	BWt[e] (% $\bar{x}$)
1 Pre		3.4	<5	2.2	13.8	26.0	39
Post	26	4.0	5	2.5	15.0	31.7	48
2 Pre		3.5	<5	2.0	13.2	21.0	37
Post	25	12.0	25	4.0	14.6	23.4	42
3 Pre		6.0	<5	3.2	11.2	7.6	62
Post	21	7.5	<25	6.0	12.9	9.2	75
4 Pre		6.8	5	4.2	11.0	7.7	63
Post	14	8.2	25	10.0	12.9	9.7	79
5 Pre		2.6	<5	2.2	11.4	15.8	38
Post	26	4.4	<5	5.2	13.1	20.3	48

[a]Triceps skinfold (mean of 3 measurements).
[b]Percentiles based on 1971–1974 Health and Nutrition Examination Survey (17).
[c]Biceps skinfold (mean of 3 measurements).
[d]Mid arm muscle circumference: obtained using the nomogram method of Gurney and Jeliffe (25).
[e]Expressed as a percentage of the National Center for Health Statistics (NCHS) mean for age (26).

of Isocal™ lowered the relative concentration of 16:0 and raised that of 18:0 with the exception of 18:0 in PE. The relative concentration of 18:1n-9 was not significantly ($P > 0.05$) different between pre-fed CP and control patients. However, the feeding of Isocal™ significantly lowered the level of 18:1n-9. The proportion of 18:2n-6 was not significantly different between pre-fed CP patients and controls. The feeding of the soy oil-based Isocal™ diet significantly raised the level of 18:2n-6. This increase in 18:2n-6 in post-fed CP patients did not result in an increase of fatty acids derived from 18:2n-6 by desaturation and elongation, i.e. arachidonic acid (20:4n-6), 22:4n-6 and 22:5n-6. The latter two fatty acids (22:4n-6 and 22:5n-6) were summed and are shown in Table 3 as C_{22}n-6. Only small amounts (< 0.3%) of 18:3n-3 were found in erythrocyte phospholipids of normal or CP patients, but the concentration of fatty acids derived from 18:3n-3, i.e. 22:5n-3 and 22:6n-3 was significantly ($P < 0.05$ and $P < 0.01$) higher in normal children than in CP patients. The consumption of the soy oil-based Isocal™ diet, rich in 18:3n-3, resulted in no significant ($P > 0.05$) increase in either 18:3n-3 or C_{22}n-3 fatty acids.

It was of interest to note that CP patient 3 appeared to be an exception to many of the observations. Data from this patient showed evidence of fatty acid desaturation and elongation. The concentration of 20:4n-6 (PE, 9.5 to 15.6%; PC, 2.3 to 4.8%), C_{22}n-6 (PE, 2.8 to 5.6%; PC, 0.2 to 0.3%) and C_{22}n-3 (PE, 1.6 to 3.4%; PC, 0.6 to 0.7%) increased with the feeding of Isocal™, a diet rich in 18:2n-6 and 18:3n-3. It is not known whether these results have any implications regarding the etiology of this patient's disorder.

The fatty acid profile of SP is shown in Table 4. SP consists primarily of saturated fatty acids from 16:0 to 24:0 with a large proportion of nervonic acid (24:1n-9). The CP patients generally showed higher than normal proportions of 16:0 and 18:0. On the other hand, proportions of 20:0, 22:0, and 23:0 in samples from CP patients pre- and post-feeding were lower than those of controls (Table 4). The feeding of Isocal™ did not have an effect on the fatty acid composition of SP—certainly not in the direction toward that found in the normal control children. No decrease in 18:1n-9 was observed after feeding as was observed in the other phospholipids; in this subclass, proportions of 18:1n-9 remained higher than the control value even after feeding ($P < 0.05$). However, a decrease in the metabolic product of 18:1n-9, i.e. 24:1n-9, was observed during feeding ($P < 0.05$), and the post-fed proportion of 24:1n-9 was significantly lower than the control value ($P < 0.01$).

Thus, the results obtained from SP membrane fractions reveal abnormalities which appear to be associated both with CP which are not affected by feeding Isocal™ and with feeding-induced alterations in membrane fatty acids. As mentioned before, dietary-induced changes in red blood cell (RBC) membrane fatty acids have been shown to parallel changes occurring in neural membrane fatty acids in experimental rats (11). Whether similar membrane fatty acid abnormalities and dietary-induced fatty acid alterations occur in other cell types is unknown.

Despite the small number of patients involved in this study, our results raise several important points. The data reveal differences between membrane fatty acid composition of samples taken from CP children and normal control children that persist despite nutritional intervention. This presumably indicates an effect of the syndrome and/or its treatment that is insensitive to our dietary intervention. Our results also reveal changes in the proportions of certain fatty acids which indicate an effect of feeding in these children. Because Isocal™ contains high proportions of 18:2n-6, we suspected that we might observe changes in erythrocyte membrane proportions of the long-chain n-6 and n-3 fatty acids. However, there is evidence to indicate that a high intake of dietary 18:2n-6 may inhibit Δ6 desaturase activity and thus suppress the synthesis of long-chain fatty acids from 18:2n-6 (35–38). It is also possible that the relatively high dietary content of 18:3n-3 inhibits the activity of Δ6 desaturase (31). Our data are consistent with such an inhibition since we observed concentrations of 18:2n-6 which increased to above-normal values during feeding while no increases were observed in the long-chain n-6 fatty acids. Despite the presence of short-chain fatty acids in the diet, one would not expect to observe short-chain fatty acids in membrane phospholipids since they would either be oxidized for energy or oxidized and converted to longer chain fatty acids in the liver (39).

It is not known whether comparable changes in membrane fatty acid composition would occur in normal children as a result of high intakes of such an enteral formula. There are no documented aberrations in lipid metabolism which are associated with CP. However, lipid metabolism is thought to vary substantially between species and to be altered by many pathologies, as well as by lipid and nonlipid dietary components (35).

In enteral and parenteral formulae, lipid is important for the provision of energy, essential fatty acids and a balanced combination of fatty acids to allow the normal incorporation and metabolism of fatty acids for their ensuing structural and functional roles. *In vivo* animal studies have shown that manipulations of dietary fatty acid composition can alter the fatty acid composition of plasma membranes (40,41), endoplasmic reticulum (42,43), mitochondrial membranes (44–46), synaptosomal membranes (47) and nuclear membranes (48). This indicates that membrane fatty acid composition is not rigidly regulated *in situ* but turns over perpetually and is dependent upon fatty acid supply. The rapidity with which these diet-induced changes are observed is consistent with fatty acyl components having a turnover independent of the complete phospholipid molecule (49). Functional correlates of diet-induced membrane lipid modifications include mitochondrial adenosinetriphosphatase (ATPase) activity (50), hormone receptor-mediated enzyme function (51), insulin receptor sensitivity (52) and the transport of numerous ions and molecules (53–56).

The formula used here and other similar enteral formulae contain greater than sufficient quantities of 18:2n-6 and 18:3n-3. Perhaps during the consumption of large amounts of the formula, these quantities may inhibit Δ6 desaturase activity. On the other hand, it may be possible that some children with CP

TABLE 3

Fatty Acid Composition of Major Erythrocyte Membrane Phospholipids

Fatty acid[a]		Phosphatidylcholine				Phosphatidylethanolamine				Phosphatidylserine and phosphatidylinositol			
		Mean (± SEM)	Pre vs post[b]	Pre vs cont.[c]	Post vs cont.[c]	Mean (± SEM)	Pre vs post[b]	Pre vs cont.[c]	Post vs cont.[c]	Mean (± SEM)	Pre vs post[b]	Pre vs cont.[c]	Post vs cont.[c]
16:0	pre	37.0 (1.17)	NS	*	NS	17.9 (2.07)	NS	NS	NS	3.9 (0.21)	NS	*	NS
	post	33.5 (1.12)				17.2 (0.97)				3.3 (0.53)			
	cont.	32.9 (0.75)				15.2 (0.75)				2.6 (0.27)			
18:0	pre	17.3 (0.34)	*	NS	*	12.6 (1.40)	NS	NS	NS	54.6 (1.91)	NS	NS	**
	post	18.6 (0.64)				11.5 (0.77)				57.5 (1.11)			
	cont.	15.8 (0.63)				12.5 (0.10)				51.8 (0.57)			
18:1n-9	pre	18.7 (1.41)	NS	NS	***	22.3 (1.36)	*	NS	NS	8.7 (0.99)	*	NS	NS
	post	15.2 (0.51)				19.3 (0.90)				6.1 (0.83)			
	cont.	21.3 (0.66)				20.7 (0.54)				7.3 (0.46)			
18:2n-6	pre	17.6 (1.00)	**	NS	**	5.4 (0.76)	*	NS	**	2.7 (0.62)	NS	NS	NS
	post	22.3 (0.98)				7.7 (0.76)				3.4 (0.84)			
	cont.	17.8 (0.74)				5.2 (0.18)				2.0 (0.25)			
18:3n-3	pre	0.3 (0.14)	NS	NS	NS	0.2 (0.06)	NS	*	NS	2.0 (0.43)	NS	NS	NS
	post	0.3 (0.07)				0.3 (0.09)				2.4 (0.44)			
	cont.	0.3 (0.03)				0.3 (0.00)				1.9 (0.21)			
20:4n-6	pre	3.1 (0.39)	NS	*	*	15.4 (1.61)	NS	NS	NS	17.3 (1.47)	NS	P=0.08	**
	post	3.5 (0.43)				16.1 (0.55)				15.8 (1.01)			
	cont.	5.0 (0.31)				17.4 (0.77)				20.2 (0.39)			
C_{22}n-6[d]	pre	0.3 (0.04)	NS	NS	NS	5.3 (0.76)	NS	NS	NS	4.1 (0.51)	NS	NS	NS
	post	0.3 (0.02)				5.7 (0.33)				3.6 (0.38)			
	cont.	0.5 (0.09)				5.9 (0.14)				4.2 (0.08)			
C_{22}n-3[e]	pre	0.6 (0.07)	NS	**	**	3.1 (0.62)	NS	NS	*	3.6 (0.81)	NS	*	***
	post	0.5 (0.11)				3.2 (0.17)				3.2 (0.30)			
	cont.	1.1 (0.09)				4.2 (0.19)				6.2 (0.29)			

[a]Minor fatty acids not listed: 20:1, 0.3%; 20:2n-6, 0.5%; 20:3n-6, 1.8%; 20:5n-3, 0.1%.
[b]Paired t-test; significant differences are indicated as: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.
[c]Weighted ANOVA; significant differences are indicated as in footnote b.
[d]Sum of 22:4n-6 and 22:5n-6.
[e]Sum of 22:5n-3 and 22:6n-3.

TABLE 4

Fatty Acid Composition of Sphingomyelin from Erythrocyte Membranes

Fatty acid[a]		Mean (± SEM)	Pre vs post[b]	Pre vs cont.[c]	Post vs cont.[c]
16:0	pre	20.9 (0.86)	P=0.07	NS	*
	post	22.8 (0.87)			
	control	19.3 (0.79)			
18:0	pre	10.7 (1.24)	NS	*	*
	post	10.6 (0.82)			
	control	7.4 (0.56)			
18:1n-9	pre	1.8 (0.27)	NS	P=0.08	*
	post	1.8 (0.15)			
	control	1.2 (0.12)			
20:0	pre	2.1 (0.07)	NS	**	**
	post	2.0 (0.07)			
	control	2.5 (0.06)			
22:0	pre	8.9 (0.55)	NS	*	**
	post	8.1 (0.38)			
	control	11.1 (0.55)			
23:0	pre	1.4 (0.12)	NS	P=0.06	P=0.09
	post	1.3 (0.16)			
	control	1.7 (0.05)			
24:0	pre	22.7 (1.77)	NS	NS	NS
	post	23.1 (1.13)			
	control	25.0 (0.25)			
24:1n-9	pre	23.7 (1.17)	*	NS	**
	post	20.7 (0.77)			
	control	25.4 (0.73)			
24:2	pre	4.0 (1.09)	NS	NS	NS
	post	5.5 (1.25)			
	control	3.2 (0.40)			

[a]Minor fatty acids not listed: 16:1, 0.6%; 17:0, 0.5%; 18:2n-6, 0.7%; 21:0, 0.4%.

[b]Paired t-test; significant differences are indicated as: *, $P < 0.05$; **, $P < 0.01$.

[c]Weighted ANOVA; significant differences are indicated as in footnote b.

have problems desaturating and elongating fatty acids.

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Selective Reduction of Fatty Acid Oxidation in Colonocytes: Correlation With Ulcerative Colitis

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Attempts were made to define which fatty acid (2:0 to 18:1) was optimally oxidized by isolated colonocytes (colonic epithelial cells) and to select inhibitors of fatty acid oxidation which would be analogous in their action to the inhibition of fatty acid oxidation observed in colonocytes involved with ulcerative colitis. Isolated colonic epithelial cells of Sprague-Dawley rats were used with 2-mercaptoacetate, dichloroacetate, 3-mercaptopropionate, 4-mercaptobutyrate, 4-sulfatebutyrate, 2-bromobutyrate, sulfite ions and nitrite ions. n-Butyrate (4:0) was maximally oxidized to CO_2 and ketone bodies (mean value 5.46 μmol/min/g dry wt). Oxidation of butyrate to CO_2 was diminished by 2-bromobutyrate, sulfite ions and all mercapto fatty acids. Both fatty acid oxidation and glucose oxidation were significantly inhibited by 2-bromobutyrate, while mercapto fatty acids and sulfite inhibited fatty acid oxidation ($p<0.01$) without significantly changing glucose oxidation. Observation with 2-mercaptoacetate and sulfite correlate with early changes of fatty acid oxidation observed in cases of ulcerative colitis, and warrant further study with isolated colonocytes of man.
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Ulcerative colitis is a disease in which colonic epithelial cells (colonocytes) fail to oxidize short chain fatty acids, particularly n-butyrate (4:0) (1) which provides 70% of the oxidative energy for the cells (2–4). Short chain fatty acids are abundantly produced through fermentation by anaerobic bacteria in the colonic lumen. In ulcerative colitis, oxidation pathways of other substrates, such as glucose and glutamine, derived from the circulation are not impaired in epithelial cells.

The cause of impaired oxidation of fatty acids in ulcerative colitis is unknown; however, selective inhibition of fatty acid oxidation with 2-bromooctanoate produced experimental colitis in rats which reproduced those histological changes observed in ulcerative colitis of man (5). Lowering levels of free CoA in colonic epithelial cells by a deficiency of pantothenic acid led to colitis in pigs which closely resembled the human disease (6,7). These observations point to impairment of fatty acid oxidation as a central feature in the genesis of ulcerative colitis.

The objectives of the study were to establish which fatty acid from 2:0 to 18:1 was optimally oxidized in isolated colonic epithelial cells and to observe whether specific inhibition of the optimally oxidized fatty acid could, in the short term, be produced by agents (8–20) (see Table 1) acting on isolated colonic epithelial cells. A third objective was to establish under what incubation conditions inhibition of fatty acid oxidation predominantly occurred. Consideration was not given as to whether any substance was likely to be found *in vivo* in humans, however it was hoped that results with appropriately reactive substances would guide a search for similar agents in cases with ulcerative colitis.

MATERIALS AND METHODS

Reagents. Sodium n-butyrate, sodium octanoate, D-glucose and DL-sodium 3-hydroxybutyrate were obtained from BDH (Melbourne, Australia); lauric acid, palmitic acid, myristic acid and oleic acid were from Fluka (Buchs, Switzerland). L-Carnitine•HCl was obtained from Otsuka Pharmaceuticals (Osaka, Japan). Bovine serum albumin, Fraction V, was obtained from Sigma Chemical Co. (St. Louis, MO). The above reagents were not purified any further, but albumin was dialyzed several times to remove dialyzable contaminants. Radioactively labelled compounds were obtained from the following sources: sodium [1-^{14}C]butyrate and sodium [1-^{14}C]octanoate from Du Pont-NEN Research Products (North Sydney, Australia); [1-^{14}C]lauric acid, [1-^{14}C]palmitic acid, [1-^{14}C]oleic acid, [6-^{14}C]glucose and D-3-hydroxy-[3-^{14}C]butyric acid from Amersham (Sydney, Australia); and [1-^{14}C]myristic acid from CEA, Biology Dept. (Paris, France).

The mercapto derivative of butyrate, 4-mercaptobutyric acid, was synthesized by Pfaltz and Bauer, Inc. (Waterbury, CT); 3-mercaptoproprionic acid, 2-bromobutyric acid and dichloroacetate were obtained from TCI (Tokyo, Japan); and sodium mercaptoacetate was obtained from Merck (Darmstadt, Federal Republic of Germany). Potassium butyrate-4-sulfate was synthesized by the method of Denner *et al.* (15) using γ-butyrolactone as starting material. Infrared and nucleur magnetic resonance (NMR) spectra of the final product confirmed that sulfation had occurred at the 4-carbon position. Purified enzymes for substrate analyses (lactate dehydrogenase, glucose-6-phosphate dehydrogenase, hexokinase, β-hydroxy butyrate dehydrogenase, glutamate dehydrogenase, alanine dehydrogenase, malate dehydrogenase, aspartate transaminase), coenzymes (NAD, NADP, NADH) and substrates (alanine, α-ketoglutarate, asparagine, aspartate and sodium lactate) were obtained from Boehringer Corporation (North Ryde, Australia). Lithium acetoacetate, L-glutamine and glutaminase were obtained from Sigma Chemical Co.

Isolation of colonic epithelial cells. Colonocytes were prepared from Sprague-Dawley rats bred in the Animal Houses of the University of Adelaide and fed on a balanced diet (Milling Industries, Adelaide). Animals weighing between 150–250 g and kept on a timed diurnal cycle, were used in the fed state as this produced optimal yield of cells. The entire colon designated as proximal or distal colon (21) was used to prepare iso-

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lated cells. Animals were killed by stunning/cervical fracture, the colon removed and flushed clear of luminal contents with 140 mM sodium chloride.

Isolated colonocytes were prepared as previously described (3,22). Aliquots of 1 mL of cells were used for experimental procedures. Viability of cells was assessed by linearity of lactate production and bacterial contribution to metabolite formation excluded in incubating cells with and without benzyl penicillin (200 U/mL) and streptomycin (200 mg/mL).

Substrate preparation and incubations. All radioactively labelled fatty acids were dried under nitrogen to remove ethanol and toluene. n-Butyrate and sodium octanoate were diluted in 500 μL distilled water and then added to unlabelled stock solutions for use with all incubations that contained albumin. Myristic, lauric, palmitic and oleic acid were converted to the sodium salt with 0.1 N NaOH at 60°C and complexed with 2.5% delipidized bovine serum albumin in Krebs-Henseleit saline. Stock solutions of acetic (2:0) to oleic (18:1) acid were each prepared fresh before use. Mercapto and bromo fatty acids (Table 1) were used as the freshly prepared sodium salt; 4-mercaptobutyric acid, 3-mercaptopropionic acid and 2-bromobutyric acid were titrated with 0.1 N NaOH to pH 7.4 and the volume adjusted with distilled water for dispensing into incubation media with the cells.

Cell suspensions of 1 mL, representing 5–12 mg dry weight of epithelial cells were incubated for 20, 40 or 60 min in conical flasks equipped with a glass center well and stoppered with suba-seals. The gas phase was O_2 and CO_2 (19:1, v/v). Incubations were carried out at 37°C in 1 or 2 mL physiological saline containing 2.5% w/v bovine serum albumin, 1 mM DTT and substrates at concentrations of 1–5 mM or as indicated in the Results section. The specific activities of the respective substrates used were as follows: [1-^{14}C]butyrate 1900 dpm/μmol, [1-^{14}C]octanoate 2400 dpm/μmol, [1-^{14}C]laurate 5020 dpm/μmol, [1-^{14}C]myristate 6250 dpm/μmol; [1-^{14}C]palmitate 2520 dpm/μmol; and [1-^{14}C]oleate 15030, dpm/μmol, [6-^{14}C]glucose, 1700 dpm/μmol. The incubation was stopped by adding 0.5 mL of 10% perchloric acid and the protein precipitate was centrifuged after cooling with ice. The supernatant was neutralized to pH 7.4 with 20% potassium hydroxide.

Analytical and radiochemical methods. Metabolites and substrates were measured enzymatically from neutralized extracts of cells according to Bergmeyer (23). $^{14}CO_2$ was trapped in 0.5 mL of 10 M NaOH injected into the center well immediately after cell proteins were precipitated with perchloric acid. Flasks were gently shaken on ice for 1.5 hr and 0.1 mL of the solution added to 5 mL of scintillant, as previously described (3). Samples were counted in a Beckman counter (Beckman Instruments, Fullerton, CA) and counts were corrected for the nonspecific activity generated by perchloric acid or volatility of the fatty acid n-butyrate.

Calculations. Results were expressed per gram dry weight rather than numbers of epithelial cells as clumping due to mucus prevented reliable counting of cells. The dry weight was obtained by drying 1 mL of the cell suspension to constancy at 100°C and correcting for the dry weight of the albumin contained in the medium. $^{14}CO_2$ generation from fatty acid or glucose were calculated from the specific activities and trapped $^{14}CO_2$ in sodium hydroxide. No correction was applied to the specific activity for the intracellular distribution of radioactive substrates.

RESULTS

Oxidation of fatty acids. The mean total of all oxidative metabolites (CO_2 and ketone bodies) for fatty acids are shown in Figure 1 and Table 2. For n-butyrate

TABLE 1

Agents Evaluated for Their Capacity to Alter Fatty Acid Oxidation in Isolated Colonic Epithelial Cells

Agents used as sodium salts	Known actions and reason for use
2-Mercaptoacetate	Inhibitor of fatty acid oxidation in liver (8–11).
Dichloroacetate	Hypolipidemic agent (12), alters fatty acid oxidation in the heart (13).
3-Mercaptopropionate	Inhibitor of fatty acid oxidation in heart (14).
4-Mercaptobutyrate	Mercapto derivative of main substrate of colonic epithelial cells in health (2–4).
4-Sulfatebutyrate	End product of detergent degradation by mammalian tissues (15).
2-Bromobutyrate	Brominated fatty acids are powerful inhibitors of fatty acid oxidation in several tissues (16,17).
Sulfite ions	Product of sulfate reduction by anaerobic bacteria.
Nitrite ions	Product of inflammatory cells (18); known modulator of fatty acid oxidation (19) and found in colon with active colitis (20).

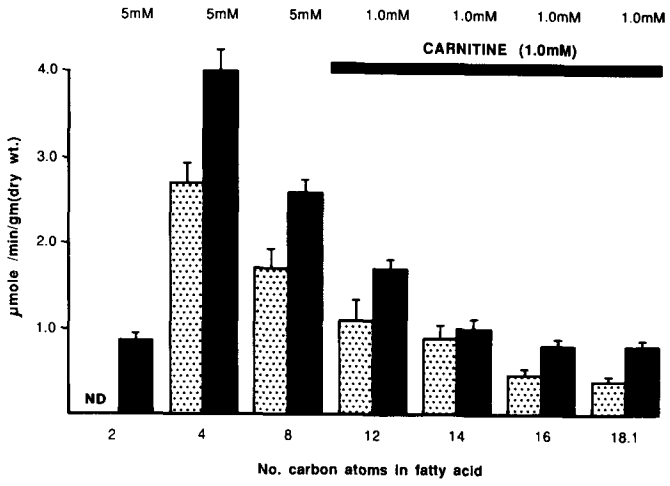

FIG. 1. Oxidation of acetic acid to oleic acid to CO_2 and acetoacetate by isolated colonic epithelial cells of the rat prepared from the same animals. Carnitine (1.0 mM) added at concentration of fatty acids indicated. Mean ± SE of four experiments each comprising pooled cells from four colons. Acetoacetate, (■); $^{14}CO_2$, (□); incubations for 40 min.

(4:0) metabolites produced by colonocytes were 5.4 µmol/min/g dry weight as compared with 1.4 µmol/min/g dry weight for 1 mM oleic acid (18:1) in the presence of carnitine. The addition of 5 mM glucose (Table 2) suppressed acetoacetate formation by 25–45% from all fatty acids (4:0 to 18:1) with a concomitant increase in appearance of β-hydroxybutyrate. Nevertheless, the total ketone body production (acetoacetate plus β-hydroxybutyrate) was slightly diminished by addition of 5 mM glucose. These observations indicated a constant relationship between glucose and fatty acid oxidation and that activation of fatty acids occurred maximally with the 4:0 in colonocytes. n-Butyrate appeared to be the most suitable fatty acid against which to compare reagents modulating oxidative metabolites.

Effect of bromobutyrate and/or nitrite on substrate oxidation. Brominated fatty acids become inhibitory to fatty acid oxidation after metabolic incorporation into cells (16,17). Colonocytes were therefore pretreated with bromobutyrate, sodium bromide and 4-mercaptobutyrate followed by addition of [1-^{14}C]butyrate. Under these conditions, linearity of CO_2 production was maintained over 40 min and ketogenesis maintained at a nonlinear rate (Fig. 2) in keeping with past observations on ketogenesis (3). Substitution of a hydrogen atom with bromine on the second carbon of n-butyrate produced 55% mean reduction of ketogenesis and 32% mean reduction of CO_2 generation.

Substitution with a sulfhydryl group at C-4 of butyrate (4-mercaptobutyrate) halved the inhibitory effect observed with substitution of bromide at C-2 (2-bromobutyrate) (Fig. 2). Sulfate substitution at C-4 of butyrate (4-sulphatebutyrate) had no effect on fatty acid oxidation (results not shown), suggesting that the action of the -SH on the fourth carbon was a specific one. Glucose oxidation, measured as the difference between total glucose utilization and lactate formation (Table 3) was 15.05 µmol/min/g dry weight without bromobutyrate as compared to 9.8 µmol/min/g dry weight with 2-bromobutyrate, which again is a one-third reduction of oxidation similar to the level of reduction of oxidation with fatty acids.

As sodium nitrite modulated fatty acid oxidation (19), this ion was used in conjunction with 2-bromobutyrate in pre-incubation experiments with colonocytes. Overall reduction of oxidative metabolites (CO_2 and ketone bodies) was by a mean value of 54% under control conditions with 2-bromobutyrate (Table 4), a percentage reduction which was also seen on addition of sodium nitrite except that ketogenesis was not significantly diminished ($p < .001$) by the addition of sodium nitrite.

Inhibition of fatty acid oxidation in colonocytes was also tested with cells that were incubated simultaneously with 2-bromobutyrate or 4-mercaptobutyrate rather than pretreating cells (Table 5). Under these conditions, the ratio between butyrate and 2-bromobutyrate or 4-mercaptobutyrate was important before the degree of inhibition was observed, as that seen with cells that were pre-treated with the same reagents. A ratio of 4:1 for 2-bromobutyrate/butyrate produced comparable reduction of oxidative metabolites observed with pre-treated colonocytes (Table 3).

As diminished fatty acid oxidation in other organs enhances glutaminolysis (25), glutamine metabolism was assessed in the presence of 2-bromobutyrate. Glutamine removal, ammonia generation and appearance of glutamate was not significantly different when fatty

TABLE 2

Effect of Glucose on Ketone Body Generation from Acetic to Oleic Acids by Isolated Colonic Epithelial Cells of Rat

	No glucose (µmoles/min/g dry wt.)		Glucose 5 mM (µmoles/min/g dry wt.)	
Fatty acid	Acetoacetate	β-hydroxybutyrate	Acetoacetate	β-hydroxybutyrate
Acetate (2:0)[a]	0.83 ± 0.08	0.1 ± 0.02	0.46 ± 0.07	0.22 ± 0.04
Butyrate (4:0)	4.0 ± 0.25	0.76 ± 0.13	2.9 ± 0.19	1.18 ± 0.11
Octanoate (8:0)	2.63 ± 0.14	0.29 ± 0.03	2.1 ± 0.15	0.71 ± 0.11
Laurate (12:0)	1.71 ± 0.10	0.46 ± 0.03	1.09 ± 0.12	0.60 ± 0.06
Myristate (14:0)	1.04 ± 0.10	0.27 ± 0.05	0.74 ± 0.05	0.36 ± 0.06
Palmitate (16:0)	0.85 ± 0.06	0.17 ± 0.05	0.68 ± 0.10	0.32 ± 0.06
Oleate (18:1)	0.85 ± 0.02	0.16 ± 0.05	0.58 ± 0.10	0.18 ± 0.01

[a]Mean ± SE of four experiments, each experiment comprising colons from four animals.

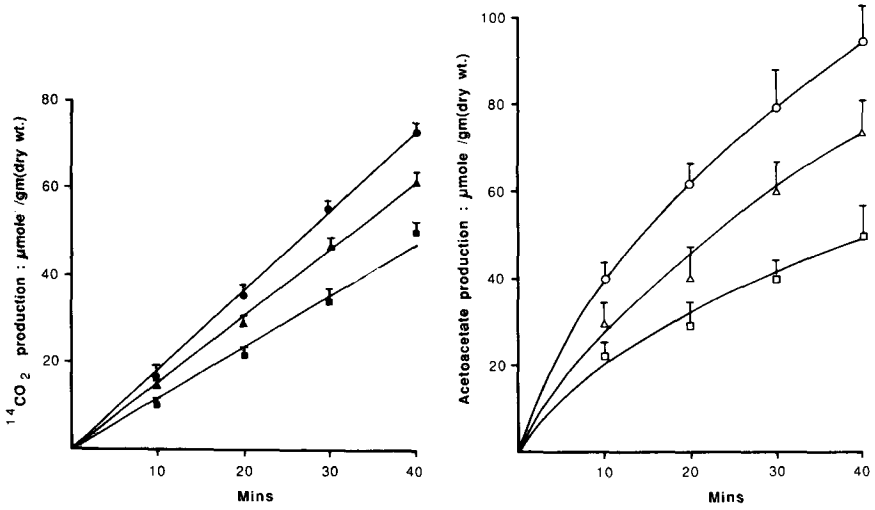

FIG. 2. Fatty acid oxidation by isolated colonic epithelial cells of [1-^{14}C]butyrate (5 mM) added after pre-treatment for 20 min with sodium bromide; 5 mM, (●○); 4-mercaptobutyrate, 5 mM, (▲△); and 2-bromobutyrate, 5 mM, (■□). Mean ± SE of four experiments each comprising pooled cells from four colons of the rat.

TABLE 3

Pre-Treatment of Colonic Epithelial Cells with 2-Bromobutyrate for 20 min. Followed by Incubation with Glucose for 40 min: Effect on Glucose Oxidized by Pre-Treated Cells[a]

	Glucose (5 mM) (µmoles/min/g dry wt)		
Condition	Glucose removed	Lactate formed	Glucose oxidized[b]
Control	6.5 ± 1.0[a]	7.1 ± 0.4	15.05 ± 4.0[c]
2-Bromobutyrate	4.6 ± 0.6	5.3 ± 0.5	9.8 ± 1.8[c]

[a]Mean ± SE of four paired experiments.
[b]Glucose oxidation was calculated by (glucose removal × 2-lactate formation) × 2.5, where 2.5 reflects oxidation through the tri-carboxylic acid cycle and is derived from the oxidation of pyruvate to oxaloacetate (24).
[c]p = < .01, Student's paired *t*-test.

TABLE 4

Pre-Treatment of Colonic Epithelial Cells with 2-Bromobutyrate for 20 min. Followed by incubation with [1-^{14}C]Butyrate for 40 min: Effect on Fatty Acid Oxidation (with or without Nitrite) by Pre-Treated Cells

	[1-^{14}C]Butyrate (5 mM)			
	Nil		Nitrite (5 mM)	
Oxidative metabolite formation (µmol/min/g dry wt.)	Sodium bromide (5 mM)	2-Bromobutyrate (5 mM)	Sodium bromide (5 mM)	2-Bromobutyrate (5 mM)
$^{14}CO_2$	2.3 ± 0.2[a,b]	1.5 ± 0.2	3.6 ± 0.4[b]	1.9 ± 0.2[c]
Acetoacetate	2.5 ± 0.4	1.1 ± 0.07	0.5 ± 0.2	0.33 ± 0.1[c]

[a]Mean ± SE of four experiments.
[b]Total of oxidative products formed without nitrite (4.8–2.6) and with nitrite (4.1–2.23) represents 54% reduction of oxidation from butyrate in presence of 2-bromobutyrate.
[c]p = < 0.001, Student's paired *t*-test.

TABLE 5

Effect of Varying Concentrations of 2-Bromobutyrate and 4-Mercapobutyrate on Oxidation of [1-^{14}C]Butyrate in Isolated Colonic Epithelial Cells when Both are Simultaneously Incubated[a]

Fatty acid or derivative of fatty acid	Oxidative metabolite (μmol/min/g dry wt)	Concentration (mM) of fatty acid or substituted fatty acid				
n-Butyrate	—	5	4	3	2	1
	$^{14}CO_2$	1.90 ± 0.14	2.2 ± 0.21	2.00 ± 0.14	1.98 ± 0.14	2.1 ± 0.13
	Acetoacetate	3.2 ± 0.23	3.3 ± 0.11	3.5 ± 0.2	3.5 ± 0.12	3.4 ± 0.1
	β-Hydroxybutyrate	0.59 ± 0.08	0.59 ± 0.08	0.61 ± 0.07	0.61 ± 0.07	0.59 ± 0.05
n-Butyrate	—	5	4	3	2	1
2-Bromobutyrate	—	—	1	2	3	4
	$^{14}CO_2$	—	2.08 ± 0.21	2.03 ± 0.1	1.83 ± 0.02	1.43 ± 0.07
	Acetoacetate	—	3.3 ± 0.16	3.0 ± 0.08	2.6 ± 0.06	1.63 ± 0.05
	β-Hydroxybutyrate	—	0.06 ± 0.08	0.48 ± 0.05	0.43 ± 0.04	0.28 ± 0.04
n-Butyrate	—	5	4	3	2	1
4-Mercaptobutyrate	—	—	1	2	3	4
	$^{14}CO_2$	—	2.17 ± 0.16	2.05 ± 0.13	1.83 ± 0.10	1.82 ± 0.07
	Acetoacetate	—	3.4 ± 0.1	3.00 ± 0.05	2.63 ± 0.07	2.0 ± 0.10
	β-Hydroxybutyrate	—	0.48 ± 0.05	0.43 ± 0.05	0.32 ± 0.02	0.20 ± 0.01

[a]Mean ± SE of four experiments each comprising cells from four colons.

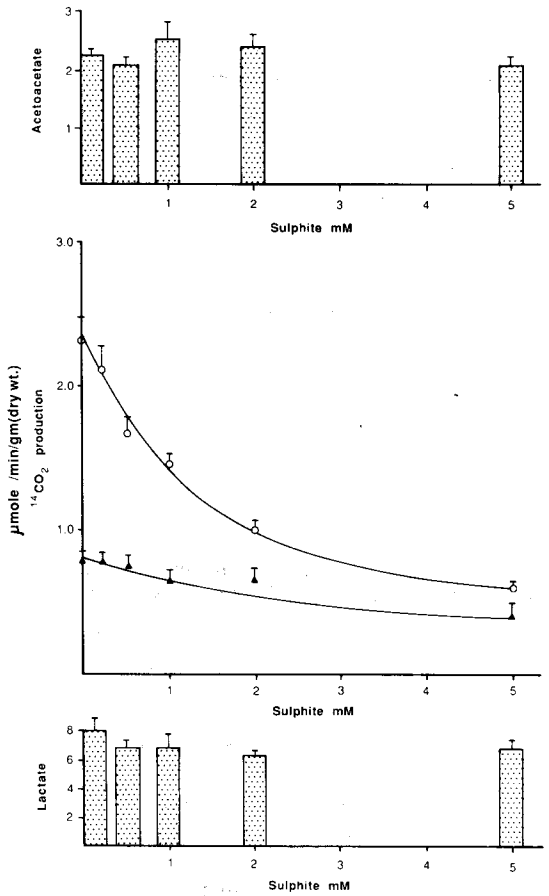

FIG. 3. Effect of varying concentrations of sodium sulfite on oxidative metabolites of glucose and butyrate produced by isolated colonic epithelial cells. Incubation with [1-^{14}C]butyrate (5 mM), (○); or [6-^{14}C]glucose (5 mM), (▲); mean ± SE four experiments each comprising colons from four rats incubated for 40 min.

acid oxidation was altered with 2-bromobutyrate (results not shown).

Effect of sulfite and mercapto fatty acids on fatty acid oxidation. Sodium sulfite reduced fatty acid oxidation markedly compared with glucose oxidation measured by $^{14}CO_2$ generation from [6-^{14}C]glucose in the same batch of cells (Fig. 3). Neither lactate production from glucose nor ketone body production from butyrate was significantly altered by sodium sulfite. At a concentration of 2 mM sodium sulfite, butyrate oxidation was reduced by a mean of 55%, while glucose oxidation was not significantly altered. Mercapto fatty acids were evaluated for their effect on fatty oxidation (Table 6), and were tested when simultaneously incubated or after pre-treatment of cells. The maximal reduction of fatty acid oxidation was observed with 2-mercaptoacetate and 3-mercaptopropionate.

Diminished fatty acid oxidation was generally observed with pre-incubated cells and the addition of sodium nitrite again diminished ketogenesis, as was previously observed with 2-bromobutyrate. Pretreatment with 2-mercaptoacetate for 20 min did not reduce oxidation of [6-^{14}C]glucose, while [1-^{14}C]butyrate oxidation to CO_2 was reduced by 34% with 5 mM mercaptoacetate (Fig. 4). Dichloroacetate had no effect on fatty acid oxidation by colonic epithelial cells (results not shown).

DISCUSSION

The observation that n-butyrate is oxidized more extensively than are longer-chain fatty acids is not unexpected, as previous manometric and enzymatic analyses had shown that n-butyrate was the preferred metabolic fuel of colonic epithelial cells (2–4). In tissue such as liver and heart butyrate is also oxidized more readily than palmitate (26), but butyrate is not abundantly available to these tissues. The present results served

TABLE 6

Effect of Mercapto Fatty Acids on CO_2 and Acetoacetate Production from Butyrate Before and After Pre-Treatment with Mercapto Fatty Acids

Conditions	Simultaneous incubation (μmol/min/g dry wt)		Pre-incubation (20 min) (μmol/min/g dry wt)	
	$^{14}CO_2$	Acetoacetate	$^{14}CO_2$	Acetoacetate
[1-^{14}C]Butyrate (5 mM)	1.84 ± 0.11(10)[a]	2.74 ± 0.12(10)	1.75 ± 0.13(10)	2.00 ± 0.15(10)
+acetate (5 mM)	1.74 ± 0.13(5)	2.26 ± 0.25(5)	1.60 ± 0.13(5)	2.02 ± 0.12(5)
+mercaptoacetate (5 mM)	1.44 ± 0.09(10)	3.57 ± 0.22(10)	0.95 ± 0.07(10)	1.87 ± 0.25(10)
+mercaptoproprionate (5 mM)	1.55 ± 0.13(5)	1.05 ± 0.11(5)	0.83 ± 0.11(5)	0.79 ± 0.05(5)
+mercaptobutyrate (5 mM)	1.75 ± 0.15(10)	1.68 ± 0.15(10)	1.53 ± 0.13(10)	0.83 ± 0.07(5)
[1-^{14}C]Butyrate (5 mM) + nitrite (5 mM)	2.76 ± 0.22(10)	0.60 ± 0.11(10)	2.18 ± 0.15(10)	0.59 ± 0.11(10)
+acetate (5 mM)	2.5 ± 0.21(5)	0.26 ± 0.22(5)	1.94 ± 0.2(5)	0.40 ± 0.08(5)
+mercaptoacetate (5 mM)	2.0 ± 0.14(10)	1.96 ± 0.32(10)	1.29 ± 0.14(10)	1.34 ± 0.21(10)
+mercaptoproprionate (5 mM)	1.72 ± 0.14(5)	0.62 ± 0.2(5)	1.78 ± 0.09(5)	0.78 ± 0.16(5)
+mercaptobutyrate (5 mM)	2.29 ± 0.17(9)	0.94 ± 0.19(9)	1.68 ± 0.13(10)	0.86 ± 0.11(10)

[a]Mean ± SE of number of experiments in brackets. Each experiment comprising colons from four animals.

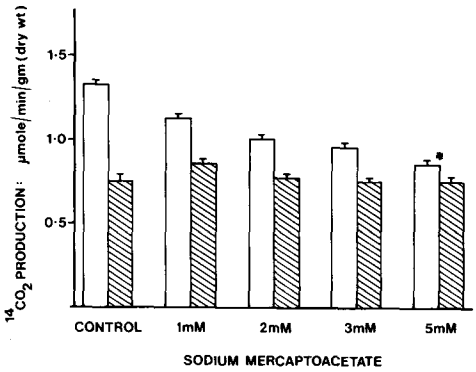

FIG. 4. Oxidation of [1-^{14}C]butyrate (5 mM), (□); and [6-^{14}C]glucose (5 mM), (□); over 40 min by isolated colonic epthelial cells pre-treated for 20 min with sodium mercaptoacetate (5 mM). Mean ± SE of four experiments each experiment with cells from four rats. *p = < .01 Student's paired *t*-test.

to direct attention towards inhibitors acting on short-chain fatty acids (4:0) rather than inhibitors of long chain fatty acid oxidation.

Bromo-substituted fatty acids of varying chain length are powerful inhibitors of fatty acid oxidation, a property investigated both with long-chain and short-chain fatty acids in a number of tissues (16,17,27,28). In general, brominated fatty acids need to be activated in cells to alkylating agents before they become inhibitory to fatty acid oxidation, which may explain why preincubation of cells with bromobutyrate is most effective in inhibiting fatty acid oxidation. Brominated fatty acids act by irreversibly inhibiting general thiolases (16), thus diminishing both CO_2 and ketone body production, which is in keeping with the branch-point action of thiolases on fatty acid oxidation and fatty acid synthesis (29). In epithelial cells not pre-treated with brominated fatty acids, the effectiveness of 2-bromobutyrate as an inhibitor depends upon the ratio of brominated to unbrominated butyrate being greater than 4:1, most likely due to the simultaneous activation of brominated and unbrominated fatty acids. That brominated fatty acids inhibit glucose oxidation to the same degree as butyrate oxidation is in keeping with the general hypoglycaemic effect that bromo-substituted fatty acids exert in intact animals (27,30), and which has prevented the introduction of brominated fatty acids in clinical use.

Nitrites were used in the present study because elevated levels of nitrite are found in the colonic lumen of acute ulcerative colitis but not quiescent disease (20) the source being activated inflammatory cells (18). Nitrites selectively modulate fatty acid oxidation in colonic epithelial cells without altering glucose oxidation (19). The addition of nitrite to 2-bromobutyrate redistributes the carbon of oxidation, but in such a way that ketogenesis is strongly diminished. Ketone bodies are important for synthesis of longer chain fatty acids, particularly in intestinal epithelial cells (31), and may thereby impair cell function.

The selective action of sodium sulfite on fatty acid oxidation without impairing the metabolic activity of the citric acid cycle has not been reported previously. In comprehensive reviews on sulfite toxicity no reference is made to the effect of sulfite on fatty acid oxidation (32,33). In the liver 1 mM sulfite stimulates oxygen consumption through the action of sulfite oxidase (34). The level of this enzyme is very low in the intestinal mucosa (35), which may account for the ulcers that sulfite can cause in the upper gastrointestinal tract (36). Sulfite has a theoretical potential to be generated by sulfate-reducing bacteria (37) acting on sulfated mucus or ingested sulfated carageenan, which is known to cause ulcers in the colon of experimental animals (38).

Mercapto fatty acids, both 2-mercaptoacetate acting on isolated hepatic mitochondria (8–11) and 3-mercaptopropionate acting upon mitochondria of the heart (16), powerfully inhibit fatty acid oxidation. The observation is now extended to unfractionated colonocytes as it is difficult to reliably produce coupled mitochondria from isolated colonic epithelial cells (39). The action of both mercapto fatty acids is by a specific action on several thiolases (8,15,40) or by inhibition of acyl-CoA dehydrogenase. In the liver accumulation of intramitochondrial acyl-CoA occurs (17) reflecting sequestration of free CoA. Accumulation of acyl-CoA,

expressed as bound CoA, occurs in colonic epithelial cells of active ulcerative colitis (41) and provides a correlation between inhibition of fatty acid oxidation and manifestation of active disease.

In overview, observations show that at least 30-50% inhibition of fatty acid oxidation occurs with mercapto fatty acids and sulfite without significantly affecting glucose oxidation in colonic epithelial cells. These observations correlate with the early phases of ulcerative colitis (1), and the present observations lend support to the view that regulation of fatty acid oxidation may be a central factor in the development of colitis.

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Metabolic Fate of Sphingomyelin of High-Density Lipoprotein in Rat Plasma

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The metabolic fate of high density lipoprotein (HDL) sphingomyelin in plasma was studied in rats over a 24-hr period after injection of HDL containing sphingomyelin which was ^{14}C-labeled in the stearic (18:0) or lignoceric acid (24:0) moiety and ^{3}H-labeled in the choline methyl groups. Decay of label in plasma followed three phases. The first two phases were similar for both isotopes and both types of sphingomyelin ($t_{1/2} \cong 10$ and 110 min). However, during the third phase (from 10 hr after injection), ^{3}H label disappeared more slowly than ^{14}C label from 18:0 sphingomyelin, whereas the $^{3}H/^{14}C$ ratio remained relatively constant when 24:0 sphingomyelin was used. Intact, doubly-labeled 18:0 sphingomyelin disappeared from HDL rapidly ($t_{1/2} = 38$ min) by tissue uptake and by transfer to very low density lipoprotein (VLDL). VLDL contained up to 12% of the sphingomyelin 1 hr after injection. This is the first demonstration of a transfer *in vivo* of sphingomyelin from HDL to VLDL. A similarly rapid transfer was also observed *in vitro*. Some nontritiated, [^{14}C]18:0 or [^{14}C]24:0 sphingomyelin was redistributed more slowly into HDL. Doubly-labeled phosphatidylcholine appeared in VLDL and HDL within 1 hr after injection and reached 1.8 and 2.1% of the injected ^{14}C and ^{3}H in VLDL at 1 hr, and 4.8 and 6.9% in HDL at 3 hr, respectively.

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Research on high density lipoprotein (HDL) metabolism has been stimulated because of the putative role of this lipoprotein in preventing the premature development of atherosclerosis. HDL renewal has been studied widely by following labeled apoproteins associated with HDL, but the time course of HDL removal from plasma is poorly documented. Furthermore, studies concerning the lipid moiety of HDL have been focused mainly on the fate of cholesterol in relation to reverse cholesterol transport and lecithin:cholesterol acyltransferase (LCAT) action (1,2). When the decay of radioactivity in rat plasma was determined for HDL simultaneously by labeling the cholesterol ester or ether and the apoproteins, removal of the lipid moiety from plasma was found to be faster than that of the apoprotein (3,4). The half-life of rat HDL phospholipids (0.7 hr) was examined by Van't Hooft *et al.* (5) and it was found to be much shorter than that of rat HDL apoproteins (10 hr), as commonly determined (1). Similar conclusions have been suggested by Bentejac *et al.* (6) using a phospholipid not involved in LCAT action, i.e., sphingomyelin.

In the present study, the time course of total plasma radioactivity in rats injected with HDL ([^{14}C]18:0, [*methyl*-^{3}H]choline)sphingomyelin showed a complex pattern and apparently followed three exponential phases. This complexity could be attributed to HDL cell uptake, intestine and liver delivery of lipoproteins, in addition to transfer or exchanges between lipoprotein fractions. In order to understand the mechanisms involved in the metabolic fate of HDL sphingomyelin, we followed the decay of total plasma radioactivity in rats injected with doubly-labeled HDL 18:0 sphingomyelin under experimental conditions slightly different from our earlier investigation and studied the time course of labeling of the main lipid classes in lipoprotein fractions for 24 hr. Parallel studies were conducted using HDL containing ([^{14}C]24:0,[*methyl*-^{3}H]choline)sphingomyelin to compare a fatty acid (24:0) specific of sphingomyelin with an ubiquitous fatty acid (18:0), both of which are present in similar proportions in rat HDL sphingomyelin, i.e. in 14.9 3.2 and 11.8 ± 1.2 mol% abundance for 18:0 and 24:0, respectively (Table 1). Experiments with tritiated bovine brain sphingomyelin (a natural mixture of sphingomyelins containing stearic and lignoceric acids along with other fatty acids) were also performed. An *in vitro* study was done to specifically examine sphingomyelin transfer from HDL to very low density lipoprotein (VLDL).

MATERIAL AND METHODS

Chemicals. [^{3}H]Methyl iodide (10 Ci/mmol) and [1-^{14}C]-18:0 fatty acid (60 mCi/mmol) were purchased from the Radiochemical Centre (Amersham, U.K.), and [1-^{14}C]24:0 (47 mCi/mmol) from Commissariat à l'Energie Atomique (Saclay, France). Bovine brain and erythrocyte sphingomyelins were obtained from Sigma Chemical Company (St. Louis, MO).

Preparation of HDL sphingomyelin. [*Methyl*-^{3}H]choline-labeled bovine brain sphingomyelin (650 mCi/mmol) was prepared according to the method of Stoffel (7). ([^{14}C]18:0 or [^{14}C]24:0, [*Methyl*-^{3}H]choline)sphingomyelin was synthesized from tritiated bovine brain sphingomyelin as previously described (6). The specific radioactivities of the final products were similar for both doubly-labeled sphingomyelins, i.e., about 125 and 50 mCi/mmol for ^{3}H and ^{14}C, respectively. Doubly-labeled 24:0 sphingomyelin was diluted with unlabeled erythrocyte sphingomyelin (8 mol% of 18:0 and 42 mol% of 24:0). The radioactivity of the diluted, doubly-labeled 24:0 sphingomyelin was equal to 10.1 and 4.1 nCi/nmol for ^{3}H and ^{14}C, respectively. Tritiated bovine brain sphingomyelin and doubly-labeled 18:0 sphingomyelin were diluted in the same way with unlabeled bovine brain sphingomyelin (50 mol% of 18:0 and 9 mol% of 24:0). Transfer to rat plasma lipoproteins and the separation and purification of labeled HDL were performed according to the procedure of Bentejac *et al.* (6).

Animals. Male Sprague-Dawley rats (Iffa-Credo, L'Arbresle, France) were fed *ad libitum* with a low-fat standard diet (UAR, Villemoisson, France) containing 73.7 (wt%) carbohydrates, 21.6 proteins and 4.7 lipids. Linoleic

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Abbreviations: HDL, high-density lipoprotein; LCAT, lecithin: cholesterol acyltransferase; LDL, low density lipoprotein; VLDL, very low density lipoprotein.

TABLE 1

Fatty Acid Composition of Lipid Classes in HDL and VLDL of Rat Plasma[a]

Fatty acid	HDL			VLDL		
	LN[b]	PL[c]	SPM[d]	LN	PL	SPM
			Mol % of total fatty acid			
16:0	15.1 ± 2.1	27.8 ± 0.8	37.4 ± 4.6	30.6 ± 0.7	24.6 ± 1.7	30.0 ± 3.3
16:1	2.6 ± 0.4	1.5 ± 0.8	2.8 ± 0.4	3.5 ± 0.2		
18:0	8.0 ± 2.6	19.4 ± 1.2	14.9 ± 3.2	4.2 ± 0.4	21.1 ± 1.2	23.0 ± 7.3
18:1	11.2 ± 1.8	9.3 ± 2.3	11.1 ± 2.9	19.3 ± 0.4	5.5 ± 0.8	3.4 ± 0.9
18:2	12.0 ± 2.5	15.8 ± 2.5	4.6 ± 0.6	31.5 ± 0.7	20.2 ± 1.4	2.9 ± 0.7
18:3	0.5 ± 0.2			1.2 ± 0.2		
20:0	0.2 ± 0.06		1.3 ± 0.2	0.2 ± 0.1		2.5 ± 0.3
20:1		0.4 ± 0.0				
20:3		0.7 ± 0.2			0.5 ± 0.1	
20:4	40.6 ± 2.2	20.1 ± 1.9	2.8 ± 0.7	4.5 ± 0.2	21.9 ± 2.8	4.1 ± 1.2
22:0			3.9 ± 0.6			6.1 ± 0.7
22:1			1.1 ± 0.3			3.0 ± 1.1
22:5	2.1 ± 1.2	0.5 ± 0.2		0.6 ± 0.2	1.2 ± 0.2	
22:6	7.7 ± 2.0	4.5 ± 0.8		4.4 ± 0.2	4.9 ± 0.4	
24:0			11.8 ± 1.2			12.8 ± 1.8
24:1			8.3 ± 1.4			12.2 ± 1.8
Total fatty acid (μg/mL plasma)	473.9 ± 17.6	188.4 ± 10.0	16.9 ± 3.4	712.0 ± 48.2	72.6 ± 5.5	8.8 ± 1.1

[a] Plasma was obtained from rats fasted overnight, and the lipoprotein fractions were separated by ultracentrifugation. Lipids from HDL and VLDL were fractionated by high performance liquid chromatography, and fatty acids were analyzed by gas chromatography. The values are means ± SE for six or seven determinations. Each determination was carried out from the pooled plasma of two rats.

[b] LN, neutral lipids.

[c] PL, phospholipids (mostly phosphatidylcholine) other than sphingomyelin.

[d] SPM, sphingomyelin.

acid (54.8 mol%) was the major fatty acid in the dietary lipids. Animals had free access to tap water. A 12-hr light-dark cycle and a temperature of 22°C were maintained in the room. Rats weighing 195–215 g were fasted overnight before the experiments.

Experiments in vivo. On the morning of the experiment, the rats were lightly anesthetized with diethyl ether and injected with 0.2–0.4 mL of phosphate-buffered saline, pH 7.4, containing HDL sphingomyelin into the penis vein. The animals received about 0.8 mg of HDL apoprotein and 120 nmol of ([^{14}C]18:0 or [^{14}C]24:0, [^{3}H]choline)sphingomyelin with an approximately equal amount of the other unlabeled molecular species from brain or erythrocyte sphingomyelin used for dilution. HDL containing tritiated bovine brain sphingomyelin was injected under similar conditions. Decay of total plasma radioactivity was followed for 24 hr at different time points after injection. A few drops (30–100 μL) of blood were collected at the tail tip into a tube containing heparin and transferred into a hematocrit micro-tube and centrifuged. Radioactivity was measured in an aliquot of the total plasma.

A study of lipid classes in lipoprotein fractions at different time points was carried out in animals injected with HDL 18:0 sphingomyelin. The aortic blood was withdrawn from the rats under ether anesthesia 15, 60, 180, 720 and 1440 min after injection. The portal vein was severed, and the liver was washed with 50 mL of chilled saline through the right ventricle. The liver was quickly removed and frozen at −20°C. When animals were injected with HDL 24:0 sphingomyelin, most of them were sacrificed at the 24 hr time point to preserve substrate.

Experiments in vitro. Sphingomyelin transfer from HDL to VLDL was studied *in vitro* by incubating HDL containing tritiated bovine brain sphingomyelin in total rat plasma under experimental conditions similar to those of the *in vivo* study described above. Approximately 30 nmol tritiated sphingomyelin (110 nCi/nmol) corresponding to 0.1 mg of HDL apoprotein was added to 1 mL of total rat plasma and incubated at 37°C in a shaking bath. After different times of incubation, the mixture was chilled to 4°C in cold water. The low temperature was maintained until the radioactivity had been measured for the lipoprotein fractions separated by ultracentrifugation. Tritium was also determined in the lipid classes of each lipoprotein fraction. Sphingomyelin transfer was assessed at 4°C in a similar way for 1 hr. To determine how much VLDL was contaminated by labeled HDL during the procedure, labeled HDL was incubated at 37°C in the presence of saline instead of plasma for 1 hr. After ultracentrifugation, no more than 1.0% of the total radioactivity was found in VLDL and was subtracted from all the results.

Separation of lipoproteins and analysis of lipids. Ultracentrifugation of lipoproteins, extraction and separation of lipids, and determination of radioactivity were carried out as previously described (6). Briefly, plasma lipoproteins (VLDL, HDL and "lipoprotein-free" fraction) were separated by ultracentrifugation, as described by Redgrave *et al.* (8), using a discontinuous KBr/NaCl gradient. Lipids were extracted from lipoprotein fractions and liver according to Folch *et al.* (9). Lipids from the "lipoprotein-free" fraction were extracted in the presence of unlabeled lysophosphatidylcholine as carrier.

Erythrocytes were washed six times with saline and lipids were extracted by the method of Rose and Oklander (10). Total lipids were fractionated by thin-layer chromatography on silica gel G (Merck, Darmstadt, Federal Republic of Germany) with chloroform/methanol/water (65:25:4, v/v/v) as the solvent mixture. Under these chromatographic conditions, ceramides and neutral lipids were not separated. Lipid classes were extracted from silica gel, and radioactivity was counted as previously described (11).

The fatty acid distribution in the lipid classes of the lipoprotein fractions was studied in rats which were fasted overnight, but not injected with labeled HDL sphingomyelin. Total lipids extracted from HDL and VLDL were first fractionated by high performance liquid chromatography using a (4.5 mm i.d. × 40 cm) 5 μm Si-60 column (Merck) and a mixture of acetonitrile/methanol/water (72:18:10, v/v/v) as the eluting solvent at a flow rate of 2 mL/min at room temperature. Under these conditions, three major lipid fractions, namely neutral lipids, phospholipids (including phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, and mostly phosphatidylcholine) and sphingomyelin were completely separated and could be collected. Small quantities of lysophosphatidylcholine which were eluted later were not collected. Pentadecanoic acid was added to lipid samples as internal standard. Fatty acid methyl esters were prepared using methanol/boron trifluoride (12) and analyzed by gas chromatography on a 30 m × 0.4 mm i.d. glass capillary column coated with Carbowax 20M (Applied Science Laboratories, State College, PA) at a constant temperature of 195°C and a nitrogen flow rate of 3 mL/min.

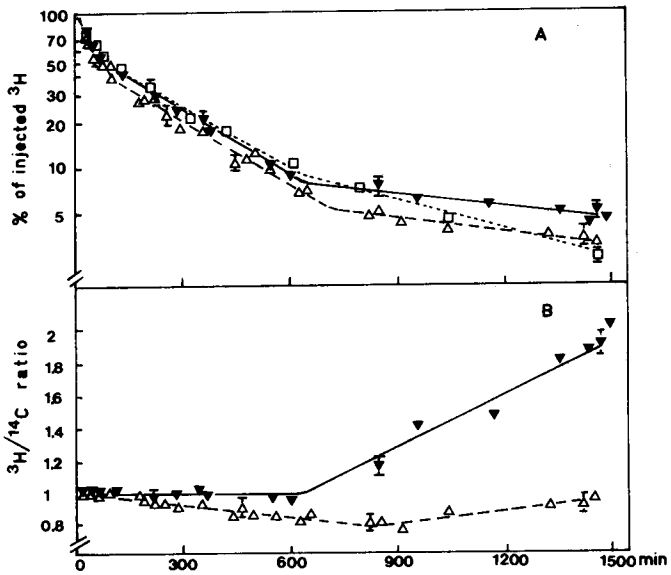

FIG. 1. Plasma radioactivity decay in rats injected with HDL containing ([^{14}C]18:0 or [^{14}C]24:0, [*methyl*-^{3}H]choline)sphingomyelin or ([*methyl*-^{3}H]choline)bovine brain sphingomyelin. ^{3}H and ^{14}C were measured directly in the total plasma obtained from blood collected at the tail tip at different time. A, logarithm of the ^{3}H radioactivity, expressed as % of the injected ^{3}H, using 18:0 (▼) and 24:0 (Δ) doubly-labeled sphingomyelin and tritiated bovine brain sphingomyelin (□); and B, ^{3}H/^{14}C ratio in 18:0 (▼) and 24:0 (△) sphingomyelin experiments. The isotopic ratio of the injected sphingomyelin is taken to be equal to 1. Values are the means ± S.E. (n=2 to 4).

RESULTS AND DISCUSSION

Lipid composition of VLDL and HDL. The fatty acid compositions of lipid classes in the two major lipoprotein fractions of the plasma of rats fasted overnight are given in Table 1. The compositions of sphingomyelin and the other phospholipids (essentially phosphatidylcholine) in HDL were very similar to those found in VLDL, as already shown in human subjects (13). The ratio (by weight) of phosphatidylcholine-to-sphingomyelin, calculated from data in Table 1, was higher in HDL (6.3) than in VLDL (4.6), as previously found in the rat (4.9–8.0 in HDL and 5.0 in VLDL) (14,6) as well as in man (5.1–8.3 in HDL and 2.9–4.0 in VLDL) (15). Very long chain saturated fatty acids were present only in sphingomyelin and the major one was 24:0, while 18:0 was a ubiquitous fatty acid in rat lipoprotein lipids. Similar observations have been made in human lipoproteins (13). Although the molar proportions of 24:0 in sphingomyelin were similar (12%) in HDL and VLDL, twice as much 24:0 was found in HDL (2.75 ± 0.66) than in VLDL (1.37 ± 0.20) when results were expressed as μg fatty acid/mL plasma. However, there was no statistically significant difference in regard to the amount of 18:0 in sphingomyelin between HDL and VLDL, i.e., 2.29 ± 0.16 and 1.98 ± 0.39 μg/mL plasma, respectively. Because the adult rat has a low LDL level and sphingoglycolipids are minor plasma lipids (16,17), 24:0 is mainly carried by HDL sphingomyelin in rat plasma.

Kinetics of the total plasma radioactivity decay. At different times after injection of labeled HDL, radioactivity was measured in the total plasma obtained from a few drops of blood taken from the tail tip. Figure 1A shows that the ^{3}H decay in the total plasma apparently followed three phases for both types of doubly-labeled sphingomyelins. Three-phase curves were also obtained based on ^{14}C values. When the experimental points of isotopic decay were fitted on a model of three-exponential decay using a nonlinear least squares program (18), the estimated $t_{1/2}$ of the first two phases were not statistically different for both isotopes and both types of sphingomyelin (18:0 sphingomyelin: $t_{1/2}$ = 7 ± 3, 8 ± 4 and 121 ± 19, 111 ± 27 min; 24:0 sphingomyelin: $t_{1/2}$ = 12 ± 5, 14 ± 5, and 98 ± 43, 107 ± 34 min for ^{3}H and ^{14}C in the first and second phases, respectively). The results obtained for the total plasma of the rats injected with HDL containing tritiated bovine brain sphingomyelin were similar to those obtained for doubly-labeled 18:0 sphingomyelin. The $t_{1/2}$ values of the third phase were 1150 ± 784 for ^{3}H and 500 ± 200 min for ^{14}C when using 18:0 sphingomyelin, and 560 ± 460 for ^{3}H and 539 ± 500 min for ^{14}C when using 24:0 sphingomyelin. These values apparently corroborate the differences observed between the plasma ^{3}H/^{14}C ratios after 10 hr (Fig. 1B). Fig. 1A shows that during the third phase the ^{3}H decay curve for tritiated bovine brain sphingomyelin was halfway between those drawn for 18:0 and 24:0 sphingomyelins. Interestingly, no difference was observed in the total plasma radioactivity of fed or fasted animals 24 hr after injection of HDL containing tritiated bovine brain sphingomyelin. In the same way, the absence of a

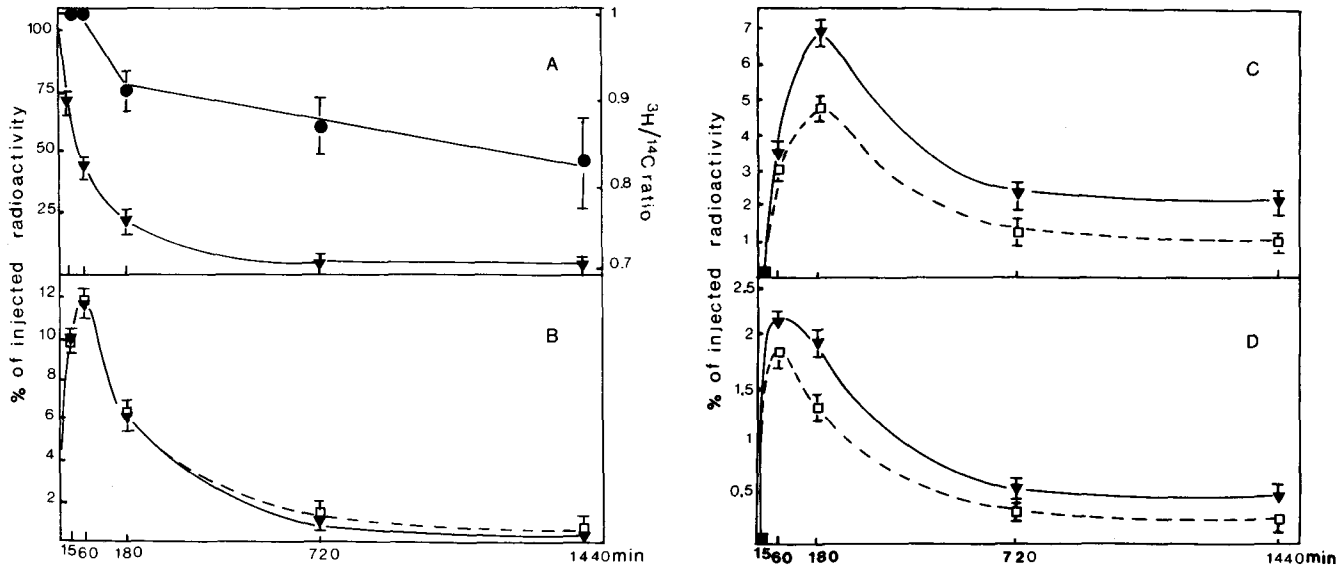

FIG. 2. Time course of the labeling of sphingomyelin and phosphatidylcholine in VLDL and HDL. Rats were injected with HDL containing ([^{14}C]18:0, [*methyl*-^{3}H]choline)sphingomyelin. Plasma lipoprotein fractions were separated by ultracentrifugation. Total lipids extracted from VLDL and HDL were fractionated by thin-layer chromatography. Radioactivity was measured in lipid classes (sphingomyelin and phosphatidylcholine). Results are expressed as % of the injected radioactivity. Values are the means ± SE (n=2 to 4). A, Time course of the labeling of sphingomyelin in HDL; ^{3}H, ▼; and ^{3}H/^{14}C ratio, ●. The isotopic ratio of the injected sphingomyelin is taken to be equal to 1. B, Time course of the labeling of sphingomyelin in VLDL; ^{3}H, ▼; ^{14}C, □; C, time course of the labeling of phosphatidylcholine in HDL; ^{3}H, ▼; ^{14}C, □; D, time course of the labeling of phosphatidylcholine in VLDL; ^{3}H, ▼; and ^{14}C, □.

difference has also been observed in the removal of ^{125}I-apoE HDL injected into fed and fasted animals (19). The $t_{1/2}$ values of the three exponential phases obtained after injection of doubly-labeled 18:0 sphingomyelin HDL were in the same order as those found in our first experiments (12.9 ± 0.3 and 12.8 ± 0.5 min in the first phase, 98 ± 13 and 105 ± 16 min in the second phase, and 862 ± 44 and 502 ± 31 min in the third phase for ^{3}H and ^{14}C, respectively) (6). The small differences observed may be due to the fact that in the previous studies lower amounts of HDL doubly-labeled 18:0 sphingomyelin and apoproteins had been injected into heavier rats. Furthermore, the nonlinear least squares program of Yamaoka *et al.* (18) had not been used for fitting of the experimental points. In the present work, the standard deviations of the $t_{1/2}$ estimations in the third phase were high, although those of the experimental values at the longest time points were very low. As a result, the fitting model used is not appropriate to describe the third phase of isotopic decay. That is also true for the first two phases, because the radioactivity recovered in the lipid classes of the lipoprotein fractions after injection of HDL 18:0 sphingomyelin (Fig. 2) indicates that complex events take place.

Kinetics of the HDL sphingomyelin removal. Rats injected with HDL containing doubly-labeled 18:0 sphingomyelin were exsanguinated at five different time points (from 0.25 to 24 hr) and plasma lipoproteins were separated by ultracentrifugation. The lipid classes of each lipoprotein fraction were fractionated by thin-layer chromatography and radioactivity was measured. Fig. 2A shows a fast, apparently exponential decrease of doubly-labeled 18:0 sphingomyelin in the plasma HDL fraction ($t_{1/2}$ = 38 min for both isotopes). During the first hour, Fig. 2A probably represents the real decay curve of HDL 18:0 sphingomyelin since the ^{3}H/^{14}C ratio remained equal to that of the injected sphingomyelin. Afterwards, there was redistribution of nontritiated, [^{14}C]18:0 sphingomyelin since the isotopic ratio decreased after 1 hr. The $t_{1/2}$ of HDL 18:0 sphingomyelin is not different from that (0.71 ± 0.03 hr) reported for [^{32}P]HDL phospholipids in total rat plasma (5), which mainly reflected removal of phosphatidylcholine involved in lecithin:cholesterol acyltransferase (LCAT) action. However, it is shorter than the $t_{1/2}$ of the total plasma radioactivity (100–110 min for both isotopes) first determined graphically from the decay curve shown in Fig. 1A based on results obtained after injection of HDL 18:0 sphingomyelin. The total plasma radioactivity decay, therefore, is not a simple reflection of the clearance of HDL 18:0 sphingomyelin.

The $t_{1/2}$ (38 min) of doubly-labeled 18:0 sphingomyelin in the plasma HDL fraction, and that (approximately 1.5 hr) of the total plasma radioactivity determined graphically in Fig. 1A for the three kinds of injected sphingomyelin as well, are shorter than those (8.5–11.5 hr) of the apoproteins first determined by Roheim *et al.* (20,21) and Eisenberg *et al.* (22) from apparently monoexponential decay curves after injection of rat ^{125}I-labeled HDL into rats. However, in more recent works (4,19,23–30), multiexponential decay curves were generally obtained under different experimental conditions. In this case, the $t_{1/2}$ determined graphically from the slowest phase was considered to be the half-life of rat HDL apoproteins and values not far from 10 hr were also obtained, even when apoproteins from rat HDL were injected in a free form (27,31). All these results suggest that HDL sphingomyelin (even that containing very long chain fatty acids, such as 24:0) is cleared faster than the apoprotein moiety. A parallel can be drawn between this fast HDL phospholipid removal and the dissociation of tissue uptake of free

TABLE 2

Radioactivity Distribution in Lipid Classes of Plasma Lipoprotein Fractions[a]

Lipoprotein fraction	Lipid class[b]	18:0-sphingomyelin			24:0-sphingomyelin		
		^3H	^{14}C	$^3H/^{14}C$	^3H	^{14}C	$^3H/^{14}C$
VLDL	SPM	0.38 ± 0.05	0.51 ± 0.06	0.74 ± 0.01	0.11 ± 0.02	0.48 ± 0.08	0.23 ± 0.02
	PC	0.49 ± 0.05	0.30 ± 0.07	1.64 ± 0.02	0.19 ± 0.02	0.07 ± 0.04	2.71 ± 1.20
	PE	—	0.03 ± 0.01	—	—	—	—
	NL+Ce	—	0.10 ± 0.02	—	—	0.32 ± 0.10	—
HDL	SPM	1.00 ± 0.01	1.20 ± 0.10	0.84 ± 0.06	1.17 ± 0.08	2.00 ± 0.19	0.58 ± 0.01
	PC	2.21 ± 0.15	1.10 ± 0.15	2.00 ± 0.20	1.07 ± 0.18	0.47 ± 0.17	2.42 ± 0.50
	PE	—	0.03 ± 0.01	—	—	—	—
	NL+Ce	—	0.30 ± 0.02	—	—	0.28 ± 0.06	—
"Lipoprotein-free" fraction	LysoPC	0.96 ± 0.06	—	—	0.21 ± 0.01	—	—
	NL+Ce	—	—	—	—	0.17 ± 0.01	—

[a] Plasma was obtained from rats 24 hr after injection with HDL containing doubly-labeled 18:0 or 24:0 sphingomyelin. Lipoprotein fractions were separated by ultracentrifugation, and total lipids were fractionated by thin-layer chromatography. ^3H and ^{14}C were counted in each lipid class. Data are percentages of injected radioactivity. The $^3H/^{14}C$ ratio of the injected dose is taken as 1. Values are means ± S.E. (n=3).

[b] SPM, sphingomyelin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; NL+Ce, neutral lipids+ceramide; LysoPC, lysophosphatidylcholine.

(32) and esterified (3,33-37) cholesterol from that of apoprotein of plasma HDL observed in the rat, a species with a small low-density lipoprotein content.

Plasma transfer of sphingomyelin. Fig. 2B shows that 18:0 sphingomyelin (10% of the injected dose) was quickly transferred from HDL to VLDL within 15 min after injection and reached a maximum (12%) at 60 min. During this period the isotopic ratio of VLDL sphingomyelin was equal to that of the injected 18:0 sphingomyelin and decreased significantly only after 3 hr. Transfer *in vivo* of doubly-labeled 24:0 sphingomyelin from HDL to VLDL probably occurred too, although it was only assessed at 3 hr and 24 hr (Table 2), because 13% of each injected isotope was found in the VLDL fraction 3 hr after injection. Transfer to (or exchange with) erythrocytes also occurred—0.44 ± 0.14 and 1.05 ± 0.32 of both isotopes injected were recovered in erythrocytes at 15 and 60 min, respectively. A sphingomyelin transfer to erythrocytes from sphingomyelin liposomes has already shown that it can take place *in vivo* (38). However, although sphingomyelin is present primarily in the outer leaflet of red blood cells (39), this transfer remained at a low rate, when compared to the observed transfer to VLDL. Moreover, since 15 min after injection of doubly-labeled 18:0 sphingomyelin HDL, sphingomyelin was the only labeled lipid class in the liver with an isotopic ratio equal to that of the injected molecule (8% of the injected radioactivity), it is likely that the liver did not release measurable amounts of labeled sphingomyelin associated with lipoproteins during this short period. Because the erythrocytes and liver did not intervene in a significant way, the appearance of radioactivity in VLDL sphingomyelin therefore appears to be the result of a plasma transfer.

When tritiated bovine brain sphingomyelin associated with rat HDL was incubated in the presence of total rat plasma, a rapid sphingomyelin transfer from HDL to VLDL was observed (Fig. 3). The transfer equalled 3.5% of the total ^3H after an incubation period of 5 min, then increased throughout the period studies, and reached

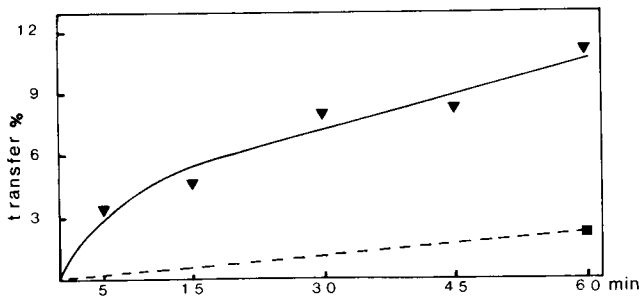

FIG. 3. *In vitro* transfer of sphingomyelin from HDL to VLDL. Rat HDL containing tritiated bovine brain sphingomyelin was incubated in the presence of total rat plasma at 37°C (▼) or 4°C (■). Lipoprotein fractions were separated by ultracentrifugation, and radioactivity was measured in each fraction. The time course of ^3H in VLDL is expressed as % of the total incubated ^3H.

11.3% at 1 hr. No transfer into the "lipoprotein-free" fraction was observed at any time since the radioactivity in this fraction was not different from that found in the control (0.9%) using saline instead of total plasma. Sphingomyelin was the only labeled lipid class (>98%) in HDL and VLDL, even at 1 hr. The phospholipid transfer from HDL to VLDL we have shown using sphingomyelin is consistent with the observed *in vitro* transfer in total rat plasma described by Rubenstein and Rubinstein (40) using ^{32}P-labeled phospholipids. However, the phospholipid transfer from HDL to VLDL appears to be lower or slower than the reverse transfer from VLDL to HDL examined under similar conditions (40,41).

A sphingomyelin transfer from HDL to VLDL induced by a phospholipid transfer protein (42), rather than being spontaneous, is likely to have occurred in the present study. Approximate values for the total external surface or the total volume of the outer shell of rat lipoprotein particles in a fixed volume of plasma can be calculated, assuming that the width of the envelope is 20 Å (1) and using the mean diameters of 433 and 110 Å (15), the mean

concentrations of 0.67 and 1.42 mg lipoprotein/mL plasma (43), and the densities of 0.965 and 1.098 for VLDL and HDL, respectively. The ratios of the surface and outer shell volume of the HDL particles to those of the VLDL particles reach 73.5 and 54.9, respectively. Assuming that sphingomyelin is in the outer shell and not in the core of the lipoproteins (44), these ratios are apparently not in favor of a high, spontaneous transfer of sphingomyelin from HDL to VLDL, since the sphingomyelin concentration in HDL was found to be only twice as high as in VLDL (Table 1), even if apoproteins are assumed to occupy a large part of the HDL surface (2). These considerations suggest that the importance and rapidity of the sphingomyelin transfer observed *in vivo* are probably due to the action of a plasma phospholipid transfer protein. This hypothesis is reinforced by the fact that the *in vitro* sphingomyelin transfer was much higher at 37°C than at 4°C (Fig. 3). Transfer proteins have been characterized in human plasma (45–48). Moreover, it is likely that exchange rather net transfer predominantly occurred since exchanges of phospholipids, including sphingomyelin between HDL and LDL (49) or VLDL and HDL (40,41), have been observed *in vitro*. Finally, changes in the composition of the HDL injected could affect the magnitude of the sphingomyelin transfer from HDL to VLDL. In the present work, the preparation of HDL *in vitro* carrying labeled 18:0 or 24:0 sphingomyelin and unlabeled bovine brain or erythrocyte sphingomyelin used for dilution involved about a four-fold increase in the concentration of the total sphingomyelin molecules (6). Decreased fluidity of the HDL membrane resulted from these changes. The fact that sphingomyelin imparts additional stability to lipoproteins, as shown, for example, in recombinant lipoproteins formed between apoprotein A-I and bovine brain sphingomyelin (50), could reduce transfer or exchange. Conversely, acceleration in the transfer may have occurred to maintain a certain degree of membrane fluidity in the HDL injected (13). However, a rapid equilibrium between labeled HDL and native plasma HDL can take place by transfer of surplus sphingomyelin. The extensive and rapid sphingomyelin transfer observed in the present work is, therefore, likely to occur normally *in vivo*.

Involvement of the liver in the labeling of lipoprotein lipids. Figures 2C and D show that, when doubly-labeled 18:0 sphingomyelin was used, doubly-labeled phosphatidylcholine was not detected in the plasma lipoproteins at 15 min, but quickly appeared thereafter, and a maximum was reached at 60 min in VLDL and at 180 min in HDL (2.1 and 6.9% of the injected ^{3}H, respectively). The $^{3}H/^{14}C$ ratios of phosphatidylcholine were similar in VLDL and HDL (1.15 ± 0.10 and 1.45 ± 0.20 at 60 and 180 min, respectively). The ^{3}H and ^{14}C labels were also measured in the lipids extracted from several tissues. The label in total lipids, with a $^{3}H/^{14}C$ ratio lower than that of the injected HDL (Fig. 4), reached a maximum of about 30% of the injected radioactivity in the liver, and only 2–3% in the small intestine, 3–12 hr after injection. The level of the total radioactivity was less than 1% in the other tissues. About half the total radioactivity in the liver was found in doubly-labeled phosphatidylcholine with a $^{3}H/^{14}C$ ratio higher than that of the injected 18:0 sphingomyelin. Since the liver is known to play a major role in the uptake of lipoproteins and in the release of

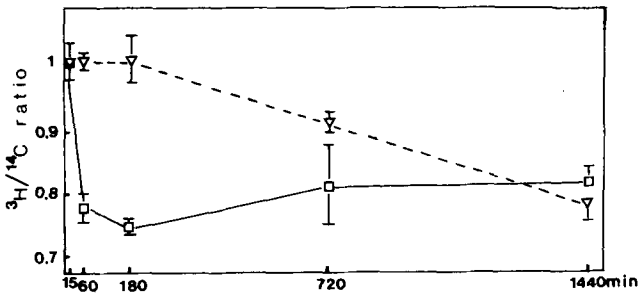

FIG. 4. Time course of the $^{3}H/^{14}C$ ratio of total lipids in the liver. Rats were injected with HDL containing ([^{14}C]18:0 (□) or [^{14}C]24:0 (▽), [*methyl*-^{3}H]choline)sphingomyelin. The isotopic ratio of injected sphingomyelin is taken to be equal to 1. Values are the means ± S.E. (n=2 to 4).

newly-synthesized lipoproteins, a preferential release of ^{3}H by the liver could occur *via* HDL and VLDL phosphatidylcholine, combined with the decrease in the isotopic ratio of total lipids in the liver of the rats injected with HDL 18:0 sphingomyelin after 15 min (Fig. 4).

There was no significant labeling of VLDL and HDL lysophosphatidylcholine. This is surprising because the lysophosphatidylcholine content in plasma lipoproteins is known to be high in the rat (7–22 wt% in total phospholipids) (51), as it is in man (3–15 wt%) (14). Furthermore, in this work the doubly-labeled phosphatidylcholine present in HDL at a high level (up to 7% of the ^{3}H injected) was likely to be utilized by LCAT to produce labeled lysophosphatidylcholine. However, some ^{3}H appeared slowly in lysophosphatidylcholine of the plasma "lipoprotein-free" fraction and reached 0.96 ± 0.06% of the injected dose at 24 hr (Table 2). Several authors have gathered evidence that lysophosphatidylcholine is a major lipid in addition to free fatty acids in the "lipoprotein-free" fraction (14). Because some tritiated lysophosphatidylcholine was detected in the liver (1.7% of the injected ^{3}H at 24 hr), it is suggested that plasma tritiated lysophosphatidylcholine, probably bound to plasma albumin, could be due to secretion by the liver. Indeed, lysophosphatidylcholine secretion by perfused rat liver (52) and cultured rat hepatocytes (53,54) has been observed. Only a small fraction of ^{14}C was redistributed in the "ceramide + neutral lipid" class of the lipoproteins and reached about 1% of the injected dose in VLDL, and in HDL as well, 1–3 hr after injection. ^{14}C radioactivity also appeared in VLDL and HDL phosphatidylethanolamine at 12 hr but remained at a very low level until 24 hr (Table 2). Taken together, the results show that the time course of [^{3}H]choline phospholipids in the plasma is the origin of the increase in $^{3}H/^{14}C$ ratio of total plasma in rats injected with 18:0 sphingomyelin after 10 hr (Fig. 1B).

Comparison of the plasma fate of HDL 18:0 and 24:0 sphingomyelin. When using 24:0 sphingomyelin, the $t_{1/2}$ of the total plasma radioactivity measured on the curve shown in Fig. 1A was slightly, but not significantly, shorter (80–90 min) than that observed when using 18:0 sphingomyelin (100–110 min). It is likely that the uptake by tissue at short times was similar for both types of sphingomyelin. However, Fig. 1B shows that the $^{3}H/^{14}C$ ratio of the total plasma increased only slightly in the third phase (after 10 hr), but always remained lower than 1.0 in the 24:0 sphingomyelin experiments, whereas it

reached 2.0 at 24 hr in the 18:0 sphingomyelin experiments. As far as the liver is concerned, Figure 4 shows that the isotopic ratio of the total lipids was close to 1.0 in rats injected with 24:0 sphingomyelin during the first 3 hours (both ^{3}H and ^{14}C in the total liver lipids reached 25% of the injected dose at 3 hr). Then, the isotopic ratio decreased slowly until 24 hr, although the radioactivity in the total liver lipids remained approximately at the same level (21% and 27% of the injected ^{3}H and ^{14}C, respectively, at 24 hr). In a different way, it decreased rapidly after the first 15 min in the 18:0 sphingomyelin experiments (Fig. 4), as a result of the different levels of the two isotopes in the total lipids (24% and 32% at 3 hr and 15% and 18% at 24 hr for ^{3}H and ^{14}C, respectively, when expressed as % of the injected dose). That suggests that the ^{3}H redistribution from the liver into the plasma, which is likely to occur in the 18:0 sphingomyelin experiments, might be less pronounced or slower for 24:0 sphingomyelin.

The radioactivities recovered in the lipid classes of the lipoprotein fractions and the "lipoprotein-free" fraction 24 hr after injection of HDL 18:0 or 24:0 sphingomyelin are compared in Table 2 and appear fairly similar. As in the 18:0 sphingomyelin experiments, we observed a redistribution of non-tritiated, [^{14}C]24:0 sphingomyelin (because the $^{3}H/^{14}C$ ratio of this lipid class was lower than that of the injected sphingomyelin) and doubly-labeled phosphatidylcholine in VLDL and HDL, and of tritiated lysophosphatidylcholine in the "lipoprotein-free" fraction. However, there were some differences between the two groups of rats. First, in the rats injected with 24:0 sphingomyelin, the $^{3}H/^{14}C$ ratio of VLDL and HDL sphingomyelin was clearly lower than that observed in the rats injected with 18:0 sphingomyelin. Liver could, therefore, utilize more specifically [^{14}C]24:0 for the resynthesis of the sphingomyelin to be secreted. In addition, less tritiated, [^{14}C]24:0 phosphatidylcholine than tritiated, [^{14}C]18:0 phosphatidylcholine was found in VLDL and HDL, and the isotopic ratio was higher for 24:0 phosphatidylcholine than for 18:0 phosphatidylcholine. In a parallel way, the $^{3}H/^{14}C$ ratio of liver phosphatidylcholine 24 hr after injection was much higher (4.8) in the 24:0 sphingomyelin experiments than the ratio (1.5) observed in the 18:0 sphingomyelin experiments. It is possible that the differences observed for phosphatidylcholine in the lipoproteins and liver, and the delay in the ^{3}H redistribution by the liver into the total plasma (Fig. 1B) after injection of 24:0 sphingomyelin as well, are linked to the fact that 24:0 is not a normal fatty acid constituents of phosphatidylcholine.

In conclusion, HDL sphingomyelin is rapidly taken up by tissues, and part of it is transferred to VLDL. Although HDL sphingomyelin is not involved in LCAT action, its renewal is fast and seems, to some extent, to be independent of that of HDL apoproteins. Additional efforts to elucidate the uptake processes and physiological roles of HDL phospholipids are in progress in our laboratory.

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METHODS

A Fluorimetric Method for Measuring Lipase Activity Based on Umbelliferyl Esters

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A new fluorimetric method for measuring lipase activity is described. The fluorescent compound umbelliferone (UMB) was esterified with various unsaturated and saturated fatty acids of different carbon chain lengths, and the non-fluorescent esters were used as substrates for measuring the activity of two lipases purified from *Rhizopus delemar* and *Candida cylindracea*. The fluorescence intensity of UMB liberated by hydrolysis of the substrates gives an indication of the lipase activity towards the respective esters. UMB was found to be somewhat more sensitive and stable than 4-methylumbelliferone (4-MU), which has been used previously. The highly sensitive method (nmoles UMB/mL/hr) described here was tested on five different esters. The method has the advantage of not requiring extraction of the lipolysis products. It also can be carried out quickly, in two to three minutes, and thus lends itself to the screening of lipase-producing micro-organisms. The method offers an interesting alternative or can be complementary to assays based on the use of 4-methylumbelliferone esters as substrates.

Lipids 25, 661–664 (1990).

Microbial lipases can be divided into two classes with respect to their substrate specificity (1). Lipases of the first group are specific to the nature of the fatty acid, while those of the second group are specific to the position of the ester linkage on the glycerol backbone. For example, the lipase of *Penicillium cyclopium* hydrolyzes short-chain triglycerides (2), whereas the lipases of *Aspergillus niger* and *Rhizopus delemar* only hydrolyze the ester linkage in position 1 of triglycerides (3,4).

Numerous methods have been described for measuring lipase activity. The most commonly used methods are based on titration of free fatty acids liberated during hydrolysis (5). However, these methods require the preparation of stable emulsions of substrates and the control of pH during hydrolysis (6). Radioisotope methods have greater sensitivity (7), but require labelled substrates. Various colorimetric methods based on the hydrolysis of esters of *p*-nitrophenol (8,9) or thioesters are also available (10). Fluorescent substrates have been utilized to assay protease (11), phosphatase (12), glycosidase (13), sulfatase and lipase activities (14,15). For example, rhodamine 6G has been useful for measuring lipases (16,17) and esters of C_6NBD for measuring phospholipases (18). A coumarin derivative (4-methylumbelliferone) also has previously been used for assaying lipase activity (19).

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Abbreviations: C_6NBD, [(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-caproyl; 4-MU, 4-methyl umbelliferone; THF, tetrahydrofuran; UMB, umbelliferone.

We describe here a sensitive fluorimetric method for measuring lipase activity using various esters of umbelliferone (7-hydroxycoumarin) and two commercially available lipases produced by *Rhizopus delemar* and *Candida cylindracea.*

MATERIALS AND METHODS

Lipases (EC 3.1.1.3) from *Rhizopus delemar* (Fluka, Buchs, Switzerland; 60 units per mg protein; one unit being equal to the activity that produces one μmole of oleic acid per min at pH 8.0 and *Candida cylindracea* (Sigma Chemical Co., St. Louis, MO; type VII, 500 units per mg protein) were used without further purification.

Chemicals. Umbelliferone, fatty acid chlorides and Triton X-100 were obtained from Sigma. Tetrahydrofuran (THF), pyridine, diethyl ether, and ethylene glycol monomethyl ether and other solvents were of chromatographic grade.

Preparation of umbelliferone esters. Oleic acid chloride (10 mmol) was dissolved in 4 mL of anhydrous pyridine and diluted with 14 mL of anhydrous THF. This solution was added to UMB (10 mmol) in THF (40 mL). The mixture was refluxed in a flask equipped with a magnetic stirrer for 6 hr at 80°C under argon in the dark. After evaporation of THF under vacuum, the residue was taken up in 100 mL of diethyl ether (in the dark under argon). The solution was subjected to three washes with water (15 mL), two washes with 10% HCl (10 mL), two washes with 1 M NaOH (10 mL), and another three washes with water (10 mL). The ether phase was dried over anhydrous magnesium sulfate. After evaporation of the ether under vacuum, the residual powder (70% yield) was kept in the dark under argon. Other fatty acid esters were obtained using the same general procedure. The relative concentrations of UMB and acid chlorides, as well as reaction times, were optimized for highest yields. The esters were recrystallized in boiling ethanol, and their purity was checked by thin-layer chromatography. ^{1}H NMR (nuclear magnetic resonance) spectra and IR spectra of the esters were in agreement with the respective structures (data not shown).

Enzyme assay. The reaction mixture contained 10 μL of a solution in ethylene glycol monomethyl ether of the particular ester at concentrations ranging from 0.1–250 mM in a total volume of 3 mL of Tris-maleate buffer (0.05 M, pH 7.4). The reaction was carried out at 30°C. At time zero, 10 μL of an aqueous solution of the lipase was added to this mixture, and the fluorescence emission (470 nm) was recorded over 2–3 min (SFM 25 spectrofluorimeter, Kontron Instruments, Hermle, Switzerland) at an excitation wavelength (320 nm) corresponding to the

absorption peak of UMB. The wavelengths chosen provide the best response under our pH and temperature conditions.

RESULTS

The fluorescence intensity of free umbelliferone (UMB) was found to be proportional to concentration over the 0–20 μM range tested. The umbelliferyl esters, dissolved in ethylene glycol monomethyl ether, showed identical low levels of fluorescence throughout the pH range within the range of concentrations (9–20 μM). There was a very weak residual fluorescence, which was probably due to the presence of traces of free UMB (Fig. 1). This basal fluorescence did not change during the 15-min incubation period at 30°C.

Under our conditions, hydrolysis of the esters led to a linear increase in fluorescence. Responses for UMB and 4-methyl umbelliferone (4-MU) were compared under optimal conditions for each substrate. [For 4-MU we used the conditions described by Jacks and Kircher (19)]. Both compounds showed essentially the same initial fluorescence which, however, remained stable only in the case of UMB with time (Fig. 2). We obtained the best response with UMB at pH 7.4 (100%) (Fig. 3). There was still a 95% response at pH 7.0, and at pH 6.0 the response was about 60%. The data were confirmed for several concentrations of UMB, ranging from 1.5 to 20 μM (Fig. 3).

Initial hydrolysis rates of umbelliferyl oleate (20 μM) were determined for enzyme concentrations ranging from 0.1 to 100 ng/mL. The hydrolysis rate of the substrate was found to be directly proportional to the enzyme concentration. Identical results were obtained with the other fatty acid esters, including 12:0, 16:0, 18:0, and 18:2.

Dependence of lipase activity upon substrate concentration was established as shown in Figure 4. For umbelliferyl oleate, maximum activity (Vmax) was reached at 20 μM substrate concentration and higher levels (Fig. 4). Maximum rates of hydrolysis were also observed for the other fluorogenic substrates above 20 μM levels.

All subsequent fluorescence measurements were carried out at a substrate concentration of 20 μM. A comparison of the lipase activities towards different fatty acid ester

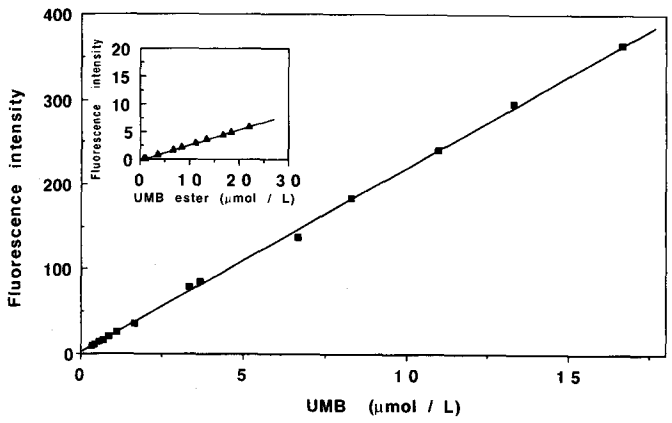

FIG. 1. Calibration of fluorescence intensity as a function of UMB concentration. Inset: fluorescence intensity as a function of UMB-ester concentration (Tris-maleate buffer; 50 mM, pH = 7.4).

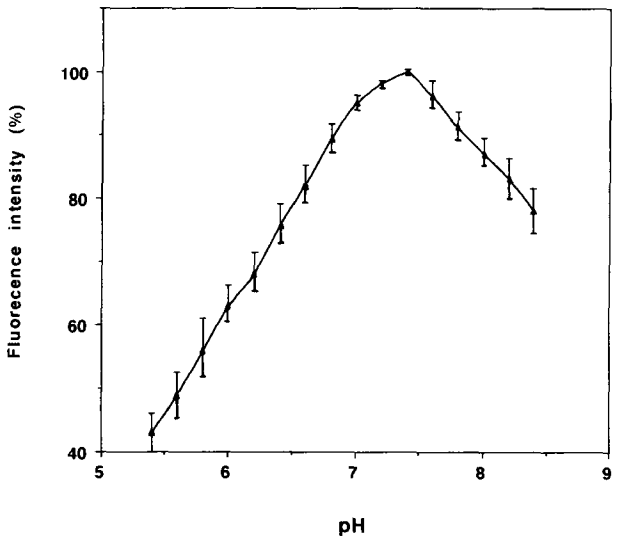

FIG. 3. pH dependence of UMB fluorescence. Several UMB concentrations (0–20 μM) were tested at each pH using 50 mM Tris-maleate buffer (λ_{Exc} = 320 nm; λ_{Em} = 470 nm).

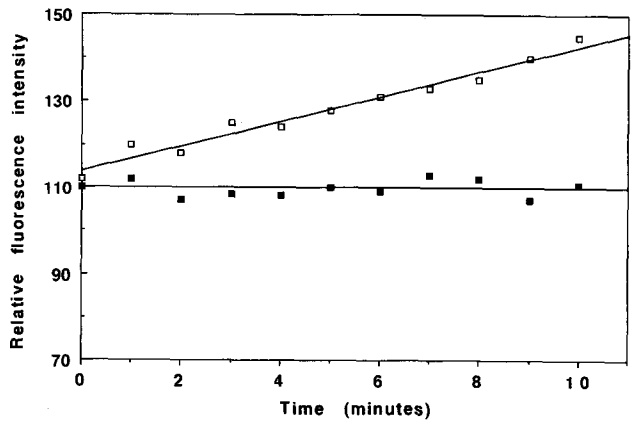

FIG. 2. Stability of UMB (■) and 4-MU (□) as a function of time. UMB (5 μM) and 4-MU (5 μM) were tested under their respective optimal conditions.

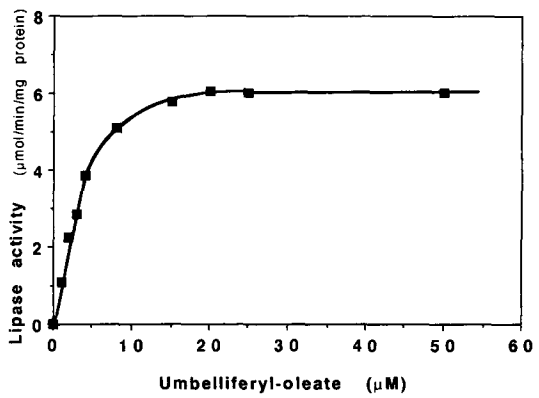

FIG. 4. Effect of different concentrations of substrate on the hydrolysis of UMB oleate. 30 ng of lipase from *Rhizopus delemar* were used.

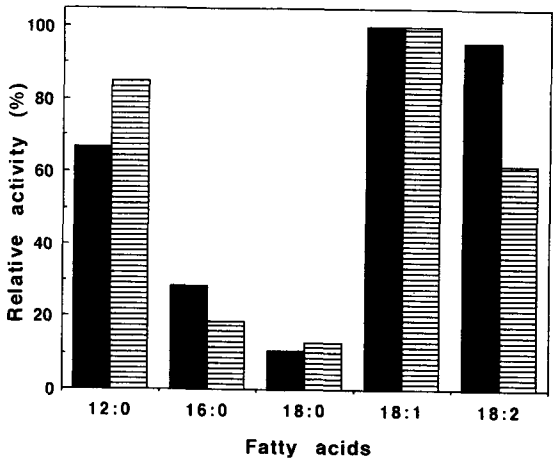

FIG. 5. Comparison of substrate specificity for *Rhizopus delemar* (solid bar) and *Candida cylindracea* (lined bar) lipases. Lipase activities observed for the 18:1 substrate were each taken as 100%.

substrates demonstrated differences in specificities between the lipases from *Rhizopus delemar* and *Candida cylindracea* (Fig. 5). It can be seen that for saturated fatty acids (12:0, 16:0, 18:0) activity increased with decreasing chain length for both lipases. In the C_{18} series, the presence of double bonds seems to enhance apparent lipase activity (6 μmol UMB/min/mg lipase for oleic and linoleic acids). This value is around ten times higher than that observed for the stearic acid ester (Fig. 5). Lipase from *Candida cylindracea* had the highest activity toward the oleic acid (18:1) ester, while lipase from *Rhizopus delemar* showed equally high activity toward both unsaturated fatty acid esters.

DISCUSSION

Goodwin and Kavanagh (20) observed that UMB and 4-MU fluorescence occur at 380 and 634 nm, respectively. We did not detect any significant differences in fluorescence between UMB and 4-MU at time zero. However, 4-MU fluorescence proved unstable and increased over time, while UMB fluorescence remained constant (Fig. 2); thus UMB appears more reliable for assay purposes than 4-MU. In addition, the highest response for UMB was obtained in a pH range lower than that reported (20). Furthermore, the response appears to be less pH dependent, although the maximum (100%) occurs at pH 7.4; activity is still 95% at pH 7.0.

Using 4-MU butyrate, Roberts (21) could detect a lipolytic activity of 10 nmoles/mL/hr. Dooijewaard-Kloosterziel and Wouters (22) used several 4-MU esters and observed a similar sensitivity. With UMB, independent of the fatty acid moiety tested, the lowest lipolytic activity we could detect was 0.3 nmoles/mL/hr. This high sensitivity will be of particular interest when one needs to measure very low levels of lipolytic activity. Fluorimetric methods are about 4,000-times more sensitive than colorimetric methods based on thioesters (23) and about 10,000-times more sensitive than titrimetric methods based on use of a pH Stat (24).

Under optimal conditions for both substrates, hydrolysis rates at the same enzyme concentration were 0.3 μmol/min/mg enzyme with 4-MU oleate, and 2.5 μmol/min/mg enzyme with UMB oleate. The presence of the methyl group may be responsible for the decrease in hydrolysis rates observed with other lipases. In addition to the gain in sensitivity, fluorimetric assays can be carried out in just two to three minutes. In contrast to radioisotope methods, the products of lipolysis also do not have to be extracted and isolated (25). The UMB esters furthermore are very stable and do not suffer spontaneous hydrolysis as has been reported for 4-MU palmitate (22). While 4-MU palmitate emulsions can be unstalbe (22) UMB emulsions remain very stable.

Calcium ions are known to increase lipase activity (21-26) by acting directly on the enzyme (27). They also can reduce the solubility of fatty acids generated during hydrolysis (25), which can be a major problem when using titrimetric methods. Fluorimetric methods avoid this problem. Under our conditions, 30 mM calcium chloride activated the lipase from *Rhizopus delemar*. Furthermore, concentrations as low as 8 μM of Triton X-100, which are known to inhibit lipase activity toward triglycerides (28), led to a 100% inhibition of activity toward our fluorigenic substrates.

Some 4-MU esters are commercially available and are suitable for routine use. But, when the lipase activity to be assayed is weak, UMB esters can be a better alternative. The wide range of UMB esters, which can be synthesized easily, offers a convenient method for the rapid screening of lipases.

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COMMUNICATIONS

Carbohydrate Content of Apolipoprotein B-48 from Rat Chylomicrons of Varying Density

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Monosaccharide composition was determined in apolipoprotein B-48 (apoB) of chylomicrons of rat mesenteric lymph. Chylomicrons were separated into three fractions based on density. Triglyceride and apolipoprotein content were determined in each. ApoB was isolated and quantified using precipitation with isopropanol. Chylomicrons were collected in lymph under normal conditions, and with Poloxalene 2930 when chylomicron secretion was inhibited. Most of the triglyceride was carried in the least dense fraction, while the highest apoB content was in the most dense fraction under normal conditions. Mannose and galactosamine contents of apoB were similar in all fractions while contents of both glucosamine and galactose were highest in the least dense fraction. When chylomicron secretion was inhibited by Poloxalene, the amount of triglyceride recovered in the least dense fraction was significantly reduced. Despite the inhibition of lipid transport in the least dense fraction of chylomicrons by Poloxalene, there was little change in apoB recoveries and in the relative content of various monosaccharides in the apoB from each of the three fractions as compared to results obtained during lipid absorption under normal conditions. In conclusion, carbohydrate composition of apoB of chylomicrons is heterogeneous and varies with chylomicron density.
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Intestinal absorption of lipids is a complex process, with the final step being secretion of absorbed lipid from the enterocytes into lymph in the form of chylomicron particles. Apolipoprotein B-48 (apoB-48) is required for this process. The absolute requirement for apoB-48 for secretion of chylomicrons is evident from the genetic disorder of abetalipoproteinemia (1).

Recently we have been interested in factors that are involved in chylomicron secretion by enterocytes and what determines the size of these particles. Our studies have demonstrated that chylomicron particles of large size contain less apoB-48 than small ones (2).

Just prior to secretion by the enterocytes, chylomicrons pass through the Golgi apparatus (3,4). It is in this subcellular compartment that the addition of terminal sugars to glycoproteins occurs (5). ApoB-48 has a considerable carbohydrate component (6,7). The present study was done to determine whether the carbohydrate component of apoB-48, in addition to the total amount of apoB in the chylomicron particle, is affected by particle size. Experiments were conducted under normal conditions and during treatment with Poloxalene 2930, a hydrophobic surfactant that inhibits secretion of large chylomicrons by the enterocytes (2). We were interested in determining whether the action of the surfactant on chylomicron secretion was correlated with changes in the carbohydrate composition of the apoB-48 component of the chylomicron particles.

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Abbreviations: ApoB, apolipoprotein B-48; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; S_f, Svedberg flotation constant.

MATERIALS AND METHODS

Male Sprague-Dawley rats were prepared with a duodenal cannula for infusion of lipids and a mesenteric lymphatic cannula for collection of intestinal lymph while under diethyl ether anesthesia as described (2). Animals were placed in restraining cages and allowed an overnight recovery period of 16–20 hr, during which time they were infused a glucose-electrolyte solution containing Na_2HPO_4 (6.75 mM), NaH_2PO_4 (16.5 mM), NaCl (115 mM), KCl (4 mM), and glucose (0.28 M) at 3.36 mL/hr and allowed 0.45% saline per os *ad libitum.* They were then infused for the next 10-hr (Period A) at the same rate a lipid emulsion containing triolein (12 mM) cholesterol and phosphatidylcholine (3 mM each), and sodium taurocholate (19 mM) in the glucose electrolyte solution, pH 6.4. Initial 2-hr lymph samples were discarded. During the next 8 hr (Period B), the lipid emulsion was supplemented with Poloxalene 2930 (0.17 mM). The initial 2-hr lymph sample of Period B was discarded. Only rats that maintained a lymph flow rate of at least 2 mL/hr throughout the entire 18-hr period were used. Six animals qualified.

Lymph samples were collected at room temperature in tubes containing preservatives with the following final concentrations: sodium azide, 0.01%; Na_2EDTA, 0.1%; and phenylmethylsulfonyl fluoride, 0.5 mM. Lymph samples in aliquots of 10 mL were transferred to centrifuge tubes, overlayered with 2.5 mL of NaCl (d=1.006), and centrifuged at 25°C (8) in a Beckman SW-41 rotor (Beckman Instruments, Fullerton, CA). Based on flotation characteristics, three density subfractions of chylomicrons were obtained as described (2): fraction I, Svedberg flotation constant (S_f) > 2000, after 15,000 rpm for 54 min; fraction II, S_f 400–2000, after 27,000 rpm for 84 min; and fraction III, S_f 20–400, after 36,000 rpm for 16 hr. Each fraction was washed once by recentrifugation.

Triglyceride content of each chylomicron fraction was determined using enzymatic kits (Sigma Chemical Co., St. Louis, MO). Total apolipoprotein content was determined using a modified Lowry procedure (9). ApoB was precipitated from the sample with 1 mL of 100% isopropanol to 1 mL of sample. The precipitation was completed overnight at 37°C. Samples were next centrifuged at 2400 rpm for 30 min and the supernatant was removed. The supernatant was passed through a 0.22 μm filter and the protein content of the filtrate was determined (9). Protein content of apoB fractions was calculated by subtracting the value of the supernatant from the value for total apolipoprotein. The supernatant was delipidated in 20 vol of ethanol/diethyl ether (3:2, v/v) overnight. The sample was then centrifuged at 2900 rpm for 30 min. The pellet was dried under N_2 and dissolved in Tris-HCl buffer,

10 mM; containing SDS, 100 mM; Na_2EDTA, 0.1%; dithiothreitol, 1.5%; glycerol, 10%; and bromophenol blue, 0.01%; pH 8.2.

Precipitated apoB was washed twice with 2 mL of 50% isopropanol and the lipid component was extracted with 2 mL of 100% isopropanol. The delipidated sample was dried under N_2 and then dissolved in buffer as described above. Various samples of isolated apoB and the supernatant fraction previously described were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a gradient gel of 4% to 22% as described previously (2). Protein standards obtained from Pharmacia Laboratory Separation (Piscataway, NJ) were used to identify various apolipoproteins.

For sugar analysis of apoB, 100 mg samples were hydrolyzed for 40 hr at 105°C with 1 mL of 40% (v/v) Dowex 50 × 2 (H) (200-400 mesh) suspension in 5 mM trifluoroacetic acid (10). The neutral sugars were washed off the resin with methanol/water (1:1, v/v), and after drying they were dissolved in 1 mL of reductive amination mixture (11) and reacted 4 hr at 105°C. After solvent removal over P_2O_5/KOH for 1 week *in vacuo,* glycamines were dissolved in 1 mL 50% ethylene glycol and aliquots analyzed on 0.9 × 55 cm column (Biorad A4) of the amino acid analyzer using orthophthalaldehyde for detection. Hexosamines were eluted from the resin with 2 mL 3M HCl and after vacuum drying they were analyzed on the 0.9 × 17 cm column of the amino acid analyzer (Dionex DC-6A, Dionex, Sunnyvale, CA; 55°C, 70 mL/hr with 0.35 M sodium citrate-0.02 M boric acid, pH 5.2) (12).

Data were analyzed statistically by the paired Student's t-test.

RESULTS AND DISCUSSION

Results for triglyceride and apoliprotein recoveries in various fractions of chylomicrons are presented in Table 1. During infusion of the lipid emulsion under normal conditions (Period A), approximately 60% of the triglyceride recovered in lymph was in the least dense chylomicron fraction (Fr I), while only 10% was in the most dense fraction (Fr III). Patterns for apolipoprotein recoveries in these three fractions were quite different compared to that observed for triglyceride. Recoveries of non-apo B apolipoproteins were quite similar in the three fractions, while apoB recovery was the least in Fr I and the greatest in Fr III. Inhibition of chylomicron secretion by Poloxalene 2930 in Period B resulted in a marked decrease in triglyceride secreton in the large, less dense chylomicrons of Fr I without causing major changes in the smaller, more dense lipoproteins of Fr II and Fr III. Thus the triglyceride contents of the three chylomicron fractions were similar. There were no significant differences of the non-apoB protein content in these three fractions, but the apoB content more than doubled in Fr III as compared to Fr I. Results are in agreement to those previously obtained using a slightly different surface active agent to inhibit chylomicron secretion (2).

Purity of apoB preparations isolated by isopropanol precipitation was analyzed by SDS-PAGE (Fig. 1). Only a single apoB band was observed in the precipitate fraction which was assumed to be apoB-48. The supernatant contained only apolipoproteins of lower molecular weight as compared to apoB. Thus the method for isolation of apoB-48 in lymph samples using isopropanol precipitation appears reliable.

Carbohydrate compositions of apoB in the various chylomicron fractions are presented in Table 2. While mannose and galactosamine contents were fairly similar in the three fractions obtained during both Period A and Period B, the contents of galactose and glucosamine tended to decrease as chylomicron fractions became more dense. For galactose content a significant difference was noted for apoB from Fr I as compared to Fr III. This was observed for both periods of collection. Galactose content for Fr II was intermediate compared to the other two fractions.

This study is the first to describe heterogeneity of the carbohydrate component of apoB-48. Galactosyl residues are added to secretory glycoproteins in the Golgi vesicles (13). Both relatively less dense and relatively more dense chylomicrons are secreted by Golgi (14). The larger the triglyceride component of a chylomicron particle the less dense and larger the particle will become. Factors

TABLE 1

Triglyceride and Apolipoprotein Content of Chylomicrons in Intestinal Lymph[a]

	Triglyceride	Non Apo B μg/rat/hr	Apo B
Period A			
Fr I	12496 ± 6239	89.4 ± 50.6	10.5 ± 4.4
Fr II	6318 ± 1088	67.7 ± 27.0	14.3 ± 7.9
Fr III	1920 ± 893[b,c]	55.6 ± 33.9	17.4 ± 8.9[d]
Period B			
Fr I	3478 ± 3186	47.3 ± 37.3	13.7 ± 8.9
Fr II	4274 ± 3142	72.0 ± 38.5	20.8 ± 9.3
Fr III	2566 ± 425	65.9 ± 26.5	31.5 ± 11.2[d]

[a]Mean ± S.D.

[b]$p < 0.005$, Fr I compared to Fr III.

[c]$p < 0.01$, Fr II compared to Fr III.

[d]$p < 0.05$, Fr I compared to Fr III.

TABLE 2

Carbohydrate Content of ApoB[a]

	Fr I	Fr II	Fr III
	Percentage of apoB by weight		
Period A			
Mannose	2.30 ± 0.59	2.66 ± 1.84	2.36 ± 0.86
Galactose	1.68 ± 0.97	0.92 ± 0.72	0.71 ± 0.81[b]
Glucosamine	2.05 ± 0.76	1.85 ± 0.38	1.21 ± 0.62
Galactosamine	0.06 ± 0.01	0.08 ± 0.02	0.03 ± 0.02
Period B			
Mannose	3.63 ± 3.36	2.05 ± 0.62	2.57 ± 1.52
Galactose	2.33 ± 1.44	0.76 ± 0.38	0.67 ± 0.46[c]
Glucosamine	2.30 ± 0.65	1.19 ± 0.72	1.41 ± 0.47
Galactosamine	0.13 ± 0.02	0.09 ± 0.01	0.03 ± 0.01

[a]Mean ± S.D.

[b]$p < 0.01$, Fr I compared to Fr III.

[c]$p < 0.05$, Fr I compared to Fr III.

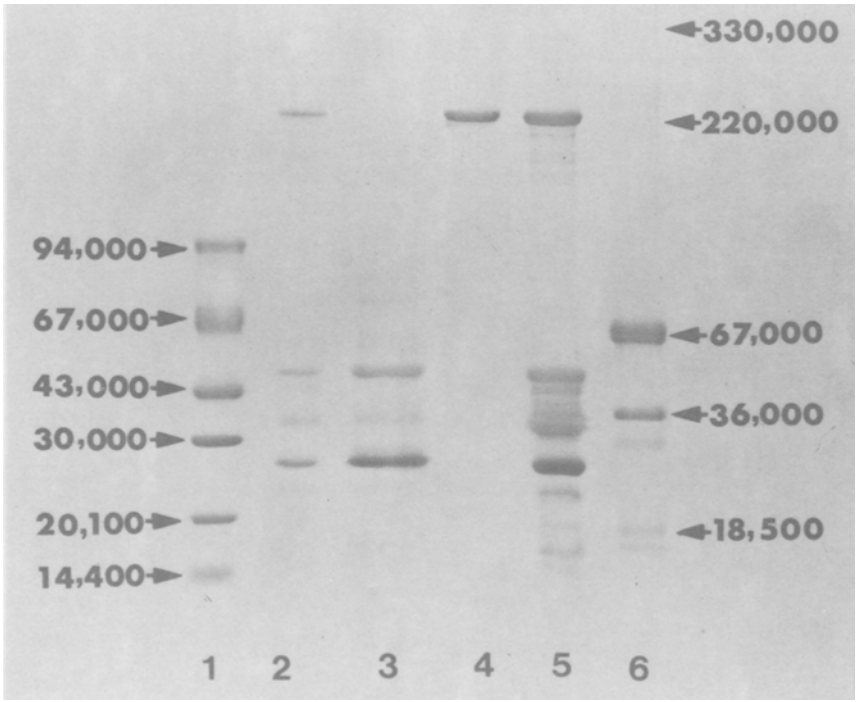

FIG. 1. SDS-PAGE of various preparations of chylomicrons from rat mesenteric lymph. (1) Small molecular weight protein standards of phospholipase B (94,000), albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (21,100), and α-lactalbumin (14,400). (2) Diluted sample of all apolipoproteins of chylomicrons. (3) Apolipoproteins of supernatant fraction after isopropanol precipitation. (4) Apo B fraction obtained from isopropanol precipitation. (5) Undiluted sample of all apolipoproteins of chylomicrons. (6) High molecular weight standards of thyroglobulin (330,000) ferritin, 1/2 unit (220,000), albumin (67,000), lactase (36,000) and ferritin (18,500).

determining the formation of chylomicrons of various sizes remains unknown. The major and consistent protein component of prechylomicrons in the Golgi apparatus, however, is apoB-48 (15), and regardless of the size of the chylomicrons formed its amount per particle remains constant (16).

Treatment with hydrophobic surfactant (Period B) markedly decreased triglyceride content in large, less dense chylomicrons (Fr I), but it did not produce any reduction of apoB recovery nor of the galactose content of the apoB for this fraction. Thus the effects of hydrophobic surfactants on chylomicron secretion do not appear to be mediated by any changes in apoB-48 being produced by the enterocytes during periods of lipid absorption.

The determining factor for the amount of galactose in apoB-48 appears to be the size or relative density of the chylomicron particle containing the apoB-48. The reason for this and any possible physiologic consequences remain unknown. It may be that relatively large chylomicrons take a long period of time to be processed and secreted by Golgi. Addition of increased amounts of galactose to apoB-48 of these chylomicron particles, therefore, may simply reflect a longer period of maturation in the Golgi complex. Thus the moderate increased content of galactose in Fr I during Period B as compared to that of Fr I of Period A may be related to the delay in secretion of large sized chylomicrons resulting from Poloxalene treatment during Period B. The addition of more galactose to apoB, which is located on the outer membrane of the chylomicron particle, would be expected to add to the hydrophilic properties of the chylomicron membrane, which may be helpful in suspending these lipid particles in the aqueous media of lymph and plasma. Regardless of how or why galactose content varies in apoB, it is clear that the carbohydrate content of apoB-48 varies according to the relative density of the chylomicron particle containing the apoB-48.

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Intermembrane Cholesterol Transfer: Role Of Sterol Carrier Proteins and Phosphatidylserine[1]

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The effect of phosphatidylserine and sterol carrier proteins on cholesterol exchange was determined using an assay not requiring separation of donor and acceptor membrane vesicles. Sterol carrier protein-2 (SCP_2, also called nonspecific lipid transfer protein), but not fatty acid binding protein (FABP, also called sterol carrier protein), enhanced the initial rate of sterol exchange between neutral zwitterionic phosphatidylcholine small unilamellar vesicles (SUV) 2.3-fold. Phosphatidylserine at 10 mol% increased the initial rate of spontaneous and of SCP_2-mediated (but not FABP-mediated) sterol exchange by 22% and 44-fold, respectively. The SCP_2 potentiation of sterol transfer was dependent on SCP_2 concentration and on phosphatidylserine concentration. The SCP_2-mediated sterol transfer was inhibited by a variety of cations including KCl, divalent metal ions, and neomycin. The data suggest that SCP_2 increase in activity for sterol transfer may be partly ascribed to charge on the phospholipid.

Lipids 25, 669-674 (1990).

Rat liver sterol carrier protein-2 (SCP_2) (1-3) and fatty acid binding protein (FABP) (4-7) belong to a class of intracellular proteins that bind cholesterol (1,2,4,7) and fluorescent sterol analogues (3,5-7). Because SCP_2 facilitates the *in vitro* intermembrane transfer of many lipid classes [gangliosides (8), neutral glycosphingolipids (8), sphingomyelin (9), phospholipids (9), and cholesterol (1, 9-13)], it has been termed a nonspecific lipid transfer protein. However, some evidence indicates a more specific role for SCP_2 in enhancement of sterol transfer during microsomal conversion of lanosterol to cholesterol (14,15), microsomal cholesterol esterification (16), and adrenal mitochondrial steroidogenesis (1,2,17). Since fatty acid binding protein (FABP) binds not only sterols, but also fatty acids and fatty acyl CoA, it has been termed fatty acid binding protein (7,18-20). It is important to note that in most of the investigations reporting activity for SCP_2 or FABP, this activity was measured by stimulation of an acceptor membrane enzyme activity. Thus, little is actually known about the mechanism of protein carrier-mediated intermembrane cholesterol transfer.

MATERIALS AND METHODS

Materials. 1-Palmitoyl-2-oleoyl phosphatidylcholine (PC), cholesterol, bovine brain L-α-phosphatidyl-L-serine (PS), and neomycin sulfate were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL), Applied Science Laboratories, Inc. (State College, PA), Sigma Chemical Co. (St. Louis, MO), and Sigma Chemical Co., respectively. Dehydroergosterol was synthesized as described earlier (21). Sterols were recrystallized in alcohol, prior to use, and purity was monitored by high performance liquid chromatography (HPLC) (21). SCP_2 was purified from rat liver (14). Prior to use, lyophylized SCP_2 (14,22) was dissolved in distilled water to make a 100 μM stock solution. Unless otherwise specified, the final SCP_2 or FABP concentration in the assay SUV suspension was 1.5 μM. Rat liver FABP was generously provided by Dr. M. E. Dempsey, University of Minnesota, while recombinant rat liver FABP was purified as described elsewhere (23).

Methods. Small unilamellar vesicles (SUV) were prepared as described earlier (24), except for the two following modifications. First, all SUV preparations were sonicated until the suspensions were clear. For control SUV (PC/sterol, 65:35, mole %) and acidic SUV (PC/PS/sterol, 55:10:35, mole %) this required 30 min and 4 min sonication time, respectively. Recovery of phospholipid in SUV (24) was similar for all SUV. Second, the buffer (10 mM PIPES/0.02% NaN_3, pH 7.4) in which SUV were sonicated and redispersed was prefiltered with a 0.2 μm filter (Millipore, Bedford, MA). In each exchange assay, total lipid concentration was near 150 μM (donor/acceptor ratio 1:10). The vesicle composition was 65 mol% phospholipid and 35 mol% sterol (dehydroergosterol in the donor, cholesterol in the acceptor vesicles). Exchange of dehydroergosterol for cholesterol between donor and acceptor membranes was monitored at 24°C in the absence and presence of SCP_2 by adaptation of the method we described previously (25,26). The procedure was adapted to continuous measurement of polarization (3) to obtain 540 data points in 3 hr or less with a Compaq-PC computer [rather than 15-20 data points taken manually as previously done (25,26)]. The excitation source was a 450 watt xenon arc; photobleaching did not occur under the conditions used (24). Inner-filter and light scattering artifacts were negligible due to use of dilute vesicle suspensions (absorbance at the excitation wavelength, 325 nm, was less than 0.1) and placing Janos GG-375 cut-off filters in the emission system.

Use of dehydroergosterol polarization to determine initial rate of molecular sterol exchange. At high concentration, fluorescent molecules such as dehydroergosterol interact to self-quench. This interaction, resulting in radiationless energy transfer, will also decrease fluorescence polarization. Such concentration (c) dependent depolarization fits the following relationship (27):

$$P = p_0/(1 + Bc) \quad [1]$$

where p_0 is a constant defined as the polarization at infinite dilution (zero concentration). $B = p_0 \times \tau \times$

[1]A portion of this work was presented as an abstract: Nemecz, G., Butko, P., and Schroeder, F. (1989) *Biophysical Journal 55,* 137a.

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Abbreviations: DHE, dehydroergosterol; FABP, fatty acid binding protein; HPLC, high performance liquid chromatography; PC, 1-palmitoyl-2-oleoyl phosphatidylcholine; PS, phosphatidylserine, SCP_2, sterol carrier protein$_2$; SUV, small unilamellar vesicles.

constant, where τ is the fluorescence lifetime. Because dehydroergosterol fluorescence lifetime is independent of concentration in an isotropic solvent (28) or a membrane system (24), B can be considered a constant. It is important to note that p_0 and B were calculated from a computed fit to experimentally obtained data from a series of SUV with varying dehydroergosterol but constant 35 mole % total sterol.

Although Equation [1] describes the dependency of P on dehydroergosterol concentration, it does not explain the molecular transfer of dehydroergosterol from donor to acceptor vesicles because the polarization of the mixture of donor and acceptor vesicles contains signal from both donor and acceptor vesicles simultaneously. In addition, polarization P, in contrast to anisotropy r, is not an additive parameter (29; also see Equation [4]). Polarization can be converted to anisotropy according to the following formula:

$$P = 3r/(2 + r) \quad [2]$$

Insertion of Equation [2] into Equation [1] reveals that anisotropy fulfills the same hyperbolic equation as polarization:

$$r = r_0/(1 + B'c) \quad [3]$$

where r_0 is the anisotropy at infinite dilution and the constant B' [B' equals (3/2) $\times$ r_0 $\times$ τ $\times$ constant] relates to B from Equation [1] as $B' = B\ (1 + r_0/2)$.

The anisotropy r of a mixture of donor and acceptor vesicles (with anisotropies r_D and r_A, respectively) is

$$r = f_D r_D + f_A r_A \quad [4]$$

where f_D, f_A are fractional fluorescence intensities (30). Due to the 10-fold excess of acceptor vesicles over donor vesicles:

$$c_D + 10c_A = c_I \quad [5]$$

where c_D, c_A are the dehydroergosterol concentrations in donor and acceptor, respectively, and the constant c_I is the initial concentration in donor [11 times the total concentration of dehydroergosterol in the lipid phase (total donor + acceptor)].Herein, c_I = 35 mol%. The relative amount x_D present in the donor vesicles as a function of time is given as:

$$x_D = c_D/c_I \quad [6]$$

The relative amount present in the acceptor population is

$$x_A = 1 - x_D = 1 - c_D/c_I \quad [7]$$

The dependence of the fluorescence intensity F on the relative concentration of dehydroergosterol in PC vesicles (containing 35 mol% sterol) can be described by an empirical equation obtained by polynomial fitting to the individual steady state polarization measurements:

$$F = 1.7\ x_D - 0.7\ x_D^2 \quad [8]$$

It is important to note that Equation [8] and subsequent mathematical treatment applies to the particular case of 35 mole% total sterol at 24°C and the 10-fold excess of acceptor over donor vesicles. Taking into account the latter fact and Equation [8], the fractional fluorescence intensities f_D and f_A of donor and acceptor vesicle populations, respectively, can be expressed as:

$$f_D = (1.7\ x_D - 0.7\ x_D^2)\ /(1.63 + 0.14\ x_D - 0.77\ x_D^2) \quad [9]$$

$$f_A = 1 - f_D \quad [10]$$

The denominator in Equation [9] appears to be due to the normalization condition $f_D + f_A = 1$, which must be fulfilled at all times. Expressing r_D and r_A by Equation [3], in combination with Equations [4] and [5], we obtain

$$r = r_0\ \{f_D/1 + Dx_D) + f_A/[1 + D(1 - x_D)/10]\} \quad [11]$$

This formula describes anisotropy r of the donor/acceptor mixture as a function of a sole variable, the relative concentration x_D of dehydroergosterol in donor vesicles. D is a new constant, $D = B'c_I$, and f_D and f_A also are functions of x_D (Eq. [8]). The number 10 in Equation [11] appears to be due to the 1:10 donor/acceptor ratio.

By applying the formula in Equation [2] on Equation [11] for many values of x_D, we obtain the dependency of polarization P on dehydroergosterol concentration x_D. The dependency can be described with the following polynomial function:

$$P = -ax_D^2 + bx_D + c \quad [12]$$

where the parameters a = 0.185, b = 0.028, and c = 0.320 for the PC/sterol (65:35) vesicles used herein at 24°C. Thus, the molecular transfer of dehydroergosterol during the exchange process can be calculated from steady state polarization of dehydroergosterol in the donor/acceptor SUV mixture.

Importantly, P changes almost linearly with x_D when x_D is not much less than 1. This means that at the initial stages of exchange, the initial rate of the polarization change $[dP/dt]_{t=0}$ is proportional to the initial rate of the sterol exchange $-[dx_D/dt]_{t=0}$. The proportionality constant can be determined from the slope of the straight line in Figure 1 (see Results and Discussion) and the experimental conditions of the measurements. The 10-fold excess of the acceptor over donor, the values of total lipid (150 μM), total sterol (52.5 μM) and dehydroergosterol concentration (4.77 μM) and the average $[dP/dt]_{t=0}$ = 0.0018 min^{-1} lead to:

$$-[dx_D/dt]_{t=0} = 30\ [dP/dt]_{t=0} \quad [13]$$

where $[dx_D/dt]_{t=0}$ is in nmol $\times$ min^{-1} and $[dP/dt]_{t=0}$ in min^{-1}. The minus sign appears to be due to the fact that polarization P and the dehydroergosterol concentration x_D in donor change in the opposite direction (while P increases, x_D decreases). Initial rates of polarization change were determined from the first 1 min for PC/PS/sterol with SCP_2 and 5 min of the record for all other SUV with or without SCP_2. The accuracy of initial rate measurements was within 5%. It is important to note that initial rates primarily reflect the fast component of the biphasic kinetics of the sterol exchange. The fast component has a rate constant one order of magnitude higher

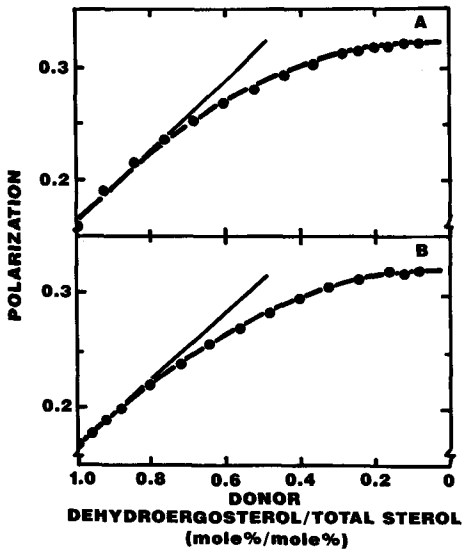

FIG. 1. Polarization of the donor/acceptor SUV mixture as a function of the relative concentration of dehydroergosterol in donor vesicles. The polarization of dehydroergosterol in the donor/acceptor mixture calculated at 24°C was plotted as a function of the ratio of donor dehydroergosterol to donor total sterol (dehydroergosterol plus cholesterol). A, PC/sterol (65:35) SUV; and B, PC/PS/sterol (55:10:35) SUV. The curve represents the best polynomial fit (Eq. [12]) (see Materials and Methods). The straight line indicates that the polarization increase can be considered linear with the decrease in the dehydroergosterol amount in donor vesicles only at the initial stages of the sterol exchange (until about 20% of dehydroergosterol leaves the donor).

than the slow component and it comprises about 10% of the exchangeable pool of sterol (3,25,26).

Turbidity determination (light scattering). Light scattering at 325 nm was measured at 90° in an SLM 4800 spectrofluorometer, with the cut-off filter removed from the emission side.

RESULTS AND DISCUSSION

Earlier we demonstrated that dehydroergosterol (DHE) and [^{3}H]cholesterol exchange kinetics between model membranes were very similar (25,26). The dehydroergosterol exchange process can be visualized as follows. At time zero, all dehydroergosterol molecules are packed in the donor vesicles. They then experience a dehydroergosterol-rich environment, such that dehydroergosterol-dehydroergosterol interactions resulting in self-quenching and energy transfer are quite probable. This results in highly depolarized fluorescence with values near 0.156 ± 0.003 (25). At the initial stages of the exchange, the acceptor vesicles contain extremely low amounts of dehydroergosterol, so that the dehydroergosterol-dehydroergosterol interactions are very rare. The fluorescence signal from acceptor vesicles exhibits high polarization near 0.348 ± 0.005, but low intensity. Under the assay conditions, donor SUV dehydroergosterol is transferred to the acceptor SUV while cholesterol from the acceptor SUV is transferred to the donor SUV in an equimolar manner. As the exchange progresses, dehydroergosterol concentration in donor vesicles decreases, while that in the acceptor vesicles increases. The process continues until equilibrium is reached, which is when all the vesicles contain the same amount of dehydroergosterol, independent of whether they originally were donor or acceptor vesicles. What is effectively observed is a continuous dilution of dehydroergosterol in vesicles, e.g., a smooth increase in the dehydroergosterol fluorescence polarization.

Changes in the initial rate of polarization change are proportional to the number of sterol molecules transfered (Fig. 1). Theoretical justification of the method is presented in Materials and Methods. Polarization change is linear until up to 20% of the donor dehydroergosterol is exchanged between phosphatidylcholine/sterol (65:35) SUV (Fig. 1, A) and between phosphatidylcholine/phosphatidylserine/sterol (55:10:35) SUV (Fig. 1, B). The slopes of the two straight line segments in Figures A and B indicate that the inclusion of 10 mole % phosphatidylserine did not significantly alter the equations derived for exchange between phosphatidylcholine/sterol (65:35) SUV (Materials and Methods).

Changes in polarization of DHE fluorescence upon mixing the donor and acceptor PC SUV in the presence or absence of SCP_2 are shown in Figure 2. The changes in polarization were not due either to instability of the donor SUV (curve 1, Fig. 2) or to addition of SCP_2 to donor alone (curve 2, Fig. 2). In both cases polarization was constant with time. Addition of SCP_2 had no effect on donor polarization for the following reasons. First, the polarization of dehydroergosterol bound to SCP_2 is 0.143 ±0.001, very similar to that in donor SUV 0.156 ± 0.003; second, the single sterol binding site of every SCP_2 is not saturated, since the ratio of SUV dehydroergosterol to SCP_2 is only 3:1 in the donor SUV/SCP_2 mixture. Third, the K_d of dehydroergosterol binding to SCP_2 is between 1 and 2 μM (3). Thus, the SCP_2 sterol binding site would not be expected to be completely saturated under the assay conditions used herein. Spontaneous exchange of dehydroergosterol between PC/sterol SUV (curve 3, Fig. 2) exhibited an initial rate of polarization change of 0.0018 ± 0.0001 min^{-1} (Table 1). SCP_2 increased the rate of polarization change in PC SUV (curve

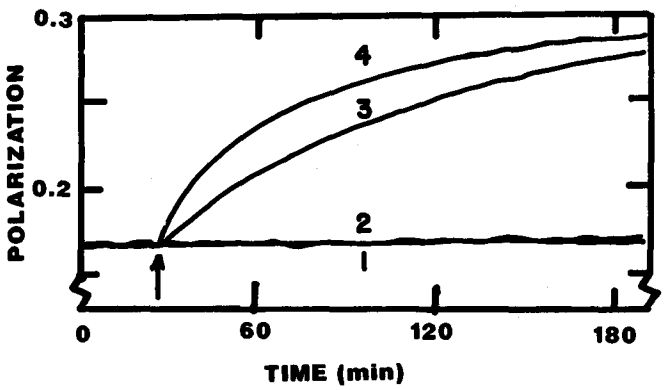

FIG. 2. SCP_2 stimulates dehydroergosterol exchange between palmitoyloleoyl phosphatidylcholine/sterol vesicles. All exchanges were performed at 24°C as described in Materials and Methods. Curve 1, PC/DHE (65:35) SUV, no acceptor SUV, no SCP_2; curve 2, no acceptor SUV, SCP_2; curve 3, spontaneous exchange of sterol between PC/sterol donor and acceptor SUV, no SCP_2; and curve 4, SCP_2-mediated exchange between PC/sterol SUV. SCP_2 (1.5 μM) and/or acceptor SUV (150 μM) were added at the time indicated by an arrow.

TABLE 1

Effect of Ionic Strength on SCP_2 Stimulated Sterol Transfer[a]

Phospholipid	Initial rate of polarization change ($min^{-1} \times min$)			
	Salt	No protein	SCP_2	FABP
Phosphatidylcholine	0	1.9 ± 0.1	4.2 ± 0.1	2.0 ± 0.1
	0.6 M KCl	1.4	2.5	
	1 mM $CaCl_2$	ND	3.2	
Phosphatidylserine	0	2.2 ± 0.1	92.3 ± 4.3	1.5 ± 0.1
	0.6 M KCl	2.2	4.0	
	1 mM $CaCl_2$	1.8	43.3	
	1 mM $MgCl_2$	2.0	47.6	

[a]Phosphatidylcholine and phosphatidylserine denote SUV containing 10 mole % of the respective phospholipid in addition to 55 mole % phosphatidylcholine and 35 mole % sterol. Protein concentration was 1.5 μM. Values represent the mean ± SEM (n = 3-7). KCl, $CaCl_2$, or $MgCl_2$ were included in the exchange buffer when indicated.

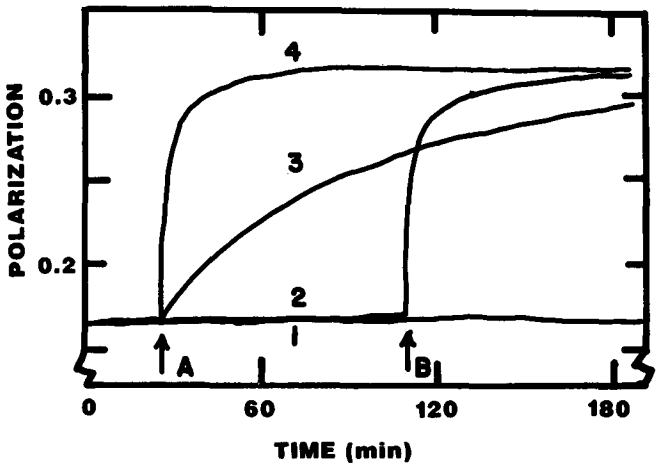

FIG. 3. Phosphatidylserine potentiates effect of SCP_2 on dehydroergosterol exchange between SUV. Curve 1, PC/PS/DHE (55:10:35) SUV, no acceptor SUV, no SCP_2; curve 2, no acceptor SUV, SCP_2; curve 3, spontaneous exchange of sterol between PC/PS/sterol donor and acceptor SUV, no SCP_2; and curve 4, SCP_2-mediated exchange between PC/PS/sterol SUV. SCP_2 1.5 μM) and/or acceptor SUV (150 μM) were added at the time indicated by arrow A (curves 3 and 4) and arrow B (curve 2).

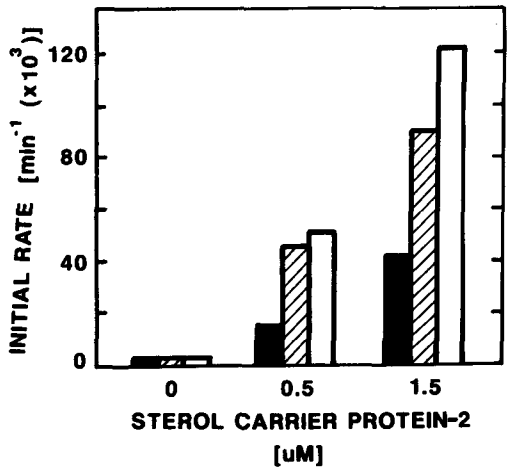

FIG. 4. Effect of SCP_2 and phosphatidylserine concentration on dehydroergosterol exchange between SUV. SCP_2 concentration was varied from 0 to 1.5 μM as indicated in the figure. SUV were comprised of phospholipid/sterol (65:35) with solid bars indicating PC/PS/sterol (60:5:35), cross hatched bars indicating PC/PS/sterol (55:10:35), and open bars indicating PC/PS/sterol (35:30:35). Otherwise, all conditions were as described in legend to Figure 2.

4, Fig. 2) 2.3-fold (Table 1). FABP (Table 1) and recombinant FABP (data not shown) were without effect.

Inclusion of 10 mole % PS did not change dehydroergosterol polarization in donor SUV in the absence (curve 1, Fig. 3) or presence (curve 2, Fig. 3) of SCP_2. PS increased the rate (curve 3, Fig. 3 *vs* curve 3, Fig. 2) of the spontaneous exchange of sterol by 22% as compared to PC SUV (Table 1). More importantly, SCP_2 enhanced the rate of polarization change (curve 4 at A and curve 2 at B, Fig. 3). The extent of this enhancement of the initial rate of sterol exchange was dependent on the concentration of phosphatidylserine in the SUV and on the SCP_2 added to the vesicles (Fig. 4). SCP_2 enhanced initial rate of polarization change by 40-fold between PC/PS/sterol (55:10:35) SUV and 60-fold between PC/PS/sterol (35:30:35) SUV (Fig. 4). In contrast, FABP (Table 1) or recombinant FABP (data not shown) did not enhance the initial rate of polarization change between PS containing SUV.

SCP_2 has a net positive charge at neutral pH, since its isoelectric point is 8.6 (14). PS bears a net negative charge at neutral pH. If electrostatic attraction between the two species accounts for the stimulatory effect of PS on the SCP_2-mediated sterol exchange, then the effect should be suppressed by screening of the charges by ions in the medium. Addition of 0.6 M KCl, 1 mM $CaCl_2$, or 1 mM $MgCl_2$ had little effect on spontaneous sterol exchange between PC or PS containing SUV (Table 1). As indicated by no change in light scattering, these ions did not cause aggregation of either PC or PS containing SUV at the concentrations and under the conditions tested. In the presence of 0.6 M KCl or 1 mM $CaCl_2$, the SCP_2-mediated sterol exchange between PC SUV was inhibited by 24% and 40%, respectively (Table 1). More important, 0.6 M KCl, 1 mM $CaCl_2$, and 1 mM $MgCl_2$ inhibited SCP_2-mediated sterol exchange between PS containing SUV by 96%, 53%, and 48%, respectively (Table 1).

Likewise, at μM concentrations the polycation neomycin also inhibited both spontaneous (Fig. 5, insert) and SCP_2-mediated (Fig. 5) sterol transfer between PS containing SUV. However, the degree of inhibition of the SCP_2-mediated sterol transfer was several orders of magnitude greater than for spontaneous sterol transfer.

From these data, the three following conclusions may be proposed. First, the ability of sterol carrier proteins to bind sterols does not necessarily allow conclusion that they enhance sterol transfer by acting as sterol carriers. SCP_2 and FABP both bind sterols (1-7) and stimulate microsomal enzymes utilizing sterol substrates (1,2, 14-20). However, only SCP_2 stimulates transfer of sterol from donor to acceptor membranes while FABP does not. Thus, sterol binding and sterol transfer enhancement by these proteins appear to be separate functions. Merely binding cholesterol is not sufficient to make a protein a sterol transfer protein. Perhaps binding of protein and cholesterol may not be relevant to the mechanism of transfer. It is certainly possible for a protein to interact with a membrane to enhance the desorption rate of sterol from the membrane without acting as a sterol carrier *per se*. The desorption rate is the rate limiting step in spontaneous sterol transfer (31). Alternately, the binding of sterol may function to deliver sterol to specific enzymes without actually enhancing intermembrane sterol transfer. The latter possibility must certainly be considered in view of the observation that another lipid binding protein, interphotoreceptor retinol binding protein, does in fact bind retinol but, surprisingly, inhibits transfer of retinol between membranes (32). Second, SCP_2 may be much more specific in its function than previously believed. SCP_2 enhances intermembrane transfer of a variety of ligands several fold (1,8-13). However, even in membranes containing acidic phospholipids the transfer of phospholipids was stimulated only 2-7-fold between model membranes (13,33). As shown in Table 1, SCP_2 stimulated sterol transfer between acidic phospholipid (PS) containing vesicles much more than between neutral zwitterionic (PC) containing vesicles (2.3- *vs* 44-fold). This observation is supported by 18-fold enhancement of sterol transfer by SCP_2 in acidic phospholipid containing monolayer membranes (13). Third, SCP_2 may facilitate transfer of sterols by interaction with membranes. This possibility is supported by the inhibition of SCP_2 mediated sterol transfer in acidic PS containing SUV by high salt and by neomycin. Other investigators have shown direct interaction of SCP_2 with acidic phospholipid containing monolayer membranes (13) and mitochondria (34). High salt concentration also inhibited protein mediated phosphatidylcholine transfer between membranes (35).

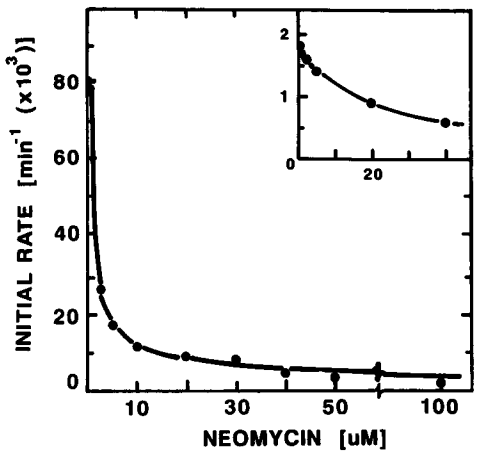

FIG. 5. Inhibition of spontaneous and SCP_2-mediated dehydroergosterol exchange by the polycationic antibiotic, neomycin. SUV were composed of PC/PS/sterol (55:10:35). All conditions were as described in the legend to Figure 2, except that neomycin was added at the indicated concentration. The inset refers to neomycin effects on spontaneous dehydroergosterol exchange.

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Vasopressin Stimulates Phospholipase D Activity Against Phosphatidylcholine in Vascular Smooth Muscle Cells[1]

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It is now clear that various hormones and agonists can stimulate the production of lipid mediators from nonphosphoinositide phospholipids. We have investigated the production of diacylglycerol from nonphosphoinositide sources, and we demonstrated that vasopressin and other vasoactive agents stimulate hydrolysis of phosphatidylcholine in a variety of cultured vascular smooth muscle cells of rat and human origin. We used vasopressin to characterize this response and found that vasopressin stimulates phospholipase D activity against phosphatidylcholine in A-10 vascular smooth muscle cells. The vasopressin-stimulated phosphatidylcholine hydrolysis is both time- and concentration-dependent. The half-maximal dose of vasopressin required for phosphatidylcholine hydrolysis ($ED_{50} \sim 1$ nM) correlates well with vasopressin binding to A-10 cells ($K_d \sim 2$ nM). The phosphatidylcholine in A-10 cells can be preferentially radiolabeled with [^{3}H]myristic acid; subsequent treatment with vasopressin stimulates a rapid increase in ^{3}H-labeled phosphatidate (~4 × control values at 3 min), and after a short lag, ^{3}H-labeled diacylglycerol rises and reaches maximal levels at 10 min (~2 × control values). Similar temporal elevations of phosphatidate and diacylglycerol occur in A-10 cells labeled with [^{3}H]glycerol. In A-10 cells radiolabeled with [^{3}H]choline, the elevation of cellular phosphatidate and diacylglycerol is concomitant with the release of [^{3}H]choline metabolites (predominately choline) to the culture medium. The temporal production of phosphatidate and diacylglycerol as well as the release of choline to the culture medium are consistent with vasopressin activating phospholipase D. In addition, vasopressin stimulates a transphosphatidylation reaction that is characteristic of phospholipase D. The transphosphatidylation reaction is detected by the production of phosphatidylethanol that occurs when A-10 cells are incubated with ethanol and stimulated with vasopressin. The phospholipase D is active in the absence of extracellular Ca^{++} whereas the vasopressin-stimulated mobilization of arachidonic acid is dependent on extracellular Ca^{++}. The data indicate that vasopressin stimulates phospholipase D which hydrolyzes phosphatidylcholine to phosphatidate. The phosphatidate is then metabolized, presumably by a phosphatidate phosphohydrolase, to produce sustained levels of cellular diacylglycerol. These sustained levels of diacylglycerol may activate protein kinase C and thereby function in the "sustained phase" of cellular responses.
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The receptor-mediated hydrolysis of polyphosphoinositides (PIP_2) by phospholipase C (PLC) is a well-recognized event in the signal transduction mechanisms of many hormones and growth factors (1–4). However, early work on the PIP_2 signaling system suggested that at least a portion of the diacylglycerol (DAG) formed after agonist stimulation was derived from nonphosphoinositide sources (5). While much is known about the PIP_2 signaling system, we have only an elementary understanding of the other signaling pathways that produce DAG. Recent studies from several laboratories have suggested or shown that a variety of agonists stimulate the production of DAG that is derived from phosphatidylcholine (PC) (see refs. 6–9 for review). Vasopressin (VP) (10–12), platelet-derived growth factor (PDGF) (13), bombesin (14), phorbol esters (TPA) (11,13,14–19), bradykinin (20), carbachol (21) and the chemotactic peptide (22,23) are among the agonists with a demonstrated ability to stimulate the hydrolysis of PC. In some cases detailed studies have shown that hydrolysis of PC is initiated by the activity of phospholipase D (PLD). An agonist-inducible PLD has been shown in hepatocytes (10), endothelial cells (24), fibroblasts (15,16), HL-60 granulocytes (22), neutrophils (23,25,26) and heart (27). In addition to PLD activity against PC, PLD has also been reported to hydrolyze phosphatidylethanolamine after agonist stimulation (28). Using cultured fibroblasts we have presented evidence that implicates the function of PLD in normal (12), as well as abnormal cellular growth behavior (11), and that protein kinase C (PKC) plays a role in regulating this PLD activity (16).

Vasopressin, when bound to cellular V_1 receptors, stimulates PIP_2 hydrolysis in vascular smooth muscle cells (VSMC) as well as other cell types (29–32). This hydrolysis of PIP_2 results in Ca^{++} mobilization and the elevation of cellular DAG. DAG, through the activation of PKC, is involved in a myriad of cellular responses (4). The importance of DAG is underscored by reports that link VP, DAG and PKC to various aspects of smooth muscle contraction (33,34), cardiomyopathy (35), hypertension (36,37) and atherosclerosis (38). In preliminary investigations we found that vasoactive agents stimulated hydrolysis of PC and produced sustained elevations of

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Abbreviations: BSA, bovine serum albumin; CDP, cytidine diphosphate; DAG, diacylglycerol; DMEM, Dulbecco's modified Eagle's medium; EGTA, ethylene glycol *bis*-(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid; FBS, fetal bovine serum; HASM, human aorta smooth muscle; PA, phosphatidate; PBS, phosphate buffered saline; PC, phosphatidylcholine; PDGF, platelet-derived growth factor; PEt, phosphatidylethanol; PIP_2, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PLC, phospholipase C; PLD, phospholipase D; RASM, rat aorta smooth muscle; SDS, sodium-dodecyl sulfate; TLC, thin-layer chromatography; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; VP, [Arg^8] vasopressin; VSMC, vascular smooth muscle cell.—The abbreviations for DAG, PA, PEt and PC are used to designate "glycerides" and as such do not differentiate between species containing ether- and/or ester-linked moieties.

cellular DAG in VSMC. Due to the importance of DAG as a second messenger in VSMC, and the possible importance of DAG in the sustained contraction of VSMC, we were interested in elucidating the pathways by which DAG is derived from the nonphosphoinositide phospholipids.

In the studies reported here we have investigated the source and dynamics of DAG production in VSMC. Using a variety of radiolabeling techniques, we show that VP, and other vasoactive agonists, stimulate the hydrolysis of PC in VSMC. This VP-stimulated hydrolysis of PC is initiated by the rapid activation of PLD and is measured by an initial increase in cellular phosphatidate (PA), and the cellular release of choline. Following the elevation of PA, a sustained elevation of cellular DAG is observed. The data indicate that DAG is derived from PC. Since the activation of PLD by vasoactive agents appears to be an initial step in the metabolic regulation of cellular DAG levels, PLD may be an important regulator of vascular cell homeostasis.

MATERIALS AND METHODS

Materials. [9,10(*N*-^{3}H]Myristic acid (41 Ci/mmol) was purchased from Amersham (Arlington Heights, IL). [*Methyl*-^{3}H]choline (80 Ci/mmol), [2-^{3}H]glycerol (11.5 Ci/mmol) 5,6,8,9,11,12,14,15-^{3}H(*N*)]arachidonic acid (100 Ci/mmol), [phenylalanyl-3,4,5-^{3}H(*N*)]vasopressin, 8-arginine (67 Ci/mmol), and [^{3}H]inositol were purchased from New England Nuclear (Boston, MA). [Arg^8]Vasopressin, angiotensin II, thrombin (bovine plasma), bovine serum albumin, choline, phosphocholine chloride, cytidine 5′-diphosphocholine, and ethylene glycol *bis*-(β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid (EGTA) were purchased from Sigma Chemical Co. (St. Louis, MO). Phospholipid standards were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL). Neutral lipid standards were purchased from Nu-Chek Prep (Elysian, MN). TPA was purchased from Chemicals for Cancer Research, Inc. (Eden Prairie, MN). Partially purified platelet-derived growth factor, prepared from human platelet-rich plasma, was a generous gift from the laboratory of Dr. R. Ross (University of Washington, Seattle, WA). Silica gel G thin-layer chromatography (TLC) plates were purchased from Analtech, Inc. (Newark, DE). Solvents were from J. T. Baker (Phillipsburg, NJ) and Burdick & Jackson Laboratories, Inc. (Muskegon, MI). Phosphatidylethanol (PEt) was synthesized from 1-palmitoyl-2-oleoyl phosphatidic acid (disodium salt) (Avanti Polar Lipids) as described (22). The authenticity of the synthetic PEt product was confirmed by demonstrating identical chromatographic behavior with PEt standards in two different TLC solvent systems. The first solvent system consisted of chloroform/methanol/NH_4OH (65:30:3, v/v/v); the second TLC system was identical to that previously described by Pai *et al.* (22).

Cell culture. A-10 smooth muscle cells (CRL 1476) were purchased from the American Type Culture Collection (Rockville, Maryland) and were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma) containing 10% fetal bovine serum (FBS) (Hy-Clone, Logan, UT) according to previously described methods (39). Primary cultures of rat aorta smooth muscle (RASM), obtained from explants, were a gift from Dr. G. Bazer (Louisiana State University Medical Center, Shreveport, LA) and were cultured in the same medium used for the A-10 cells. Human abdominal aorta smooth muscle cells (HASM) were obtained from Drs. W. McKeehan and H. Sawada (W. Alton Jones Cell Science Center, Inc., Lake Placid, NY) and were prepared and cultured as described by Hoshi *et al.* (40). All cell cultures were maintained in plastic cultureware from Corning or Linbro. HASM were used at passages of less than 5; RASM cells were used at passages of less than 15; A-10 cells were used at passages of less than 26. Experiments were conducted when the cell densities were approximately 70–90% of confluence.

Radiolabeling and treatment of cell cultures. Radiolabeling methodologies followed previously described protocols (11,12) but used slight modifications. Cells were radiolabeled with [2-^{3}H]glycerol (1 μCi/mL) or [^{3}H]choline (1 μCi/mL) for 48 hr in medium containing 10% FBS. Cultures to be radiolabeled with [^{3}H]myristic acid (0.5 μCi/mL) or [^{3}H]arachidonic acid (2 μCi/mL) were prewashed one time with serum-free medium and incubated in serum-free medium that contained radiolabel and 0.1 mg bovine serum albumin (BSA)/mL for 1 hr. After incubation with radiolabeled compounds, all cultures were washed one time with serum-free medium and were then incubated in radioisotope-free, serum-free medium containing 1 mg BSA/mL for another 1 hr. Vasopressin was added to cultures in acetic acid. The final concentration of acetic acid was 0.001%. TPA was diluted into medium to give a final concentration of 50 ng/mL (acetone vehicle was 0.05%). Thrombin (1 U/mL) was added to cultures using phosphate buffered saline (PBS) as a vehicle (final PBS concentration = 0.01%). Partially purified PDGF was diluted in a solution of 2 mg BSA/mL PBS and further diluted with serum-free medium just prior to addition to cell cultures. For investigations studying the effects of extracellular Ca^{++}, cells were incubated with EGTA (10 mM) in serum-free medium containing 1 mg BSA/mL, pH = 7.2. After incubations with agonists, all cultures were washed once with ice-cold PBS, and fixed with ice-cold methanol containing acetic acid (2%).

Extraction of cellular lipids and analyses of lipid metabolites. Cells were scraped from the culture dish in ice-cold methanol containing acetic acid (2%), and lipids were extracted by the method of Bligh and Dyer (41). Phospholipids were resolved using a TLC solvent system of chloroform/methanol/acetic acid/water (50:25:8:4, by vol) and silica gel G plates. Neutral lipids were resolved by TLC using a solvent system of hexane/diethyl ether/acetic acid (60:40:1, v/v/v). The silica gel areas corresponding to commercial standards (I_2 vapor visualization) were scraped, and the radiolabeled lipids were quantitated by liquid scintillation spectrometry (11,12). Radiolabeled phosphatidate was quantitated in a similar manner after resolution in a TLC system using chloroform/pyridine/70% formic acid (50:25:7, v/v/v). Total [^{3}H]-choline metabolites released to the culture medium were quantitated by counting an aliquot of the medium mixed with ACS scintillation fluid (Amersham). Resolution of the water-soluble [^{3}H]choline metabolites was achieved with TLC (Silica gel G) using a solvent system containing 0.9% NaCl/methanol/concentrated NH_4OH (50:50:5, v/v/v) (42). Radiolabeled metabolites comigrating

TABLE 1

Preferential Labeling of Phosphatidylcholine in A-10 Vascular Smooth Muscle Cells[a]

Lipids	Incorporation of radiolabeled precursor			
	[^{3}H]Myristic acid	[^{3}H]Arachidonic acid	[^{3}H]Choline	[^{3}H]Glycerol
	% of Total ^{3}H			
Polar lipids				
Lysophosphatidylcholine	0.2	0.5	0.5	0.7
Sphingomyelin	1	0.5	13	0.7
Phosphatidylcholine	77	65	86	63
Phosphatidylserine + phosphatidylinositol	0.5	8	0.1	9
Phosphatidylethanolamine	2	16	0.1	12
Neutral lipids	21	9	<0.1	14
	% of Total [^{3}H]phospholipid			
Phosphatidylcholine	95	72	85	73

[a]A-10 cells were seeded into 60 mm dishes at approximately 5×10^4 cells/dish. Cells were radiolabeled with either [2-^{3}H]glycerol (1 μCi/mL), [^{3}H]choline (1 μCi/mL), [^{3}H]myristic acid (0.4 μCi/mL), or [^{3}H]arachidonic acid (0.5 μCi/mL) as described in Materials and Methods. After a 1-hr incubation with serum-free medium containing 1 mg BSA/mL, cell cultures were washed twice with ice-cold PBS, and scraped in methanol containing 2% acetic acid. Cellular lipids wre extracted after the addition of organic solvents. Specific lipids were resolved by TLC, scraped from the TLC plate, and the radioactivity contained in each lipid was determined by scintillation spectrometry.

with commercial standards were quantitated as described previously (42). Hormone-stimulated hydrolysis of phosphoinositides was assayed by quantitating polyinositol-phosphates produced in cells that had been radiolabeled with [^{3}H]inositol (43).

Transphosphatidylation reaction. A-10 cells were prelabeled with [^{3}H]myristic acid and treated with vasopressin (93 nM) in the presence of 0.7% ethanol for 20 min. The incubation was terminated, cellular lipids were extracted, and PEt was resolved from other lipids in a TLC system using chloroform/methanol/concentrated NH_4OH (65:30:3, v/v/v) (phosphatidylethanol $R_f = 0.72$). The radiolabeled lipid that comigrated with phosphatidylethanol was scraped from the TLC plate and quantitated by scintillation spectrometry.

Binding of vasopressin to A-10 cells. A-10 cells were cultured in 12 well dishes and were used at ~70% of confluence. Cells were washed twice with serum-free medium and the binding was initiated in DMEM medium containing 1 mg/mL BSA and [^{3}H]VP. The cells were incubated with 0.3–42 nM [^{3}H]VP for 30 min at 37°C in the presence or absence of 10 μM VP. At the end of the incubation period, the medium was removed and the cells were quickly washed one time with ice-cold PBS and two times with DMEM. The cells were solubilized in 2% sodium-dodecyl sulfate (SDS), and the [^{3}H]VP bound was quantitated by scintillation spectrometry in ACS cocktail.

RESULTS

Characterization of the biosynthetic labeling of phosphatidylcholine in VSMC. In order to investigate the hormonal activation of PC hydrolysis, we first developed experimental protocols that provided a preferential radiolabeling of PC. Thus, following treatment with agonists, we were able to analyze the metabolites resulting from the hydrolysis of PC. Table 1 shows the radioactivity profiles of cellular lipids after radiolabeling with various phospholipid precursors. Eight-six percent of the [^{3}H]-choline was preferentially incorporated into PC; 13% of the [^{3}H]choline was found in sphingomyelin. When included in the incubation medium of A-10 cells for various periods of time, [^{3}H]glycerol, [^{3}H]myristic acid and [^{3}H]-arachidonic acid were also preferentially incorporated into PC. After incubating A-10 cells with [^{3}H]glycerol for 24 hr, 73% of the ^{3}H found in the phospholipids was incorporated into PC. After a 1-hr labeling period, the radiolabeled fatty acids were also primarily incorporated into PC. Almost all (95%) of the [^{3}H]myristic acid incorporated into the phospholipids was found in PC, and 72% of the [^{3}H]arachidonic acid localized in the phospholipids was incorporated into PC (Table 1). Of the radiolabels and protocols used to label the cellular phospholipids, myristic acid was the most effective "tool" for selectively labeling the PC.

With the exception of choline, each of the radiolabeled phospholipid precursors also labels the neutral lipids to varying degrees (Table 1). Triacylglycerols were the predominant radiolabeled neutral lipid (data not shown). In experiments described below, we found no evidence that the triacylglycerols play a role in the responses measured. With these protocols, we radiolabeled the cellular PC and examined both hydrophilic as well as the hydrophobic metabolites produced after agonist-stimulated phospholipid hydrolysis.

Various agonists stimulate hydrolysis of phosphatidylcholine in vascular smooth muscle cells from different sources. VSMC respond to a variety of agonists by hydrolyzing PC. The data in Table 2 show that this response is functional in an established cell line, in cells derived from a primary culture of VSMC from rat aorta and in VSMC established from primary culture of human abdominal aorta. When evaluated as a function of an increased accumulation of [^{3}H]choline metabolites released

TABLE 2

Agonist-Induced Hydrolysis of Phosphatidylcholine and Accumulation of Diacylglycerol in Vascular Smooth Muscle Cells[a]

	Response to agonist (% of control[b])				
	Rat aorta smooth muscle		A-10 Smooth muscle cell line		Human aorta smooth muscle
Agonist	[^{3}H]Choline[c]	[^{3}H]DAG[d]	[^{3}H]Choline	[^{3}H]DAG	[^{3}H]Choline[e]
Vasopressin (93 nM)	1.9	3.6	2.4	4.5	
PDGF[f] (50 ng/mL)	2.0	3.0	1.8	3.2	
TPA (50 ng/mL)	2.2	4.9	2.2	2.4	2.2
Thrombin (1 U/mL)			1.5		
Angiotensin II (100 ng/mL)	1.3	1.9	NR		
Fetal bovine serum (10%)					1.9

[a] Cellular labeling, harvest and lipid analyses were performed as described in Table 1.

[b] Data represent "fold increase" over control (control = 1). Values were obtained from comparison of means derived from 3–4 replicate determinations. NR = no response.

[c] [^{3}H]Choline-labeled metabolites, with the exception noted below, were quantitated after a 20-min incubation with agonists.

[d] [^{3}H]Glycerol-labeled diacylglycerol was quantitated after a 10-min exposure to agonists.

[e] [^{3}H]Choline-metabolites released by human vascular smooth muscle cells were quantitated after a 1-hr treatment with TPA.

[f] Partially purified PDGF was used such that the stated concentration reflects the mass of PDGF in the treatment medium.

TABLE 3

Analysis of Radiolabeled Choline Metabolites in A-10 Vascular Smooth Muscle Cells and in the Culture Medium of A-10 Cells Incubated with or without Vasopressin[a]

	Cellular radiolabeling with [^{3}H]choline		[^{3}H]Choline metabolites released to the culture medium	
Metabolites[b]	Control	Vasopressin	Control	Vasopressin
Total lipid-soluble ^{3}H	307700 ±12600	296660 ±3450	ND[c]	ND
Total water-soluble ^{3}H	28397 ±974	27497 ±276	4136 ±123	11477 ±256

[a] A-10 vascular smooth muscle cells (100 mm dishes) were radiolabeled with [^{3}H]choline, manipulated, and incubated with or without vasopressin (93 nM) for 20 min as described in Materials and Methods. Data are cpm/dish and represent the mean (±SD) of triplicate determinations. Repeated experiments yielded similar results.

[b] Total lipid-soluble radioactivity was determined by liquid scintillation counting of an aliquot of the material recovered in the organic phase of cellular extracts. Total water-soluble radioactivity was quantitated by liquid scintillation counting of an aliquot of the material recovered in the aqueous phase of an extract of cells or culture medium.

[c] ND = not determined.

to the medium, each of the three different VSMC cell cultures hydrolyzes PC in response to agonists (Table 2). The responses to VP, PDGF and TPA were characterized by <2-fold increases in [^{3}H]choline metabolites released to the culture medium. In contrast, the responses to thrombin and angiotensin II were attenuated and showed a 1.5- and 1.3-fold increase over control values, respectively. In addition to the PC hydrolysis response, elevated DAG levels were also found in the VSMC treated with VP, PDGF, serum and TPA (Table 2). When examined in cells labeled with [^{3}H]glycerol, cellular responses were observed as approximate 2- to 5-fold increases in cellular DAG levels after a 10-min treatment with agonists. These data (Table 2) demonstrate that the hydrolysis of PC and elevation of cellular DAG are characteristic of several different cultured VSMC and that this response is initiated by a variety of agonists.

Choline is released to the culture medium of vasopressin-treated A-10 cells. In order to gain insight on the enzyme activity induced by the agonists (*i.e.*, phospholipase C or phospholipase D), A-10 cells were radiolabeled with [^{3}H]choline, treated with vasopressin and the choline metabolites were analyzed. Table 3 shows that ~90% of the [^{3}H]choline is incorporated into lipophilic

components and that treatment with vasopressin (93 nM; 20 min) stimulates a 2.8-fold increase in [^{3}H]choline-labeled metabolites released to the culture medium. These metabolites were analyzed by TLC and found to be: 80% choline, 16% phosphocholine and 4% glycerolphosphocholine plus CDPcholine (CDP = cytidine diphosphate). A similar relative composition was found for the ^{3}H released by untreated A-10 cells. The finding that choline is the predominant metabolite released to the culture medium indicates the presence of an agonist-inducible PLD. The relative composition of the intracellular water-soluble metabolites was found to be: 81% phosphocholine, 14% glycerophosphocholine plus CDPcholine and 5% choline. There was no change in the relative composition of the intracellular metabolites after treatment with vasopressin.

Pharmacokinetics of VP stimulation of phosphatidylcholine hydrolysis in VSMC. The ability of VP to stimulate hydrolysis of PC was investigated with regard to concentration dependency, binding to cellular receptors, and the time course of evolution of PC metabolites. The data of Figure 1 show that VP induces PC hydrolysis in a concentration-dependent manner in both A-10 and RASM cells. After a 20-min treatment with VP and subsequent quantitation of [^{3}H]choline metabolites released to the medium, we found that both VSMC cell types demonstrate maximal responses at approximately 100 nM VP and that the ED_{50} for the VP-stimulated hydrolysis of PC is ~1 nM. Consistent with previous reports (29), we also found that VP (93 nM) stimulates the hydrolysis of PIP_2 and the elevation of cellular inositol phosphates in A-10 cells (data not shown).

Further analyses showed that A-10 cells possess specific, saturable receptors. Figure 2 illustrates the binding of VP to A-10 cells. Under the experimental conditions employed, specific binding was maximal at concentrations of VP above ~20 nM. As derived from Scatchard analyses of the binding data, A-10 cells possess ~75,000 binding sites per cell with a K_d of ~2 nM. The data indicate the presence of high affinity binding sites. The close correlation between the ED_{50} (~1 nM) for the VP induced PC hydrolysis and the K_d for VP binding to A-10 cells (~2 nM) supports the conclusion that VP stimulates PC hydrolysis in A-10 cells.

The stimulation of PC hydrolysis by VP in VSMC is time-dependent. Figure 3 shows that when A-10 or HASM cells are prelabeled with [^{3}H]choline and treated with VP or TPA, PC degradation is indicated by a time-dependent accumulation of [^{3}H]choline metabolites in the culture medium. In A-10 cells treated with VP, the PC-hydrolysis response continued through a 20-min incubation. Experiments with A-10 cells using longer time points showed that the maximal increase of ^{3}H-labeled choline metabolites was found at 20 min (~2.5 × control values). When treated with TPA, HASM also demonstrates a time-dependent degradation of PC (Fig. 3). The longer-lasting effect of TPA, a stable DAG analog, is illustrated in HASM by the nearly linear response that continues for at least 1 hr (Fig. 3).

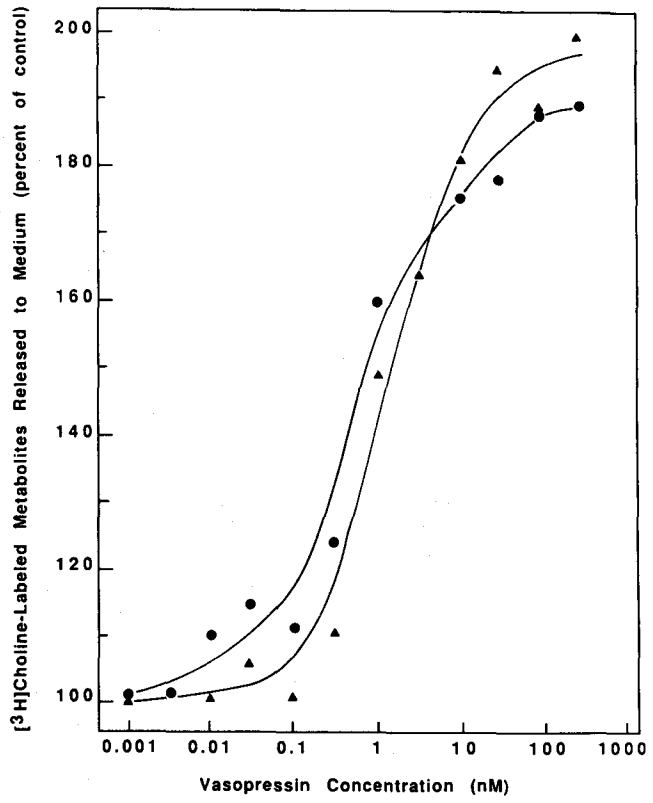

FIG. 1. Concentration dependency of vasopressin-stimulated hydrolysis of phosphatidylcholine in vascular smooth muscle cells. A-10 (●—●) and smooth muscle cells from rat aorta (▲—▲) were seeded in 24 well plates, labeled with [^{3}H]choline, treated with various concentrations of vasopressin (0.001–186 nM) for 20 min, and the [^{3}H]choline-labeled metabolites released to culture medium were quantitated by scintillation spectrometry. The ED_{50} for both varieties of vascular smooth muscle cells is approximately 1 nM. Data points represent the mean of 3–4 determinations. All standard deviations were less than 12% of the respective mean. Repeated experiments yielded similar results.

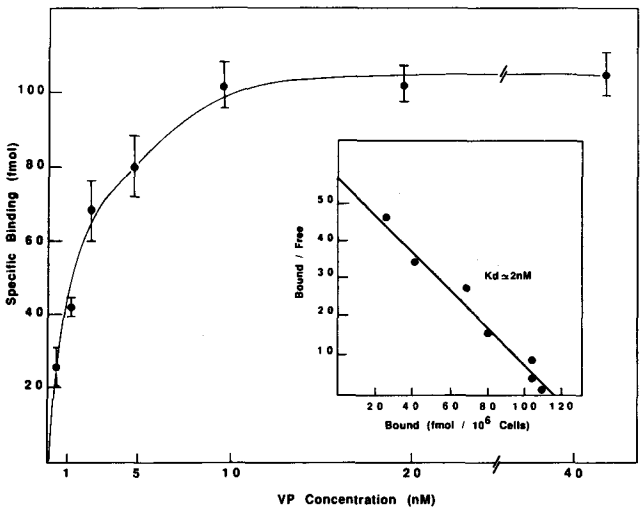

FIG. 2. Binding of vasopressin to A-10 vascular smooth muscle cells. A-10 cells were seeded into 12 well plates and were used at ~70% of confluence. The cells were washed just prior to the experiment and binding was initiated by the addition of [^{3}H]vasopressin. Specific binding of [^{3}H]vasopressin was measured at 37°C with a 30-min incubation. Scatchard analysis (inset) indicates: K_d ~ 2 nM and approximately 75,000 binding sites/cell. Data points represent the mean n = 3 ± SD (error bars). A repeated experiment yielded similar results.

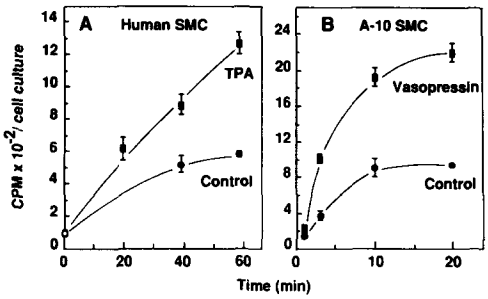

FIG. 3. Time course of agonist-stimulated hydrolysis of phosphatidylcholine in vascular smooth muscle cells. A-10 and vascular smooth muscle cells derived from human abdominal aorta were seeded into 24-well plates, radiolabeled with [^{3}H]choline, manipulated as described in Materials and Methods, and incubated ± vasopressin (93 nM) or ± TPA (50 ng/mL) for the time periods indicated. [^{3}H]Choline metabolites released to the medium were quantitated by counting an aliquot of the culture medium in ACS scintillation fluid. Data points represent the mean of four replicate determinations; error bars represent the standard deviation of the mean. Similar results were obtained from replicate experiments.

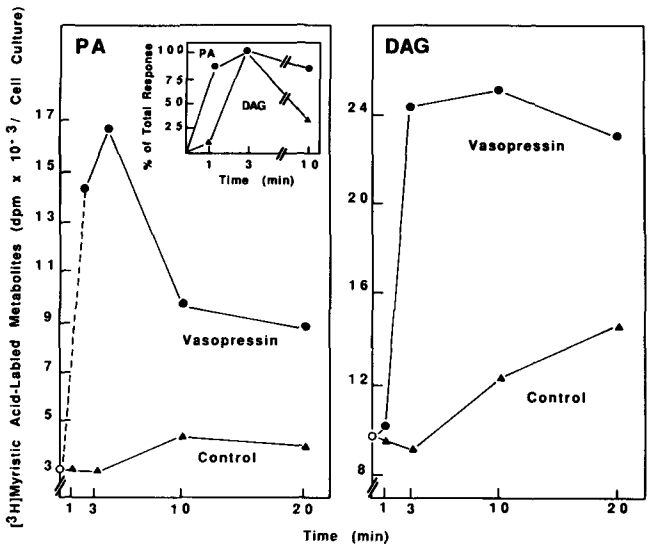

FIG. 4. Comparison of time courses for vasopressin-stimulated elevation of phosphatidic acid and diacylglycerol in vascular smooth muscle cells radiolabeled with [^{3}H]myristic acid. A-10 cells were seeded in 60 mm dishes, labeled with [^{3}H]myristic acid and treated with vasopressin for the times shown. Lipids were extracted from washed cell cultures. Diacylglycerol (DAG) and phosphatidate (PA) were quantitated following resolution by thin-layer chromatography. Data points represent the mean of 2-3 replicates. Coefficients of variation were all less than 10% of the respective means. Repeated experiments yielded similar results.

Concomitant with the release of [^{3}H]choline is the time-dependent generation of radiolabeled PA and DAG. In A-10 cells prelabeled with [^{3}H]myristic acid (to preferentially label the aliphatic moieties of PC), VP stimulates an increase in [^{3}H]myristic acid-labeled PA (Fig. 4). This elevation in cellular PA is rapid (~80% of the maximal response after 1 min) and maximal within 3 min. Cellular levels of [^{3}H]myristic acid-labeled DAG also rose sharply but only after a lag period of approximately 1 min. Over the course of several repeated experiments, we found that VP stimulated 2-6 fold increases in PA and

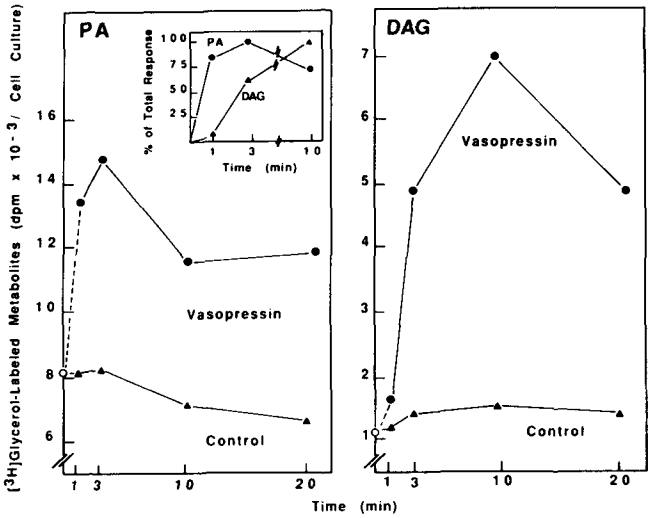

FIG. 5. Comparison of time courses for vasopressin-stimulated elevation of phosphatidic acid and diacylglycerol in vascular smooth muscle cells radiolabeled with [^{3}H]glycerol. A-10 cells were seeded in 60 mm dishes, labeled with [^{3}H]glycerol and were treated with vasopressin (93 nM) for the times shown. Lipids were extracted from washed cell cultures. Diacylglycerol (DAG) and phosphatidate (PA) were quantitated following resolution by thin-layer chromatography. Data points represent the mean of 2-3 replicates. Coefficients of variation were all less than 13% of the respective means. Similar results were obtained from repeated experiments.

2-3 fold increases in DAG. The relationship of the time courses for the rapid production of PA and the initial lag prior to the elevation of DAG levels are illustrated in Figure 4 (inset). Similar results were obtained in A-10 cells radiolabeled with [^{3}H]glycerol (Fig. 5). An increase in the cellular [^{3}H]glycerol-labeled PA level was obvious after a 1-min treatment with VP, and a maximal level of radiolabeled PA (2-3 × control levels) was found at 3 min. Maximal increases in the levels of [^{3}H]glycerol-labeled DAG (3-5 × control values) were found after a 10-min treatment with VP (Fig. 5). These time course experiments show that, in A-10 cells treated with VP, [^{3}H]PA is produced rapidly and reaches nearly maximal levels by 1 min, whereas the [^{3}H]DAG increases markedly only after a lag period of 1 min (see Fig. 4, inset). This pattern of evolution of the PC metabolites is consistent with a precursor-product relationship in which PLD produces an immediate increase in PA, and DAG is produced by a subsequent metabolism of the PA. Since [^{3}H]myristic acid is the most effective agent for selectively radiolabeling PC, these results also provide support for the role of PC as a precursor of the DAG that results from treatment of A-10 cells with VP.

In conjunction with these time-course experiments, the ^{3}H-labeled triacylglycerols were also resolved by TLC and quantitated. These tests were aimed at finding hormone-stimulated, time-dependent decreases in the radioactivity associated with the triacylglycerols. However, from these experiments we found no evidence that triacylglycerols contribute to the metabolite pools measured after treatment with VP (data not shown).

Vasopressin stimulates phospholipase D in A-10 cells. Thus far, all of the data indicate the presence of an agonist-inducible PLD in the VSMC. To examine the

TABLE 4

Analysis of Vasopressin-Stimulated Phospholipase D Activity Against Phosphatidylcholine in A-10 Vascular Smooth Muscle Cells: Evidence Derived from Analysis of Various Metabolites of Phosphatidylcholine[a]

Treatment	Phosphatidylcholine metabolism (% of control)[b]	
	Phosphatidate	Phosphatidylethanol
Control	100	100
Vasopressin	217	98
Control + ethanol	82	90
Vasopressin + ethanol	182	429

[a]Cells were labeled with [^{3}H]myristic acid and manipulated as described in Table 1. After a 10 min treatment with VP (100 ng/mL) in the presence or absence of ethanol (1%), lipids from washed cell cultures wre extracted, and PC metabolites were resolved by thin-layer chromatography and quantitated by scintillation spectrometry.

[b]Phospholipase D activity is measured as increases in metabolites derived from phosphatidylcholine. Data are presented as % of control; control values = 100%. Values represent the mean of determinations from three cell cultures. Standard deviations were all less than 10% of the respective means. Repeated experiments yielded similar results.

hormone-activated PLD mechanism further, we employed the ability of PLD to catalyze not only the hydrolysis of choline from PC, but also a transphosphatidylation reaction in which an exogenous alcohol acts as an acceptor for PA (22,44). Thus, in the presence of exogenous ethanol, activation of PLD will result in the elevation of both PA and the product of the transphosphatidylation reaction, phosphatidylethanol (PEt). This catalytic activity has been used previously as a marker for PLD activity (16,22,45–47).

We found that VP stimulates a transphosphatidylation reaction in A-10 cells (Table 4). In cells that had been prelabeled with [^{3}H]myristic acid, VP had no effect on he cellular PEt levels, whereas the VP treatment increased cellular PA levels by ~2 fold. In the presence of ethanol, the VP-induced increase in PA was reduced and cellular PEt levels increased 4.3 times over control values. These results are consistent with the ability of PLD to catalyze the transphosphatidylation reaction and suggest that PEt is generated at the expense of PA. Since [^{3}H]myristic acid preferentially radiolabels PC (Table 1), these data lend additional support for a response whereby agonists induce hydrolysis of PC. Our finding that VP induces the transphosphatidylation reaction strengthens the evidence that indicates VP activates PLD against PC.

Cellular phospholipase D activity is insensitive to extracellular Ca^{++}. The hormone-induced PLD was also examined with regard to a possible co-factor role for Ca^{++}. VP stimulates arachidonic acid metabolism in A-10 cells (31). It is well-known that the ability of phospholipase A_2 to stimulate arachidonic acid metabolism is Ca^{++}-dependent (48,49). With this in mind, we used arachidonic acid mobilization as a "positive control" for experiments that examined the activity of A-10 cells exposed to Ca^{++}-free medium. A-10 cells were labeled with either [^{3}H]choline, [^{3}H]myristic acid or [^{3}H]arachidonic acid and treated with VP in the absence or presence of EGTA. The data in Table 5 show that the chelation of the Ca^{++} in the culture medium had no effect on the VP-stimulated generation of [^{3}H]myristic acid-labeled DAG or PA, nor did the absence of extracellular Ca^{++} effect the release of [^{3}H]choline metabolites to the culture medium. Our experiments confirm a previous finding (31) that VP stimulates a release of [^{3}H]arachidonic acid-labeled metabolites to the medium (Table 5). However, the release of arachidonic acid metabolites to the culture medium was inhibited by approximately 65% when Ca^{++} was excluded from the treatment medium containing VP. These results indicate that the VP-induced hydrolysis of PC is insensitive to extracellular Ca^{++} whereas a maximal response of arachidonic acid metabolism is dependent on extracellular Ca^{++}.

TABLE 5

Analysis of Vasopressin-Stimulated Activation of Phospholipase D in Vascular Smooth Muscle Cells in the Absence of Extracellular Ca^{++}[a]

Treatment	Metabolic response (% of control[b])			
	[^{3}H]Choline release	[^{3}H]Myristic acid-labeled metabolites produced		[^{3}H]Arachidonic acid metabolites released
		Phosphatidate	Diacylglycerol	
Control	100	100	100	100
Vasopressin	175	453	215	503
EGTA	110	93	95	86
EGTA + vasopressin	195	413	195	169

[a]A-10 vascular smooth muscle cells were seeded in 60 mm dishes, radiolabeled, and incubated ± vasopressin (93 nM), ± EGTA (10 mM). [^{3}H]Choline release (20 min + agonist) and [^{3}H]diacylglycerol (10 min + agonist) were measured as described in Table 2. Radiolabeled phosphatidate was quantitated, as described in Figure 4, after a 3-min incubation with agonist. [^{3}H]Arachidonic acid metabolism was assessed by quantitating the ^{3}H released to the culture medium after a 10-min treatment.

[b]Data are presented as % of control [acetic acid (0.001%) control = 100] and are derived from means of 2–3 replicate determinations.

DISCUSSION

Collectively our results demonstrate that, in vascular smooth muscle cells, PC is hydrolyzed upon treatment with VP and other agonists. Vasopressin activates PLD that hydrolyzes PC, and this activity results in the elevation of cellular PA with a subsequent elevation of DAG. Thus, the VP-stimulated PLD generates lipid mediators that are important regulators of cellular physiology. The PC-hydrolysis response appears to be a common mechanism in VSMC and is observed after treatment of different VSMC, including human vascular smooth muscle cells, with a variety of vasoactive agonists. The conclusions are supported by the following: i) The ability to preferentially radiolabel PC allows a selective view of the hormone-stimulated metabolism of PC. In experiments using cells radiolabeled with [^{3}H]myristic acid, we found that VP stimulates a rapid increase in cellular ^{3}H-labeled PA followed by elevations of ^{3}H-labeled DAG. The rapid evolution of PA that contains [^{3}H]myristic acid indicates that VP can stimulate PLD activity against PC in VSMC. In cells radiolabeled with [^{3}H]glycerol the rapid elevation of [^{3}H]glycerol-labeled PA and subsequent elevation of [^{3}H]glycerol-labeled DAG is similar to the pattern seen in cells radiolabeled with [^{3}H]myristic acid. The temporal relationship of the evolution of PA and DAG indicates the activation of a hormone-stimulated PLD and suggests that PA serves as precursor of DAG. This suggestion is strengthened by finding similar patterns of PA and DAG production regardless of whether the cells were radiolabeled with [^{3}H]myristic acid or [^{3}H]glycerol. ii) The close correlation of the K_d for VP binding to A-10 cells and the ED_{50} for the VP-stimulated hydrolysis of PC support the conclusion that VP is the agent that stimulates PLD activity against PC in A-10 cells. iii) The hydrolysis of PC by PLD is further supported by the finding that, after a 20-min incubation with VP, the majority of the water-soluble metabolites released to the culture medium are choline and not phosphocholine. iv) The finding that VP stimulates a transphosphatidylation reaction, a characteristic of PLD, is an additional indicator of a hormone-activated PLD. The accumulation of PEt, a transphosphatidylation product of PLD, has been used in various cell culture models as an indicator of PLD activity (16,21,46,47). That PEt can be derived from PC is further support for a hormone-sensitive PLD that hydrolyzes PC. v) Our results are consistent with a previous report (27) that demonstrates PLD is insensitive to extracellular Ca^{++}. vi) The apparent activation of PC hydrolysis by different vasoactive agonists, in different cultured smooth muscle cells, indicates that the hormonal stimulation of PLD is a common signal transduction response in VSMC. In this context, it is reasonable to draw attention to the human VSMC. While small quantities of these cells precluded more extensive analyses, the data obtained indicate that the pathways characterized in the A-10 VSMC also function in human VSMC.

It has been previously reported that PLD is active in the absence of Ca^{++} (27,50). Our results are consistent with those findings. Chelation of Ca^{++} from the culture medium had no effect on the ability of VP to activate PLD. From similar studies using myocytes from chick heart, extracellular Ca^{++} was not required for PLD activation by carbachol (27). In A-10 cells, VP also stimulates PIP_2 hydrolysis and Ca^{++} mobilization (29). While we have demonstrated that PLD activity is not dependent on Ca^{++} influx, we have not investigated the mobilization of internal Ca^{++} as an important element in the VP-stimulated activity of PLD. Indeed, Ca^{++} mobilization from intracellular sources may be important in the PLD activity described in HL-60 granulocytes (46).

While our experiments show that extracellular Ca^{++} is not required for PLD activity in A-10 cells, the activity of phospholipase A_2 appears to be Ca^{++} sensitive. In the absence of extracellular Ca^{++}, the VP-stimulated release of radiolabeled arachidonic acid metabolites to the culture medium was reduced by 70%. This result is consistent with Ca^{++} serving a cofactor role for phospholipase A_2 (48,49). The finding that PLD activity was maximally active in the absence of extracellular Ca^{++}, while arachidonic acid metabolism was significantly reduced, indicates that the VP activation of PLD is not dependent on prior metabolism of arachidonic acid, nor on metabolites of arachidonic acid.

With the data from the time course studies (Figs. 3, 4 and 5), we suggest that the elevation of cellular DAG results from a secondary metabolism of the PA produced by PLD. While not proven by these studies, the suggestion that DAG is derived from PA is supported by the consistency of the time courses for the evolution of the PC metabolites in cells labeled with either [^{3}H]glycerol or [^{3}H]myristic acid. This suggestion correlates well with findings reported by other investigators. From studies on vascular endothelial cells, Martin (24) showed that DAG could result from the hydrolysis of PC by PLD coupled with a subsequent metabolism by phosphatidate phosphohydrolase. Several investigators (16,22,27) have suggested that PA, produced through agonist stimulation of PLD, is a precursor of DAG. Our findings, in a system using VSMC, coupled with the report of a similar hormone-stimulated PLD activity in vascular endothelial cells (24), lead to the speculation that "second messengers" derived from the hydrolysis of PC by PLD could be an important element of signal transduction mechanisms in vascular tissues.

Our findings complement and extend those of other investigators. Grillone *et al.* (31) reported that VP stimulates a transient PLC activity against PC in A-10 cells. Concomitant with that metabolism, a modest rise in cellular DAG (35% increase over control) was reported. Evidence for this PLC activity was derived from a clonal variety of A-10 cells which were treated with VP for 0–8 min at room temperature (31). Our data include longer time points and experimental incubations at 37°C. An integration of our data with that of Grillone *et al.* (31) suggests that VP stimulates both PLD and PLC in A-10 cells. With respect to such a dual phospholipase activity, Martinson *et al.* (21) have shown carbachol can induce a rapid activation of both PLD and PLC, and that the PLC activity was transient while PLD activity was sustained. Collectively, the data show that VP can induce a rapid stimulation of PLC and PLD activity against PC; the PLC activity is transient (31), while the PLD activity is sustained (this report). Such a regulation of phospholipase activity could provide lipid mediators that are temporally distinct and thereby affect rapid *vs* sustained cellular responses.

We suggest that the inability to detect PLD activity in previous studies using A-10 cells (31) is likely due to the difference in experimental protocol and is related to the lower incubation temperatures used by Grillone *et al.* (31). Data supporting such an explanation are presented by Griendling *et al.* (51) who showed that changes in incubation temperatures could markedly affect the time course for production of cellular phospholipase metabolites in VSMC. With these data, we are led to the speculation that experimental protocols that employ reduced incubation temperatures might be used to achieve a finer biochemical dissection of the signaling pathways that use PLC and/or PLD. We are presently investigating the temperature sensitivity of the PLD.

In this work we have described a rapid rise in cellular PA that results from the activity of PLD. While this PA appears to serve as a precursor pool for DAG, we cannot rule out other possible functions. Jackowski and Rock (52) have presented evidence that PA can activate PLC *in vitro*. Thus, the elevation of cellular PA may also play a role in the regulation of PLC. The hormonally modulated level of PA is also notable because PA is mitogenic for different cell lines (53–56) and because PA can induce contraction of isolated smooth muscle cells (57). Our findings are particularly intriguing in light of reports that demonstrate that exogenous PLD can increase the contractile force of rabbit heart (58). More research will be required to define the possible regulatory role of PA in A-10 VSMC.

Results from several studies, when paired with the findings presented here, suggest that vasoactive hormones can stimulate biphasic elevations in cellular DAG in VSMC cells. In primary cultures of rat aorta VSMC, angiotension II stimulates PLC causing an initial rise in DAG derived from PIP_2, and subsequently a PLC hydrolyzes phosphatidylinositol to produce a more sustained elevation of DAG (51). In A-10 cells, an early peak of DAG results from the activity of PLC on PC (31), and a more sustained peak of DAG (Maximal at 10 min) results from PLD activity against PC (this report). Similar sustained productions of DAG have been reported by others (59–62). Such a pattern of DAG production is consistent with the biphasic response phenomena described by Zawalich *et al.* (63) and Kojima *et al.* (64). This biphasic cellular response correlates with, and may be activated by, the biphasic waves of cellular DAG (63–65). Thus, the biphasic elevation of DAG may play an important role in the cellular activation of PKC.

Our finding that TPA can stimulate hydrolysis of PC and elevate DAG levels in VSMC also implicates a role for PKC in the activation of PLD. While we cannot define the role of PKC with certainty, we have recently shown that an inhibitor of PKC, staurosporine, can be used to inhibit the VP-stimulated PLD activity against PC in REF52 fibroblasts (16). With regard to the regulation of PKC, specific diacylglycerol species or ether-containing diradylglycerols may serve as regulatory signals (46, 66,67). Another possible regulatory mechanism exists with respect to the selective "down modulation" of specific subtypes of PKC (68) by specific diacylglycerols.

For some time the hydrolysis of PIP_2 and the activation of PKC have been thought to regulate various aspects of cellular growth control mechanisms (2,3). While the roles of PIP_2 hydrolysis and PKC activation in regulating cellular growth are not precisely understood, the combination of our data with that from others leads to some intriguing speculation. VP, PDGF, thrombin and TPA stimulate PC hydrolysis, and we have shown or suggested that these agents also activate PLD in VSMC. These agonists also stimulate PLD activity in cultured fibroblasts (11,12) and other cell lines (7). VP, PDGF and thrombin also affect PIP_2 metabolism and cellular DAG levels in a variety of cells including VSMC, hepatocytes, and fibroblasts (1,4,11,12,30,60,61,69). In addition, these agonists are mitogenic in different cultured cell systems (2,3,12,60,70,71) and can activate PKC (61,63,65,72–75). These commonalities lead to a working model in which cellular growth regulation is affected by a series of complex interactions of second messengers derived from: i) sequential hydrolysis of PIP_2 and PC by PLC, and by ii) the activity of PLD against PC. The dual activation of these phospholipases results in biphasic elevations of DAG that may affect the activity of specific PKC isozymes. The relationships of the temporal production of specific DAGs and the activation of PKC are presently under investigation.

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Comparison of the Effect of Six Compactin-Related Compounds on Cholesterol Synthesis in Five Human Cell Types

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We have investigated the effect of six compactin-related compounds—mevinolin, compactin, ML-236A, monacolin X, monacolin L and dihydromonacolin L—on cholesterol synthesis in human umbilical vein endothelial cells, human small intestine epithelial cells, human hepatoma cell line HEP G2, normal human skin fibroblasts and in skin fibroblasts from a patient with familial homozygous hypercholesterolemia. The inhibition of cholesterol synthesis was found to depend on both the cell type and the type of compound used. The most effective compounds were mevinolin and compactin. Monacolin X, monacolin L and ML-236A were less effective, and dihydromonacolin L was the least efficacious. Endothelial and epithelial cells were sensitive to very low concentrations of inhibitors (IC_{50}=1.0–30 pg/mL), HEP G2 cells required higher concentrations (IC_{50}=0.01–66 ng/mL) and fibroblasts needed even higher concentrations (IC_{50}=0.1–200 ng/mL). Lactone and acid forms of the inhibitors were equally active. None of the inhibitors had any effect on either protein or fatty acid synthesis in any of the cell types studied. It can be concluded that different compactin-related compounds show a range of potencies as cholesterol synthesis inhibitors and a dose-dependent tissue-selectivity.
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Hypercholesterolemia is a major risk factor in the development of atherosclerosis. It has been demonstrated in numerous studies that elevated blood cholesterol levels are typically associated with the development of atherosclerosis. The lowering of serum cholesterol levels can result in slowing and even regression of the development of atherosclerosis (for review see 1). Three approaches are used to treat hypercholesterolemia. The first is restriction of dietary cholesterol consumption and absorption. The large-scale programs which have focused on the restriction of cholesterol consumption have provided promising results (2); however, this approach is far from optimal since more than 70% of plasma cholesterol is derived from *de novo* cholesterol synthesis (3). A second approach is to stimulate cholesterol degradation and efflux, using bile acid sequestrants, ileal bypass or plasmapheresis (4). The main complication of these methods is the compensatory enhancement of cholesterol synthesis, which significantly reduces the efficiency of treatment (4). Another approach seems to be a most promising one—the inhibition of cholesterol synthesis alone or in combination with the first and/or second types of approaches.

The most effective pharmacologic inhibitors of cholesterol synthesis are compactin-related compounds. These compounds are the competitive inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, a rate-limiting enzyme of the cholesterol biosynthesis pathway (5). Many of these compounds have been highly effective as hypocholesterolemic drugs when studied both experimentally and clinically (1,6,7). However, while these drugs are beginning to be broadly used, many aspects of their action remain unclear. These include the optimal drug choice, drug dose and drug tissue selectivity (8). Additional unknowns are the mechanism of induction of HMG-CoA reductase and HMG-CoA synthase overproduction (9), causes of the elevated plasma high-density lipoprotein levels (6,7) and drug effects on cholesterol absorption (10). A valuable model to study these and other aspects of the compactin-related compound action is cultured human cells as this can overcome problems of species specificity of animal studies. In the present work we used five different types of cultured human cells to assess the possibility of tissue-selectivity, to find optimal drug concentrations, and to compare the relative effectiveness of six compactin-related compounds.

MATERIALS AND METHODS

Materials. All compactin-related compounds were a product of microbiological synthesis; their purification and characterization were described previously (11–14). All compounds except ML-236A were in lactone form; acid forms were obtained as previously described (15). Cell culture media, balanced salt solutions, fetal calf serum and antibiotics were from Flow (Irvine, Scotland). Cell culture plasticware was from Costar (Cambridge, MA). Digitonin, cholesterol and trichloroacetic acid were from Sigma (St. Louis, MO). Organic solvents and thin-layer chromatography (TLC) plates were from Merck (Darmstadt, FRG). Sodium 2-[^{14}C]acetate (specific radioactivity, 40–60 mCi/mmol) and 1-[^{14}C]leucine (specific radioactivity, 50–60 mCi/mmol) were from Amersham (Buckinghamshire, England).

Cells. The isolation and maintenance of human small intestine epithelial cells (enterocytes) have been previously described (16). Human umbilical vein endothelial cells were isolated and cultured according to Gimbrone *et al.* (17). Normal human skin fibroblasts were obtained from the skin of healthy volunteers; fibroblasts from the skin of a patient with familial homozygous hypercholesterolemia were a kind gift of Dr. I. Fuki. The human hepatoma cell line HEP G2 was a kind gift of Dr. A. Shnyra. Fibroblasts and HEP G2 cells were grown in a humidified incubator at 37°C using Leibovitz L-15 medium containing 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 μg/mL kanamycin and 2.5 μg/mL fungisone. Cells were grown to confluency on 24-well

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Abbreviations: DPS, digitonin-precipitable sterols; FCS, fetal calf serum; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; LDL, low density lipoprotein; MEM, minimum essential medium; TCA, trichloroacetic acid; TLC, thin-layer chromatography.

plates (area 2 cm^2). Human enterocytes were used in suspension at a concentration of 2×10^6 cells per well.

Cholesterol synthesis. The incubation mixture contained 10 μCi sodium 2-[^{14}C]acetate, indicated concentrations of compactin-related compounds and appropriate medium in a final volume of 0.5 mL. Since the cholesterol synthesis rate in the endothelial cells was very low, 50 μCi [^{14}C]acetate was added to the incubation mixture in these experiments. All compactin-related compounds, except ML-236A, were stored at −20°C in dimethylsulfoxide stock solutions containing 4 mg/mL. Immediately prior to use, these solutions were diluted with the appropriate medium. Preliminary experiments showed that the low dimethylsulfoxide levels had no effect on cholesterol synthesis in any of the cell types. The stock solution of compound ML-236A (4 mg/mL) was prepared in Hanks balanced salt solution.

Cells were incubated for 2 hr at 37°C in air (when bicarbonate-free media were used) or 5% CO_2, 95% air (when bicarbonate-containing medium was used) with shaking at 60 rpm in an orbital shaker. After incubation, the cells were washed twice with balanced salt solution and dissolved in 1 M NaOH. Samples were saponified by incubation for 2 hr at 100°C in 5 M KOH and 50% ethanol. Cholesterol was precipitated with digitonin according to Sperry and Webb (18). Preliminary experiments showed that [^{14}C]acetate incorporation into digitonin-precipitable sterols (DPS) was linear for at least 3 hr for all cell types.

Fatty acids and protein synthesis. In order to determine the rate of fatty acid synthesis, cells were incubated as described above, dissolved in 1 M NaOH and saponified. Lipids were extracted according to Folch *et al.* (19), and fatty acids were isolated by thin-layer chromatography as described previously (16).

For protein synthesis determinations, cells were incubated in minimum essential medium (MEM) without leucine, containing 10 μCi/mL 1-[^{14}C]leucine, 2 mM L-glutamine, 100 μg/mL kanamycin, 2.5 μg/mL fungisone for 2 hr at 37°C in 5% CO_2, 95% air. After the incubation, cells were washed twice with MEM, scraped with a rubber policeman and lysed by freezing-thawing. Protein was precipitated with 10% trichloroacetic acid (TCA) and washed twice with 5% TCA. [^{14}C]Leucine incorporation into the TCA-precipitable fraction was determined. Cell protein content was determined according to Bradford (20).

Data analysis and statistics. The rate of cholesterol synthesis was determined by the amount of [^{14}C]acetate incorporated into digitonin-precipitable sterols in 2 hr normalized per mg cell protein. Since use of [^{14}C]acetate may result in the underestimation of the absolute rate of cholesterol synthesis (21), results were expressed relative to controls. Dose-dependence curves were approximated using computerized polynomial regression analysis, and theoretic values of the doses required for inhibition of cholesterol synthesis by 50% of maximal inhibition (IC_{50}) were calculated from these approximated curves. Maximal inhibition of cholesterol synthesis refers to the point(s) where the maximal effect of the compound was observed. Statistical significance was calculated using Student's t-test.

Experiments were performed in triplicate and reproduced 2-3 times. The coefficients of variation were less than 15%.

RESULTS

Six compactin-related compounds were studied in this work: mevinolin, compactin, ML-236A, monacolin X, monacolin L and dihydromonacolin L. The structures of these compounds are presented in Figure 1. The effect of these compounds on cholesterol synthesis was tested on five human cell types: human umbilical vein endothelium, human enterocytes, the human hepatoma cell line HEP G2, normal human skin fibroblasts, and human skin fibroblasts lacking the low-density lipoprotein (LDL) receptor.

All compactin-related compounds appeared to be effective inhibitors of cholesterol synthesis in human umbilical vein endothelial cells (Fig. 2). Mevinolin, compactin and monacolin X inhibited cholesterol synthesis by 90% while monacolin L, ML-236A and dihydromonacolin L reduced synthesis by 70-80% (Table 1). All compounds were effective at very low concentrations: IC_{50} values for all compounds, except dihydromonacolin L, were 1-13 pg/mL (Table 2).

A more pronounced difference between the different compounds was seen in human enterocytes (Fig. 3). Mevinolin was the most effective, inhibiting cholesterol synthesis by 75%, followed by compactin and monacolin X (54-56% inhibition), while the other compounds were less effective (Table 1). The IC_{50} values for all compounds, except dihydromonacolin L, were similar to those of endothelial cells (Table 2).

Significant cholesterol inhibitory differences were also found in HEP G2 cells (Fig. 4). Mevinolin was the most effective compound followed by compactin, monacolin X, ML-236A and monacolin L. Dihydromonacolin L was nearly inactive (Table 1). The IC_{50} values found for the hepatoma cells were much higher than those for endothelial cells and enterocytes (Table 2).

In normal human skin fibroblasts, the relative inhibition of the compounds was similar to that in hepatoma cells (Fig. 5, Table 1). Again, mevinolin was the most active compound. Compactin, monacolin X and ML-236A were less active and dihydromonacolin L was the least. With the exception of monacolin X, the IC_{50} values for all the compounds were higher when compared to the hepatoma cells (Table 2).

In human skin fibroblasts lacking LDL receptor activity (Fig. 6), the maximal inhibition of the compounds

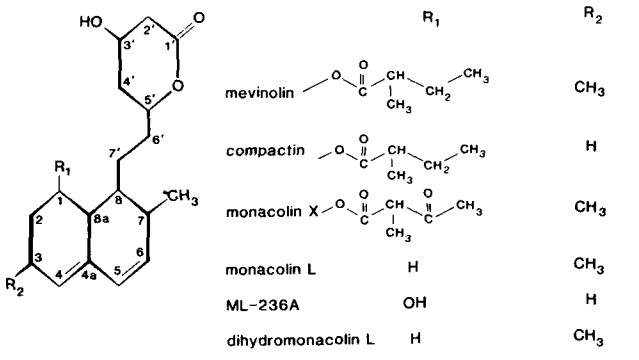

FIG. 1. Structures of the compactin-related compounds. Double bound 4-4a is saturated in dihydromonacolin L.

TABLE 1

Maximal Inhibition of Cholesterol Synthesis by Compactin-Related Compounds

Cells	Maximal inhibition (% of control)[a]					
	Mevinolin	Compactin	Monacolin X	ML-236A	Monacolin L	D-Monacolin L
Endothelium	90 ± 2	89 ± 1	92 ± 3	81 ± 3	75 ± 3	70 ± 6
Enterocytes	76 ± 6	54 ± 4	56 ± 2	25 ± 12	37 ± 7	36 ± 6
HEP G2	90 ± 5	57 ± 8	41 ± 7	31 ± 7	35 ± 4	19 ± 16
Fibroblasts normal	80 ± 4	70 ± 4	47 ± 3	47 ± 12	50 ± 9	22 ± 13
Fibroblasts ldl−	90 ± 1	88 ± 3	70 ± 3	58 ± 4	49 ± 2	39 ± 5
p[b]		0.09[c]	0.04[c]	0.02[d] 0.001[g]	0.43[e]	0.03[f] 0.007[g]

[a]Mean ± SEM of triplicate determinations.
[b]According to Student's t-test.
[c]vs mevinolin.
[d]vs monacolin X.
[e]vs ML-236A.
[f]vs monacolin L.
[g]vs mevinolin and compactin.

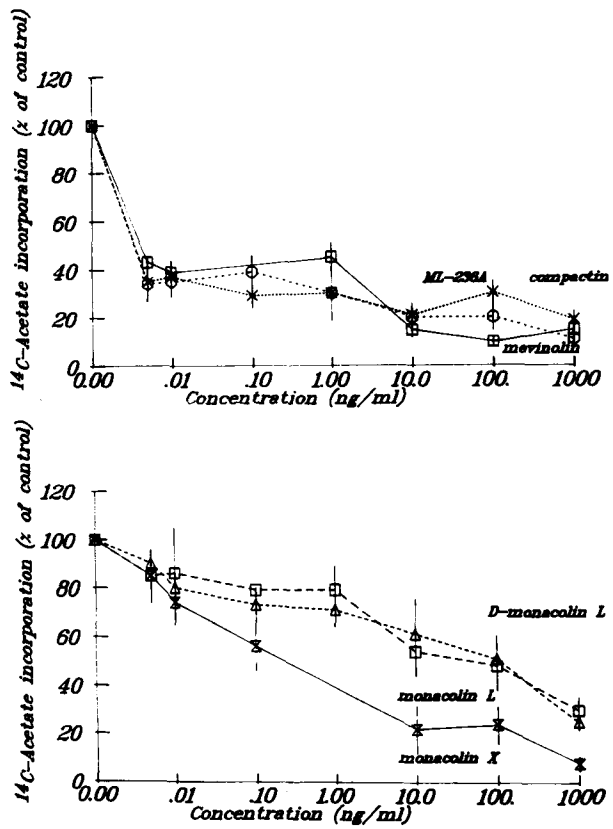

FIG. 2. Dose-dependence of cholesterol synthesis inhibition in human umbilical vein endothelial cells by compactin-related compounds. Human umbilical vein endothelial cells were incubated for 2 hr at 37°C and 5% CO_2 with the 100 μCi/mL [^{14}C]acetate and the indicated concentration of each compactin-related compound. Incorporation of the [^{14}C]acetate into digitonin-precipitable sterols was measured as described in Materials and Methods. Each point represents the mean ± standard error of mean of triplicate determinations. The basal level of cholesterol synthesis was 7760±935 dpm/mg cell protein. (The value is not normalized for the higher concentration of [^{14}C]acetate.)

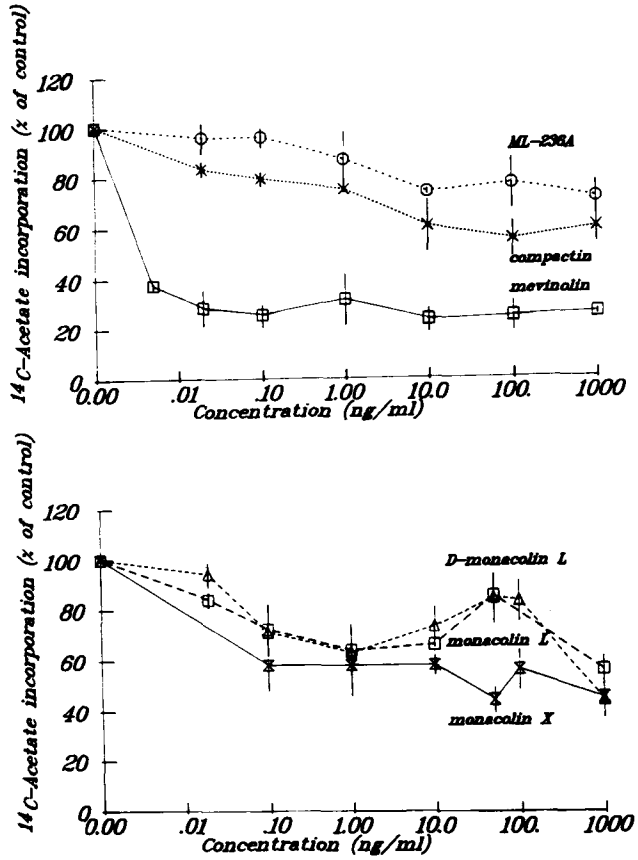

FIG. 3. Dose-dependence of cholesterol synthesis inhibition in human enterocytes by compactin-related compounds. Human enterocytes were incubated for 2 hr at 37°C with the 20 μCi/mL [^{14}C]acetate and the indicated concentration of each compactin-related compound. Incorporation of the [^{14}C]acetate into digitonin-precipitable sterols was measured as described in Materials and Methods. Each point represents the mean ± standard error of mean of triplicate determinations. The basal level of cholesterol synthesis was 7289±2525 dpm/mg cell protein.

TABLE 2

Doses of 50% Inhibition of Cholesterol Synthesis by Compactin-Related Compounds

Cells	IC_{50} (ng/mL)[a]					
	Mevinolin	Compactin	Monacolin X	ML-236A	Monacolin L	D-Monacolin L
Endothelium	0.006	0.002	0.013	0.002	0.001	3.16
Enterocytes	0.005	0.003	0.007	5.0	0.031	0.007
HEP G2	0.011	0.13	25.1	66.1	0.70	63.1
Fibroblasts normal	0.1	1.58	3.1	100.0	2.51	199.5
Fibroblasts ldl−	1.0	11.22	100.0	79.5	56.2	125.9

[a]Values of IC_{50} were calculated as described in Materials and Methods.

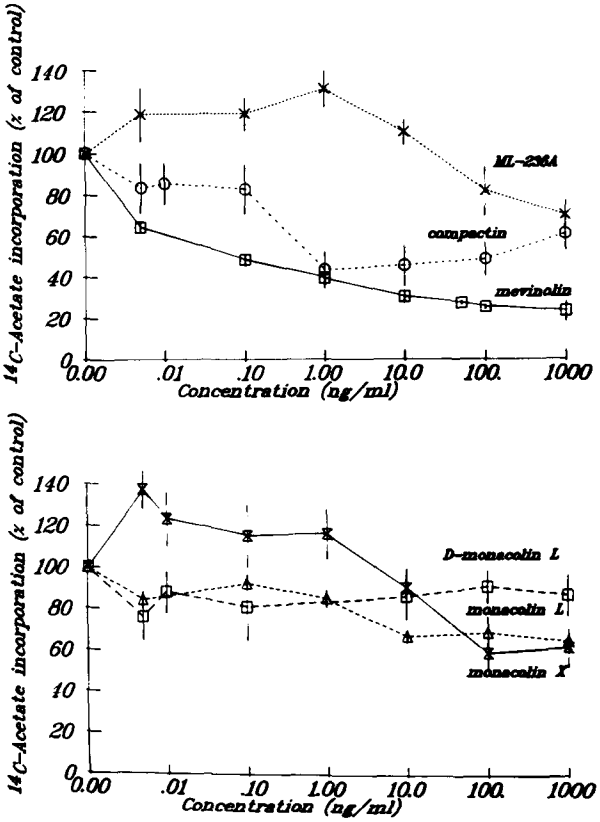

FIG. 4. Dose-dependence of cholesterol synthesis inhibition in human hepatoma cells HEP G2 by compactin-related compounds. Human hepatoma cells were incubated for 2 hr at 37°C with the 20 μCi/mL [^{14}C]acetate and the indicated concentration of each compactin-related compound. Incorporation of the [^{14}C]acetate into digitonin-precipitable sterols was measured as described in Materials and Methods. Each point represents the mean ± standard error of mean of triplicate determinations. The basal level of cholesterol synthesis was 577284±101733 dpm/mg cell protein.

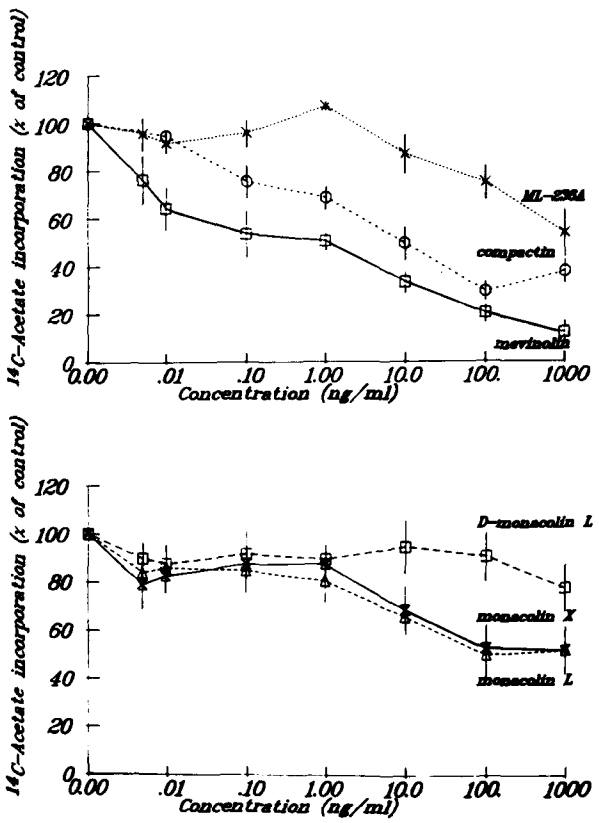

FIG. 5. Dose-dependence of cholesterol synthesis inhibition in human skin fibroblasts by compactin-related compounds. Human skin fibroblasts were incubated for 2 hr at 37°C with the 20 μCi/mL [^{14}C]acetate and the indicated concentration of each compactin-related compound. Incorporation of the [^{14}C]acetate into digitonin-precipitable sterols was measured as described in Materials and Methods. Each point represents the mean ± standard error of mean of triplicate determinations. The basal level of cholesterol synthesis was 41567±4449 dpm/mg cell protein.

(Table 1) and the IC_{50} values (Table 2) were higher or equal to those of normal fibroblasts. The rank order of inhibition for the compounds remained the same.

In order to compare the effect of different compactin-related compounds on cholesterol synthesis in different cells, two parameters were selected: the maximal inhibition of cholesterol synthesis and the dose required for 50% inhibition of cholesterol synthesis (IC_{50}). The values for maximal inhibition of cholesterol synthesis are presented in Table 1. The maximal inhibition of cholesterol synthesis by each compound varied from one cell type to another, but the rank order of cholesterol synthesis inhibition for the compounds was similar in all cell types. The most effective inhibitors of cholesterol synthesis were mevinolin and compactin. Monacolin X, monacolin L and ML-236A were less effective, and dihydromonacolin L was the least effective.

The IC_{50} values (Table 2) varied considerably from one

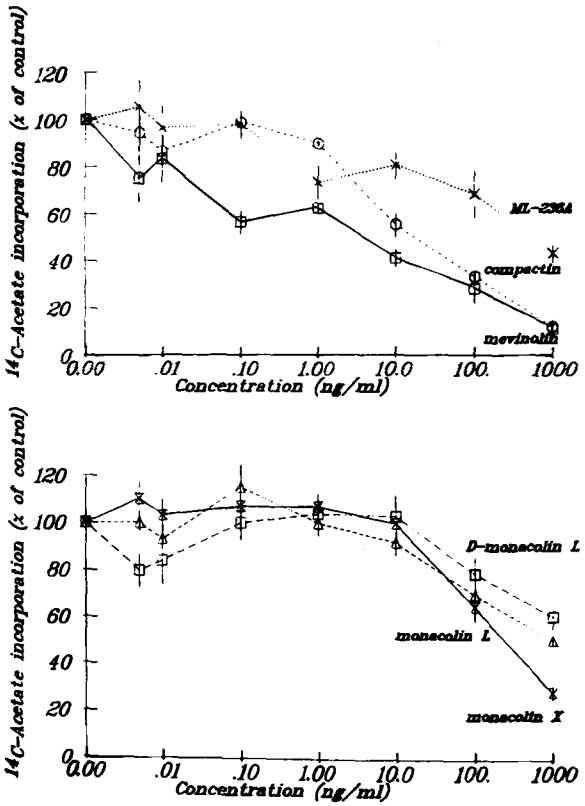

FIG. 6. Dose-dependence of cholesterol synthesis inhibition in human skin fibroblasts lacking LDL receptor by compactin-related compounds. Cells were incubated for 2 hr at 37°C with the 20 μCi/mL [^{14}C]acetate and the indicated concentration of each compactin-related compound. Incorporation of the [^{14}C]acetate into digitonin-precipitable sterols was measured as described in Materials and Methods. Each point represents the mean ± standard error of mean of triplicate determinations. The basal level of cholesterol synthesis was 38742±2561 dpm/mg cell protein.

compound to another; however, a pattern could be observed. Endothelial cells and enterocytes were sensitive to very low drug doses, hepatoma cells to much higher levels, and fibroblasts to even higher concentrations. With few exceptions, this relationship was found for all compounds. No correlation could be established between the sensitivity of the cells to compactin-related compounds and the basal rate of incorporation of [^{14}C]-acetate into digitonin-precipitable sterols.

The lactone forms of all compounds, except ML-236A, were used in this study. When comparing the effects of lactone and acid forms of the five compounds on cholesterol synthesis in hepatoma cells, no statistically significant differences were found (not shown). The biochemical specificity of the compactin-related compounds was evaluated by measuring their effect on fatty acid and protein synthesis. When using concentrations higher than their corresponding cholesterol synthesis IC_{50} values, neither protein nor fatty acid synthesis was inhibited by any of the compactin-related compounds (not shown).

DISCUSSION

Compactin-related compounds are very effective inhibitors of cholesterol synthesis (5-8,11-15,22-26). Their effects on cholesterol synthesis have been studied in animal cells (22-24) and in cell-free enzyme systems (11,13,14,24). However, information on the effects of these compounds on human cells is still scanty. The literature reports only investigations on human lymphocytes and fibroblasts (25,26).

In the present work the specific inhibition of cholesterol synthesis in five human cells types by six compactin-related compounds was demonstrated. The rank order of the different compounds in respect to their cholesterol synthesis inhibition was similar in all cells tested. The most effective inhibitors were mevinolin and compactin, followed by monacolin X, monacolin L and ML-236A, and finally dihydromonacolin L. Apparently, the effectiveness of these compounds depends on their structure rather than on the cell used. The most potent compounds shared a common structural feature, namely a 2-methylbutanoyl moiety, in the position designated R_1 (Fig. 1). This moiety has some similarity with the structure of HMG-CoA, the substrate of HMG-CoA reductase, and thus may be able to interact with HMG-CoA reductase. Another portion of the compactin-related compound that is also reminiscent of HMG-CoA is the 1′ − 5′ moiety (Fig. 1). However, i) conversion of lactone to the β-hydroxy acid form did not affect the activity of the compounds and ii) compounds which possess this moiety may differ significantly in respect to their activity as cholesterol synthesis inhibitors. It may be therefore assumed that despite the possible intracellular conversion of the lactone to the acid form (27), the R_1 structure may be as important as the 1′ − 5′ portion to exhibit potency. The relationship between different inhibitors observed in this work is consistent with the data obtained in HMG-CoA reductase preparations (13,14).

A dose-dependent tissue selectivity was seen in the compactin-related compounds. Endothelial cells and enterocytes were sensitive to very low drug levels, hepatoma cells required higher levels, and fibroblasts needed even higher concentrations of the compactin-related compounds. The nature of this phenomenon is unknown. Certainly it cannot be explained by the differences in HMG-CoA reductase activity, since there was no correlation between basal cholesterol production and the IC_{50} for the compactin-related compounds in different cells. Perhaps the tissue selectivity may be due to a different localization of the HMG-CoA reductase within different cells or differences in the intracellular conversion of the lactone to the acid form (27). The tissue selectivity is not related to the different properties of HMG-CoA reductase itself (e.g., presence of different isoenzymes, etc.) since only one copy of HMG-CoA reductase gene has been found in the human genome (28). It is therefore clear that this phenomenon, which could not be demonstrated in the HMG-CoA reductase assay, can be observed in the cell model.

The difference between the normal and LDL receptor defective fibroblasts is of particular interest, since it may represent a functional regulatory link between the LDL receptor and HMG-CoA reductase. An altered LDL receptor was described in compactin-resistant Chinese hamster cell mutants (29). A difference in the IC_{50} and the maximal compactin inhibition of cholesterol synthesis in hepatocytes as well as normal and LDL receptor defective fibroblasts has also been demonstrated by Tsujita

et al. (22). These authors have also demonstrated that another compactin-related compound, CS-514, possesses an even more pronounced tissue selectivity. Together with the results of the present work, it is possible to speculate that tissue selectivity is a characteristic of all compactin-related compounds. In spite of the fact that the tissue selectivity of the compounds tested in the present study is not high enough to suggest that one of them may function as a tissue selective drug, our findings open a way for the search of compounds that would be able to inhibit cholesterol synthesis in one tissue without affecting the pathway in others. The use of cultured human cells demonstrates the convenience of this type of model for human *in vitro* screening of different drugs.

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Molecular Species Composition of Phosphatidylinositol from the Brain, Retina, Liver and Muscle of Cod (*Gadus morhua*)

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The molecular species composition of phosphatidylinositol (PI) purified from four different tissues from cod was found to show large tissue-specific differences. In brain 18:0/20:5 was the most abundant species (40.8%) followed by 18:1/20:5 (13.5%). In retina, 24–26% each of PI was the 16:0/22:6 and 18:0/20:4 species with 16–18% each of 18:0/20:5 and 18:0/22:6. In liver, almost half of the PI was 18:0/20:4 with 18% 18:1/20:4. In contrast, muscle contained almost 40% of 18:0/22:6 with 10–14% each of 18:0/20:4, 18:0/20:5 and 18:1/22:6.
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The development of increasingly sophisticated high performance liquid chromatography (HPLC) and gas liquid chromatography (GLC) methods for molecular species analysis of phospholipids has shown the great complexity of the lipid components of biomembranes. However, most such analyses have focused on the major phospholipids, phosphatidylcholine (PC) and phosphatidylethanolamine (PE). Relatively few analyses of phosphatidylinositol (PI) are available, though it was known from fatty acid composition data that PI is rich in stearic and arachidonic acids. Positional analysis showed that in PI from rat liver most stearic acid was in the 1-position of the glycerol and most arachidonic acid in the 2-position (1) and this was also the case in PI, phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate from bovine brain (2). There was, therefore, strong evidence that 18:0/20:4 was the major molecular species in PI from terrestrial animals. This was confirmed directly in PI from rat liver, where 57.8% was 18:0/20:4 (3), in rat erythrocytes (44.1%) (4) and in human erythrocytes (45.3%) (5). However, arachidonic acid is the major polyunsaturated fatty acid (PUFA) in terrestrial animals so it is not surprising that arachidonyl species of phospholipids predominate. The 18:0/20:4 species was also of major significance in PC, PE and phosphatidylserine (PS) from rat liver (3) and from rat erythrocytes (4), and in PE and PS from human erythrocytes (5).

In both freshwater and particularly seawater fish, eicosapentaenoic acid 20:5n-3 and docosahexaenoic acid 22:6n-3 are the major PUFAs (6,7). PC and PE from cod roe and PC, PE and PS from the brain and retina of trout are dominated by 22:6n-3 containing molecular species and, to a lesser extent, 20:5n-3 containing species (8,9). However, even in tissues such as cod roe where n-3 PUFA predominate, PI contained 36.7% 18:0/20:4, but there was only 0.7% of this species in each of PC and PE (8). About 40% of trout retina PI was also 18:0/20:4, but in trout brain PI 18:0/20:5 was the major species (42.3%) (9). This paper shows that the 18:0/20:5 species is also predominant in PI from the brain of a marine fish, cod, and that there are also large differences in the composition of PI in other tissues.

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Abbreviations: BHT, butylated hydroxytoluene; GLC, gas liquid chromatography; HPLC, high performance liquid chromatography; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PUFA, polyunsaturated fatty acid; TLC, thin-layer chromatography. Molecular species are abbreviated as follows: *e.g.*, 18:0/20:4 PI is 1-stearoyl-2-eicosatetraenoyl-*sn*-glycero-3-phosphoinositol.

MATERIALS AND METHODS

Materials. Cod *(Gadus morhua)* (300–600 g weight) were obtained from the Marine Station, Millport on the Firth of Clyde, and maintained in a seawater aquarium at 10–14°C on a chopped squid diet. Fish were killed by decapitation and the eyes, liver and a fillet of muscle removed and frozen at −70°C until required. Brains were removed and used immediately. *Bacillus thuringiensis* type strain IAM 12077 was obtained from the National Collection of Industrial and Marine Bacteria Ltd. (Aberdeen, Scotland).

Butylated hydroxytoluene (BHT) was obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). 3,5-Dinitrobenzoylchloride was from Aldrich Chemical Co. (Gillingham, Dorset, U.K.) and was recrystallized from carbon tetrachloride before use. Standard lipids were obtained from Sigma and from Nu-Chek Prep (Elysian, MN), as detailed in Bell and Tocher (9). Merck thin-layer chromatography (TLC) and HPTLC plates coated with Silica Gel 60, Analar grade glacial acetic acid, carbon tetrachloride, propan-2-ol and pyridine were purchased from BDH Ltd. (Poole, Dorset, U.K.). All other solvents of HPLC grade were from Rathburn Chemicals (Walkerburn, Peeblesshire, Scotland). Ultrasphere ODS and Ultrasphere C8 HPLC columns (25 × 0.46 cm, 5 micron particle size) were obtained from Altex/Beckman (Beckman Instruments U.K., High Wycombe, Bucks, U.K.).

Extraction and purification of lipids. All solvents apart from those used for TLC and HPLC contained 0.01% (w/v) BHT. Samples were stored at −20°C under nitrogen between preparative procedures.

The brains were taken from 20 cod, the meninges removed and the remaining tissue (8.3 g) homogenized in 10 volumes of chloroform/methanol (2:1, v/v) using a Polytron tissue disrupter. The retinas from 40 frozen cod eyes (5.7 g tissue) and 20 chopped frozen livers (89 g) were each homogenized in 10 volumes of chloroform/methanol (2:1, v/v) and finely chopped frozen muscle fillets (211 g) from 20 fish in 5 volumes of solvent. After homogenization, total lipid was extracted by the method of Folch *et al.* (10).

Neutral lipid was first removed from the brain and liver extracts by preparative TLC in hexane/diethyl ether/acetic acid (70:30:1, v/v/v). Phospholipids were eluted from the origin with chloroform/methanol/water (5:5:1, v/v/v), dried by rotary evaporation under vacuum at 35°C and finally under a stream of nitrogen. Phospholipid classes were separated by TLC in chloroform/ethanol/water/triethylamine (30:35:6:35, v/v/v/v) (11) and the lipids visualized under UV light after spraying with 0.1% (w/v), 2′,7′-dichlorofluorescein in methanol containing 0.01%

(w/v) BHT. Phospholipids were eluted from the silica gel with three 40 mL washes of chloroform/methanol/water (5:5:1, v/v/v) and dried as before. 2′,7′-Dichlorofluorescein was removed from lipids by extracting with a solution of 2% (w/v) $KHCO_3$. Phosphatidylinositol was further purified by TLC in chloroform/acetone/methanol/acetic acid/water (10:4:2:2:1, v/v/v/v/v) (12), detected and extracted as before. All phospholipids were finally checked for purity by HPTLC in methyl acetate/propan-2-ol/chloroform/methanol/0.25% (w/v) KCl (25:25:25:10:9, v/v/v/v/v) (13) and lipids detected by spraying with 3% (w/v) copper acetate in 8% (v/v) phosphoric acid and charring at 160°C for 15 min.

Fatty acid methyl esters were prepared by esterification in 2 mL of 1% (v/v) concentrated sulfuric acid in methanol at 50°C for 16 hr under nitrogen. The fatty acid composition of the phospholipids was determined by GLC of fatty acid methyl esters in a Packard 436 chromatograph fitted with a CP Wax 52CB fused silica capillary column (50 m × 0.32 mm id) (Chrompack U.K. Ltd., London) using hydrogen as carrier gas (14).

Preparation of 3,5-dinitrobenzoyl derivatives. One-mg portions of PI were hydrolyzed with a PI-specific phospholipase C prepared from *B. thuringienis* (15) using a two phase system of 1 mL diethyl ether and 1 mL 0.1 M sodium borate buffer (pH 7.5) at room temperature under nitrogen for 5 hr (16). At the end of the incubations, 1,2-diacylglycerols were extracted and purified by TLC in hexane/diethyl ether/acetic acid (50:50:1, v/v/v) (8). Amounts of PI remaining after phospholipase C digestion were <2% of the starting material. Diacylglycerols were derivatized in dry pyridine with 3,5-dinitrobenzoyl chloride at 60°C for 45 min under nitrogen, extracted and washed as described by Takamura *et al.* (17). The purity of the product was checked by HPTLC in hexane/diethyl ether/acetic acid (70:30:1, v/v/v).

Separation of molecular species. The 1,2-diacyl-3-dinitrobenzoyl-*sn*-glycerols were separated by HPLC at 19–21°C on reverse phase columns using a Pye Unicam 4010 pump (Pye Unicam, Cambridge, U.K.) and three isocratic solvent systems. An ODS column was used with methanol/propan-2-ol (95:5, v/v), flow rate 1.0 mL/min and acetonitrile/propan-2-ol (80:20, v/v), flow rate 1.0 mL/min (17); a C8 column was used with methanol/water/acetonitrile (93:5:2, v/v/v), flow rate 1.2 mL/min (8). Peaks were detected at 254 nm with a Pye Unicam 4020 detector and quantified using a Shimadzu C-R3A (Anachem, Luton, Bedfordshire, U.K.) recording integrator.

Peaks were identified from plots of $\log_{10}$ (relative retention time) *vs* the effective carbon number on the C-1 position of the glyceride (13,17) using 16:0/22:6 as a reference peak. Each sample was chromatographed three times in each of the solvent systems and the standard deviations calculated. Where final peak areas were calculated by difference, the standard deviations of the contributing peaks were added to give the final error. For clarity, results are given to one decimal place.

RESULTS AND DISCUSSION

The molecular species compositions of PI from the muscle, liver, brain and retina of cod were all markedly different (Table 1). Stearoyl species were the most abundant in each tissue but oleoyl species were also prominent in brain, liver and muscle, and palmitoyl species in retina. Trace amounts of diPUFA species were found in retina and liver.

In brain, 18:0/20:5 was the most abundant species (40.8%) followed by 18:1/20:5 (13.5%). No other species was present at greater than 10%. Other species, including 18:0/20:4, 18:0/22:6, 16:0/20:5 and 16:0/22:6, were present between 5.1 and 9.1%, and another 17 minor species were found. Phosphatidylinositol from cod brain was remarkably similar to that from trout brain (9). In retina, 18:0/20:4 was the most abundant stearoyl species, but this was not as marked as in the retina of trout (9); 18:0/20:5 and 18:0/22:6 were almost as prominent. However, 16:0/22:6 was the single most abundant species in PI from cod retina, a situation in contrast to that in the trout.

Liver was the only tissue in which 18:0/20:4 was the dominant species, comprising almost half the sample. Another arachidonoyl species, 18:1/20:4, was the next most abundant (18.3%) and another 28 species were present at up to 5.8% each. Fatty acid analyses of PC, PE and PS from cod liver revealed only 2.0–4.5% arachidonate (unpublished observations). In muscle, 18:0/22:6 (39.7%) was the most abundant species while the two stearoyl/C_{20} PUFA species—18:0/20:5 and 18:0/20:4—comprised 13.4% and 11.0%, respectively; 18:1/22:6 was almost as abundant and another 19 species were found. Muscle had, by some 15%, the lowest total of 18:0/C_{20} PUFA species. In PI from cod roe, 18:0/20:4 was found to be the main species (8), and in microsomes prepared from the gills of cod arachidonic acid was found to be the major PUFA (19).

We conclude that phosphatidylinositol isolated from tissues of a species containing large amounts of n-3 PUFA has a much more variable molecular species composition than had previously been thought from results obtained from terrestrial animals where arachidonic acid is the major PUFA. There is a marked tissue specificity in the composition of PI, but the means by which this is achieved is not understood. However, it is clear that the specificities of the enzymes involved in *de novo* synthesis of PI, the phospholipases and transacylases involved in retailoring PI *in situ* in the biomembrane, and the PI-transfer proteins must be determined primarily by genetic means rather than by the availability of substrate PUFAs, since the structural phospholipids PC, PE and PS do not show the same selectivity as PI.

Prostaglandins are found in most tissues where they have diverse roles. Although other phospholipid classes may provide fatty acids for eicosanoid synthesis, in fish tissues arachidonate is overwhelmingly concentrated in PI (8,19,20). The main prostaglandin in fish tissues is PGE_2 (6), *i.e.*, derived from 20:4n-6, which is strongly suggestive that in fish the substrate for eicosanoid synthesis must come from PI. The present findings are particularly intriguing in the light of recent findings that protein kinase C has a distinct and, in some cases, very precise tissue specificity (21). Diacylglycerol formed from PI is a cofactor for this enzyme and it may be that tissue specificity of protein kinase C is related to the tissue specificity of PI *via* the diacylglycerol formed from PI by phosphoinositidase C. The same argument could hold for phosphoinositidase C itself since there is now compelling evidence that this enzyme also has marked tissue specificity (22).

TABLE 1

Molecular Species Compositions of Phosphatidylinositols from Cod Tissues[a]

	Species	Mole %			
		Brain	Retina	Liver	Muscle
PUFA/PUFA	20:5/22:6; 22:6/20:5	—	0.2 ± 0.1	0.1 ± 0.0	—
	22:6/22:6	—	0.3 ± 0.1	—	—
	20:5/20:4; 20:4/20:5	—	—	0.1 ± 0.0	—
sat/PUFA	16:0/20:5	5.7 ± 0.2	1.9 ± 0.2	1.7 ± 0.7	1.5 ± 0.2
	16:0/22:6	5.1 ± 0.4	26.4 ± 0.3	1.7 ± 0.3	5.0 ± 0.3
	16:0/20:4	2.0 ± 0.1	3.6 ± 0.2	5.8 ± 0.6	1.2 ± 0.1
	16:0/22:5	0.2 ± 0.1	—	0.8 ± 0.1	1.6 ± 0.2
	16:0/18:2	0.4 ± 0.1	0.3 ± 0.1	—	0.3 ± 0.2
	18:0/20:5	40.8 ± 0.3	16.5 ± 0.7	5.2 ± 0.2	13.4 ± 0.5
	18:0/22:6	8.1 ± 0.5	17.8 ± 0.5	2.2 ± 0.1	39.7 ± 0.7
	18:0/20:4	9.1 ± 0.1	23.8 ± 0.9	49.1 ± 1.5	11.0 ± 0.5
	18:0/22:5	0.2 ± 0.1	—	1.1 ± 0.1	4.7 ± 0.4
	18:0/18:2	—	—	0.2 ± 0.0	0.2 ± 0.0
	18:0/22:4	—	—	—	0.4 ± 0.1
monoene/PUFA	16:1/20:5	—	—	0.1 ± 0.0	—
	16:1/22:6	—	—	0.2 ± 0.1	—
	16:1/20:4	—	—	1.1 ± 0.1	—
	16:1/22:5	—	—	—	0.2 ± 0.1
	16:1/18:2	—	0.2 ± 0.0	tr	0.2 ± 0.0
	18:1/20:5	13.5 ± 0.5	1.7 ± 0.3	2.5 ± 0.1	2.5 ± 0.1
	18:1/22:6	3.7 ± 0.1	2.6 ± 0.3	1.9 ± 0.1	10.4 ± 0.4
	18:1/20:4	3.1 ± 0.0	3.0 ± 0.2	18.3 ± 0.3	3.8 ± 0.6
	18:1/22:5	1.0 ± 0.2	0.5 ± 0.1	—	tr
	18:1/18:2	—	—	—	1.7 ± 0.2
	20:1/20:5	1.2 ± 0.2	—	2.6 ± 0.3	0.2 ± 0.0
	20:1/22:6	0.7 ± 0.3	—	0.6 ± 0.1	0.5 ± 0.2
	20:1/20:4	1.1 ± 0.3	0.3 ± 0.1	0.3 ± 0.1	0.8 ± 0.3
	20:1/22:5	1.2 ± 0.1	tr	0.6 ± 0.1	0.5 ± 0.1
sat/sat	14:0/16:0; 16:0/14:0	—	0.5 ± 0.1	0.9 ± 0.1	—
	16:0/16:0; (14:0/18:0)	0.2 ± 0.1	—	1.4 ± 0.1	—
sat/monoene	14:0/16:1	—	—	0.5 ± 0.1	—
	16:0/18:1; (14:0/20:1)	0.5 ± 0.1	0.4 ± 0.0	0.6 ± 0.1	tr
	18:0/16:1	0.2 ± 0.1	—	—	—
	18:0/18:1	1.3 ± 0.2	0.1 ± 0.0	—	—
monoene/monoene	18:1/18:1; (16:1/20:1)	0.4 ± 0.1	—	0.2 ± 0.0	—
	18:1/20:1; 20:1/18:1	0.3 ± 0.1	—	—	—

[a]The 1,2-diacyl-3-dinitrobenzoyl-glycerols were prepared and chromatographed by isocratic, reverse phase HPLC as described in the methods section. Peaks were detected at 254 nm with a Pye Unicam 4020 detector and quantified using a Shimadzu C-R3A recording integrator. The first solvent (methanol/propan-2-ol) gave the basic molecular species composition and the other two solvent systems resolved most coeluting species. Each sample was chromatographed three times in each of the solvent systems and the standard deviations calculated. Where final peak areas were calculated by difference, the standard deviations of the contributing peaks were added to give the final error. For clarity, results are given to one decimal place. Compositions are corrected to 100%, tr, <0.1%; —, not detected. The following fatty acid isomers were identified by GLC: 16:1n-9 and n-7, 18:1n-9 and n-7, 20:1n-9 and n-7, 24:1n-9 and n-7, 18:2n-6, 20:4n-6, 20:5n-3 22:4n-6, 22:5n-3 and n-6 and 22:6n-3. The method could not resolve molecular species containing n-9 or n-7 isomers of monounsaturated fatty acids nor n-3 or n-6 isomers of polyunsaturated fatty acids. Molecular species are abbreviated as follows: *e.g.*, 18:0/20:4 PI is 1-stearoyl-2-eicosatetraenoyl-*sn*-glycerol-3-phosphoinositol. The most saturated fatty acid is assumed to be in position 1 of the glyceride (18). In coeluting pairs, the bracketed species are likely to be minor components based on the fatty acid composition of the phospholipids.

Few analyses of phospholipids have been carried out in tissues from marine mammals which consume high levels of n-3 PUFA, so it is unclear whether similar tissue specific differences in PI composition might occur in such species. However, in PI isolated from the platelets of five species of phocid seals, arachidonic acid was by far the major PUFA, with small amounts of eicosapentaenoic acid and trace amounts of docosahexaenoic acid (23).

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In Vitro Incorporation of Elongated Fatty Acyl Products into Lipid Classes in the Housefly, *Musca domestica* L. and the American Cockroach, *Periplaneta americana* (L.)

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The *in vitro* incorporation of elongated fatty acyl products into various lipid classes was studied in the American cockroach, *Periplaneta americana* (L.) and the housefly *Musca domestica* L. Stearoyl-CoA (18:0-CoA) and linoleoyl-CoA (18:2-CoA) were each elongated in microsomal preparations from abdominal epidermal tissue of the adult cockroach. Incorporation of radioactive tracer into different lipid classes was determined by thin-layer chromatography (TLC). In the American cockroach, 40–45% of the total radioactive label was incorporated into the free fatty acid fraction, with smaller amounts in the triglyceride (12–31%) and phospholipid (12–19%) fractions. Of the elongated products analyzed by radio-high performance liquid chromatography (HPLC), 53–60% was found in the free fatty acid fraction. In the housefly, the substrates 18:0-CoA and 18:1-CoA were used to determine into which lipids the elongated products would become incorporated. The saturated fatty acyl elongated products were found mainly in the free fatty acid (41%), triglyceride (23%), and acyl-CoA (17%) fractions. The monounsaturated fatty acyl elongated products were found in the triglyceride (44%), free fatty acid (11%), acyl-CoA (35%) and phospholipid (10%) fractions in three-day-old males. In three-day-old females, the elongated products were found in the triglyceride (45%), free fatty acid (28%), acyl-CoA (11%) and phospholipid (15%) fractions. From these data, it is not possible to determine the identity of the substrate for the conversion of the elongated fatty acyl products to the corresponding hydrocarbon (Hy). In the cockroach, incubations with 18:0-CoA and with 18:2-CoA resulted in small incorporations into 25:0 Hy and into 27:2 Hy, respectively. In the housefly, incubations with 18:1-CoA resulted in a very small production of 27:1 Hy in mature males and 23:1 Hy in mature female houseflies. These data support the idea that the preparation of subcellular fractions results in an uncoupling of fatty acid chain elongation from the conversion of the fatty acid to the corresponding hydrocarbon in both insects.
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The fate of desaturated and elongated fatty acyl products in microorganisms (1), plants (2,3) and insects (4) has been previously investigated to gain an understanding of the metabolism of prostaglandins and essential fatty acids. It has been suggested that prostaglandins in insects play an important role in reproduction (5). Arachidonic acid (20:4), the precursor for prostaglandins, is incorporated mainly into the phospholipid (ca. 70%) fraction and into triglycerides (ca. 18%) in the housefly, *Musca domestica* L. (5). Linoleate, synthesized by many insects, is found rather evenly distributed between the four major phospholipid fractions and the three neutral lipid fractions of housefly microsomes (6).

Desaturated and elongated products of stearic acid are also involved in the biosynthesis of hydrocarbons (7) which, in turn, accomplish several important functions in different insects including the housefly, *Musca domestica* L., and the American cockroach, *Periplaneta americana.* The elongation of 18:0 and 18:1 (housefly) and 18:0 and 18:2 (cockroach) to chain lengths up to 28 and 30 carbons has been demonstrated (8,9). Most of these very long chain fatty acids act as precursors to the very long chain hydrocarbons. These compounds serve to coat the surface of the insect to protect it from injury and prevent desiccation. In some insects, for example, in the housefly, the 24:1 fatty acyl moiety serves as the precursor to the major sex pheromone component, *(Z)*-9-tricosene (Z9-23 Hy). Z9-23 Hy is synthesized by the following sequence of reactions: i) the desaturation of stearoyl-CoA (18:0-CoA) to oleoyl-CoA (18:1-CoA); ii) the elongation of 18:1-CoA to a 24:1 acyl moiety; and iii) the conversion of the 24:1 acyl moiety to Z9-23 Hy by either decarbonylation or decarboxylation. In the pea leaf, *Pisum sativum* (10), and in the eared grebe *Podicebs nigricollis* (11), the conversion of the elongated fatty acyl product to the corresponding hydrocarbon occurs *via* decarbonylation. The fatty acyl-CoA is converted into an aldehyde which is subsequently converted to the hydrocarbon with the loss of the carboxyl carbon as carbon monoxide. In insects, it is not known whether the conversion of the elongated fatty acyl products to the corresponding hydrocarbons occurs by a decarbonylation or decarboxylation mechanism. Further, the incorporation of these elongated fatty acyl moieties into different lipid classes has not been reported. Because the chain elongation reactions require the acyl-CoA derivatives of the various fatty acids, there is ample opportunity for many competing reactions. To address the question of the *in vitro* fate of the elongated products formed from 18:0-CoA and 18:1-CoA (housefly), and 18:0-CoA and 18:2-CoA (cockroach), the lipid composition from microsomal preparations from both the housefly and the cockroach were examined. We were specifically interested in which lipid fractions the elongated fatty acids would be found.

MATERIALS AND METHODS

Insects. Insects were maintained as described earlier (8,9).

Radioactive materials. [1-^{14}C]Stearoyl-CoA (5 μCi/μmol at 4.4 nmol/μL), [9,10-^{3}H]oleoyl-CoA (5.2 μCi/μmol at 3.0 nmol/μL) and [1-^{14}C]linoleoyl-CoA (2.5 μCi/μmol at 3.9 nmol/μL) were prepared from the corresponding fatty acids (New England Nuclear, Boston, MA) by the procedure of Bergstrom and Retiz (12).

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Abbreviations: HPLC, high performance liquid chromatography; Hy, hydrocarbon; TLC, thin-layer chromatography.

Chemicals. Malonyl-CoA, NADPH, sucrose, dithiothreitol, magnesium chloride, sodium ascorbate, potassium hydrogen phosphate monobasic, potassium cyanide and perchloric acid were purchased from Sigma Chemical Company (St. Louis, MO). Biosil A was obtained from Biorad (Richmond, CA). BF_3 in methanol was purchased from Eastman Kodak Company (Rochester, NY).

Preparation and extraction of microsomes. Microsomes were prepared from whole housefly and abdominal epidermal cockroach tissues using the same protocols as described earlier (8,9). The microsomes were extracted by the procedure of Bligh and Dyer (13) and methyl esters of the total microsomal fatty acids were prepared (14). The fatty acid composition was determined by gas chromatography (15), and gas chromatography/mass spectrometry was used to identify the various fatty acids (16).

Elongation reaction assay. The elongation reaction assay conditions were identical to those previously reported (8,9), except for some modifications as indicated in the text. The reaction was stopped by the addition of 10% perchloric acid (final concentration was 1% perchloric acid, v/v).

Isolation and identification of elongation products. The reaction mixture containing perchloric acid was centrifuged at 2,000 rpm for 10 min to isolate a protein pellet that contained both protein and lipid fraction. The supernatant, which contained no radioactivity, was discarded. The lipid was then extracted from the pellet using chloroform/methanol (1:2, v/v) (13). The various lipid classes were separated on silica gel-H thin-layer chromatographic (TLC) plates using double development in petroleum ether/diethyl ether/acetic acid (83:17:1, v/v/v). The hydrocarbon fraction was collected and saved for further analysis. The other lipid bands were scraped from the plate into test tubes and extracted using appropriate solvents. The solvent was evaporated under a gentle stream of N_2, and the fatty acids were esterified with BF_3/methanol reagent (14). The fatty acid methyl esters were chromatographed on silica gel-H TLC plates using a hexane/diethyl ether (80:20, v/v) solvent system to remove impurities and unesterified fatty acids. The methyl ester fractions were scraped from the TLC plates and extracted with diethyl ether. The methyl esters were analyzed by radio-HPLC using a Spectra-Physics SP 8700 solvent delivery system (Spectra-Physics, St. Albans, U.K.) connected to a 15 cm × 4.6 mm LC-8 (Alltech Associates, Ontario, Canada) reverse phase column (3 μ particle size) (8). The hydrocarbon fraction was passed through a 20% silver nitrate Biosil A column as described (17), and fractionated by radio-HPLC using acetonitrile/hexane (99:1, v/v). The protein pellet containing the unextracted acyl-CoAs was then hydrolyzed by adding 1 mL of 1 N KOH, and heating at 80°C in a sand bath for 2 hr. The mixture was acidified to pH 2-3 with 2 mL of 0.5 N HCl and extracted by the procedure of Bligh and Dyer (13). The methyl esters were prepared and analyzed as described above. In a separate experiment, we neutralized the protein pellet by bringing the pH to about 7.0. We observed that about 1/3 of the counts that had precipitated upon perchloric acid addition were solubilized. This would be consistent with at least 1/3 of the counts in the protein precipitate being in the form of acyl-CoA. The remaining 2/3 of the counts probably represent either acyl-CoA or free fatty acid bound tightly to protein.

RESULTS

Table 1 shows the total incorporation of radioactivity into different lipid classes by microsomes prepared from abdominal epidermal tissues of the American cockroach. The substrates were 18:0-CoA and 18:2-CoA. This radioactivity represents the unreacted substrate as well as the elongated products. The greatest total incorporation of the radiolabel from the incubations with the 18:0-CoA and 18:2-CoA substrates was found in the free fatty acid fraction (40-45%), with lesser amounts in the phospholipid (12-32%) and triglyceride (12-19%) fractions.

The incorporation of elongated fatty acyl products formed from 18:0-CoA into the various lipid fractions was examined by radio-HPLC of the methylated fatty acids. These products ranged from 20 to 28 carbons (Table 2). Sixteen percent of the substrate was converted to elongated products. Of this 16% elongated products, there was significantly more 26:0 (4.6%) formed compared to the 22:0 (2.3%), 24:0 (2.3%) and 28:0 (1.1%). This 26:0 fatty acyl moiety is the immediate precursor for the 25:0 hydrocarbon, one of the two major hydrocarbons in the American cockroach. After extracting the hydrocarbon fraction from the TLC separation of the neutral lipids, this fraction was chromatographed on the radio-HPLC, and as we have previously demonstrated (9), the only hydrocarbon observed was 25:0 Hy. However, less than 1% of the total radioactivity was recovered in the hydrocarbon fraction. About 60% of the products formed from the elongation of 18:0-CoA were free fatty acids. The diglyceride fraction did not contain any elongated products. Despite the addition of the Δ9 desaturase inhibitor, KCN (8,9), desaturation of 18:0 to 18:1 was observed and the desaturated product, 18:1, was found mainly in the phospholipid (60%) fraction.

Table 3 shows the results of radio-HPLC analysis of the incorporation of the individual products from the elongation of 18:2-CoA into various lipid classes. The total amount of substrate elongation was about 41%, and the

TABLE 1

Distribution of Radioactivity in Different Lipid Classes Using [1-^{14}C]18:0-CoA and [1-^{14}C]18:2-CoA Substrates and 4 mg Microsomal Preparation from the American Cockroach[a]

	nmoles/assay	
Lipid class	18:0	18:2
Hydrocarbon	0.12 ± 0.07	0.71 ± 0.09
Triglyceride	3.42 ± 0.17	2.99 ± 0.26
Fatty acid	10.52 ± 0.48	10.65 ± 0.30
Diglyceride	1.31 ± 0.07	1.35 ± 0.54
Phospholipid	5.46 ± 0.41	2.96 ± 0.91
Acyl-CoA	1.68 ± 0.21	1.37 ± 0.41

[a]Microsomes were prepared from adult cockroaches as described in Materials and Methods. The incubation conditions for the elongation reactions were: 10 μM Acyl-CoA; 0.2 mM malonyl-CoA; 1 mM NADPH; 0.25 mM sucrose; 2 mM $MgCl_2$; 2 mM sodium ascorbate; 1 mM dithiothreitol; 0.1 mM potassium phosphate buffer (pH 7.2); 4 mg protein; 2 mL incubation volume; 45 min incubation time. The reaction was started by the addition of protein. The separation of the different lipid fractions is described in Materials and Methods. Each value represents the mean from eight experiments ± standard deviation.

TABLE 2

Radio-HPLC Analysis of the Incorporation of Individual Product Formed from [1-^{14}C]18:0-CoA into Different Lipid Classes in the American Cockroach[a]

Lipid class	Unreacted substrate 18:0	Desaturated product 18:1	Elongated fatty acyl product 20:0	22:0	24:0	26:0	28:0	Total elongated product
			nmoles/assay					
Expt. A								
Total Lipid	11.1	5.8	1.1	0.5	0.5	1.0	0.2	3.30
Expt. B								
Triglyceride	2.3 ± 0.2	1.0 ± 0.1	0.3 ± 0.1	0.1 ± 0	0	0.2 ± 0	0	0.6
Fatty acid	5.2 ± 0.6	0.9 ± 0.1	0.8 ± 0.1	0.3 ± 0	0.3 ± 0	0.5 ± 0.1	0	1.9
Diglyceride	0.1 ± 0	0	0	0	0	0	0	0
Phospholipid	3.5 ± 0.4	2.6 ± 0.6	0.2 ± 0.1	0.1 ± 0	0.1 ± 0	0.1 ± 0	0	0.5
Acyl-CoA	0.4 ± 0.1	0.9 ± 0.4	0.1 ± 0	0.1 ± 0	0.03 ± 0	0.01 ± 0	0	0.24
Total	11.5	5.4	1.4	0.6	0.43	0.81	0	3.24

[a]Samples were prepared for radio-HPLC analysis as described in Materials and Methods. The assay conditions are described in Table 1. Each value represents the mean from four experiments ± standard deviation.

TABLE 3

Radio-HPLC Analysis of the Incorporation of Individual Product Formed from [1-^{14}C]18:2-CoA into Different Lipid Classes in the American Cockroach[a]

Lipid class	Unreacted substrate 18:2	Elongated fatty acyl product 20:2	22:2	24:2	26:2	28:2	30:2	Total elongated product
		nmoles/assay						
Expt. A								
Total lipid	11.8	3.9	0.9	0.7	0.9	1.4	0.4	8.22
Expt. B								
Triglyceride	1.0 ± 0.0	1.0 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.04 ± 0.0	0	1.5
Fatty acid	4.9 ± 0.1	1.9 ± 0.1	0.8 ± 0.1	0.5 ± 0.0	0.6 ± 0.0	1.0 ± 0.0	0	4.9
Diglyceride	0.8 ± 0.0	1.2 ± 0.0	0	0	0	0	0	1.2
Phospholipid	1.4 ± 0.1	0.9 ± 0.1	0.1 ± 0.0	0	0.1 ± 0.0	0	0	1.1
Acyl-CoA	0.14 ± .02	0.04 ± 0	0.02 ± 0	0.04 ± 0	0.02 ± 0	0.16 ± .02	0.1 ± 0	0.4
Total	8.24	5.04	1.12	0.64	0.82	1.2	0.1	9.1

[a]Samples were prepared for radio-HPLC analysis as described in Materials and Methods. The assay conditions are described in Table 1. Each value represents the mean from four experiments ± standard deviation.

products ranged from 20 to 30 carbons in chain length. Of this 41% elongation, the total amount of 28:2 (7.1%) synthesized was greater than that of 22:2 (4.4%), 24:2 (3.4%), 26:2 (4.6%) and 30:2 (2.1%), consistent with earlier studies (9). The 28:2 fatty acyl moiety is the immediate precursor to the other major hydrocarbon (27:2 Hy) in the American cockroach, and radio-HPLC of the hydrocarbon fraction isolated from the TLC separation of the neutral lipids showed that, similar to the incubation with 18:0-CoA, the only hydrocarbon formed was 27:2 Hy. About 53% of the elognated products formed from the substrate 18:2-CoA were free fatty acids, similar to the results with 18:0-CoA (60%).

The data in Table 4 show the total incorporation of unreacted 18:0, desaturated 18:1 and elongated products into different lipid classes by microsomes prepared from two-day-old whole male houseflies. A negligible amount of radioactivity was detected in the hydrocarbon fraction, and the largest amount (45%) was incorporated into the free fatty acid fraction.

TABLE 4

Total Incorporation of [1-^{14}C]18:0-CoA into Different Lipid Classes Using Microsomal Preparation from Three-day-Old Male Whole Flies and [1-^{14}C]18:0-CoA Substrate[a]

Lipid class	nmoles/assay	Lipid class	nmoles/assay
Hydrocarbon	0.06 ± 0.03	Diglyceride	7.5 ± 0.45
Triglyceride	24.3 ± 3.6	Phospholipid	19.8 ± 2.16
Free fatty acid	52.8 ± 4.35	Acyl-CoA	13.8 ± 1.05

[a]Microsomes were prepared from three-day-old whole flies as described in Materials and Methods. The incubation conditions were: 50 μM acyl-CoA; 0.1 M potassium phosphate buffer (pH 7.2); 0.25 mM sucrose; 2 mM $MgCl_2$; 2 mM sodium ascorbate; 1 mM dithiothreitol; 1 mM NADPH; 0.2 mM malonyl-CoA; 3.3 mg protein; 3 mL total incubation volume. The incubation time was 45 min. The reaction was started by the addition of protein. The separation of the different lipid fractions is described in Materials and Methods. Each value represents the mean from 3-4 experiments ± standard deviation.

TABLE 5

Radio-HPLC Analysis of the Incorporation of Individual Products Formed from 18:0-CoA into Different Lipid Classes Using Microsomal Preparation from Three-Day-Old Male Whole Houseflies[a]

Lipid class	Unreacted substrate 18:0	Desaturated product 18:1	Elongated fatty acyl products 20:0	22:0	24:0	26:0	28:0	Total elongated product
			nmoles/assay					
Total lipid	115.7	3.3	15.5	9.2	5.3	0.9	0.2	31.1
Triglyceride	17.0	0.5	3.9	2.0	1.1	0	0	6.9
Fatty acid	40.1	0.6	7.1	3.6	1.5	0.2	0.2	12.5
Diglyceride	4.8	0	2.0	1.2	0.3	0	0	3.3
Phospholipid	16.8	0.8	0.9	0.8	0.7	0.2	0	2.4
Acyl-CoA	8.1	0	1.4	0.9	0.6	2.4	0	5.1
Total	86.8	1.9	15.3	8.5	4.2	2.8	0.2	30.2

[a]Incubations were as described in Table 4. Samples were prepared for radio-HPLC analysis as described in Materials and Methods. Each value represents a pool of 3–4 samples.

Table 5 shows the results of radio-HPLC analysis of the incorporation of the elongated and desaturated products into different lipid classes. The elongated products (20:0 to 28:0) were observed mainly in the free fatty acid (41%), the triglyceride (23%), and the acyl-CoA (17%) fractions.

Oleoyl-CoA (18:1-CoA) is the precursor for the 24:1 fatty acyl moiety which, in turn, is the immediate precursor for the major sex pheromone component, Z9-23 Hy, in mature female flies. Therefore, the fate of the elongated products, particularly the 24:1 formed from 18:1-CoA, was studied using mature male and female houseflies in an attempt to determine if this fatty acid was handled differently in males as compared to females. The total incorporation of unreacted 18:1-CoA plus elongated fatty acyl products into microsomes from three-day-old males and females is shown in Table 6. Most of the label was found in either the phospholipid (37.8 and 34.8%) or free fatty acid (27.5 and 27.9%) fractions, with the acyl-CoA fraction containing the third most label (14.0 and 16.6%).

Table 7 shows the result of radio-HPLC analysis of the distribution of unreacted 18:1-CoA and the elongated products into different lipid classes in three-day-old male and female houseflies. In the males, most (37%) of the unreacted 18:1 was incorporated into the phospholipid fraction followed by the free fatty acid (26%), acyl-CoA (15%), diglyceride (9%) and triglyceride (8%) fractions. A similar trend was observed in females. With the exception of the diglyceride fraction, elongated products were observed in all lipid fractions in both male and female insects. In the females, 6.2% of the substrate was elongated, but no significant amounts of elongated products beyond 24:1 were observed. However, in the males the elongated products, 26:1 and 28:1, were formed and incorporated only into the triglyceride fraction. In experiments designed to measure incorporation of 18:1-CoA into the hydrocarbon fraction, three-day-old males made 27:1 Hy, and three-day-old females made 23:1 Hy (data not shown).

We determined the fatty acid composition of the total microsomal lipid extract, and these data are shown in Table 8. In the cockroach, the three major fatty acids were 16:0, 18:1 and 18:2. In the housefly, both sexes contained

TABLE 6

Distribution of Radioactivity in Different Lipid Classes Using Microsomal Preparations from Three-Day-Old Male and Female Whole Flies and [9,10-^{3}H]18:1-CoA Substrate[a]

Lipid class	Three-day-old male nmoles/assay	Three-day-old female nmoles/assay
Hydrocarbon	0.9 ± 0.5	3.9 ± 1.2
Triglyceride	17.3 ± 1.5	13.1 ± 4.0
Free fatty acid	41.3 ± 4.2	41.9 ± 2.0
Diglyceride	12.9 ± 2.0	15.3 ± 2.0
Phospholipid	56.7 ± 5.0	52.2 ± 11.3
Acyl-CoA	21.0 ± 2.6	24.9 ± 6.0

[a]The experimental conditions were as described in Table 4. Each value represents the mean of four values ± standard deviation.

major amounts of 16:0, 16:1, 18:1 and 18:2, but females contained more 18:2 than males, and males contained more 16:1 than females. Fatty acids of chain length longer than 20 carbons were not found in either the cockroach nor the housefly microsomal lipids.

DISCUSSION

Spates *et al.* (6) have noted that there are only small amounts of 20, 22 and 24 carbon fatty acids present in various lipid fractions from housefly microsomes. The lipids with the highest percentages of these longer chain fatty acids were lysoPE, lysoPC and diglyceride. These lipids represent only small components of the total lipid from housefly microsomes. When we determined the fatty acid composition of the total microsomal fraction, we could find only traces of fatty acids of chain length of 20 carbon atoms or longer (Table 8).

It is well established that cuticular *n*-alkanes and alkenes are synthesized directly from very long chain fatty acids which are formed by chain elongation of stearate and oleate (in the housefly), and stearate and linoleate (in the American cockroach) (18,19). The hydrocarbons present on the epidermal layer of insects range in chain length from 21 to 55 carbons (3); however, the

TABLE 7

Radio-HPLC Analysis of the Incorporation of Individual Product Formed from [9,10-^{3}H]18:1-CoA Into Different Lipid Classes in Three-Day-Old Male and Female Houseflies[a]

Lipid class	Unreacted substrate 18:1	Elongated fatty acyl product 20:1	22:1	24:1	26:1	28:1	Total elongated product
Three-day-old male							
Triglyceride	12.2 ± 2.6	2.3 ± 0.2	1.5 ± 0.5	0.11 ± 0.09	0.9 ± 0.5	0.2 ± 0.0	5.0 ± 0.5
Fatty acid	39.3 ± 4.7	1.1 ± 0.2	0.2 ± 0.0	0	0	0	1.3 ± 0.0
Diglyceride	12.8 ± 2.0	0	0	0	0	0	0
Phospholipid	55.1 ± 4.8	0.9 ± 0.0	0.2 ± 0.0	0.02 ± 0.01	0	0	1.1 ± 0.0
Acyl-CoA	21.8 ± 3.8	3.8 ± 2.0	0.2 ± 0.0	0.02 ± 0.01	0	0	4.0 ± 0.2
Three-day-old female							
Triglyceride	10.7 ± 3.9	2.7 ± 0.6	1.2 ± 0.6	0.15 ± 0.06	0.3 ± 0.0	0	4.4 ± 0.6
Fatty acid	41.4 ± 8.7	2.1 ± 1.2	0.6 ± 0.3	0.03 ± 0.03	0	0	2.7 ± 0.6
Diglyceride	16.1 ± 2.1	0	0	0	0	0	0
Phospholipid	53.9 ± 11.6	1.2 ± 0.3	0.3 ± 0.0	0.03 ± 0.03	0	0	1.5 ± 0.3
Acyl-CoA	28.1 ± 4.8	0.6 ± 0.3	0.3 ± 0.0	0.15 ± 0.15	0	0	1.1 ± 0.3

[a]Incubation conditions were as described in Table 4. Samples were prepared for radio-HPLC analysis as described in Materials and Methods. Each value represents the mean from three experiments ± standard deviation.

TABLE 8

The Fatty Acid Composition of Cockroach and Housefly Microsomes[a]

Fatty acid	Cockroach nMole %	Male housefly nMole %	female housefly nMole %
14:0	—	—	1.2
14:1	—	0.9	1.0
14:0 MB[b]	—	0.6	—
15:0	0.5	0.8	0.8
15:1	—	1.7	1.1
16:0	14.1	22.7	20.9
16:1	2.2	24.7	20.5
16:0 MB	1.0	1.5	1.8
16:1 MB	4.3	1.4	1.4
18:0	8.1	3.0	2.6
18:1	41.9	21.9	20.3
18:2	21.9	14.6	18.7
20:0	0.4	0.4	2.0
20:1	4.3	2.9	3.6
20:2	0.9	0.1	0.1
20:4	0.9	0.3	0.3

[a]Gas chromatography-mass spectrometry was utilized to confirm the identity of some of the minor components.

[b]MB, methyl branched fatty acids.

absence of very long chain fatty acids (C_{22}-C_{30}) in most insects (17,20,21) suggests that elongation activity is tightly coupled to enzyme activities which convert the acyl moieties into hydrocarbons. Because we have observed the elongated fatty acids being incorporated mainly into triglyceride, phospholipid or the free fatty acid fractions, the coupling between elongation activity and the conversion into hydrocarbon must have been disrupted during tissue homogenization. The concentration of these acids will build up allowing the competing reactions of acyl transfer to occur, therefore, producing the incorporations we have observed.

In a previous publication (9), we have demonstrated that microsomes from the cockroach can make 25:0 Hy from 18:0-CoA and 27:2 Hy from 18:2-CoA. Further, we have observed 18:1-CoA to be the precursor for 23:1 Hy in microsomes from mature female houseflies and for 27:1 Hy in microsomes from mature male houseflies (unpublished data). Preliminary *in vitro* data (unpublished data) using the substrates 24:1-CoA, 24:1 fatty acid and 24:1 aldehyde have shown that 24:1-CoA is converted to 23:1 Hy in the housefly. Furthermore, there was an absolute requirement for NADPH in the conversion of 24:1-CoA to 23:1 Hy. This suggests the mechanism for the conversion of the elongated fatty acyl products to the corresponding hydrocarbon is *via* decarbonylation. In current experiments, if most of the elongated fatty acyl products had been found in the fatty acyl-CoA fraction, then we would have been able to provide more data in support of the presumed decarbonylation mechanism. However, because the elongated products were found as fatty acyl-CoA, free fatty acid, triglyceride and phospholipid, additional experiments are needed to ascertain which of these lipids is the direct precursor for hydrocarbon biosynthesis.

The data presented in this report show that *in vitro*, about 94% of the elongated fatty acids appear in three lipid fractions in the cockroach. In the housefly, depending upon the initial substrate and upon the sex of the fly, between 71% and 87% of the elongated fatty acids appeared in the three tissue lipids.

These data suggest that *in vitro* there are significant competing reactions so that much of the elongated fatty acids appear in either triglyceride, free fatty acid or phospholipid fractions. These data further support the idea that *in vivo* the biosynthesis of hydrocarbon is sufficiently coupled to fatty acid chain elongation so that only very small amounts of the elongated fatty acids become incorporated into these tissue lipids. *In vitro* elongation and subsequent conversion of the elongated products into hydrocarbon occurred only in relatively low amounts providing additional support for the hypothesis that these systems are coupled *in vivo*. Because *in vitro* there is such a strong competition for the various acyl-CoA derivatives by the acyltransferases, which incorporate these fatty

acids into triglyceride and phospholipid, and by acyl-CoA hydrolases, which convert the acyl-CoAs into free fatty acids, the substrate concentrations for hydrocarbon synthesis are reduced significantly, thus allowing only small amounts of hydrocarbon to be synthesized *in vitro*.

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Effects of Triton WR 1339 and Heparin on the Transfer of Surface Lipids from Triglyceride-Rich Emulsions to High Density Lipoproteins in Rats[1]

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The influence of lipolytic mechanisms on the transfer of phospholipids and unesterified cholesterol from artificial emulsions, serving as chylomicron models to other plasma lipoproteins, mainly high density lipoproteins (HDL) were tested *in vivo*. The emulsions labeled with radioactive lipids were injected into the bloodstream of rats (controls) and the results were compared with those obtained from rats that had previously been treated with Triton WR 1339 or heparin. Plasma clearance and the distribution of cholesteryl esters, phospholipids and unesterified cholesterol in the different plasma lipoprotein fractions were then determined. Whereas virtually no cholesteryl esters were found in d > 1.006 g/mL density fraction of the three experimental groups, 2.8 ± 1.3% of the injected phospholipids were in the 1.063–1.210 g/L density fraction of the Triton treated rats, and 12.6 ± 5.4% of the heparin treated rats, as compared to 10.1 ± 1.7% in controls. This indicates that lipolysis directly influences phospholipid transfer to HDL. In contrast, free-cholesterol transfer to HDL, besides being less pronounced than phospholipid transfer, was enhanced by Triton and diminished by heparin, indicating that lipolytic mechanisms were not important determinants in this process.
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The lipid constituents of plasma lipoproteins are continuously exchanged between the various lipoprotein classes. This exchange process can be enhanced greatly in the presence of lipid transfer or exchange proteins (1,2). To date, at least two different lipid transfer proteins have been identified. Cholesteryl ester transfer protein shuttles cholesteryl esters, triacylglycerols and phospholipids, whereas phospholipid transfer protein is specifically involved in the exchange of phospholipids. The transfer processes can substantially alter the composition and metabolism of plasma lipoproteins (1,2).

Net transfer of phospholipids and unesterified cholesterol from the triglyceride-rich lipoproteins, namely chylomicrons and very low density lipoproteins (VLDL), to other lipoprotein fractions, particularly high density lipoprotein (HDL), has previously been demonstrated (3–6). Incorporation of surface components into HDL_3 decreases the density of this fraction, which then approaches the density of HDL_2. After a fatty meal, both HDL_3 and HDL_2 are present at elevated concentrations (7,8). Although *in vitro* experiments have suggested that simultaneous hydrolysis of triacylglycerols in the lipoprotein particles is a prerequisite for the shift of surface lipids to occur (9,10), the role of lipolysis in these processes has not previously been tested *in vivo*.

In our previous studies (11,12) we developed protein-free emulsions with lipid compositions similar to those of chylomicrons obtained from lymph. When injected into the bloodstream of rats, the emulsions adsorbed circulating apolipoproteins and then mimicked chylomicrons. After hydrolysis of their triacylglycerols by lipoprotein lipase, the remnant particles containing the bulk of the original cholesterol ester mass, as well as residual triacylglycerols, were rapidly taken up by the liver. Based on a subsequent study, we suggested that the metabolized artificial triglyceride-rich emulsions shared identical receptor sites with natural chylomicron remnants on hepatic cells (13,14). Also, Triton WR 1339 and heparin were capable of inhibiting or stimulating lipolysis of the emulsions, respectively, as expected for chylomicrons. In those experiments, heparin treatment accelerated and Triton blocked the removal of the emulsion particles from the plasma (15).

In the present study, the influence of lipolysis on the transfer of the surface components of the chylomicron-like emulsions to native HDL and low density lipoproteins (LDL) was evaluated *in vivo* by injecting emulsions containing radio-labeled phospholipids, unesterified cholesterol or cholesteryl esters into control and Triton or heparin-treated rats.

MATERIALS AND METHODS

Preparation of the emulsions. Glyceryl trioleate, cholesteryl oleate and cholesterol were from Nu-Chek Prep (Elysian, MN). Egg lecithin was from Lipid Products (Surrey, U.K.). All lipids used were 99% pure as judged by thin-layer chromatography.

The chylomicron-like emulsions were prepared as described previously (11). In short, dried lipid mixtures composed of 2% cholesterol, 23% lecithin, 6% cholesteryl oleate and 69% triolein were prepared with [^{3}H]phosphatidylcholine, [^{14}C]cholesteryl oleate or [^{14}C]cholesterol (Amersham, U.K.) added. The mixtures were emulsified by sonication in 8 mL of a 2.785 M NaCl solution (density, 1.101 g/mL) with a Branson Cell Disruptor at 55°C for 30 min using a 1-cm probe with a continuous output of 70–80 watts. Purification of the crude emulsions was by ultracentrifugation using discontinuous NaCl gradients with densities of 1.065, 1.020 and 1.006 g/mL and a SW 41 rotor (Beckman Instruments, Inc., Palo Alto, CA) at 12000 rpm at 22°C for 15 min. The coarse lipid layer that initially floated to the top (first run) was discarded and replaced with a corresponding volume of the 1.006 g/mL solution. The emulsion fraction that was utilized in the present study was recovered from the top of the gradient after a second centrifugation at 36000 rpm for 25 min. The lipid composition of the emulsions was assayed using standard laboratory procedures (16–18).

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Abbreviations: FCR, fractional clearance rate; HDL, high density lipoproteins; LDL, low density lipoproteins; VLDL, very low density lipoproteins.

TABLE 1

Plasma Removal of the Emulsion Phospholipids, Cholesteryl Esters and Unesterified Cholesterol[a]

Treatment groups	Removal from plasma (FCR, min^{-1})		
	Phospholipids	Cholesteryl esters	Cholesterol
Control	0.05 ± 0.03 (n = 12)	0.11 ± 0.03 (n = 8)	0.10 ± 0.03 (n = 10)
Triton WR 1339[b]	0.04 ± 0.01 (n = 8)	0.03 ± 0.00[d] (n = 8)	0.04 ± 0.01[d] (n = 8)
Heparin[c]	0.05 ± 0.03 (n = 9)	0.25 ± 0.06[d] (n = 10)	0.23 ± 0.03[d] (n = 7)

[a]Emulsions with radioactive lipids were injected in controls and rats pre-treated with Triton WR 1339 or heparin. Blood was sampled from an implanted arterial cannula at 2 min intervals for 10 min. Fractional clearance rates (FCR) were calculated from the exponential curves fitted to the data from radioactivity remaining in the plasma. Results are means ± S.D. for numbers of observations in parentheses.

[b]600 mg/Kg body weight.

[c]250 U/Kg body weight.

[d]$p < 0.001$ (Student t-test) comparison with control.

Clearance studies. In order to determine plasma disappearance rates, emulsions labeled with [^{3}H]phosphatidylcholine, [^{14}C]cholesteryl ester or [^{14}C]cholesterol were injected as bolus through polyethylene cannulae, that were inserted under diethyl ether anesthesia into the carotid arteries of rats using technique described previously (11-13). Clotting was prevented by prior treatment of the cannulae with silicon. The animals were allowed to recover from anesthesia in individual cages for at least 2 hr. Non-fasted male Wistar rats weighing 250-350 g were used. After injection, 0.4-mL blood samples were drawn at 2 min intervals over a period of 10 min.

Aliquots (0.1 mL) of blood plasma were extracted with chloroform/methanol (2:1, v/v), and the solvent phase was transferred into counting vials and dried under a stream of nitrogen. Radioactivity was measured in scintillation solution (PPO/DM-POPOP/Triton X-100/toluene, 5 g/0.5 g/333 mL/667 mL) (19) using a Beckman LS-100C Spectrometer. Plasma clearance kinetics [fractional clearance rates (FCR)] of the different isotopes were computed from monoexponential curves fitted by least square procedure. FCR was calculated by the equation $FCR = \ln 2/t_{1/2}$ [min^{-1}].

Transfer studies. Emulsions labeled with [^{3}H]phospholipid and [^{14}C]cholesteryl oleate or [^{14}C]cholesterol were injected into the carotid arteries of controls and of rats treated with Triton WR 1339 (600 mg/kg of body weight), or heparin (250 U/kg of body weight) 10 min prior to the injection of the emulsions.

Fifteen minutes after injection of the emulsions, the rats were exsanguinated at the carotid artery. Blood was collected and plasma fractionated by ultracentrifugation in discontinuous density gradients at 41000 rpm at 20°C for 24 hr in a SW 41 Beckman rotor (20). The lipids of each lipoprotein fraction of a given density were extracted with 25 volumes of chloroform/methanol (2:1, v/v) and radioactivity was measured as described above. Rat plasma volume was estimated based on the relationship $V = 0.175 \times W^{0.725}$, where V equals the plasma volume and W the body weight (21).

In vitro *transfer studies.* In order to determine whether Triton affects lipid transfer due to inhibitory action on lipolysis or whether Triton directly affects donor or recipient particles, an assay of lipid transfer was performed *in vitro* in the absence of lipoprotein lipase. For this purpose, 55 mg of Triton WR 1339 was added to six test tubes containing 3.5 mL of plasma at 37°C. Ten minutes later, 150 μL of emulsion containing labeled phospholipids was added. After 15 min incubation under agitation, the plasma lipoprotein fractions were separated by ultracentrifugation and the percentage of radioactivity was determined in each fraction as described above. Control incubations were also done without Triton. Similar experiments were performed with plasma from rats that had previously been treated with heparin (250 U/kg of body weight) for 10 min before samples were taken.

RESULTS AND DISCUSSION

The emulsions used contained 1.9% cholesterol, 10.4% phospholipids, 11.2% cholesteryl ester and 76.5% triacylglycerols (% weight).

Data obtained on plasma clearance of emulsion constituents are shown on Table 1. The cholesteryl esters can, indeed, be considered as the real markers of plasma disappearance of the emulsion particles (11), because they cannot be selectively removed from the particles due to the virtual absence of a cholesteryl ester transfer protein in the rat (1). Thus, the fact that in control rats phospholipid clearance was 55% slower than cholesteryl ester clearance (Table 1) strongly suggests that phospholipids were being detached from the surface of the emulsion particles and incorporated into other lipoprotein classes that are removed from plasma at slower rates than are chylomicrons. On the other hand, free cholesterol clearance was not statistically different from cholesteryl ester clearance, suggesting that if any transfer had occurred it was minimal.

In the current study, triacylglycerol clearance was not measured. However, in previous studies utilizing

emulsions with the same composition (11-13,15), we demonstrated that in control rats triacylglycerol clearance was always faster than cholesteryl ester clearance, indicating lipolysis. When rats were treated with Triton (same dose utilized in this study), lipolysis was virtually abolished, as is indicated by identical FCR of triacylglycerols and cholesteryl esters (15). When the animals were treated with heparin, the rate of triacylglycerol removal was so fast that it was not possible to measure it, indicating that lipolysis had been markedly enhanced (15). The data in Table 1 also show that upon Triton treatment, removal of the emulsions as measured by cholesteryl ester FCR was nearly four times slower. This appears to result from decreased generation of remnant particles capable of interacting with the liver receptors (15). On the other hand, accelerated generation of remnants caused by heparin accounts for removal of cholesteryl esters at faster rates. In Triton-treated rats, differences in phospholipids and cholesteryl ester removal rates from plasma were abolished, which suggests diminished dissociation of phospholipids from the emulsion particles. In contrast, upon heparin treatment the differences in clearance were accentuated. This could be interpreted as being part of a trend towards enhanced dissociation of phospholipids.

The data in Table 2 show that in the three experimental situations (control, Triton and heparin treatment), only trace amounts of cholesteryl esters were moved from the emulsion fraction into the 1.006-1.063 and 1.063-1.210 g/L density fractions. Lack of cholesteryl ester transfer protein activity in rat probably accounts for this. The results confirm the notion that cholesteryl esters can be used as markers for removal of emulsion particles. Moreover, the data exclude the possibility that emulsion particles may increase their density while circulating in plasma due to lipolysis or other factors. The increased levels of cholesteryl ester radioactivity remaining in plasma at the 1.006 density in the group of rats treated with Triton and the decreased levels in heparin-treated animals reflect delayed or accelerated plasma clearance, respectively, as illustrated in Table 1.

TABLE 2

Distribution of [^{14}C]Cholesteryl Ester Radioactivity from Chylomicron-Like Emulsions in Different Plasma Lipoprotein Fractions[a]

Plasma lipoprotein density (g/mL)	Control (n = 4)	Triton[b] (n = 4)	Heparin[c] (n = 4)
d<1.006	11.6 ± 3.0	39.3 ± 6.4	2.7 ± 2.0
1.006<d<1.063	0.1 ± 0.0	0.2 ± 0.2	0.1 ± 0.0
1.063<d<1.210	0.1 ± 0.14	0.1 ± 0.0	0.1 ± 0.0
d>1.210	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.2
Total	12.1 ± 4.1	39.7 ± 6.4	3.1 ± 2.0

[a]Measured 15 min after the injection of the emulsions in controls and rats treated with Triton WR 1339 or heparin. The values are given as percent of the radioactivity (means ± S.D. for numbers of observations in parentheses) injected arterially and recovered in each plasma fraction. Blood samples were corrected for total plasma volume (21).

[b]600 mg/Kg body weight.

[c]250 U/Kg body weight.

Table 3 shows the results of the phospholipid transfer experiments. Conceivably, transfer of phospholipids to HDL and LDL *in vivo* depends on several factors, such as the number of donor and recipient particles and the activity of the phospholipid transfer protein, which, in contrast to the cholesteryl ester transfer protein, has been shown to be high in rats (1). Transfer proteins are believed to act by promoting lipid exchange during particle collision. Finally, lipolysis could influence phospholipid transfer by removal of triacylglycerols from particles and by "shrinking" of the core. Excess phospholipid molecules accumulating on the particle surface could then fuse with HDL (22).

Formation of remnants by lipoprotein lipase action is an obligatory step for chylomicron removal by liver receptors. Consequently, inhibition or stimulation of the lipoprotein lipase action causes either impairment or enhancement of removal of the emulsion particles from plasma, respectively (13,15). Therefore, the much larger number of emulsion particles remaining in the plasma upon Triton treatment (Table 1) would favor interaction with native HDL particles. On the other hand, the smaller number of emulsion particles remaining in the plasma upon heparin treatment would tend to minimize the interaction with HDL, as the emulsion particles are being cleared from plasma at a much faster rate. Table 3 shows that the "number of particle effect" was totally overcome by the effect of lipoprotein lipase action as far as phospholipid transfer is concerned. There was a clear-cut decrease of the phospholipid shift to the 1.006-1.063 and 1.063-1.210 g/L density fractions in the Triton-treated animals. However, it would be possible that the inhibition of transfer by Triton was elicited not by its inhibitory action on lipolysis, but rather by the detergent modifying the HDL surface in a way so that it would less readily accept phospholipids originating from the triglyceride-rich emulsions. Another possibility is that Triton may interact with the phospholipid transfer protein and

TABLE 3

Distribution of [^{3}H]Phospholipid Radioactivity from Chylomicron-Like Emulsions in Different Plasma Lipoprotein Fractions[a]

Plasma lipoprotein density (g/mL)	Control (n = 6)	Triton[b] (n = 5)	Heparin[c] (n = 8)
d<1.006	5.5 ± 1.2	34.6 ± 5.6[d]	2.2 ± 2.3[d]
1.006<d<1.063	6.9 ± 2.5	3.5 ± 1.1[e]	7.7 ± 3.4
1.063<d<1.210	10.3 ± 1.7	2.8 ± 1.3[f]	12.6 ± 5.4
d>1.210	0.8 ± 0.3	0.2 ± 0.0	0.6 ± 0.3
Total	23.3 ± 4.4	41.1 ± 4.9	23.1 ± 7.0

[a]Determined 15 min after injection of the emulsions in controls and rats treated with Triton WR 1339 or heparin. The values are given as percent of the radioactivity (means ± S.D. for numbers of observations in parentheses) injected arterially and recovered in each plasma fraction. Blood samples were corrected for total plasma volume (21).

[b]600 mg/Kg body weight.

[c]250 U/Kg body weight.

Student t-test (comparison with control):

[d]$p < 0.0005$.

[e]$p < 0.001$.

[f]$p < 0.005$.

suppress its activity. In the *in vitro* experiments designed to clarify this point, after incubation of the labeled emulsions with plasma, 44% of the radioactive phospholipids remained at the <1.006 density fraction, 10% transferred to 1.006-1.063, and 42% transferred to the 1.063-1.210 density fraction. When Triton was added to the incubates, the percentage of phospholipids that remained at the <1.006 fraction increased to 60%, 9% were at 1.006-1.063 g/L, and the amount that moved to the 1.063-1.210 g/L range decreased to 26%. When these experiments were repeated in the presence of lipoprotein lipase, i.e., with plasma taken from rats pretreated with heparin, the amount of phospholipids remaining at the <1.006 fraction decreased to 19%, 31% moved to 1.006-1.063, and 39% to the 1.063-1.210 g/L fraction. When Triton was added to the plasma from heparin treated rats, phospholipids remaining at the <1.006 fraction increased to 66%, and only 9% and 14%, respectively, moved to the 1.006-1.063 and 1.063-1.210 fractions. These results show that, although Triton treatment had really diminished the transfer of phospholipids from the emulsions to the other plasma lipoprotein fractions in the absence of lipoprotein lipase (40% more phospholipids were retained at the emulsion density upon addition of Triton), this effect was much greater in the presence of the enzyme (three times more phospholipids were retained in the 1.006 fraction with Triton). These results clearly show that the effect of Triton is mostly due to its inhibitory action on lipolysis rather than to surface effects on the lipoprotein particles that could affect the ability of the particles to accept lipids from the emulsions or to interact with transfer proteins.

The results obtained upon lipoprotein lipase stimulation by heparin reaffirm the importance of the enzyme for bringing about phospholipid transfer. Although the phospholipid transfer values were not statistically greater than those of the controls, it must be pointed out that the fractional clearance rate of the cholesteryl ester was about twice as high as in controls (0.25 and 0.11, respectively). Thus, as the particles rapidly disappeared from plasma, the amount of phospholipid disappearing from the <1.006 g/L density fraction must actually represent the relative enhancement of the transfer process in the heparin-treated animals.

The results of the transfer of radioactive free cholesterol are shown in Table 4. As reported earlier by others (4,5), transfer of unesterified cholesterol from chylomicrons to the 1.063-1.210 g/L fraction was several times lower than for phospholipids. In addition, if lipolysis would have had any influence on the transfer of unesterified cholesterol, it would be totally obscured by the "number of particles effect"—the transfer seen upon Triton treatment was enhanced six-fold as compared to controls, whereas heparin administration decreased cholesterol radioactivity that was shifted to the 1.063-1.210 g/L density fraction.

In conclusion, although in the current study net transfer cannot be distinguished from exchange of lipids, because radioactivity rather than mass transfer was measured, it is clear that altering lipolysis rates by treatment with Triton or heparin has a strong influence on the transfer of phospholipid molecules from triglyceride-rich emulsions. On the other hand, transfer of unesterified cholesterol does not seem to be affected by lipolysis.

TABLE 4

Distribution of the [^{14}C]Cholesterol Radioactivity From Chylomicron-Like Emulsions in Different Plasma Lipoprotein Fractions[a]

Plasma lipoprotein density (g/mL)	Control (n = 4)	Triton[b] (n = 4)	Heparin[c] (n = 5)
d<1.006	3.8 ± 0.8	31.0 ± 2.7[d]	1.9 ± 1.6[e]
1.006<d<1.063	1.1 ± 0.6	3.0 ± 0.4[d]	0.6 ± 0.5
1.063<d<1.210	1.3 ± 0.2	6.5 ± 1.2[d]	0.8 ± 0.2[e]
d>1.210	0.2 ± 0.0	1.1 ± 0.4	0.2 ± 0.2
Total	6.4 ± 1.4	41.8 ± 6.8	3.5 ± 1.8

[a] Determined 15 min after the injection of the emulsion in controls and rats treated with Triton WR 1339 or heparin. The values are given as percent radioactivity (means ± S.D. for numbers of observations in parentheses) injected arterially and recovered in each plasma fraction. Blood samples were corrected for total plasma volume (21).

[b] 600 mg/Kg body weight.

[c] 250 U/Kg body weight.

Student t-test (comparison with control):

[d] $p < 0.005$.

[e] $p < 0.05$.

[f] $p < 0.01$.

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Effects of Bile Acid Feeding on Hepatic Deoxycholate 7α-Hydroxylase Activity in the Hamster

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In order to investigate the effects of bile acid feeding on hepatic microsomal deoxycholate 7α-hydroxylase activity, three different bile acids were administered (0.2% w/w in chow) to hamsters for two weeks. Deoxycholate 7α-hydroxylase activity was increased markedly by feeding of cholic acid (CA) and slightly by deoxycholic acid (DCA). Chenodeoxycholic acid (CDCA) had little effect on the enzyme activity. Feeding each of the bile acids significantly inhibited the activity of cholesterol 7α-hydroxylase in the order CDCA ≥ DCA > CA. There was no correlation between deoxycholate 7α-hydroxylase activity and cholesterol 7α-hydroxylase activity. It is concluded that the activity of deoxycholate 7α-hydroxylase is up-regulated by feeding DCA and CA and that the mechanism seems to be different from that of cholesterol 7α-hydroxylase. The increased activity of hepatic deoxycholate 7α-hydroxylase by CA and DCA should be beneficial in minimizing the toxic effects of DCA in the hamster.
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Deoxycholic acid (DCA) is a secondary bile acid formed by the bacterial 7α-dehydroxylation of cholic acid (CA) in the large intestine. DCA thus formed is absorbed from the intestine by passive diffusion, extracted by the hepatocytes, conjugated with taurine or glycine, and excreted into bile to undergo enterohepatic circulation (1). DCA serves as a detergent to solubilize biliary and dietary lipids and participates in the digestion and absorption of lipids in the small intestine. On the other hand, DCA is reported to increase intestinal absorption and biliary excretion of cholesterol and to form supersaturated or lithogenic bile (2). Furthermore, DCA is reported to be a promotor in colon carcinogenesis (3).

With respect to acute toxicity, DCA is reported to be toxic at least in some animals (4-8), although pharmacological doses of DCA are not generally considered to be harmful in man. In animals with low DCA tolerance, DCA is likely to be detoxified by hepatic microsomal enzymes (4-6). 7α-Hydroxylation of DCA is shown to occur in rats (9), mice (10), prairie dogs (4), and hamsters (5,6), though there was little capacity of deoxycholate 7α-hydroxylation in humans (11,12). Our recent studies have dealt with 7α-hydroxylation of DCA *in vivo* (5) and *in vitro* (6) in the hamster. Approximately 21% of DCA administered intravenously or intraduodenally was hydroxylated at the 7α-position and converted to conjugated CA and excreted in bile (5). Glycine and taurine conjugated DCA were equally good substrates for deoxycholate 7α-hydroxylase while unconjugated DCA was not (6). The enzyme activity was decreased in the hamster and increased in the rat by phenobarbital treatment (6). The enzyme might play an important role in minimizing the toxicity of DCA in these rodents.

To date, little is known concerning the effect of bile acid administration on the enzyme activity in these animals. The aim of the present study was to investigate whether or not bile acid feeding influences hepatic microsomal deoxycholate 7α-hydroxylase activity in the hamster.

MATERIALS AND METHODS

Reagents. All solvents were of analytical grade and were distilled before use. Sephadex LH-20 was purchased from Pharmacia Fine Chemicals AB (Uppsala, Sweden). Dimethylethylsilylimidazole (DMES-imidazole) was purchased from Tokyo Kasei Kogyo (Tokyo, Japan), and TMSI-H (hexamethyldisilazane/trimethylchlorosilane/pyridine, 2:1:10) was purchased from Gasukuro Kogyo Co. (Tokyo, Japan). Piperidinohydroxypropyl Sephadex LH-20 (PHP-LH-20) was prepared according to the procedure described by Goto *et al.* (13). Bond-Elut silica cartridge columns were obtained from Analytichem International (Harbor City, CA). Cholylglycine hydrolase and ethylenediaminetetraacetic acid (EDTA) were obtained from Sigma Chemical Co. (St. Louis, MO). Nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), was purchased from Kojin Co., Ltd. (Tokyo, Japan). Sodium taurodeoxycholate, glycodeoxycholate and nordeoxycholate were generous gifts from Dr. Erwin H. Mosbach, Beth Israel Medical Center, New York. Sodium glycodeoxycholate was further purified by chromatography on a PHP-LH-20 column and recrystallized before use. Sodium deoxycholate, cholate, and chenodeoxycholate were obtained from Nakarai Chemical Co., Ltd. (Kyoto, Japan). Coprostanol was purchased from Steraloids Inc. (Wilton, NH). 7α-Hydroxycholesterol and 5α-cholestane-3β,7β-diol were synthesized as described previously (14). Bile acids used in this study were checked for purity by thin-layer chromatography on precoated silica gel G plates (thickness, 0.2 mm; Merck, Darmstadt, West Germany) using isopropanol/isooctane/dioxane/acetic acid (7:10:6:2, v/v/v/v) (15) and n-butanol/acetic acid/water (10:1:1, v/v/v) (16) as solvent systems. Bile acids used were also checked by gas-liquid chromatography (GLC) as described below. Purities of the bile acids were better than 98%.

Animals. Twenty-eight female Golden Syrian hamsters, six-weeks-old, with a mean weight of 96 g, were purchased from Hamster Misaki (Kagawa, Japan). The animals were kept in individual cages and had free access to food and water. The temperature of the vivarium was maintained at 23°C with light periods from 8 a.m. to 8 p.m. After two weeks of acclimation, the animals were randomly

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Abbreviations: ALP, alkaline phosphatase; CA, cholic acid (3α,7α,12α-trihydroxy-5β-cholanoic acid); CDCA, chenodeoxycholic acid (3α,7α-dihydroxy-5β-cholanoic acid); DCA, deoxycholic acid (3α,12α-dihydroxy-5β-cholanoic acid); DMES, dimethylethylsilyl; EDTA, ethylenediaminetetraacetic acid; GLC, gas-liquid chromatography; GOT, glutamate oxaloacetate transaminase; GPT, glutamate pyruvate transaminase; 7KDCA, 7-ketodeoxycholic acid (3α,12α-dihydroxy-7-oxo-5β-cholanoic acid); LCA, lithocholic acid (3α-hydroxy-5β-cholanoic acid); NADPH, nicotinamide adenine dinucleotide phosphate, reduced form; PHP-LH-20, piperidinohydroxypropyl Sephadex LH-20; TMS, trimethylsilyl.

divided into four groups (seven animals each). Each group was fed for two weeks a Standard Powder Chow containing only 0.075% of cholesterol (Oriental Yeast Co., Ltd., Tokyo, Japan) with or without one of the following bile acids: chow (control group); 0.2% sodium deoxycholate (DCA-fed group); 0.2% sodium cholate (CA-fed group); or 0.2% sodium chenodeoxycholate (CDCA-fed group). The animals were fasted for 24 hr prior to sacrifice. All animals were killed between 9 and 10 a.m. Under ether anesthesia, the abdomen and the chest were opened. Blood was obtained by cardiac puncture, and bile was aspirated from the gallbladder with a 50-μL microsyringe (Hamilton Co., Reno, NE). The liver was resected, weighed, and rinsed with ice-cold homogenizing medium, and a 1.5-g portion of the liver was used for measuring the activities of deoxycholate 7α-hydroxylase and cholesterol 7α-hydroxylase. A small aliquot of the liver was used for cholesterol determination.

Preparation of liver microsomes. A liver homogenate, 20% (w/v), was prepared in 0.1 M potassium phosphate buffer, pH 7.4, containing 0.25 M sucrose, 2.5 mM neutralized EDTA, and 75 mM nicotinamide, in a Potter-Elvehjem homogenizer with a loosely fitting pestle (17). The homogenate was centrifuged at 800 × *g* for 10 min and the supernatant was centrifuged at 20,000 × *g* for 10 min. The microsomal fraction was obtained by centrifugation of the 20,000 × *g* supernatant fluid at 100,000 × *g* for 1 hr. The microsomal pellet was suspended in the homogenizing medium. A small aliquot was used for microsomal protein determination according to Lowry *et al.* (18).

Determination of microsomal deoxycholate 7α-hydroxylase activity. The methods for determining the rate of conversion of glycodeoxycholate or taurodeoxycholate to conjugated cholic acids have been described previously (6). In brief, 450 nmol of each substrate was added to an incubation mixture containing, in a volume of 2.5 mL, potassium phosphate buffer, pH 7.4, 0.25 mmol; $MgCl_2$, 10 μmol; NADPH, 4 μmol; and 1 mL of the microsomal suspension (17). After 10 min of preincubation, the mixture was incubated for 30 min at 37°C in air and terminated by addition of four volumes of ethanol. Twenty μg of nordeoxycholic acid was added as an internal standard. After enzymatic hydrolysis with clostridial cholylglycine hydrolase (19), the free bile acids were extracted with ethyl acetate, esterified with 5% ethanolic hydrochloride and silylated with DMES-imidazole. The derivatives were analyzed by GLC using a 15 m × 0.2 mm i.d. fused silica capillary column (Hicap CBP1, Shimadzu Co., Kyoto, Japan) as described previously (20).

Assay of microsomal cholesterol 7α-hydroxylase activity. Endogenous microsomal cholesterol was used as the only substrate for the enzyme. The assay system consisted of 0.5 mL of the microsomal suspension, 0.1 M potassium phosphate buffer, pH 7.4, and 1 μmol NADPH, in a total volume of 1.0 mL. The enzyme reaction was conducted for 15 min at 37°C. Two hundred pmol of 5α-cholestane-3β,7β-diol was added as an internal standard. After extraction with chloroform/methanol (2:1, v/v), purification on a Bond-Elut silica cartridge column, and derivatization, the actual mass of 7α-hydroxycholesterol was assayed by gas-liquid chromatography/selected ion monitoring as described previously (14).

Analysis of bile acids in gallbladder bile. Gallbladder bile (20 μL) was deproteinized with 1 mL of isopropanol. Bile samples corresponding to 2 μL of original bile were hydrolyzed with cholylglycine hydrolase (19), and deconjugated bile acids were analyzed by GLC as their ethyl ester-DMES ether derivatives as reported previously (6,20). Ten μg of nordeoxycholic acid was used as an internal standard.

Determination of serum and liver cholesterol concentrations. One hundred miligram of liver was hydrolyzed with 10% KOH in 95% ethanol and 100 μL of serum was hydrolyzed with 5% KOH in 95% ethanol at 70°C for 2 hr. Lipids were extracted with n-hexane. The cholesterol concentration was determined as trimethylsilyl (TMS) ether derivative by GLC (21,22). Two hundred μg of coprostanol was used as an internal standard.

Serum transaminases and alkaline phosphatase. Serum glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT), and alkaline phosphatase (ALP) activities were analyzed with an autoanalyzer (Olympus-5000, Olympus, Tokyo).

Statistical methods. Results are expressed as mean ± S.D. The significance of differences among the group means was evaluated by one-way analysis of variance followed by Student's *t*-test (23).

RESULTS

No significant differences were found in the initial body weight, final body weight, and food intake among the four groups. The liver weight was significantly increased in DCA-fed and CA-fed groups (Table 1).

Bile acid composition of gallbladder bile. The results are summarized in Table 2. Bile of the control group contained approximately 55% of CA, 23% of CDCA, and 14% of DCA. In DCA-fed hamsters, the percentage of DCA was increased to two times the control value. CA administration led to a significant increase in the percentage of DCA, presumably reflecting 7α-dehydroxylation of cholic acid by intestinal bacteria. When CDCA was fed, the percentages of CA and DCA were decreased significantly and those of CDCA and LCA were increased markedly.

Serum and liver cholesterol concentrations. The effect of bile acid feeding on serum and liver cholesterol concentrations is shown in Table 3. The serum cholesterol level was markedly elevated (56–80% increase) by feeding each of the bile acids. Liver cholesterol concentration was increased to about five times the control value in DCA- and CA-fed hamsters, while it was increased slightly in the CDCA-fed group.

Hepatic deoxycholate 7α-hydroxylase activity. The activity of hepatic deoxycholate 7α-hydroxylase in each group is shown in Table 4. In the control group, the activities of 7α-hydroxylation of taurodeoxycholate and glycodeoxycholate were 706 ± 92 and 688 ± 78 pmol/min per mg protein, respectively. Administration of CA significantly increased the activity about two-fold. Feeding of DCA resulted in a small increase of the enzyme activity. In the CDCA-fed group, the activity was almost the same as that in the control. There was a significant correlation (slope = 0.98, intercept = 45.6, n = 28, r = 0.97, $P<0.01$) between the 7α-hydroxylase activity of taurodeoxycholate and that of glycodeoxycholate in each group (Fig. 1).

TABLE 1

Effect of Bile Acid Feeding on Food Intake, Weight Gain, and Liver Weight in Female Hamsters[a]

Group	Food intake (g/day)	Initial body weight (g)	Final body weight (g)	Weight gain (g)	Liver weight (g)
Control	10.1 ± 1.2	134.0 ± 4.4	146.6 ± 8.4	12.6 ± 8.9	4.5 ± 0.7
DCA-fed	9.7 ± 0.8	134.0 ± 4.4	150.6 ± 5.4	16.6 ± 3.3	6.0 ± 0.4[b]
CA-fed	9.8 ± 1.3	132.9 ± 3.3	143.7 ± 9.4	10.9 ± 8.2	5.6 ± 0.7[b]
CDCA-fed	9.2 ± 0.9	132.4 ± 3.9	141.1 ± 10.0	8.7 ± 7.8	4.7 ± 0.5

[a]Three different bile acids were given (0.2% w/w in chow) to hamsters for two weeks. Values represent the mean ± S.D. for seven animals. DCA, deoxycholic acid; CA, cholic acid; CDCA, chenodeoxycholic acid.
[b]$P<0.01$, significantly different from the value of the control group.

TABLE 2

Effect of Bile Acid Feeding on Bile Acid Composition of Gallbladder Bile in Female Hamsters[a]

Group	Cholic acid (%)	Chenodeoxycholic acid (%)	Deoxycholic acid (%)	Lithocholic acid (%)	7-Ketodeoxycholic acid (%)
Control	54.8 ± 5.8	23.0 ± 2.5	13.8 ± 4.3	4.6 ± 1.4	3.8 ± 1.2
DCA-fed	52.2 ± 6.4	12.0 ± 3.3[c]	28.2 ± 7.8[c]	3.7 ± 1.1	3.9 ± 3.1
CA-fed	58.2 ± 10.2	7.6 ± 7.3[c]	30.0 ± 16.5[b]	1.4 ± 0.8[c]	2.7 ± 1.5
CDCA-fed	13.7 ± 5.3	60.1 ± 7.6[c]	4.0 ± 0.9[c]	21.4 ± 3.5[c]	0.8 ± 0.3[c]

[a]Bile acid composition was analyzed by GLC as ethyl ester-DMES ether derivatives. Values represent the mean ± S.D. for seven animals.
[b]$P<0.05$, [c]$P<0.01$, significantly different from the value of the control group.

TABLE 3

Effect of Bile Acid Feeding on Serum and Liver Cholesterol in Female Hamsters[a]

Group	Serum cholesterol (mg/dl)	Liver cholesterol (mg/g wet liver)
Control	98.2 ± 23.8	3.10 ± 0.21
DCA-fed	165.9 ± 32.4[c]	16.76 ± 7.52[c]
CA-fed	177.4 ± 44.2[c]	14.07 ± 7.35[c]
CDCA-fed	153.5 ± 11.6[c]	4.19 ± 0.95[b]

[a]The blood and liver were obtained at the sacrifice. Cholesterol concentration was analyzed by GLC as TMS ether derivatives. Values represent the mean ± S.D. for seven animals.
[b]$P<0.05$, [c]$P<0.01$, significantly different from the value of the control group.

TABLE 4

Effect of Bile Acid Feeding on Hepatic Microsomal Deoxycholate 7α-Hydroxylase Activity in Female Hamsters[a]

Group	Taurodeoxycholate 7α-hydroxylase activity	Glycodeoxycholate 7α-hydroxylase activity
	(pmol/min per mg protein)	
Control	706 ± 92	688 ± 78
DCA-fed	823 ± 194	849 ± 116[b]
CA-fed	1314 ± 265[c]	1355 ± 276[c]
CDCA-fed	643 ± 119	692 ± 92

[a]Hepatic microsomal deoxycholate 7α-hydroxylase activity was determined as described in Materials and Methods. Values represent the mean ± S.D. for seven animals.
[b]$P<0.05$, [c]$P<0.01$, significantly different from the value of the control group.

Hepatic cholesterol 7α-hydroxylase activity. Hepatic microsomal cholesterol 7α-hydroxylase activity in each group is summarized in Table 5. Feeding of either DCA or CDCA significantly decreased the activity. Administration of CA had a mild but significant inhibitory effect on the enzyme. There was no correlation between activities of deoxycholate 7α-hydroxylase and cholesterol 7α-hydroxylase.

Hepatotoxicity. Feeding of CA or DCA had no adverse effect on GOT, GPT, and ALP (Table 6). Administration of CDCA increased serum levels of GOT and GPT, suggesting of hepatotoxicity of CDCA and LCA.

DISCUSSION

In order to investigate the effects of bile acid feeding on hepatic microsomal deoxycholate 7α-hydroxylase activity, three different bile acids were fed to hamsters for two weeks. Animals ingested about 9.6 g of chow containing 0.2% of each bile acid, *i.e.*, 19.2 mg of bile acid was given daily. The dose was 4.3 times that of endogenous bile acid synthesis (4.52 ± 1.17 mg/day; ref. 24) and was considered to be large enough to change bile acid metabolism.

As DCA and CDCA are known to be toxic in hamsters (5,25), it would be important to know whether they exhibit hepatotoxicity under the experimental conditions. Judging from the serum levels of liver enzymes (Table 6), CA and DCA were not hepatotoxic and CDCA showed only a mild to moderate adverse effect at the dose of 0.2% of each bile acid in chow. Liver damage by CDCA has also been reported in humans, which has been taken to rationalize abandonment of CDCA treatment.

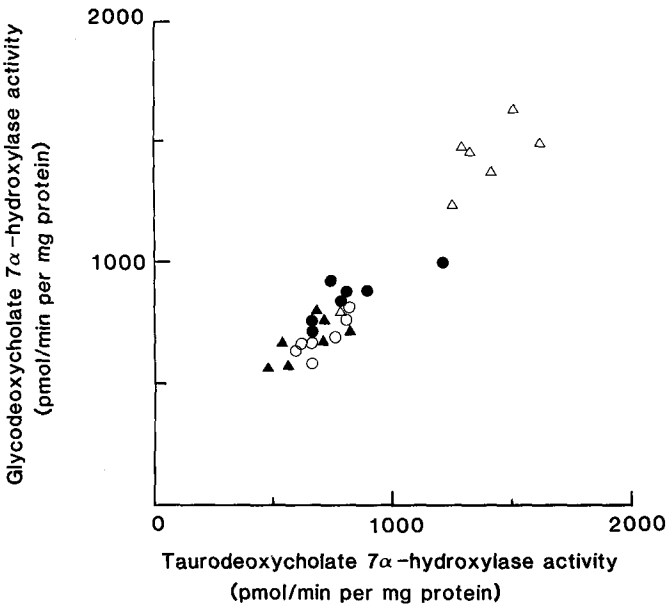

FIG. 1. Correlation between the 7α-hydroxylase activity for taurodeoxycholate and for glycodeoxycholate. Details of the assay are described in Materials and Methods. Control group (○), DCA-fed group (●), CA-fed group (△) and CDCA-fed group (▲). Positive correlation was found (n=28, r=0.97, P<0.01, y=0.98x+45.6).

TABLE 5

Effect of Bile Acid Feeding on Hepatic Cholesterol 7α-Hydroxylase Activity in Female Hamsters[a]

Group	Cholesterol 7α-hydroxylase activity (pmol/min per mg protein)
Control	12.41 ± 2.99
DCA-fed	3.07 ± 3.08[c]
CA-fed	6.20 ± 5.66[b]
CDCA-fed	2.45 ± 0.70[c]

[a]Hepatic microsomal cholesterol 7α-hydroxylase activity was determined as described in Materials and Methods. Values represent the mean ± S.D. for seven animals.

[b]P<0.05, [c]P<0.01, significantly different from the value of the control group.

TABLE 6

Effects of Bile Acid Feeding on Serum Transaminases and Alkaline Phosphatase[a]

Group	GOT (KU[b])	GPT (KU[b])	ALP (KAU[c])
Control	61.7 ± 9.6	52.9 ± 5.4	14.9 ± 4.1
DCA-fed	67.7 ± 16.5	60.7 ± 8.2	11.7 ± 1.6
CA-fed	61.7 ± 10.2	63.1 ± 8.6	13.7 ± 1.2
CDCA-fed	137.1 ± 83.1	294.0 ± 159.3[d]	15.1 ± 5.1

[a]Values represent the mean ± S.D. for seven animals.

[b]KU: Karmen unit.

[c]KAU: King-Armstrong unit.

[d]P<0.01, significantly different from the value of the control group.

Biosynthesis of bile acids was decreased by administration of each of the bile acids as shown in Table 5. CDCA and DCA were the most potent inhibitors of cholesterol 7α-hydroxylase and CA had a moderate effect. Heuman *et al.* (26) showed that the activity of cholesterol 7α-hydroxylase could be a function of monomeric hydrophobicity of biliary bile acids in the rat. Thus, DCA and CDCA should inhibit the enzyme activity more efficiently than CA (26). It also should be pointed out that the inhibition of the enzyme activity by DCA might be the result of the combined effects of DCA and its 7α-hydroxylated product, CA, and that the effects of CDCA might result from negative feedback (27) and from the hepatotoxicity described above.

In control animals, gallbladder bile contained approximately 55% of CA, 23% of CDCA and 14% of DCA. This composition was quite similar to reported values except for the percentage of DCA being slightly higher than that we reported previously (24,28). This might be due to the qualitative and quantitative differences in intestinal bacterial populations (28). Feeding of either CA or DCA resulted in an enrichment of DCA in the gallbladder bile (Table 2). Administration of CA was considered to increase the load of DCA by bacterial 7α-dehydroxylation in the intestine. Feeding of CDCA inhibited synthesis of CA and resulted in decreased proportions of CA and DCA in the gallbladder bile. Consequently, the percentage of CDCA in the gallbladder bile of CDCA-fed hamsters was increased markedly.

We speculated that deoxycholate 7α-hydroxylase activity might be affected by increasing or decreasing the load of deoxycholate. Danielsson and Rutter (29) reported that the activity of deoxycholate 7α-hydroxylase increased as embryos and suckling rats grew older. It is thus suggested that the enzyme activity might be induced by a prolonged exposure of the liver to deoxycholate. In the present study, however, the deoxycholate 7α-hydroxylase activity was increased significantly in CA-fed animals and only slightly in DCA-fed hamsters. The reason why the activity did not increase as much by DCA feeding than by CA feeding is not clear. It is not likely that the discrepancy could be due to the hepatotoxic effect of DCA, because DCA and CA did not adversely affect serum levels of liver enzymes (Table 6), at least not under the conditions employed. It is thus speculated that CA itself may induce the enzyme activity independently of DCA. Presently, the regulatory mechanism of the enzyme by bile acids is not fully understood. However, increased deoxycholate 7α-hydroxylase activity due to feeding of CA and DCA should be beneficial and serve a purpose in minimizing the known toxicity of DCA (5).

In CDCA-fed hamsters, the activity of deoxycholate 7α-hydroxylase was about the same as that in control animals. 7α-Hydroxylation of LCA, as well as DCA, was shown to occur in rats (30) and hamsters (25). It is possible that deoxycholate 7α-hydroxylase might also catalyze hydroxylation of LCA just as does cholesterol 7α-hydroxylase, which catalyzes hydroxylation of sitosterol or coprostanol as well as cholesterol (31). The enzyme activity might be influenced by the increased load of LCA in CDCA-fed hamsters.

The effects of dietary bile acids on deoxycholate 7α-hydroxylase activity in rats was reported by Danielsson (32), who observed a slightly decreased enzyme activity

upon feeding of taurocholate and taurodeoxycholate. The rat is known to have efficient hepatic microsomal 6β-hydroxylase(s) for LCA, CDCA, and DCA (31), and the bile acid metabolism in both species cannot be compared directly. There clearly appear to be species differences in the regulation of the enzyme, as we have recently shown that administration of phenobarbital affects the enzyme activity inversely in the rat and the hamster (6).

In the present study, there was a significant correlation between the 7α-hydroxylation of taurodeoxycholate and that of glycodeoxycholate in all groups (Fig. 1). This result was consistent with our recent report (6) and supports the hypothesis that the same enzyme may catalyze 7α-hydroxylation of both taurodeoxycholate and glycodeoxycholate. On the other hand, cholesterol 7α-hydroxylase activity did not correlate with deoxycholate 7α-hydroxylase activity. It is suggested that the regulatory mechanism of deoxycholate 7α-hydroxylase by bile acids should differ from that of cholesterol 7α-hydroxylase in the hamster.

Feeding of all bile acids increased the level of serum cholesterol significantly. Liver cholesterol concentration was increased markedly in DCA- and CA-fed hamsters, and slightly in the CDCA-fed group. These results are consistent with those reported previously (33,34). Liver cholesterol concentration did not correlate with the activity of deoxycholate 7α-hydroxylase indicating that the enzyme activity seems not to be influenced directly by the liver cholesterol concentration.

In conclusion, deoxycholate 7α-hydroxylase activity was increased markedly by feeding CA and slightly by feeding DCA. CDCA had no significant effect on the enzyme. Deoxycholate 7α-hydroxylase and cholesterol 7α-hydroxylase were independently regulated by administering each of the bile acids. The mechanism of deoxycholate 7α-hydroxylase regulation by bile acids is far from being understood. It is quite likely, however, that the enzyme plays an important role in minimizing the toxic effects of DCA in the hamster.

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Nonspecific and Metabolic Interactions Between Steroid Hormones and Human Plasma Lipoproteins

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Previous observations demonstrated that steroid hormones associate with plasma lipoproteins. The objective of this study was to estimate the relative importance of lipoproteins as steroid hormone binding agents in comparison to sex hormone binding globulin, corticosteroid binding globulin, and albumin in both normal and hyperlipidemic human plasma. The 16 steroid hormones and related metabolites included in the study were: androstanediol, androstenediol, androstenedione, androsterone, corticosterone, cortisol, dehydroepiandrosterone, deoxycorticosterone, dihydrotestosterone, estradiol, estriol, estrone, 17α-hydroxyprogesterone, pregnenolone, progesterone, and testosterone. The binding activity of these 16 steroid hormones with purified high density lipoprotein (HDL), low density lipoprotein and very low density lipoprotein were separately evaluated by equilibrium dialysis incubations to yield 48 steroid hormone-lipoprotein combinations for further study. In incubations with HDL, six steroid hormones (androstenediol, dehydroepiandrosterone, dihydrotestosterone, estradiol, pregnenolone, and progesterone) were identified as non-equilibrium, apparently due to metabolic conversion of the steroid hormones. The metabolic activity for the three Δ^5-3β hydroxy steroids and estradiol appears to be fatty acid esterification by lecithin:cholesterol acyltransferase. The computer program TRANSPORT, which was used to evaluate only the nonspecific steroid hormone-lipoprotein association levels in a 16 × 6 matrix at simultaneous equilibrium, indicated that lipoprotein-bound steroid hormones ranged from 1% for cortisol to 56% for pregnenolone in normal human blood. Simulated projections of the increase in nonspecific steroid hormone association with lipoproteins during hyperlipidemia are also presented. These results demonstrate how lipoproteins are likely to be important in the transport and metabolism of steroid hormones in human plasma.
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It is currently believed that plasma steroid hormones (SH) in the blood are mostly bound to either high affinity globulins, mainly sex hormone binding globulin (SHBG) and corticosteroid binding globulin (CBG), or proteins with low affinity, primarily albumin (1,2). Furthermore, it is generally thought that the free or unbound SH, which in many cases consists of only 1–3% of the plasma total (3,4), is the biologically active form of SH which enters cells by "passive diffusion" (5,6). Recently, we discovered that plasma SH are also associated with high density lipoprotein (HDL), low density lipoprotein (LDL), and very low density lipoprotein (VLDL) lipoproteins (LP) (7). An evaluation of the biological significance of this discovery requires a knowledge of SH-LP association levels relative to other SH dispositions in the plasma. This question can be addressed by utilizing SH-LP association data in the computer program TRANSPORT (8) to simulate "normal" male and female association patterns of numerous different SH among six binding agents (SHBG, CBG, albumin, HDL, LDL, and VLDL) at simultaneous equilibrium. However, during the process of data collection, it became apparent that some SH-LP associations were non-equilibrium due to additional (second-order) SH-LP interactions (7). In a recent study, a detailed analysis of the second-order interaction between dehydroepiandrosterone (DHEA) and HDL revealed that DHEA was being converted to DHEA-fatty acid esters during interaction with HDL_3 (9). We now report our final evaluation of 16 different SH for their level of nonspecific association (first-order interaction) as well as the occurrence of second-order SH-LP interactions. In addition, computer simulations of purely first-order association of these 16 SH among the six principal lipoprotein and protein SH binding agents in normal and hyperlipidemic human plasma are presented.

MATERIALS AND METHODS

In preliminary experiments comparing high and low specific activity estradiol and progesterone, significant differences, particularly in partition coefficient data, were encountered. Therefore, the following radiochemicals of low specific activity commercially available from New England Nuclear (Boston, MA) were employed for both partition coefficient analysis and equilibrium dialysis: [1,2-^{3}H]5α-androstane-3α,17β-diol (spec. act., 30.1 Ci/mmol); [1,2-^{3}H]androst-5-ene-3β,17β-diol (spec. act., 46.9 Ci/mmol); [19-^{3}H]androst-4-ene-3,17-dione (spec. act., 74.0 Ci/mmol); [9,11-^{3}H]-androsterone (spec. act., 53.3 Ci/mmol); [1,2-^{3}H]corticosterone (spec. act., 46.6 Ci/mmol); [1,2,6,7-^{3}H]cortisol (spec. act., 86.0 Ci/mmol); [1,2-^{3}H]dehydroepiandrosterone (spec. act., 58.0 Ci/mmol); [4-^{14}C]deoxycorticosterone (spec. act., 60.0 mCi/mmol); [1,2-^{3}H]dihydrotestosterone (spec. act., 50.6 Ci/mmol); [6,7-^{3}H]estradiol (spec. act., 42.0 Ci/mmol); [2,4,6,7-^{3}H]estriol (spec.

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General Abbreviations: B, steroid bound; B/U, B divided by U; CBG, corticosteroid binding globulin; chol, cholesterol; DPM, disintegrations per min; DTNB, dithiobis-(2-nitrobenzoic acid); E, exponent of base 10; HDL, high density lipoproteins; LDL, low density lipoproteins; LP, lipoproteins; SH, steroid hormone(s); SHBG, sex hormone binding globulin; U, steroid unbound; VLDL, very low density lipoproteins.

Steroid Abbreviations: AADOL, androstanediol; AEDOL, androstenediol; AEDON, androstenedione; AND, androsterone; B, corticosterone; DHEA, dehydroepiandrosterone; DHT, dihydrotestosterone; DOC, deoxycorticosterone; E2, estradiol; E3, estriol; E1, estrone; F, cortisol; HP, 17α-hydroxyprogesterone; P5, pregnenolone; P4, progesterone; T, testosterone.

TABLE 1

Plasma Concentration Values, Lipoprotein Binding Values, and Octanol-Water Partition Coefficients of 16 Steroid Hormones and Related Metabolites

Steroid + abbreviation	Plasma steroid concentration (nM/L)		Steroid-lipoprotein binding values[a,b]			
	M	F(lut)	HDL B/U	LDL B/U	VLDL B/U	Log P_c[c]
Androstanediol (AADOL)	0.41	0.068	5.450	3.095	0.980	4.16
Androstenediol (AEDOL)	4.3	2.4	1.401	1.006	0.390	3.55
Androstenedione (AEDON)	4.1	5.4	0.636	0.413	0.244	2.68
Androsterone (AND)	2.0	1.5	4.912	3.147	1.246	3.69
Corticosterone (B)	12.0	13.0	0.387	0.125	0.059	1.85
Cortisol (F)	400.0	400.0	0.167	0.072	0.049	1.86
Dehydroepiandrosterone (DHEA)	24.0	17.0	1.830	1.648	0.698	3.23
Deoxycorticosterone (DOC)	0.20	0.20	1.509	0.580	0.284	2.59
Dihydrotestosterone (DHT)	1.7	0.65	4.822	2.427	0.855	3.66
Estradiol (E2)	0.084	0.72	3.058	1.550	0.728	3.57
Estriol (E3)	0.037	0.032	0.184	0.083	0.033	2.45
Estrone (E1)	0.081	0.46	4.590	2.800	0.915	3.13
17α-Hydroxyprogesterone (HP)	5.4	5.9	1.339	0.971	0.362	3.17
Pregnenolone (P5)	2.4	4.4	18.358	18.611	8.561	4.22
Progesterone (P4)	0.57	38.0	6.292	4.799	2.979	3.87
Testosterone (T)	23.0	1.3	1.241	0.942	0.228	3.17

[a]Normal male and female (luteal phase) SH plasma concentration values were taken from the corresponding files listed in Dunn *et al.* (4). Steroid-Lipoprotein binding values were determined at LP concentrations of: HDL, 60 mg chol/dL; LDL, 130 mg chol/dL; and VLDL, 20 mg chol/dL.

[b]Five SH-HDL values were corrected to fit a model which assumes only first order interactions. The original experimental values of AEDOL-HDL (2.409), DHEA-HDL (4.789), E2-HDL (3.623), and P5-HDL (45.455) were replaced with values obtained in the presence of 0.1 mM dithio*bis*(2-nitrobenzoic acid), where second-order SH-LP activity was inhibited. The non-equilibrium experimental value for DHT-HDL (1.691), which also reflected second-order activity, was replaced with an estimated first-order activity value generated from a structure-activity model.

[c]The symbol P_c represents the SH octanol-water partition coefficient.

act., 90.0 Ci/mmol); [6,7-^{3}H]estrone (spec. act., 42.0 Ci/mmol); [1,2-^{3}H]17α-hydroxyprogesterone (HP) (spec. act., 40.0 Ci/mmol); [7-^{3}H]pregnenolone (spec. act., 10.5 Ci/mmol); [1,2-^{3}H]progesterone (spec. act., 45.0 Ci/mmol); [1,2-^{3}H]testosterone (T) (spec. act., 52.0 Ci/mmol).

Before use, radioactive steroids were purified by chromatography over Sephadex LH-20 columns which were 1 cm in diameter and contained 0.85 g dry weight resin solvated in benzene/methanol (90:10, v/v). Afterwards, column equilibration, steroid loading, and steroid elution solvents were isooctane/benzene/methanol (62:20:18, v/v/v) for estradiol (E2), estriol (E3), and estrone (E1); isooctane/benzene/methanol (90:5:5, v/v/v) for dihydrotestosterone (DHT), HP, progesterone (P4), and T; and cyclohexane/benzene/methanol (60:40:10, v/v/v) for all other radioactive steroids. Peak fractions were dried under N_2 and stored in methanol at −15°C. For partition coefficient experiments, only radioactive steroids were used. For equilibrium dialysis experiments, unlabeled analytical grade steroids [all purchased from Steraloids, Inc. (Wilton, NH), except for 5β-androstane-3α,17β-diol from Sigma Chemical Co. (St. Louis, MO)] were adjusted to "normal" male plasma concentration values and then tracer steroids were added to a final concentration of 10,000 DPM/mL.

Purified lipoprotein fractions were prepared essentially according to Lindgren (10); specific modifications and procedures have been previously detailed (7,9). Precise methods employed for octanol/water partition coefficient analysis, equilibrium dialysis, and radioactive counting have also been presented in detail (7). Briefly, in equilibrium dialysis experiments, a purified lipoprotein solution and a purified steroid solution, both adjusted to concentrations normally found in human plasma (see Table 1), are introduced into opposing chambers that are separated by a treated membrane permeable only to the steroid. If only nonspecific SH-LP interactions occur, equilibrium is generally achieved in 10–12 hr at 37°C, as indicated by radioactive counts from both the SH and LP chambers. At this point, it is assumed that the DPM in the SH chamber is identical to the disintegrations per min (DPM) of unbound SH present in the LP chamber. Consequently, the relative amount of SH bound to LP in the LP chamber can be determined by subtracting the DPM present in the SH chamber from the total DPM present in the LP chamber, and the ratio of bound/unbound steroid (B/U) in the LP chamber can be calculated. Experimental B/U values are used to determine the initial apparent association constants (K_a') for specific SH-LP equilibrium associations; and these initial K_a' values, in conjunction with the experimental LP and SH concentrations, serve as input for the TRANSPORT program which simulates the simultaneous distribution of multiple ligands among multiple binding agents as previously described (7). The B/U value in the LP chamber divided by the molar concentration of unbound LP binding agent in the LP cham-

ber designates the initial K_a' for each SH-LP combination (7). In the process of TRANSPORT analysis, the final K_a' values were determined by iteration of the TRANSPORT program, each time inputing the revised values for unbound LP binding agents in a 16 × 6 matrix until convergence.

Projected levels of SH-LP association during simulated hyperlipidemia were derived from SH-LP first order equilibrium binding experiments of androstenedione, androsterone, dehydroepiandrosterone, and estrone (at "normal" male plasma levels) against HDL, LDL, and VLDL separately, over a wide range (HDL, 30–600 mg chol/dL; LDL, 60–4000 mg chol/dL; VLDL, 10–1000 mg chol/dL) of concentrations. The experimental B/U data from each of these 12 SH-LP pairs were used to generate model second-degree polynomial curves by weighed polynomial regression. The assignment of weights for each experimental (Y_i coordinate) value in a series, $Y_1, Y_2, \ldots, Y_n$, was according to the formula: $W_i = Y_n(10)/Y_i$. In addition, in order to force the polynomial curve through the origin, coordinates (0,0) were assigned a weight equal to 100 times the weight of Y_1. The adequacy of these model polynomial curves were judged by comparing the experimental values to their corresponding model values. These comparisons were evaluated by polynomial regression, analysis of variance, F-test. A plot of the experimental data points against the corresponding theoretical binding values in these 12 analyses resulted in calculated F values ranging from 484 to 11,530, which indicates a good fit in all 12 cases. For the model equations ($B/U = a + b[LP] + c[LP]^2$) in each LP class, a linear relationship was obtained between SH-LP B/U values *vs* the coefficient of the first power (b). This observation permitted a means for generating a complete set of 48 second-degree polynomial equations, encompassing all 16 SH, from B/U values presented in Table 1. This was accomplished by first calculating coefficient "b", and then solving for coefficient "c" by means of simultaneous equations with coefficient "a" set equal to zero. These 48 model polynomial equations were created for determining the B/U and corresponding K_a' values of each SH at any given HDL, LDL, or VLDL concentration in a 16 × 6 matrix. Linear and polynomial regression analyses were performed according to Dowdy and Wearden (11).

RESULTS AND DISCUSSION

Since the direct measurement of LP associated SH by radioimmunoassay proved to be inaccurate and essentially unachieveable (12), computer simulation of SH-LP associations, based on the law of mass action and utilizing the TRANSPORT program, was selected as a means of estimating SH-LP associations. The mathematical model underlying this analysis assumes that association and dissociation of ligands and binding agents are the only interactions occurring in the system; furthermore, allosteric or cooperative effects between or among the various binding elements are assumed to be negligible. In such a system, the association of any one species of ligand with any particular type of binding agent can be defined by an association constant. If the association is specific, then the association constant is a true constant which is invariable over a wide range of ligand and binding agent concentrations; alternatively, for nonspecific association that is non-saturable within and beyond the physiological range, an apparent association constant must be determined for each particular set of ligand and binding agent concentrations. Thus, the valid use of TRANSPORT for estimating SH-LP association requires not only a determination of whether SH-LP associations are specific or nonspecific, but also a demonstration that the binding elements in a large multicomponent system do not interact to alter the elementary SH-LP associations.

As described previously, the association between SH and LP was found to be nonspecific as determined by Scatchard analysis (7). Evaluation for the occurrence of either positive or negative interference was accomplished by measuring the B/U values of SH with HDL, LDL, and VLDL individually, and comparing the sum of those values with B/U value of SH incubated against a LP solution containing the same concentrations of HDL, LDL, and VLDL combined together (7). In the absence of interference, the sum of individual LP-B/U values should equal the combined LP-B/U value; and, a regular ratio of B/U values should be maintained between HDL, LDL, and VLDL based on surface area and surface activity considerations. Of the 16 SH evaluated for additivity and regularity, six [AEDOL (androstenediol), DHEA, DHT, E2, P5 (pregnenolone), and P4] gave aberrant patterns. These six SH were recognized to have additional (second-order) interactions with LP that were different from nonspecific (first-order) SH-LP association.

For the 10 SH which gave additive and regular B/U values among the three LP classes, it appeared that the SH-LP associations were strictly nonspecific and thus dependent only on the physical/chemical composition of LP. This concept was tested by use of structure-activity models (12,13) which were based on correlations between two types of nonspecific interactions—the chemical partitioning of SH between water and octanol, and the chemical partitioning of SH between the aqueous media and organic LP surfaces in equilibrium dialysis experiments (B/U values). Structure-activity models proved useful for identifying specific SH-LP interactions that exhibited irregularity. When the SH that were not additive were evaluated by this test, it became apparent the SH-HDL interactions were the primary source of deviation; and this observation was confirmed in extended incubations of up to 96 hr, which characteristically failed to achieve equilibrium.

Two types of non-equilibrium profiles were distinguishable among the six SH that exhibited second-order interactions with HDL during prolonged incubation. The lipophilic profile, observed in incubations of HDL with AEDOL, DHEA, E2, and P5, which was characterized by a continual increase in SH-HDL association over time; and the hydrophilic profile, observed in DHT-HDL and P4-HDL incubations, which was characterized by a continual decrease in SH-HDL association over time. These two profile types are illustrated in Figure 1, where typical P4-HDL and E2-HDL incubation results are presented. Preincubation (37°C) of

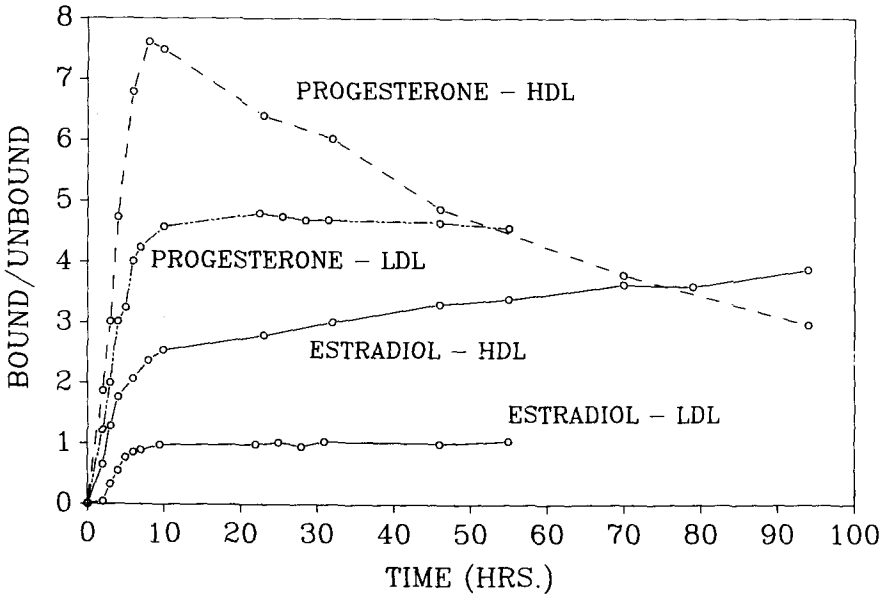

FIG. 1. Equilibrium dialysis binding of estradiol (E2) and progesterone (P4) with HDL and LDL lipoprotein fractions. Initial steroid concentrations were 0.084 nM for E2 and 0.57 nM for P4. Initial LP concentrations were 17.8 μM for HDL and 1.88 μM for LDL. For both E2 and P4, association with LDL achieved an equilibrium after 10 hr of incubation and maintained it. The E2-HDL association is non-equilibrium as the original E2 appears to become more lipophilic over time. The P4-HDL association is also non-equilibrium, but here the original P4 becomes more hydrophilic over time.

E2, P4, VLDL, and LDL for up to four days prior to experiments did not alter the levels or patterns of binding.

Of the four lipophilic SH-HDL interactions, E2-HDL was the weakest, exhibiting an approximately 50% increase in B/U between 12 and 72 hr of incubation; in contrast, AEDOL-, DHEA-, and P5-HDL incubations of similar duration exhibited 8–10-fold increases (manuscript in preparation). In all four cases, the active HDL subfraction was HDL_3 with no activity apparent in HDL_2; and this HDL_3 activity was completely inhibited in the presence of 1 mM dithio*bis*(2-nitrobenzoic acid) (DTNB). In the case of DHEA and P5, the lipophilic products of SH-HDL interaction have been positively identified as sterol fatty acid esters, and the fatty acid compositions of these sterol esters are very similar to those of native HDL_3 cholesteryl esters (9; and manuscript in preparation). DHEA- and P5-esters have also been identified in fresh human plasma, and are present in significantly large quantities (9,14). A number of observations suggest that these lipophilic compounds are produced *via* the lecithin:cholesterol acyltransferase reaction (9). This suggestion is entirely compatible with the *in vitro* demonstration that the 3β-hydroxyl group and the *trans* configuration of the A and B steroid rings are the only requirements for LCAT activity (15).

The inhibition of all four SH-HDL second-order lipophilic interactions by DTNB established a method for determining their purely first-order SH-HDL B/U values, which are presented in Table 1. This manipulation allowed the creation of a catalogue with 14 first-order SH-HDL B/U values. A structure-activity model relating these B/U values with the respective SH octanol-water partition coefficients was created to estimate the theoretical first-order B/U values for DHT- and P4-HDL. The projected value for P4-HDL was very close to that which was determined experimentally, but the projected DHT-HDL value was substantially different. These theoretical DHT-HDL and experimental P4-HDL B/U values are presented in Table 1, and were used to complete the catalogue of 16 SH-HDL B/U values. Since SH interactions with LDL and VLDL were determined to be strictly first-order, the respective SH-LDL and SH-VLDL B/U values presented in Table 1 were all experimentally derived. From the data in Table 1, three structure-activity models were constructed from a general model based on an often cited method (16), which utilized octanol/water partition coefficients as the structural parameter of steroids and the binding affinity (B/U) of steroids as the activity parameter. The statistics obtained from this analysis are presented in Table 2.

For TRANSPORT analysis, first-order SH-LP binding values from Table 1 were combined with the binding values of the 16 SH with SHBG, CBG, and albumin (4) in a 16 × 6 matrix to produce the simultaneous first-order binding distribution patterns presented in Table 3. Basically, three input files are required for TRANSPORT analysis: normal male/female SH concentrations; normal male/female binding agent concentrations; and association constants for each possible SH-binding agent combination. The SH concentrations, the protein (SHBG, CBG, albumin) concentrations, and

TABLE 2

Linear Regression and Correlation Statistics Obtained from the Paired Variables Log LP-B/U and Log P_c for 16 Different SH

LP class	a ± SE[a]	t_a[b]	b ± SE[a]	t_b[b]	r[c]
VLDL	3.532 ± 0.100	35.440	1.015 ± 0.141	7.212	0.888
LDL	3.127 ± 0.080	38.877	1.065 ± 0.134	7.955	0.905
HDL	2.883 ± 0.104	27.825	1.011 ± 0.154	6.561	0.869
HDL_{cor}[d]	2.896 ± 0.091	31.795	1.144 ± 0.150	7.643	0.898

[a]Slope "b" and y-intercept "a" plus standard errors "SE" of regression line defined as y = a + bx.
[b]Calculated t statistics of a and b for testing the null hypotheses (H_o: $\alpha = 0$; H_o: $\beta = 0$), where the critical value for $t_{0.001,14} = 5.363$.
[c]r Refers to the sample correlation coefficient.
[d]Five experimental HDL-B/U values corrected to reflect only first-order SH-HDL binding (see Table 1, footnote [b]).

TABLE 3

Simulated Equilibrium Distribution of 16 Steroid Ligands Among Six Binding Agents in "Normal" Male and Female Blood Plasma

		Protein binding agents			Lipoprotein binding agents			
Steroid ligand	Sex[a]	SHBG % bound	CBG % bound	Albumin % bound	HDL % bound	LDL % bound	VLDL % bound	Free % unbound
AADOL	M	13.43	0.02	78.39	4.24	2.40	0.74	0.78
	F	26.60	0.01	66.50	3.59	2.03	0.60	0.66
AEDOL	M	57.04	0.06	32.06	4.01	2.88	1.10	2.86
	F	75.63	0.03	18.21	2.28	1.63	0.60	1.63
AEDON	M	2.74	1.36	79.63	4.52	2.94	1.70	7.11
	F	6.18	1.30	76.89	4.36	2.83	1.58	6.87
AND	M	0.56	0.41	67.87	14.88	9.52	3.72	3.03
	F	1.31	0.40	67.49	14.79	9.45	3.54	3.01
B	M	0.09	77.71	17.34	1.20	0.39	0.18	3.10
	F	0.21	77.39	17.50	1.21	0.39	0.17	3.12
F	M	0.08	89.31	5.98	0.60	0.26	0.22	3.56
	F	0.18	89.10	6.04	0.60	0.26	0.22	3.60
DHEA	M	3.07	0.07	78.40	6.41	5.80	2.74	3.50
	F	6.90	0.07	75.50	6.17	5.57	2.43	3.37
DOC	M	0.78	36.30	54.71	3.68	1.41	0.67	2.44
	F	1.81	35.63	54.42	3.66	1.40	0.64	2.43
DHT	M	57.43	0.22	35.21	3.79	1.91	0.66	0.79
	F	75.92	0.12	19.94	2.15	1.08	0.36	0.45
E2	M	18.45	0.04	68.62	6.24	3.16	1.44	2.04
	F	34.58	0.03	55.09	5.01	2.53	1.12	1.64
E3	M	0.45	0.16	89.06	1.46	0.66	0.26	7.95
	F	1.05	0.15	88.54	1.45	0.65	0.25	7.91
E1	M	5.92	0.06	66.50	13.62	8.30	2.63	2.97
	F	12.82	0.05	61.70	12.64	7.69	2.35	2.75
HP	M	0.30	40.94	50.49	3.02	2.19	0.81	2.25
	F	0.69	40.48	50.58	3.02	2.19	0.78	2.26
P5	M	0.23	0.07	41.82	22.84	23.14	10.64	1.24
	F	0.54	0.07	41.76	22.81	23.10	10.48	1.24
P4	M	0.21	13.99	59.33	11.11	8.46	5.13	1.77
	F	0.47	13.39	57.50	10.78	8.32	7.83	1.71
T	M	43.53	3.58	45.87	2.54	1.94	0.48	2.05
	F	64.33	2.24	29.03	1.61	1.22	0.28	1.30

[a]For both male and female simulations, the input binding agent concentrations (M/L) for LP, CBG, and albumin were: HDL, 1.78E-5; LDL, 1.88E-6; VLDL, 7.23E-8; CBG, 7.0E-7; and albumin, 5.6E-4. Plasma SHBG (M/L) was input as 2.8E-8 for the male and 3.7E-8 for the female. The "normal" male and female (luteal phase) SH concentration sets presented in Table 1 were used as male and female SH input.

the SH-protein K_a values were taken from TRANSPORT as presented in Dunn *et al.* (4). For the three LP binding agents, the initial SH-LP K_a' values were derived from the formula (SH-B/U)/[unbound LP], where SH-B/U values are those of Table 1 and initial molar concentrations of unbound LP are determined from the starting LP concentration in the incubation, the corresponding SH molar concentrations, and SH-B/U values assuming, for computational purposes, that no more than one SH molecule is bound to any one LP particle (7). Final molar concentrations of unbound LP and protein binding agents in multi-component first-order SH-LP equilibrium simulations are then solved as described previously (7), where five iterations were sufficient to produce convergence. The percentage of each available LP and protein binding agent which contains no SH at equilibrium can be determined by dividing the final concentration of each unbound binding agent by the total binding agent concentration available in the system. For the luteal phase female (Table 3), the percentages of each binding agent that contains no associated SH were: SHBG, 83.62%; CBG, 46.55%; albumin, 99.99%; HDL, 99.95%; LDL, 99.64%; and VLDL, 93.13%; and for the male (Table 3) the percentages were: SHBG, 47.44%; CBG, 47.17%; albumin, 99.99%; HDL, 99.97%; LDL, 99.78%; and VLDL, 96.92%. Under normal conditions (Table 3) the large percentages of HDL, LDL, and VLDL that contain no SH serve to justify the use of a mathematical assumption, for computational purposes, that each LP particle has a single binding site.

The concentration of unbound LP and the B/U value are the two parameters required for calculating the initial K_a'; therefore, it is necessary to establish the number of binding sites (LP particles) available in the system. The program TRANSPORT as originally written for the protein binding agents SHBG, CBG, and albumin, assumes that each protein molecule has one specific binding site for SH. Thus the molar concentration of any protein binding agent is directly related by default to the concentration of binding sites. For the calculation of SH-LP association constants, the TRANSPORT default of one binding site per LP particle was used because in all cases more than 93% of LP particles contained no SH. If one assumes that many molecules of SH can bind to an LP particle, the Poisson probability equation (11) can be used to predict the level of association by random distribution of ligands among binding agents. In Table 3, VLDL of the luteal female has the highest degree of SH association with 6.87% of the VLDL particles containing SH. For this case, Poisson probability (λ = 0.0687) predicts that less than 0.3% of the VLDL particles would contain two or more SH molecules. The small fraction of LP particle with more than one SH imposes only slight correction to the default calculation of the molar concentration of unbound LP. As described previously (7), a model which assigned three binding sites per LP particle was tested and produced sharply different K_a' values as compared to the default model, but the final simulated SH distribution patterns were the same because the reduction in K_a' values was balanced by the increased concentration of binding sites in the system.

The analyses presented in Table 3 demonstrate that all SH are associated to some extent with LP in normal human plasma. In the case of plasma P5, more than 56% is projected to be bound to LP on the basis of first-order interaction. However, since P5 also has especially strong second-order lipophilic interaction with HDL, an even greater percentage of total plasma P5 will be associated with LP; support for this prediction can be deduced from the report by Jones and James (15). Other SH with substantial LP association of the first-order are androsterone (AND), E1, and P4; in each case about 1/4 of the SH is associated with LP. The ultimate impact of second-order lipophilic conversion of AEDOL and DHEA by HDL on their final distribution among LP and protein binding agents cannot be accurately assessed, but in these two cases, LP association would also be increased substantially in comparison to the values given in Table 3. The mere existence of both specific and nonspecific SH-LP associations provides implicit evidence that SH, to varying degrees, enter cells *via* well known cell surface LP receptor pathways (17). Clearly, any future study or treatise concerning the disposition, conveyance, or transport of steroid hormones in the blood should include SH-LP interactions as an important consideration.

A point of some importance, which can be gleaned from TRANSPORT analyses, is that massive changes in plasma SH concentrations impart only minor effects on overall SH distribution (compare male and female

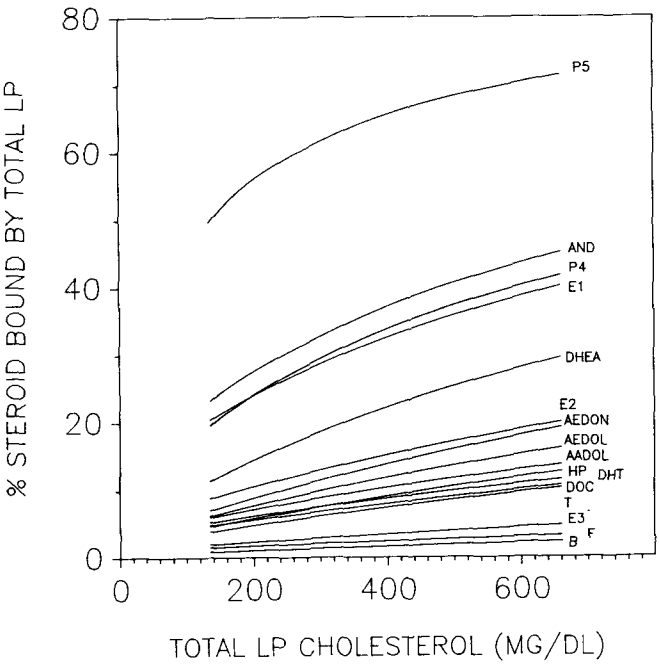

FIG. 2. Projected increase in first-order association of SH with LP during simulated hyperlipidemia. In the Figure, total plasma cholesterol at any point was the sum of HDL, which was held constant at 60 mg/dL, and the β-liproproteins (LDL + VLDL), which varied in concentration but with a constant LDL/VLDL ratio of 13:2. For each of eight discrete total plasma cholesterol values, which ranged from 130 to 1200 mg chol/dL, the LP and protein binding data were analyzed by TRANSPORT in an otherwise "normal male" 16 × 6 matrix and the resulting % SH bound to HDL, LDL, and VLDL were added to produce the % SH bound to plasma total lipoproteins (y-axis).

TABLE 4

Coefficients of Model Second Degree Polynomial Equations Obtained by Regression Analysis of 12 SH-LP Equilibrium Binding Experiments

SH-LP combination	N[a]	F[b]	Coefficients[c] a(× 10^{-4})	b(× 10^{-2})	c(× 10^{-5})
AEDON-HDL	8	2,681	.275982	1.431374	−1.250646
AEDON-LDL	10	1,096	−0.438497	.366846	−0.136061
AEDON-VLDL	10	477	2.220578	1.184872	−0.036206
AND-HDL	8	484	66.144880	7.230065	−5.034828
AND-LDL	10	908	77.182620	1.470832	−0.203400
AND-VLDL	10	2,492	−1.402483	3.684665	−1.549254
DHEA-HDL[d]	6	2,991	−0.374292	2.901957	1.452362
DHEA-LDL	6	2,218	−2.369234	1.337380	−0.591064
DHEA-VLDL	6	11,530	−0.658882	3.256951	−1.253946
E1-HDL	8	4,097	7.781610	6.744209	−4.008225
E1-LDL	10	1,546	43.887310	1.444291	−0.183653
E1-VLDL	10	1,765	2.646381	3.593703	−1.018865

[a]The number of different observations at variable LP concentration values, measured in triplicate, from which experimental B/U numbers were obtained.
[b]Calculated F values from the polynomial regression analysis are used to indicate the goodness of fit of the model equations to the experimental data. The critical F value for β=0.05, with 2 degrees of freedom for regression, and 6 observations is: $F_{0.05,2,4} = 6.944$; and for 10 observations, $F_{0.05,2,8} = 4.459$
[c]Coefficients for equations of the form: $B/U = a + b\,[LP] + c\,[LP]^2$.
[d]Experimental data was obtained in the presence of 1 mM DTNB.

binding patterns of P4), whereas relatively small changes in plasma binding agent concentrations may profoundly influence the distribution patterns of SH. Plasma concentrations of SHBG are known to vary over a 20-fold range depending on physiological circumstances (1); an even greater range in LP concentrations can occur between normal humans and some hyperlipidemic patients. Figure 2 illustrates the theoretical change in first-order LP association of four SH during simulated type II hyperlipidemia. As described in the Methods section, this figure was generated based on the binding analyses of AEDON, AND, DHEA, and E1 with each of the three LP classes in incubation experiments over a wide range of LP concentrations. In this study, a quadratic level, rather than linear or cubic, gave the best fit for polynomial regression, where weighted least squares means were used to generate model equations. The coefficients of these 12 model second-degree polynomial equations are presented in Table 4. A graphic presentation of the model E1-VLDL equation in Table 4, as well as the experimental observations, has been published (7). Data generated from the equations in Table 4 were employed to derive model equations for all 48 SH-LP first-order interactions, which were used to simulate the disposition of SH during hyperlipidemia (Fig. 2).

For the simulation in Figure 2, HDL concentration is held constant at 60 mg HDL-chol/dL while LDL and VLDL concentrations are increased, but with a constant ratio of 6.5 for LDL/VLDL cholesterol. The simulation in Figure 2 is the product of a theoretical model based on numerous mathematical assumptions. Other sets of mathematical assumptions or constructs could also be useful for simulating the disposition of multiple SH in human plasma. The prudent interpretation of Figure 2 or other simulations should focus on the overall pattern rather than the quantitative treatment of specific components within the pattern. There are two major aspects in simulation pattern from mass action models: first, lipoprotein binding is of minor importance for estriol and the corticosteroids (B,F,DOC) compared to pregnenolone in particular, and also progesterone, estrone, and dehydroepiandrosterone, which have high LP affinity; second, the fraction of SH bound to LP will increase in cases of hyperlipidemia. Although our computer simulations of plasma SH patterns have a sound theoretical basis, it is difficult to test their accuracy because precise evaluation of SH plasma disposition by direct chemical analysis is not currently feasible.

Our experimental evidence indicates that at normal or below normal lipoprotein concentrations, the LP and protein binding agents are sufficiently dilute to allow the SH full access to binding sites. In cases of extreme hyperlipidemia, interactions between LP particles become increasingly significant and reduce the accessibility of binding sites; thus, compromising the basis of TRANSPORT analysis. In the model construct of the Figure 2 simulation, the binding site accessibility on SHBG, CBG, and albumin was kept constant during hyperlipidemia, but in actuality, some masking of these protein binding sites could also be expected. Indeed, a recent report has demonstrated that physiological concentrations of free fatty acids can interfere with SH binding to albumin (18). Therefore, the simulation presented in Figure 2 represents a conservative estimate of the increase in SH binding by LP during hyperlipidemia because simulations which

factor in a reduced SH accessibility to SHBG, CBG, and albumin would project enhanced SH-LP binding. Clearly, in many clinical cases of hyperlipidemia, where LP concentrations are increased substantially relative to SHBG, CBG, and albumin, a much larger fraction of the total SH will become associated with LP compared to the values of the normal male in Table 3. It is not known if such a shift in SH disposition among the plasma binding agents could influence SH metabolic regulation.

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Effects of Dietary Fats on Prostanoid Production and Aortic and Plasma Fatty Acid Composition in Rats

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Male Sprague-Dawley rats were fed diets with 10%, 30%, or 50% of energy derived from fat for two weeks. The fats used were beef tallow, olive oil, peanut oil and butter. Aortic prostacyclin (PGI_2) production, platelet aggregation and thromboxane A_2 (TXA_2) production and plasma and aortic phospholipid (PL) content were measured. Butter- and beef tallow-feeding reduced aortic PGI_2 production and collagen-induced TXA_2 production in a dose-dependent manner as the level of fat in the diet increased. Neither olive oil nor peanut oil had any effect on aortic PGI_2 production or collagen-induced TXA_2 production. Butter-feeding also resulted in a decrease in collagen-induced platelet aggregation; however, none of the other fats had any effect on collagen-induced platelet aggregation. The observed decreases in aortic PGI_2 and collagen-induced TXA_2 production were paralleled by similar decreases in aortic and plasma PL arachidonic acid content and an increase in eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Only the most saturated fats, butter and beef tallow, had significant inhibitory effects on prostanoid production and platelet aggregation.

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Much interest has been shown in the relationship between dietary fat and prostanoid production in relation to the regulation of haemostatic function and risk of occlusive vascular disease. Fish oils, rich in long-chain n-3 polyunsaturated fatty acids (PUFA) have been shown in numerous animal studies to modify haemostatic function by reducing the formation of arachidonic acid-derived prostanoids, thromboxane A_2 (TXA_2) and prostacyclin I_2 (PGI_2) (1–5). In recent studies, we have demonstrated similar changes in prostanoid production in response to dietary saturated fat in the form of butter (6).

Increasing the proportion of butter (10%, 30%, 50% energy) in the diets of rats resulted in a dose-dependent decrease in arterial PGI_2 production which was accompanied by dose-dependent decreases in arachidonic acid in plasma and aortic PL (6). Lard, however, did not affect any of these parameters, possibly due to its higher proportion of PUFA (6). The aim of the present study was to examine the effects of other types of common dietary fats (beef tallow, olive oil and peanut oil) on prostanoid production and platelet aggregation.

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Abbreviations: ADP, adenosine diphosphate; DGLA, dihommo-γ-linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; ETA, eicosatrienoic acid; KRB, Krebs Ringer bicarbonate buffer; 6-keto $PGF_{1\alpha}$, 6-keto prostaglandin $F_{1\alpha}$; PGI_2, prostacyclin I_2; PL, phospholipid; PPP, platelet-poor plasma; PRP, platelet-rich plasma; P/S ratio, ratio of polyunsaturated to saturated fatty acids; PUFA, polyunsaturated fatty acid; RIA, radioimmunoassay; TXA_2, thromboxane A_2; TXB_2, thromboxane B_2; 20:3n-9, a 20 carbon fatty acid with three methylene-interrupted double bonds, the first one being nine carbons (n-9) from the methyl terminus.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats weighing 100–200 g were used in these studies. They were housed in a temperature-controlled room with a 12-hr light-dark cycle. Food and water were available *ad libitum* and body weights were recorded three times per week.

Diets. The nutrient composition of the experimental diets is shown in Table 1, and the fatty acid composition is given in Table 2. The diets were prepared from wholemeal flour and skim milk powder (providing all of the carbohydrate and protein and little of the fat), and beef tallow, olive oil, peanut oil, or butter (providing most of the fat). Protein remained constant at 20% energy, and carbohydrate levels varied inversely with the level of fat in the diet. Vitamins and minerals were added to all diets in accordance with the recommendations by the American Institute of Nutrition (7).

Platelet aggregation. After two weeks on the diets, rats were fasted overnight and anaesthetized with sodium pentobarbitone (60 mg/kg intraperitoneally) (Civa Chemicals, Hornsby N.S.W., Australia). Blood was removed by cardiac puncture and collected into 3.8% tri-sodium citrate (blood/citrate, 9:1, v/v). The blood was then centrifuged at 150 × *g* for 10 min at room temperature, and platelet-rich plasma (PRP) was removed. The remaining blood was centrifuged at 3,000 × *g* for 15 min to obtain platelet-poor plasma (PPP). The platelet count was determined using a Coulter Counter (Model IV Plus) and adjusted with PPP to give a count of 600,000 platelets/μL. The PRP was then further diluted with incomplete Tyrodes buffer (ionic composition, mmol/L: Na^+, 142.8; K^+, 2.7; Cl^-, 139.6; HCO_3^-, 11.9; $H_2PO_4^-$, 0.3) to give a count of 300,000 platelets/μL. Platelet aggregation studies were performed using a Payton dual channel aggregometer.

TABLE 1

Composition of the Experimental Diets

	Butter (%)		(Beef tallow/olive oil/peanut oil) (%)		
	10	50	10	30	50
Constituents (g/100 g)					
Wholemeal flour	73.1	4.5	73.5	43.8	4.8
Skim milk powder	19.9	61.3	20.0	39.8	64.9
Butter	2.5	29.7			
Beef tallow/olive oil/ peanut oil			2.1	12.0	25.8
Choline chloride	0.1	0.1	0.1	0.1	0.1
Mineral mix	3.4	3.4	3.3	3.3	3.4
Vitamin mix	1.0	1.0	1.0	1.0	1.0
Composition (% energy)					
Protein	20	20	20	20	20
Carbohydrate	70	30	70	50	30
Fat	10	50	10	30	50

TABLE 2

Fatty Acid Composition (%) of the Experimental Diets

	Beef tallow (%)			Olive oil (%)			Peanut oil (%)		
Fatty acid	10	30	50	10	30	50	10	30	50
12:0	0.7	0.5	0.6	—	—	—	—	—	—
14:0	4.6	4.5	5.4	0.7	0.4	0.3	0.3	0.2	0.2
14:1	0.4	0.5	0.5	—	—	—	—	—	—
15:0	0.1	0.4	0.1	—	—	—	—	—	—
16:0	28.1	25.1	26.5	20.1	16.8	15.4	13.8	10.3	9.6
16:1	3.1	4.1	4.2	1.0	1.6	1.5	0.3	0.1	0.1
17:0	1.0	1.4	1.3	tr	0.3	0.2	0.8	0.3	3.2
18:0	12.5	16.9	17.6	2.6	2.8	2.8	2.9	2.8	2.7
18:1	30.2	39.2	39.4	50.1	64.2	67.6	34.0	44.5	45.2
18:2n-6	17.0	5.8	3.0	23.7	12.9	11.4	41.8	34.6	32.2
18:3n-3	1.8	1.2	1.0	1.6	0.8	0.6	0.6	1.0	1.0
20:0	0.1	0.1	0.1	—	—	—	—	—	—
20:1	0.6	0.8	0.6	—	—	—	2.7	1.8	1.6
Saturated	47.1	48.9	51.6	23.4	20.3	18.7	17.8	13.6	15.7
Monoenoic	34.3	44.6	44.7	51.1	65.8	69.1	39.8	50.8	51.1
Polyenoic	18.8	7.0	4.0	25.3	13.7	12.0	42.4	35.6	33.2
n-6/n-3 ratio	9.4	4.8	3.0	14.8	16.1	19.0	69.7	34.6	32.2
P/S ratio	0.40	0.14	0.08	1.08	0.67	0.64	2.38	2.62	2.11

PRP (500 μL) was stirred at 1,000 rpm at 37°C, and 100 μL of the appropriate aggregating agent was added: collagen (Hormon-Chemie, Munich, West Germany), 0.08, 0.17 mg/mL (for the experiments involving beef tallow, olive oil and butter) or 0.05, 0.1 mg/mL (peanut oil) and adenosine diphosphate (ADP), Sigma Chemical Co., St. Louis, MO, 2.5, 5.0 mM. All aggregating agents were diluted in complete Tyrodes buffer (ionic composition, mmol/L: Na^+, 142.8; K^+, 2.7; Ca^{++}, 1.8; Mg^{++}, 1.1; Cl^-, 145.5; HCO_3^-, 11.9; $H_2PO_4^-$, 0.3). Platelet aggregation was monitored continuously for 4 min. In order to quantitate platelet aggregation, the maximum change in light transmittance was expressed as a percentage of the light transmittance through PPP. Five minutes after the addition of collagen, a 200 μL aliquot of the aggregation sample was mixed with 200 μL of ice-cold ethanol, centrifuged and the supernatant stored at −20°C for subsequent radioimmunoassay (RIA) of thromboxane B_2 (TXB_2, Sigma Chemical Co., Amersham, U.K.), the stable metabolite of TXA_2.

Aortic PGI_2 production. Immediately after blood collection the rat was perfused with Krebs Ringer Bicarbonate buffer (KRB, pH 7.4, containing 5.5 mM glucose) to flush out the remaining blood, leaving the artery preparation free of adhering blood thrombi. The abdominal aorta was carefully dissected free of connective tissue and the distal 2 cm segment above the bifurcation removed. This was then split longitudinally and preincubated in KRB at 37°C for 15 min in a shaking water bath. The artery strip was then transferred to another vial and incubated for a further 15 min, after which a 100 μL aliquot of the incubation medium was taken and stored at −20°C for RIA of 6-keto prostaglandin $F_{1\alpha}$, ($PGF_{1\alpha}$, Sigma Chemical Co.), the stable metabolite of PGI_2. PGI_2 values are expressed as ng of 6-keto $PGF_{1\alpha}$/aorta strip and not as ng of 6-keto $PGF_{1\alpha}$/mg of tissue as it made no difference to the overall results and the conclusions drawn from them.

Phospholipid (PL) fatty acid analysis. Fatty acid composition of plasma and aortic PL was determined as previously described (8). Lipid extracts were prepared from plasma and thoracic aorta by chloroform/methanol extraction (8). PLs were separated by thin-layer chromatography, and the concentration of the component fatty acids determined by capillary gas-liquid chromatography using diheptadecanoyl phosphatidylcholine (Sigma Chemical Co.) as an internal standard as previously described (9). The identification of 20:3n-9 (eicosatrienoic acid, ETA) was based on identical retention times with 20:3n-9 isolated from essential fatty acid-deficient rat liver when separated on a 50 m × 0.32 mm bonded phase capillary column coated with CPSil 88 (Chrompak, Middleburg, The Netherlands). On this column 20:3n-9 was separated clearly from 20:2n-6, 20:3n-6 and 22:0.

Statistical analysis. Differences between groups were established using the unpaired Student t-test. Results are expressed as mean ± SEM. As experiments testing each fat type were carried out on different batches of rats, statistical comparisons were done only within, and not between, batches of rats.

RESULTS

The average weight gain of the rats was 6.0–7.5 g/rat/day, and there were no major differences in weight gain between the different dietary groups.

Increasing the level of butter in the diet of rats from 10% to 50% energy significantly reduced aortic PGI_2 production ($p<0.001$, Fig. 1), consistent with our previous data (6). Similarly, as the level of beef tallow in the diet rose from 10% to 30% and 50% energy, there was a dose-dependent decrease in aortic PGI_2 production (Fig. 1). This decrease was statistically significant on the 50% beef tallow diet ($p<0.02$). Neither olive oil nor peanut oil had any effect on aortic PGI_2 production as the level of these fats rose in the diet.

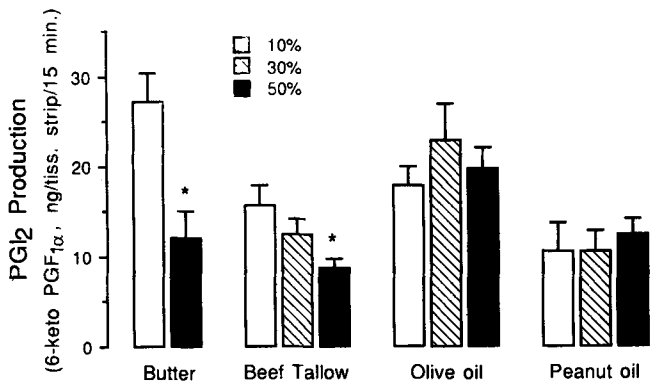

FIG. 1. Aortic PGI_2 production in rats fed butter, beef tallow, olive oil or peanut oil (ng 6-keto $PGI_{1\alpha}$/aorta strip/15 min). *Significant difference from respective 10% diet, $p < 0.02$.

TABLE 3

Collagen-induced Platelet Aggregation (%, mean ± SEM)

Fat source	[Collagen] (mg/mL)	Fat level (%) 10	30	50
Butter (n=6)	0.08	21.0 ± 1.5		15.7 ± 1.5[a]
	0.17	29.5 ± 2.6		25.5 ± 1.8
Beef tallow (n=9)	0.08	24.5 ± 1.6	20.9 ± 1.4	20.7 ± 1.6
	0.17	26.0 ± 1.3	28.3 ± 1.4	24.8 ± 1.6
Olive oil (n=5)	0.08	60.4 ± 4.7	50.8 ± 5.2	58.4 ± 3.9
	0.17	48.3 ± 2.4	45.2 ± 1.1	56.1 ± 2.4[b]
Peanut oil (n=5)	0.05	44.6 ± 7.2	50.9 ± 4.6	47.8 ± 6.7
	0.10	33.0 ± 3.2	34.5 ± 3.1	41.8 ± 4.8

[a]Significant difference from 10% $p<0.05$.

[b]Significant difference from 30% $p<0.01$.

Butter at the 50% energy level significantly reduced collagen-induced platelet aggregation at the lower concentration (0.083 mg/mL collagen, $p<0.05$, Table 3), but not at the higher concentration (0.167 mg/mL collagen). In contrast, increasing the level of beef tallow or peanut oil in the diet of rats did not have any significant effect on collagen-induced platelet aggregation (Table 3). Similarly, increasing the level of olive oil in the diet did not affect collagen-induced platelet aggregation except in one instance: there was a statistically significant increase in platelet aggregation at the higher collagen concentration in the rats fed 50% olive oil compared to those fed 30% olive oil (Table 3). Whether this apparent effect has any physiological significance or was a chance observation is not clear. ADP-induced platelet aggregation was not affected by the type or amount of fat used.

The 50% butter diet markedly reduced collagen-induced TXA_2 production at both levels of collagen (0.083 mg/mL, $p<0.01$, 0.167 mg/mL, $p<0.005$, Table 4). Similarly, increasing the level of beef tallow in the diet resulted in a dose-dependent decrease in collagen-induced TXA_2 production (Table 4). In contrast, increasing the level of olive oil or peanut oil in the diets of rats had no effect on collagen-induced TXA_2 production (Table 4).

TABLE 4

Collagen-induced TXA_2 Production (ng/mL PRP, mean ± SEM)

Fat source	[Collagen] (mg/mL)	Fat level (%) 10	30	50
Butter (n=6)	0.08	99.3 ± 9.6		29.9 ± 3.0[a]
	0.17	124.4 ± 12.3		68.5 ± 5.1[a]
Beef tallow (n=9)	0.08	136.5 ± 7.5	104.3 ± 9.4[a]	70.0 ± 8.0[a,b]
	0.17	159.5 ± 10.9	145.4 ± 11.0[a]	102.5 ± 10.6[a,b]
Olive oil (n=5)	0.08	164.0 ± 11.6	145.6 ± 14.6	153.8 ± 13.3
	0.17	79.3 ± 10.2	75.2 ± 6.4	77.5 ± 9.2
Peanut oil (n=5)	0.05	130.7 ± 22.3	149.6 ± 17.1	132.4 ± 13.2
	0.10	93.3 ± 20.3	118.2 ± 23.7	80.1 ± 6.2

[a]Significant difference from 10% $p<0.02$.

[b]Significant difference from 30% $p<0.05$.

TABLE 5

Fatty Acid Composition (%) of Plasma Phospholipids from Beef Tallow-Fed Rats (mean ± SEM)

Fatty acid	Diet (%) 10 (n=9)	30 (n=10)	50 (n=11)
16:0	23.4 ± 0.8	22.0 ± 0.5	21.1 ± 0.4[a]
16:1	1.0 ± 0.1	0.9 ± 0.05	0.9 ± 0.08
18:0	21.2 ± 0.5	24.2 ± 0.3[a]	24.6 ± 0.5[a]
18:1	10.9 ± 0.2	11.4 ± 0.4	12.4 ± 0.4[a]
18:2n-6	12.2 ± 0.5	11.0 ± 0.4	12.8 ± 0.4[b]
20:3n-9	0.5 ± 0.03	1.2 ± 0.08[a]	2.2 ± 0.1[a,b]
20:3n-6	0.8 ± 0.05	1.2 ± 0.1[a]	1.7 ± 0.1[a,b]
20:4n-6	23.8 ± 0.5	20.2 ± 0.4[a]	14.6 ± 0.5[a,b]
20:5n-3	0.3 ± 0.04	0.5 ± 0.04[a]	1.3 ± 0.1[a,b]
22:5n-3	0.6 ± 0.04	0.7 ± 0.05	1.0 ± 0.09[a,b]
22:6n-3	5.3 ± 0.3	6.7 ± 0.2[a]	7.4 ± 0.3[a]

[a]Significant difference from 10% $p<0.05$.

[b]Significant difference from 30% $p<0.01$.

Increasing the level of beef tallow in the diet of rats resulted in a dose-dependent decrease in the level of arachidonic acid in plasma PL, but a dose-dependent increase in the proportion of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and ETA (20:3n-4) (Table 5). This result is similar to that obtained when butter was used as the source of dietary fat (6). The plasma PL fatty acid composition of the rats in the olive oil experiment is shown in Table 6. The 50% olive oil-fed rats had significantly higher levels of arachidonic acid than both 10% and 30% olive oil-fed rats. There were no significant differences in plasma PL levels of EPA and DHA as the level of olive oil in the diet rose. The plasma PL fatty acid composition of the rats in the peanut oil experiment is shown in Table 7. There were no significant differences in the level of arachidonic acid and EPA in plasma PL when comparing any of the groups in the peanut oil experiment. DHA levels were significantly lower in the rats fed the 50% peanut oil diet than in those fed peanut oil at the two lower levels.

TABLE 6

Fatty Acid Composition (%) of Plasma Phospholipids from Olive Oil-Fed Rats (mean ± SEM)

Fatty acid	Diet (%)		
	10 (n=5)	30 (n=5)	50 (n=4)
16:0	27.4 ± 1.2	26.0 ± 0.7	22.1 ± 0.5[a,b]
16:1	0.9 ± 0.1	0.7 ± 0.1	0.4 ± 0.03[a,b]
18:0	18.8 ± 0.7	21.3 ± 0.9	22.7 ± 0.6[a]
18:1	10.3 ± 0.4	10.7 ± 0.3	11.5 ± 0.3[a]
18:2n-6	12.0 ± 0.5	10.6 ± 0.5	9.6 ± 0.5[a]
20:3n-9	0.4 ± 0.04	0.3 ± 0.02	0.4 ± 0.03[b]
20:3n-6	0.7 ± 0.02	0.6 ± 0.06	0.6 ± 0.04
20:4n-6	24.4 ± 0.5	25.4 ± 0.6	28.0 ± 0.4[a,b]
20:5n-3	0.2 ± 0.07	0.1 ± 0.02	0.1 ± 0.01
22:5n-3	0.3 ± 0.03	0.3 ± 0.02	0.4 ± 0.05
22:6n-3	4.6 ± 0.3	4.0 ± 0.2	4.2 ± 0.2

[a]Significant difference from 10% $p<0.05$.
[b]Significant difference from 30% $p<0.05$.

TABLE 7

Fatty Acid Composition (%) of Plasma Phospholipids from Peanut Oil-Fed Rats (mean ± SEM)

Fatty acid	Diet (%)		
	10 (n=5)	30 (n=5)	50 (n=5)
16:0	25.1 ± 1.0	22.7 ± 1.4	23.6 ± 1.0
16:1	1.6 ± 0.1	1.1 ± 0.2	0.9 ± 0.2[a]
18:0	18.9 ± 0.8	21.7 ± 0.8[a]	24.5 ± 1.8[a,b]
18:1	9.0 ± 0.5	8.8 ± 0.6	8.2 ± 0.6
18:2n-6	12.5 ± 0.8	13.4 ± 0.8	13.6 ± 0.1
20:3n-9	0.1 ± 0.05	trace	0.2 ± 0.2
20:3n-6	0.5 ± 0.07	0.2 ± 0.1[a]	0.2 ± 0.1[a]
20:4n-6	28.6 ± 1.2	27.6 ± 1.1	26.5 ± 3.1
20:5n-3	—	0.2 ± 0.2	—
22:4n-6	—	0.2 ± 0.1	—
22:5n-6	0.1 ± 0.08	0.3 ± 0.2	0.3 ± 0.2
22:5n-3	0.1 ± 0.08	0.2 ± 0.1	—
22:6n-3	3.5 ± 0.3	3.6 ± 0.3	2.0 ± 0.2[a,b]

[a]Significant difference from 10% $p<0.05$.
[b]Significant difference from 30% $p<0.05$.

In view of the dose-dependent changes in plasma PL fatty acid composition with increasing levels of beef tallow in the diet, the effects of these dietary changes on aortic PL fatty acid composition were determined (Table 8). Although they were of smaller magnitude, the changes in aortic PL levels of arachidonic acid, EPA, DHA and ETA mirrored the changes seen in plasma PL: *i.e.*, decreased arachidonic acid as the level of beef tallow in the diet increased, and increased levels of EPA, DHA and ETA. Since we have shown previously (6) that changes in aortic PL fatty acid composition are similar but less pronounced than those seen in plasma PL, measurements of aortic PL fatty acid composition were not performed for the diets where no changes in plasma PL fatty acid composition were observed (*i.e.*, olive oil and peanut oil).

TABLE 8

Fatty Acid Composition (%) of Aortic Phospholipids from Beef Tallow-Fed Rats (mean ± SEM)

Fatty acid	Diet (%)		
	10 (n=9)	30 (n=10)	50 (n=11)
16:0	25.0 ± 0.7	23.7 ± 0.7	22.9 ± 0.6[a]
16:1	2.1 ± 0.1	1.8 ± 0.08[a]	2.0 ± 0.2
18:0	19.3 ± 0.3	19.5 ± 0.3	19.7 ± 0.4
18:1	16.9 ± 0.2	17.6 ± 0.2	19.4 ± 0.2[a,b]
18:2n-6	4.7 ± 0.1	5.1 ± 0.2	4.7 ± 0.1
20:3n-9	1.6 ± 0.1	1.9 ± 0.1	2.3 ± 0.1[a,b]
20:3n-6	1.7 ± 0.09	1.9 ± 0.07	1.7 ± 0.09
20:4n-6	20.6 ± 0.4	19.8 ± 0.3	18.1 ± 0.3[a]
20:5n-3	0.3 ± 0.02	0.6 ± 0.02[a]	0.9 ± 0.04[a,b]
22:4n-6	2.8 ± 0.1	2.5 ± 0.1	2.2 ± 0.08[a,b]
22:5n-6	0.7 ± 0.04	0.6 ± 0.03	0.6 ± 0.03
22:5n-3	1.1 ± 0.07	1.4 ± 0.05[a]	1.8 ± 0.08[a,b]
22:6n-3	3.2 ± 0.2	3.6 ± 0.1	3.7 ± 0.1[a]

[a]Significant difference from 10% $p<0.05$.
[b]Significant difference from 30% $p<0.05$.

DISCUSSION

Diets containing increasing levels of beef tallow were associated with dose-dependent changes in plasma and aortic PL fatty acid composition and reductions in arterial PGI_2 production, as observed previously with butter-feeding (6), and platelet TXA_2 production in response to collagen. However, these changes did not occur with olive oil- or peanut oil-feeding, similar to our previous observations with lard-feeding (6).

The most consistent predictor of these results was the P/S ratio of the diet. Only those diets with low P/S ratios (butter and beef tallow) produced changes in tissue PL fatty acid composition and prostanoid production. This suggests that the diets with the lowest P/S ratios are providing insufficient linoleic acid to maintain tissue levels of arachidonic acid. The extremely high levels of saturated and monounsaturated fatty acids combined with the very low level of PUFAs in the 50% butter and beef tallow diets would exacerbate this effect since saturated and monounsaturated fatty acids can reduce the conversion of linoleic acid to arachidonic acid (10). The observation that ETA (20:3n-9) is raised in these two diets is further support for a relative deficiency of linoleic acid accompanied by an enhanced conversion of oleic acid to ETA. However, it must be noted that while a low P/S ratio is necessary for effects on prostanoid production to be observed, the actual PUFA affected is also very important as very small amounts of preformed arachidonic acid administered orally to rats are sufficient to reverse the effects of butter-feeding without markedly altering the P/S ratio of the diet (11).

The mechanism by which the beef tallow and butter diets exert their effects on prostanoid production is probably through decreased availability of the prostanoid precursor, arachidonic acid. The dose-dependent increase in the levels of EPA in plasma and aortic PL may provide an additional or alternative explanation for the decreased prostanoid production, as this fatty acid competitively inhibits arachidonic acid metabolism by cyclooxygenase (12).

The results of the present study show that increasing the level of saturated fat in the diet of rats leads to substantial decreases in both PGI_2 and TXA_2 production but no consistent changes in platelet aggregation to collagen. This may appear to conflict with previous work regarding the effect of saturated fat on platelet aggregation. For example, Renaud and co-workers (13,14) have shown that long-term feeding of saturated fat is associated with an increase in platelet aggregation. They have postulated that this effect is mediated through an increase in ETA levels—a fatty acid shown to promote platelet aggregation *in vitro* (15). Our experiments which were only of a short-term duration showed that as the level of saturated fat increased there was a reduction in the level of arachidonic acid in aortic and plasma PL and an increase in ETA and dihomo-γ-linolenic acid (DGLA). The increase in the n-3 fatty acids is probably due to the increased proportion of n-3 PUFA relative to n-6 PUFA in the highly saturated fats. The n-6/n-3 ratio decreases from 9.4 in the 10% beef tallow diet to 3.0 in the 50% beef tallow diet (Table 2) which is likely to lead to an increased synthesis of n-3 PUFA (16). These complex changes in aortic, plasma and platelet (17) PL fatty acids could result in an overall increase in thrombosis tendency, as decreased arachidonic acid, increased EPA, DHA and DGLA are all associated with a reduction in TXA_2 production, and increased ETA is associated with an increase in platelet aggregation (15). Therefore, the interaction of these PL fatty acid changes would result in little net effect on platelet aggregation. This coupled with a reduction in PGI_2 production may shift the balance toward an increased thrombosis tendency. However, it may be necessary to conduct studies of longer duration for clear-cut effects on platelet function to become evident.

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Embryonic Fatty Acid Composition as a Function of Yolk Fatty Acid Composition in Eggs of the Lesser Spotted Dogfish (*Scyliorhinus canicula* L.)

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Yolk and embryonic total lipids were extracted from spotted dogfish eggs at two developmental stages. Total lipids were fractionated into neutral lipids (NL) and polar lipids (PL), and the fatty acid composition of each group was determined. Yolk lipid composition was found to be quantitatively different (NL/PL≅1) from embryo lipid composition (NL/PL≅0.5), for both stages of development. However, individual fatty acid composition did not differ from younger to older eggs for either yolk or embryo. There were significant differences ($p < 0.05$) in major fatty acid groups from yolk and embryonic PL for saturated fatty acids, monounsaturated fatty acids (MUFA) and n-3 polyunsaturated fatty acids (PUFA) for younger eggs, and for MUFA and n-3 PUFA for older eggs. For NL, only MUFA composition from the oldest eggs showed differences between yolk and embryo. Results are discussed in terms of embryonic needs for highly unsaturated fatty acid (HUFA) biosynthesis, as well as to provide some explanations for the unusually high levels of 20:4n-6 in both yolk and embryonic neutral lipids and polar lipids.
Lipids 25, 724-728 (1990).

The embryos of oviparous fishes are totally dependent on the nutrients stored in the yolk for successful development. Therefore, yolk is generally accepted to be ideally suitable to satisfy embryonic needs as both building blocks and fuel. With regard to lipid composition fish, like mammals, cannot desaturate beyond the Δ9 position of any acyl chain (1). Therefore, most fishes have dietary requirements for linoleic and linolenic acids and, in addition, highly unsaturated fatty acids (HUFA) must be derived from there or taken up from the diet (2) or, in the particular case of fish eggs, from yolk. Moreover, since it has been shown that marine teleosts have little ability to convert exogenous linolenate (18:3n-3) to HUFA (1), even broader dietary requirements are imposed on them (3). Recently it has been reported (4) that larvae of the gilthead sea bream showed very low desaturation and elongation activities during the yolk sac stage. Therefore, since the embryonic rates of HUFA biosynthesis would be unable to sustain rapid embryonic development, the bulk of HUFA needs during embryonic development must be stored in the yolk. In consequence, minor differences should be expected in yolk and embryo HUFA composition, unless there is a preferential use of some particular fatty acid for fueling purposes.

In the present paper, the fatty acid composition of neutral lipids (NL) and polar lipids (PL) from yolk and embryo of dogfish egg is presented. Eggs of the dogfish provide a suitable model for such studies since they exhibit a clear separation between yolk and embryo (5). Such defined developmental separation should help in avoiding the problems currently associated with whole egg analyses, which are mainly due to the simultaneous variation in yolk and embryo composition.

MATERIALS AND METHODS

Eggs. Adult spotted dogfish (*Scyliorhinus canicula* L.) were caught by trawling on the northern coast of Anglesey, North Wales, U.K., during the spring of 1989 (April-June). The fish were maintained in filtered sea water at ambient temperature. Dogfish are known to lay eggs frequently in the days following their capture (6). Tanks were inspected for eggs every morning. Eggs were collected and left hanging by the tendrils in separate tanks at 13°C, which was the temperature used in all experiments. Sixty eggs whose ages were within the narrow range of 90-104 days were distributed randomly amongst four vessels. Each vessel was kept at a different oxygen tension: 100% air saturated sea water; 50% and 20% air saturated sea water; and a regime alternating 100% air saturation for 22 hr with 0% air saturation for 2 hr per day. At two-week intervals, three eggs were taken from each vessel and analyzed for carbohydrate, lipid and protein contents (7). The different oxygen tensions were employed in an attempt to exacerbate metabolic expenditure of fuel reserves. The various oxygen tension regimes were maintained for 10 weeks; after which three eggs from each vessel were analyzed for fatty acid composition, and the results compared with those obtained at the beginning of the experiment. The starting age of the eggs (90-104 days) corresponded to inconspicuous yolk consumption, while by the end of the experiment (160-174-days-old) active yolk consumption was taking place (7,8).

Analytical methods. Total lipid was extracted by the method of Folch *et al.* (9). Briefly, samples for lipid analysis were placed in chloroform/methanol (2:1,v/v) containing 0.01% (w/v) of butylated hydroxytoluene to prevent oxidation of lipids. Samples in chloroform/methanol were thoroughly homogenized and the suspension was filtered through Whatman No. 4 filter paper, prewashed in chloroform/methanol, and the filtrate collected. The chloroform phase was dried under nitrogen and taken up in a small volume of chloroform.

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Abbreviations: FAME, fatty acid methyl esters; HUFA, highly unsaturated fatty acids; MUFA, monounsaturated fatty acids; NL, neutral lipids; PL, polyunsaturated acids; PUFA, polyunsaturated fatty acids; TLC, thin-layer chromatography.

NL and PL were separated by column chromatography on silica gel (Merck, Darmstadt, Federal Republic of Germany; 0.063 0.200 mm); NL were eluted with chloroform and PL with methanol. The fractions were dried under nitrogen and relative proportions determined gravimetrically. NL were separated by thin-layer chromatography (TLC) on Merck silica gel 60 plates using petroleum ether (40-60°C)/diethyl ether/acetic acid (85:15:1, by vol) for 90 min at room temperature (10). Spots were made visible by brief exposure to iodine vapor.

Fatty acid methyl esters (FAME) were prepared by the method of Morrison and Smith (11), and separated according to Holland *et al.* (10). FAME were analyzed by capillary gas-liquid chromatography using a Carlo Erba Vega 6180 instrument (Carlo Erba, Milano, Italy), fitted with a 30 m 0.32 mm I.D. Wax 30W column (Durabond; J & W Scientific, Folsom, CA). FAME were identified using standards (Sigma Chemical Co., St. Louis, MO) and graphical techniques. In addition, samples were hydrogenated to verify carbon chain length. All statistical comparisons were made by Student's *t*-test (12).

RESULTS

Yolk and embryo total lipids and fatty acid composition were measured at two developmental stages. The starting ages corresponded to the time at which the embryo was easily separable from yolk, and yolk consumption inconspicuous. The experiment was terminated before yolk exhaustion. Appreciable yolk consumption was shown by the decrease in dry weight, protein, lipid and carbohydrate contents (7).

Fatty acid composition for yolk and embryonic NL and PL are given in Tables 1 and 2, respectively. There were no statistically significant changes in either NL or PL (for both yolk and embryo) between younger and older eggs in fatty acid composition when comparison was made for each individual fatty acid.

Comparison of individual fatty acids between yolk and embryonic NL composition demonstrated significantly ($p < 0.05$) lower values for embryonic monounsaturated fatty acids (MUFA), especially 16:1n-7, 18:1n-7 and 18:1n-9, regardless of egg age. The opposite trend was found for 22:6n-3 fatty acid content. However, when fatty acids were grouped according to their degree of unsaturation, no significant differences were found in the total content between yolk or embryonic saturates, n-6 and n-3 PUFA; however, differences persisted for MUFA from older eggs (Fig. 1).

When fatty acid composition from yolk and embryonic PL were analyzed, significant differences ($p < 0.05$) were found for saturated fatty acids, MUFA and n-3 PUFA for the youngest eggs, and MUFA and n-3 PUFA for the oldest eggs. Higher values for embryonic 16:1n-7, 18:1n-7, and 18:1n-9 fatty acids were found, regardless of the age of the eggs, but the opposite trend for 22:6n-3, with lower embryonic values (Fig. 1; Tables 1 and 2).

TABLE 1

Fatty Acid Composition of Yolk Neutral Lipid and Polar Lipid

	Neutral lipid		Polar lipid	
	Younger eggs	Older eggs	Younger eggs	Older eggs
13:0	—	0.3 ± 0.1	—	—
14:0	0.9 ± 0.1	1.0 ± 0.3	0.2 ± 0.1	0.2 ± 0.1
15:0	0.4 ± 0.1	0.4 ± 0.1	0.1 ± 0.1	0.2 ± 0.1
16:0	16.6 ± 0.6	16.8 ± 1.8	20.0 ± 0.7	22.1 ± 1.0
17:0	0.6 ± 0.1	0.7 ± 0.1	0.6 ± 0.1	0.8 ± 0.1
18:0	3.1 ± 0.1	3.6 ± 0.8	4.8 ± 0.7	5.6 ± 0.9
19:0	—	—	0.2 ± 0.1	0.2 ± 0.1
20:0	0.1 ± 0.1	0.2 ± 0.1	0.1 ± 0.0	0.1 ± 0.0
16:1n-7	7.7 ± 0.6	6.8 ± 2.6	2.0 ± 0.4	2.3 ± 0.3
18:1n-9	15.1 ± 0.9	11.4 ± 3.6	6.4 ± 0.5	5.4 ± 0.8
18:1n-7	9.3 ± 0.3	9.1 ± 0.6	4.9 ± 0.1	5.4 ± 0.5
20:1[a]	3.4 ± 0.3	2.4 ± 0.4	1.1 ± 0.2	0.9 ± 0.2
22:1n-11	0.2 ± 0.1	0.2 ± 0.0	—	—
18:2n-6	0.7 ± 0.1	0.7 ± 0.1	0.5 ± 0.1	0.5 ± 0.1
20:2n-6	0.8 ± 0.1	0.6 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
20:3n-6	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.0
20:4n-6	2.3 ± 0.4	3.6 ± 1.7	8.7 ± 0.4	9.3 ± 0.4
22:3n-6	0.5 ± 0.1	0.5 ± 0.1	0.3 ± 0.0	0.3 ± 0.1
22:4n-6	2.0 ± 0.4	2.5 ± 0.4	1.3 ± 0.1	1.3 ± 0.1
18:3n-3	0.3 ± 0.1	0.3 ± 0.1	0.1 ± 0.1	0.2 ± 0.0
18:4n-3	0.4 ± 0.1	0.3 ± 0.0	—	0.1 ± 0.0
20:5n-3	10.5 ± 0.5	10.0 ± 0.4	8.5 ± 0.3	8.9 ± 0.5
22:3n-3	0.8 ± 0.2	1.1 ± 0.2	0.8 ± 0.1	1.0 ± 0.1
22:5n-3	7.2 ± 0.5	5.8 ± 0.4	4.1 ± 0.5	3.2 ± 0.4
22:6n-3	16.3 ± 1.7	20.7 ± 5.2	34.7 ± 1.4	31.7 ± 0.3

Composition as percentage of weight. Values are means of three yolk analyses. Mean ± SD.

[a]Predominantly 20:1n-11 and 20:1n-9.

TABLE 2

Fatty Acid Composition of Embryonic Neutral Lipid and Polar Lipid

	Neutral lipid		Polar lipid	
	Younger eggs	Older eggs	Younger eggs	Older eggs
13:0	—	—	—	—
14:0	0.4 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	0.3 ± 0.1
15:0	0.4 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.2 ± 0.1
16:0	17.8 ± 1.3	16.7 ± 0.2	23.6 ± 1.1	21.3 ± 0.8
17:0	0.6 ± 0.1	0.6 ± 0.1	0.5 ± 0.1	0.6 ± 0.1
18:0	3.4 ± 1.1	3.4 ± 0.4	9.2 ± 0.7	6.8 ± 0.7
19:0	1.2 ± 0.1	0.2 ± 0.1	—	0.2 ± 0.1
20:0	0.4 ± 0.1	0.1 ± 0.1	3.2 ± 1.4	—
16:1n-7	4.7 ± 0.3	4.9 ± 0.5	4.2 ± 0.5	3.3 ± 0.3
18:1n-9	11.7 ± 0.7	8.9 ± 1.2	12.5 ± 2.4	7.1 ± 0.4
18:1n-7	6.3 ± 0.3	7.5 ± 0.9	6.1 ± 0.3	7.2 ± 0.7
20:1[a]	1.9 ± 0.1	2.1 ± 0.3	1.2 ± 0.2	1.8 ± 1.0
22:1n-11	—	0.3 ± 0.0	—	0.5 ± 0.3
18:2n-6	0.9 ± 0.1	0.6 ± 0.2	1.7 ± 0.8	0.5 ± 0.1
20:2n-6	1.0 ± 0.5	2.7 ± 3.0	—	1.3 ± 0.4
20:3n-6	—	0.2 ± 0.2	—	0.2 ± 0.1
20:4n-6	5.4 ± 1.4	5.8 ± 0.6	7.5 ± 0.2	9.2 ± 0.4
22:3n-6	—	0.4 ± 0.1	—	0.2 ± 0.0
22:4n-6	2.5 ± 0.2	2.2 ± 0.3	1.1 ± 0.1	1.5 ± 0.1
18:3n-3	—	0.2 ± 0.1	0.2 ± 0.1	—
18:4n-3	0.6 ± 0.4	0.2 ± 0.1	—	—
20:5n-3	10.1 ± 0.3	9.0 ± 0.1	5.6 ± 1.7	6.1 ± 0.4
22:3n-3	—	1.2 ± 0.2	0.5 ± 0.0	0.7 ± 0.2
22:5n-3	6.2 ± 0.4	5.4 ± 0.4	3.3 ± 0.3	2.9 ± 0.8
22:6n-3	23.5 ± 1.4	25.8 ± 2.6	18.1 ± 3.0	27.8 ± 1.1

Composition as percentage of weight. Results are means of three embryo analyses. Mean ± SD.

[a]Predominantly 22:1n-11 and 22:1n-9.

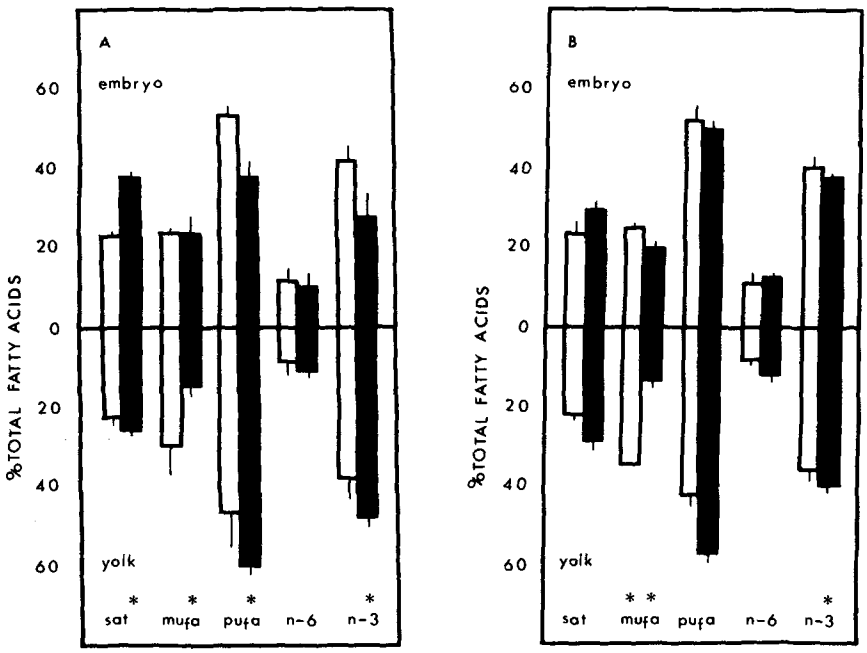

FIG. 1. Major fatty acid groups of neutral lipids (open frame) and polar lipids (shadowed frame) from embryo and yolk of dogfish eggs 90–104-days-old (A) and 160–174-days-old (B). Paired comparisons for yolk and embryonic fatty acid composition were made by the Student's *t*-test. *Denotes significant differences ($p < 0.05$) between yolk and embryo.

TABLE 3

Yolk and Embryo Total Lipid Composition

Age (days)	Yolk		Embryo	
90-104	45 ± 9	55 ± 9	28 ± 7	72 ± 7
160-174	52 ± 2NS	48 ± 2NS	39 ± 3NS	61 ± 3NS

Composition as percentage of total lipids. Results are the mean of three egg analyses. Mean ± SD. NS, nonsignificant ($p < 0.05$) difference between data for younger and older eggs.

Total lipid composition for yolk and embryo for the two developmental stages assayed are shown in Table 3. Neutral lipid and polar lipid concentrations for yolk and embryo did not differ significantly between younger and older eggs. However, yolk NL content was found to be significantly higher ($p < 0.05$) than embryo NL content at both sampling times. These results show that most of the embryonic lipids are devoted to membrane constituents, the role commonly assumed to be played by PL. TLC analysis of both yolk and embryo NL showed triglycerides as the main constituents, followed by minor amounts of free cholesterol and cholesterol esters. No free fatty acids were detected in yolk or embryo NL (results not shown). The fatty acid compositions of eggs held under reduced oxygen tension (results not shown) did not differ statistically from those of control eggs.

DISCUSSION

Fatty acid analyses showed that linoleic and linolenic acids accounted for about 1% of yolk and embryonic total fatty acids, while the sum of all fatty acids of the linoleic and linolenic families is close to 10% and 40%, respectively, for both yolk and embryonic total fatty acid composition. Apparently, the bulk of embryonic HUFA needs are already deposited in the yolk. Therefore, there is no apparent requirement for embryonic elongation and saturation of linoleic and linolenic acids before hatching, since the embryonic needs for HUFA can be satisfactorily met from yolk reserves. This is in agreement with the reported very low desaturation and elongation activities in eggs and larvae of other species (4). Indeed, this seems to be the general pattern, since fatty acid compositions reported to date indicate identical strategies of very low amounts of precursors deposited in yolk (4,13-15).

The fatty acid composition of the polar lipid fractions from dogfish eggs as recorded by the present study is generally similar to that reported for several species, except for minor differences in specific fatty acids, which may possibly be due to differences in parental diet (4,13-15). Moreover, when fatty acids are grouped according to their degree of unsaturation, a more common general pattern appears. However, the unusually high content of 20:4n-6 in both yolk and embryo PL of dogfish differs markedly from the norm. The fatty acid composition of neutral lipid fractions showed wide differences when compared to they fatty acid composition of NL for other species, approximately to the same degree as there are differences between the same species themselves (4,13-15). The proportion of 20:4n-6 is higher in dogfish egg NL than reported for other fish lipids of this type.

The reason for such unusually high levels of 20:4n-6 in both NL and PL fractions of yolk and embryo could be related to parental diet. Adult dogfish feed opportunistically on a wide range of macrobenthic invertebrates, mainly crustacea and mollusca (16), whose lipids can contain relatively high proportions of 20:4n-6 (17). Thus adult dogfish will inevitably have a significant input of 20:4n-6 in their diets that could be carried over to the eggs. The presence of very low amounts of the 20:n-6 precursor acids, 18:2n-6, 18:3n-6 and 20:3n-6, would argue in favor of the dietary origin as opposed to an endogenous biosynthetic origin.

However, the 20:4n-6 content in a related species—the spiny dogfish (18)—does not support this view. Therefore, the high content of 20:4n-6 could be related to other causes, such as the development of the nervous system and prominent external gills. Both of these tissues have been shown to contain relatively high concentrations of 20:4n-6 acid (19,20). Regardless, it would seem that apart from being specifically important for the function of the rectal gland of the adult dogfish (20), 20:4n-6 acid might be important for embryonic development.

The total lipids of the yolk of the dogfish eggs appear to consist of almost 50% of NL and PL. However, polar lipids are clearly predominant in dogfish embryos. Therefore, a certain degree of transformation of yolk lipid components appears to occur in which yolk lipid constituents are chanelled into embryonic PL, the excess being either derived into NL constituents or used as fuel. Conclusive evidence cannot be drawn because most consumption of yolk lipid during the time considered does not result in embryonic growth, but provides fuel for metabolic processes (7).

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Myocardial Changes in Newborn Piglets Fed Sow Milk or Milk Replacer Diets Containing Different Levels of Erucic Acid

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This study was undertaken to determine whether the neonate was more susceptible to the effects of dietary erucic acid (22:1n-9) than the adult. Newborn piglets were used to assess the safety of different levels of 22:1n-9 on lipid and histological changes in the heart. Newborn piglets showed no myocardial lipidosis as assessed by oil red 0 staining, but lipidosis appeared with consumption of sow milk and disappeared by seven days of age. Milk replacer diets containing soybean oil, or rapeseed oil mixtures with up to 5% 22:1n-9 in the oil, or 1.25% in the diet, gave trace myocardial lipidosis. Rapeseed oil mixtures with 7 to 42.9% 22:1n-9 showed definite myocardial lipidosis in newborn piglets, which correlated to dietary 22:1n-9, showing a maximum after one week on diet. The severity of the lipidosis was greater than observed previously with weaned pigs. There were no significant differences among diets in cardiac lipid classes except for triacylglycerol (TAG), which increased in piglets fed a rapeseed oil with 42.9% 22:1n-9. TAG showed the highest incorporation of 22:1n-9, the concentration of 22:1n-9 in TAG was similar to that present in the dietary oil. Among the cardiac phospholipids, sphingomyelin and phosphatidylserine had the highest, and diphosphatidylglycerol (DPG) the lowest level of 22:1n-9. The low content of 22:1n-9 in DPG of newborn piglets is not observed in weaned pigs and rats fed high erucic acid rapeseed oil. The relative concentration of saturated fatty acids was lowered in all cardiac phospholipids of piglets fed rapeseed oils, possibly due to the low content of saturated fatty acids in rapeseed oils. The results suggest that piglets fed up to 750 mg 22:1n-9/kg body weight/day showed no adverse nutritional or cardiac effects.
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The effect of dietary erucic acid (22:1n-9) has previously been studied extensively in rats, and to a lesser extent in pigs, monkeys and dogs, to assess its safety for humans (1–4). In rats, lower growth rate and digestibility, myocardial lipidosis and necrosis, reduced rates of fatty acid oxidation, and accumulation of 22:1n-9 in tissue lipids were observed which were attributed to 22:1n-9 (1–4). Based on the results with rats, the no effect level of 22:1n-9 was determined to be 5% in the oil, or 1% of the diet (4). A limit of 5% (4) or 2% (5) 22:1n-9 in the fat portion of food products for man was therefore established as safe in many Western countries.

In the U.S., inclusion of oils containing no more than 2% 22:1n-9 was permitted for use in the adult population but not for use in infant formulas (5), because of concern that infants would be exposed to greater amounts of 22:1n-9 from the same canola oil, and might be more susceptible to the effects of 22:1n-9 than an adult. In all of the previously published reports (1–4), weaned or older animals were fed diets containing different levels of 22:1n-9 for various periods of time. There are no reports on evaluating the effect of feeding diets containing 22:1n-9 in newborns from birth to weaning.

This study was therefore undertaken to provide data on this question. The early time period after birth is of particular interest because of the known change in energy utilization from mainly carbohydrates in the fetal state to mainly fatty acids after birth (6,7). This raised the possibility that 22:1n-9 may affect the heart of the newborn more severely with commencement of β-oxidation than an older animal, which has a fully functional β-oxidation system. The pig was chosen as the experimental animal because there are many anatomical, physiological, nutritional and biochemical similarities between pigs and human (8,9). In particular, the coronary vascular distribution and the response to atherosclerosis are similar (9).

In this report, results are presented for newborn piglets fed milk replacer diets containing up to 42.9% 22:1n-9 from birth to two weeks of age. The growth, and histological and lipid changes in the hearts of piglets are compared with sow reared piglets. Furthermore, the results are discussed in relation to those observed previously in older weaned pigs (10,11) and rats (12).

MATERIALS AND METHODS

Animals. Newborn Yorkshire male and female piglets used were raised at the Animal Research Center specific pathogen free facility. Litters were from first or second parities, and gestations were allowed to proceed naturally to birth (average 114 days). Piglets were weighed and ear-notched to identify litter and position within the litter. Males were not castrated. Sows were fed a standard gestation/lactation ration. Colostrum and milk samples were taken for fatty acid analysis (Table 1).

Housing and rearing. All piglets were left with the sow for one day to permit maximum consumption of colostrum. They were then taken into a separate room and housed individually in banks of six stainless steel cages fitted with eating bowls, wire mesh flooring and feces collection pans, described previously (13). Piglets on the same diet received feed from a common reservoir. Animals were initially fasted for 8 hr and then fed every 2 hr at the rate of solid intake equivalent to 7% of body weight per day. Piglets were weighed every three days and the feeding level adjusted. Water was available *ad libitum.* A 24 hr light regime was used and the room temperature was set at 31–33°C for the first week and then reduced to 28°C. Daily feed intake, feed refusal, feces texture and general health of piglets were recorded. The apparent

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Abbreviations: C, cholesterol; DPG, diphosphatidylglycerol; EFA, essential fatty acids; HEAR, high erucic acid rapeseed; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PUFA, polyunsaturated fatty acids; PS, phosphatidylserine; SP, sphingomyelin; TAG, triacylglycerol.

TABLE 1

Fatty Acid Composition of Sow's Colostrum and Milk and Dietary Oils

Fatty acids	Sow colostrum[a]	Sow milk[a]	Soybean oil	Canola oil	HEAR oil
			(area %)		
≤ 14:0	1.6	2.1	0.1	0.1	0.1
16:0	22.7	23.3	9.9	5.2	3.2
16:1	4.1	4.6	0.2	0.3	0.2
18:0	5.7	6.1	3.6	2.2	1.2
18:1n-9	32.8	32.4	23.8	51.6	19.0
18:1n-7	1.9	2.8	1.5	2.6	0.8
18:2n-6	25.0	22.9	50.0	22.8	14.3
18:3n-3	1.3	1.1	8.7	10.0	8.4
20:0	0.2	0.2	0.4	0.8	0.8
20:1n-9	0.5	0.6	0.4	2.1	6.3
22:0	0.1	0.1	0.4	0.5	0.7
22:1n-9	0.1	0.1	0	0.8	42.9
24:0	0.2	0.1	0.1	0.2	0.3
24:1	0.1	0.1	0	0.3	1.1
n-6 PUFA[b]	1.6	1.7	0	0	0
n-3 PUFA[c]	0.7	0.7	0	0	0

[a] Colostrum and milk were each collected from three sows, and the values are averaged. Percent lipid on wet weight basis: colostrum 4.6 ± 0.6% (X ± SD) and milk 15.4 ± 8.4%; and on dry weight basis: colostrum 18.8 ± 4.2% and milk 59.1 ± 11.7%.

[b] The n-6 PUFA (polyunsaturated fatty acids) include 20:3n-6, 20:4n-6, 22:4n-6 and 22:5n-6.

[c] The n-3 PUFA include 20:5n-3, 22:5n-3 and 22:6n-3.

digestibilities of dietary fats were determined from total fecal collections carried out on individual piglets over 24 hr periods (14).

Dietary oils. Canola oil (cv. Westar) and high erucic acid rapeseed (HEAR) oil (cv. S80514) were obtained from CSP Foods Ltd. (Altona, Manitoba). The oils were fully refined, bleached and deodorized, and contained 0.04% antioxidant (G50C; Griffith Laboratories Ltd., Scarborough, Ontario). Dietary oils with 2.3, 4.7, 7.0, 11.7 and 20.7% 22:1n-9 were prepared by mixing appropriate amounts of canola and HEAR oils. The fatty acid composition of the oils is shown in Table 1.

Diets. The composition of the milk replacer (21% solids) was as described previously (15), except for the addition of citric (440 ppm) and folic acids (28 ppm). Oil was incorporated at 25% (or about 50% of calories) and immunoglobulin at 16% by weight of the diet on a dry matter basis. The immunoglobulin preparation (94.7% solids, 6.0% ash) contained 48.8% protein, of which 28.0% was pure γ-globulin (16). The digestible energy content of the diet was 4539 kcal/kg of dry milk replacer (15) or 95.3 kcal/dL of final milk replacer. Formalin (40% solution) was added at 0.1% as a preservative.

Experimental design. In Experiment I, piglets from five litters were sampled at birth from sows farrowed on a raised platform to prevent suckling. An average weight male and female was killed at birth from each litter for histological analyses; the remainder of the piglets were returned to the sow. After 24 hr, the piglets were weighed and histological analyses were done on the heaviest and the lightest male and female piglet from each litter.

In Experiment II, six litters were selected which had at least three males and four females or four males and three females. Two litters were randomly allotted to each of three diets (sow milk, canola oil and HEAR oil) at one day of age, to permit among-litter comparisons on a within-litter basis, since the primary objective was to determine when maximum myocardial lipidosis occurred. Male and female piglets within a litter were killed alternately at the time periods indicated in Table 3. For Experiments III, IV and V, piglets from six, four and three litters, respectively, were used. Piglets were placed on experimental diets balanced for both sex, time and diet to permit within-litter comparisons.

Pathology. Within 2 hr of removal from the diet, each piglet was anesthetized with a 5:1 mixture of ketamine hydrochloride (Rogarsetic-Rogar/STB, Montreal, Quebec) and acepromazine maleate (Atravet-Ayerst Lab., Montreal, Quebec), and killed by exsanguination. The heart was removed immediately and bisected in a vertical plane from apex to base, passing through the middle of the right and left ventricle. One half was fixed in formalin, and the other half was used for lipid analyses.

Following 36-48 hr of fixation, the heart was divided into right and left ventricle and interventricular septum. Each area was then sliced longitudinally into two halves. One half was routinely processed, embedded in paraffin and 6 μm sections were stained with hematoxylin and eosin. The second half was frozen by immersion in liquid nitrogen and 8–10 μm sections were made on a cryostat (Reichert Histostat, Scientific Instruments, Buffalo, NY). These sections were stained with oil red 0 and examined histologically for the presence of myocardial lipid.

A quantitative measurement of the amount of myocardial lipidosis was made by examining the stained frozen sections at a 200-fold magnification. Approximately 40 consecutive fields were examined and the area of lipidosis was estimated expressed in percent. The average percentage of the 40 fields was then given a numerical score from 0–7 as follows: 0, 0%; 1, >0 to 1%; 2, >1 to 5%; 3, >5 to 10%; 4, >10 to 25%; 5, >25 to 50%; 6, >50 to 75%; and 7, >75 to 100%. All tissues were examined without knowledge of diet treatment associations.

Lipid analyses. Half the heart used for lipid analyses was frozen immediately between blocks of dry ice. The frozen section was weighed, pulverized at dry ice temperature and the total lipids extracted with chloroform/methanol (2:1) (17). The cardiac lipid classes were quantitated using the Iatroscan method (18). Only the lipid classes of the left ventricle were isolated by 3-directional thin-layer chromatography (TLC) (19) using Silica Gel H plates (Analtech Inc., Newark, DE), converted to their fatty acid methyl esters (17) and analyzed by GLC using fused silica capillary columns (20).

Statistical analyses. All the data was analyzed by analyses of variances. For the growth data, initial weight at the beginning of the experiment was used as a covariate. In Experiment II, diets were confounded with litters leaving only three degrees of freedom to test for diet effects. Whenever the litter effects were not significant ($P>0.05$), this term was pooled with the among-piglet within-litter term to provide a more powerful test of the diet effect. Correlations were computed for cardiac TAG and the average lipidosis score in Experiment II, and for dietary level of 22:1n-9 and the average lipidosis score for piglets killed at 7, 10 or 13 days of age in Experiments III, IV and V.

TABLE 2

Intake of 22:1n-9 by Piglets Fed Milk Replacer Containing Different Levels of 22:1n-9[a]

% 22:1n-9 in dietary oil	Age of piglet (days)[b]					SEM[c]
	1-4	4-7	7-10	10-13	13-16	
	(mg 22:1n-9/kg body weight/day)					
0.8%	129(9)[d]	154(7)	140(5)	136(3)	108(2)	13
2.3%	264(12)	351(12)	336(8)	350(4)	—	20
4.7%	620(12)	753(12)	747(6)	—	—	29
7.0%	890(6)	1114(6)	1196(4)	1219(2)	—	71
11.7%	1720(12)	1733(12)	1845(8)	1702(4)	—	111
20.7%	2838(6)	2965(6)	2930(4)	3360(2)	—	135
42.9%	4705(10)	4222(8)	5138(6)	4916(3)	6495(2)	529

[a]Calculations are based on measured milk replacer consumed by each piglet, composition of diet (21% solids, 25% oil in diet per dry matter), and average body weight of piglet between the two age periods. Final milk replacer provided 95.3 kcal/dL.

[b]Piglets were one day of age when placed on experiment.

[c]SEM is that of the 1-4 age group.

[d]Number of piglets/diet/time.

RESULTS AND DISCUSSION

Growth and feed consumption. The results show that piglets can be successfully reared with milk replacers, provided proper facilities and diet preparations are employed. No diarrhea, the common cause of death in artificially reared piglets (21), was observed in these studies. Lack of immunoglobulins (21) or incorrect dispersion of fat/oil in the milk replacer (22) can lead to poor growth. The fat globule size distribution of the present milk replacer was 0-1 μm 35%; 1-2 μm, 34%; 2-3 μm, 23%; 3-4 μm, 5%; and 4-6 μm, 4%; which is very similar to the distribution found in sow milk (23).

Piglets fed the milk replacer diets grew as well as piglets left with the sow (Fig. 1). There were no significant diet effects ($P>0.05$) on body weight in any of the experiments, partly because of the experimental design since piglets were fed at 7% body weight. However, all the diets were completely consumed without measurable spillage, including the HEAR oil diet, which resulted in similar growth in all piglets. Based on measured consumption of milk replacer (average consumption in Fig. 1), the amount of 22:1n-9 consumed per kg body weight per day by the piglets fed the different rapeseed oil containing diets was determined (Table 2).

There were no significant differences ($P>0.05$) in the apparent digestibilities of the different dietary oils which contained up to 42.9% 22:1n-9. At 15 days on the milk replacers, the digestibility of the oils was 95%, and of 22:1n-9 in HEAR oil was 89%. The apparent digestibility of similar HEAR oils was also found to be high in humans (99%), but lower in older pigs (80%) and young Sprague-Dawley rats (77%) (3,4). In the young rat, the apparent digestibility of 22:1n-9 was only 50% (3,4). This would indicate that the piglet is a more appropriate model than the rat for evaluating milk replacers for humans.

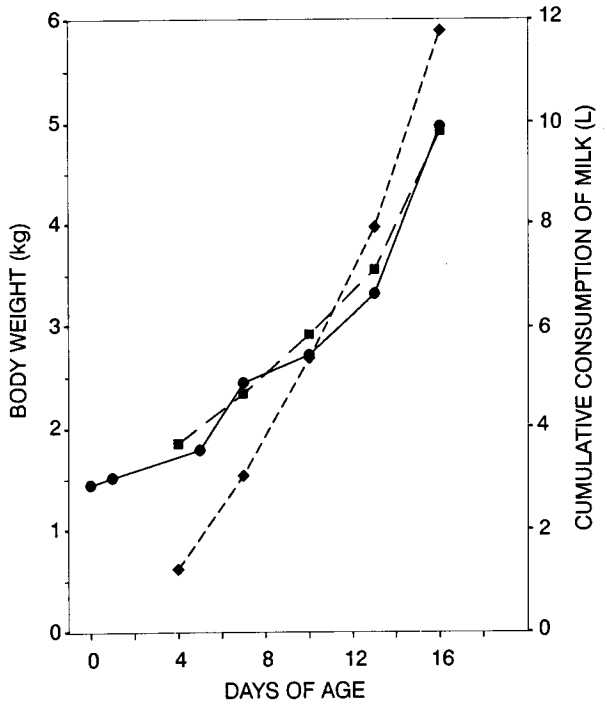

FIG. 1. Body weights of piglets left with the sow (●——●) (Pooled SEM=0.14) and those fed milk replacer containing rapeseed oil with different levels of erucic acid (22:1n-9) (■——■) (Pooled SEM=0.29). Cumulative milk consumption of piglets (◆———◆) fed milk replacer diets (Pooled SEM=0.18). Values for artificially reared piglets are an average of all rapeseed oil containing diets.

Histological Changes

Myocardial lipidosis in nursing piglets. No piglets at birth showed myocardial lipidosis prior to nursing (Table 3, Experiment I). After one day of nursing, 80% of the piglets examined showed myocardial lipidosis with a range in scores from 0 to 2.4. The lipid was unevenly distributed, occurring mainly subjacent to the epicardium and endocardium, and within the papillary muscles; none appeared in the auricles. There was no significant ($P>0.05$) sex or weight effect, but a significant ($P<0.01$) litter effect. In addition, five out of six piglets in Experiment II and all three piglets in Experiment IV taken from the sow

TABLE 3

Myocardial Lipidosis in Piglets Nursing the Sow or Fed Milk Replacer Containing Oils with Different Levels of Erucic Acid

Experiment[a]	Diet[b] (%22:1n-9)	n[c]	Age of piglet[d]								Significance[e]			
			0	1	3	5	7	10	13	16	D	L	A	S
			Incidence/(Severity)[f]											
I	Newborn	10	0											
	Sow milk	20		16 (1.0)								**	***	NS
II	Sow milk	2		2 (1.4)	2 (0.4)	1 (1.0)	0	0	0	0	**	NS	NS	NS
	Canola oil (0.8%)	2		1 (0.4)	2 (0.6)	2 (0.3)	0	1[g] (0.1)	0	0				
	HEAR oil (42.9%)	2		2 (1.0)	1 (0.5)	2 (3.2)	2 (4.4)	1 (0.9)	2 (0.9)	2 (3.5)				
III	Soybean oil	6					5 (0.7)	5 (0.6)			NS	NS	NS	NS
	RSO mixture (4.7%)	6					3 (0.4)	3 (0.5)						
IV	Sow milk	3		3 (1.2)										
	RSO mixture (7.0%)	2					1 (1.3)	2 (1.5)	1 (0.4)		*	NS	NS	NS
	RSO mixture (11.7%)	2					1 (0.7)	2 (1.7)	2 (0.8)					
	RSO mixture (20.7%)	2					2 (2.9)	2 (3.7)	2 (1.0)					
V	RSO mixture (2.3%)	4					4 (0.9)	0	3 (0.6)		*	NS	NS	NS
	RSO mixture (11.7%)	2					2 (2.7)	2 (1.3)	0					

[a] For protocol of experiments see Methods, Experimental design.

[b] HEAR, high erucic acid rapeseed oil; RSO, rapeseed oil. The RSO mixture was prepared by mixing HEAR oil and canola oil.

[c] n, Number of piglets/diet/age.

[d] All piglets nursed for one day. Age 0 were piglets killed after birth and did not nurse.

[e] Effect of diet (D), sex (S), litter (L) and age (A); NS, not significant; *P<0.05; **P<0.01; ***P<0.001; —, not applicable all piglets on sow milk.

[f] Incidence, number of piglets affected. Severity is based on percent of tissue area stained with oil red 0; for scoring system see Methods, Pathology.

[g] One piglet died. Cause of death, rupture of the colon.

at one day of age showed myocardial lipidosis. Based on the results of Experiment II, myocardial lipidosis disappeared by seven days of age in piglets left with the sow.

Myocardial lipidosis in newborn nursing piglets has not been reported previously. In a recent review, Barer (24) reported unpublished data from his laboratory which showed myocardial lipidosis in newborn rats during the first and last week of nursing but absent during mid-weaning and at 25 days of age. The occurrence of myocardial lipidosis in piglets during the first week after birth appears to be related to the low capacity of the fetus to oxidize fatty acids (7) which increased rapidly after birth (6). Extensive β-oxidation was demonstrated at two days (25) and four weeks (26) of age using perfused pig hearts. The results suggest that the piglet adapts to sow milk high in fat by the end of the first week, and any lipidosis beyond the first week is probably diet related.

Diet related myocardial lipidosis. The feeding of HEAR oil, with 42.9% 22:1n-9, resulted in a high incidence and severity of myocardial lipidosis at the time when this lesion disappeared in nursing piglets (Table 3, Experiment II). The nature of the lesion caused by 22:1n-9 was the same as that observed in one-day-old piglets left with the sow. There was a significant correlation of the myocardial lipidosis scores (Table 3) to the content of 22:1n-9 in the diet after 6 ($P<0.01$), 9 ($P<0.05$) and 12 ($P<0.05$) days on diet. Maximum myocardial lipidosis in piglets fed rapeseed oils with 7-42.9% 22:1n-9 was difficult to assess because of the variability between piglets, but it appeared to occur at four to nine days on diet. This variability in myocardial lipidosis with an apparent maximum after one week on diet was also observed when three-week-old pigs (11) were fed HEAR oils. Weaned four- to nine-week-old pigs showed no myocardial lipidosis specific to 22:1n-9 (4,27-29). The apparent reoccurrence of myocardial lipidosis at 16 days of age in piglets fed HEAR oil (Table 3, Experiment II) may be due to hormonal development during this period, as suggested by Barer (25).

The piglets consuming diets with ≤4.7% 22:1n-9 in the oil portion of the milk replacer showed myocardial lipidosis which was not significantly different ($P>0.05$) from those fed soybean oil (Table 3). These results suggest that the no effect level of 22:1n-9 in the newborn piglet was <5% 22:1n-9 in the oil fed at 25% dry matter,

TABLE 4

Cardiac Lipid Class Composition of Piglets

Lipid class[a]	0 day	Diet			LSD[b] (P<0.05)	Sigificance	
		Sow milk	Canola oil	HEAR oil		Piglet[c]	Diet
		(mg/g wet weight heart)[d]				(Significance)[e]	
CE	0.4[f]	0.2[g]	0.4[g]	0.4[g]	0.13	**	*
TAG	0.5	0.3	0.5	1.1	0.63	***	*
FFA	0.02	0.03	0.03	0.04	0.02	NS	NS
C	1.1	1.3	1.3	1.3	0.38	***	NS
DPG	1.2	1.4	1.2	1.3	0.13	**	*
PE	2.7	2.9	2.7	2.8	0.53	***	NS
PI	0.7	1.0	0.7	0.7	0.13	*	**
PS	0.4	0.3	0.4	0.4	0.13	**	NS
PC	6.0	6.7	6.2	6.6	0.73	**	NS
SP	0.6	0.7	0.7	0.7	0.15	***	NS
LPC	0.2	0.2	0.2	0.2	0.07	***	NS

[a]For abbreviation of lipid classes see Footnote to Title page.

[b]LSD, least significant difference of the mean for corresponding diets. Data from day 0 were excluded from analysis comparing diets.

[c]Assessment of piglet within litter variation.

[d]Average heart weights: 9.6 g (birth), 11.5 g (1 day), 13.7 g (4 days), 15.3 g (7 days), 17.1 g (10 days), 19.3 g (13 days) and 24.1 g (16 days).

[e]NS, not significant; *P<0.05; **P<0.01; ***P<0.001.

[f]Data represent the mean of six piglets (three males, three females). The data were combined because there were no significant sex effects.

[g]Data represent the means of 12 piglets (six males, six females) killed after 2, 4, 6, 9, 12 and 15 days on diet. Data were combined because there were no significant age or sex effects and no significant interactions involving diet (P>0.05).

or at 1.25% of total dry matter content. This corresponds to a consumption of 620 to 750 mg 22:1n-9/kg body weight/day for the newborn piglets (Table 2). By comparison, weaned pigs (four- to 6-week-old) could consume up to 2800 mg 22:1n-9/kg body weight/day without showing evidence of myocardial lipidosis (28). These results indicate that the newborn piglet was more affected by dietary 22:1n-9 than a weaned pig. The ability of the weaned pig to metabolize 22:1n-9 was confirmed by a study which showed that 22:1n-9 and 16:0 were equally well oxidized in perfused hearts from four-week-old pigs (26). On the other hand, weaned rats showed myocardial lipidosis when consuming more than 1400 mg 22:1n-9/kg body weight/day (30). Differences between species may be related to differences in body size and metabolic rate.

Heart necrosis. Focal myocardial necrosis, a frequent occurrence in rats fed any diet and, in particular, HEAR oils (1-4), was not observed in any of the piglets fed sow milk or any of the milk replacer diets containing up to 42.9% 22:1n-9 for 15 days. This was not unexpected, since occurrence of myocardial necrosis in pigs was rare (4,27-29) and observed only in a mild form after one year of feeding HEAR oil (29).

Lipid Changes

Heart weight and lipid classes. There were no significant (P>0.05) differences in heart weight due to diet; average heart weights are given as a footnote in Table 4.

Only the cardiac lipid composition of piglets in Experiment II was examined because these diets represented the extremes of 22:1n-9 fed in the milk replacers. There were no significant (P>0.05) age and sex differences and for this reason, only the diet means are presented in Table 4. There were only a few diet differences (P<0.05) in cardiac phospholipids; however, there were numerous litter differences. Cardiac TAG was significantly higher in piglets fed HEAR oil as compared to sow milk or canola oil. By comparison, the content of cardiac TAG was generally lower in newborn piglets than weaned pigs fed a similar HEAR oil (36% 22:1n-9) for four days (3.2 mg/g wet weight) (11), even though the newborn piglet is undergoing a major transition from utilizing mainly carbohydrates to fat for energy production (6,7). Furthermore, the values of cardiac TAG in piglets and pigs were considerably lower than about 30 mg/g generally observed in rats fed HEAR oil diets for one week (12).

Correlation of TAG and histological staining. For piglets fed HEAR oil, there was a strong correlation between the cardiac TAG content and the lipidosis score determined histologically (r=0.82). The correlation was weak for piglets fed canola oil (r=−0.15) or sow milk (r=0.48). The inability to measure increased levels of TAG in hearts with low lipidosis scores of 1 or 2, as found in piglets fed sow milk or canola oil, was not surprising since oil red 0 staining showed only a few lipid droplets which were not evenly distributed in the heart. The results demonstrated that histological staining is a more sensitive and a better test of choice, particularly when small or localized lipid accumulation in tissues is to be assessed.

Fatty acid changes in cardiac TAG. The fatty acid composition of cardiac TAG (Table 5) reflects the large influence of dietary fatty acids, selective utilization of essential fatty acids (EFA) and metabolism of long chain monounsaturated fatty acids. The relative concentration of

TABLE 5

Fatty Acid Composition of Cardiac Triacylglycerol (TAG) of Piglets

Fatty acids	Diets[a,b]							LSD (P = 0.05)	Significance		
	Sow milk			Canola oil		HEAR oil					
	1 day	7 day	16 day	7 day	16 day	7 day	16 day		Diet	Age	Sex
	(area %)										
14:0	0.8	2.1	1.7	0.5	0.5	0.2	0.3	0.5	*	NS	NS
16:0	27.9	36.2	39.1	13.2	14.6	6.6	9.9	10.4	***	NS	NS
16:1n-7	1.8	2.3	3.8	0.5	1.2	0.8	1.3	1.0	*	NS	NS
18:0	9.2	23.2	17.9	8.9	13.7	3.7	8.0	14.8	*	NS	NS
18:1n-9	30.2	18.1	21.1	43.9	39.7	15.4	17.0	19.8	**	NS	NS
18:1n-7	4.5	1.9	2.5	4.4	4.4	2.2	2.4	2.3	*	NS	NS
18:2n-6	14.8	6.0	7.8	15.3	13.4	10.0	11.4	6.2	*	NS	NS
18:3n-3	0.8	0.6	0.2	3.7	3.0	2.5	2.3	1.2	***	NS	NS
20:0	0.2	0.8	0.4	0.6	1.2	0.6	0.7	0.5	NS	NS	NS
20:1n-9	0.7	1.2	0.5	2.1	2.3	7.2	7.4	2.0	***	NS	NS
20:4n-6	1.9	0.6	1.1	0.9	0.5	0.6	0.4	0.8	NS	NS	NS
22:0	0.1	0.3	0.1	0.2	0.6	0.6	0.5	0.3	*	NS	NS
22:1n-9	0.1	0.2	tr	0.4	1.1	42.6	30.9	11.5	***	NS	NS
24:0	0.2	0.2	tr	0.4	0.4	0.3	0.2	0.2	NS	NS	NS
24:1n-9	0.2	0.1	tr	1.0	0.7	2.3	2.4	1.0	***	NS	NS

[a]Abbreviations: HEAR, high erucic acid rapeseed; LSD, least significant difference; NS, not significant ($P < 0.05$); *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; tr, trace (<0.05%).

[b]All values are means of two piglets/diet/time.

20:1n-9, 22:1n-9 and 24:1n-9 in cardiac TAG (Table 5) was similar to that present in dietary oil (Table 1). On continued feeding, 22:1n-9 in cardiac TAG decreased (Fig. 2), possibly due to increased peroxisomal oxidation (31). The EFA fatty acids 18:2n-6 and 18:3n-3 were lower in cardiac TAG than in the TAG fed due to selective utilization of EFA during growth and development (15). By selective removal of the EFA, the remaining saturated fatty acids were higher in cardiac TAG. The relative fatty acid composition of cardiac TAG present in newborn piglets (this study), weaned pigs (11) and rats (12,32) was similar despite large differences in total content of cardiac TAG in rat and pig hearts.

Fatty acid changes in cardiac phospholipids. In general, there were very few sex differences and they showed no consistent pattern; therefore, only the averages are presented (Tables 6 and 7). A few age differences were observed. In DPG, the level of saturates and 18:2n-6 significantly increased with age, while the concentrations of 18:1 (n-9 and n-7) and 20:4n-6 decreased significantly. In SP, 14:0, 16:0 and 20:0 significantly increased with age, while the C_{22} n-3 PUFA in PE and PS decreased.

There were numerous diet differences in the cardiac phospholipids of nursing piglets and those fed milk replacer diets containing canola or HEAR oils (Tables 6 and 7). In general, the total saturated fatty acids were significantly lower in all phospholipids except DPG of piglets fed the two milk replacer diets. The decrease was due to 16:0 in PC and PE, 18:0 in PI and PS, and 18:0 and 24:0 in SP. Older pigs fed diets containing corn or HEAR oil for eight weeks in general showed no significant differences in the content of saturated fatty acids in cardiac PC, PE and SP, with the exception of an increase in 16:0 in DPG of pigs fed HEAR oil (10). Weaned rats (three-week-old) likewise showed no significant diet differences in saturated fatty acids of cardiac

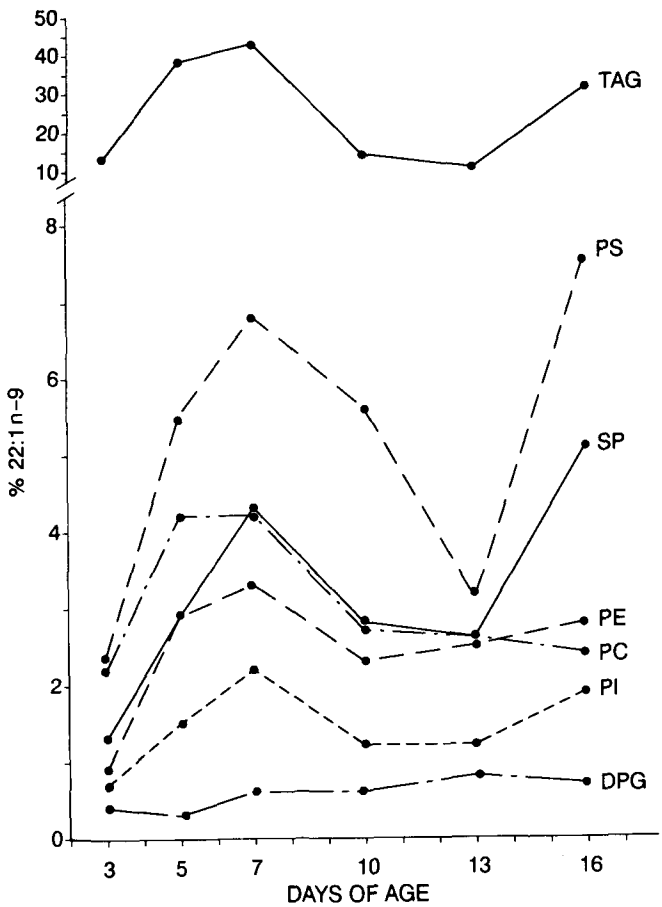

FIG. 2. The concentration of erucic acid (22:1n-9) in the different cardiac lipids of piglets fed a milk replacer diet containing HEAR oil with 42.9% 22:1n-9. Piglets were one day of age at start of experiment and were fed for 2, 4, 6, 9, 12 or 15 days (Experiment II).

TABLE 6

Fatty Acid Composition of the Major Cardiac Phospholipids of Piglets[a]

			Saturated fatty acids			Monounsaturated fatty acids					Polyunsaturated fatty acids				
		DMA	16:0	18:0	Σ Sat.	18:1 n-9	18:1 n-7	20:1 n-9	22:1 n-9	Σ Mono.	18:2 n-6	18:3 n-3	20:4 n-6	Σ C_{22} PUFA	Σ C_{22} n-3
										(area %)					
Phosphatidylcholine (PC)															
Sow milk	−1 d	2.9	25.9	13.8	41.0	23.0	8.0	0.2	tr	33.4	12.0	0.2	6.8	0.5	1.2
	−7 d	2.9	28.2	15.3	44.7	19.6	5.6	0.2	tr	26.8	16.0	0.2	6.5	0.6	1.0
	−16 d	3.4	29.6	14.0	44.7	19.3	5.3	0.2	tr	26.4	15.3	0.2	7.3	0.6	0.9
Canola oil	−7 d	2.7	19.2	16.4	36.5	26.4	5.8	0.7	0.1	33.9	19.1	1.9	3.6	0.5	0.8
	−16 d	1.6	19.4	18.4	38.7	25.9	6.8	0.7	0.1	33.8	20.7	1.7	1.9	0.3	0.3
HEAR oil	−7 d	3.2	15.1	15.2	31.3	20.9	4.2	2.2	4.2	33.3	19.8	2.7	6.4	0.4	0.7
	−16 d	2.7	18.1	18.6	37.8	18.8	4.4	2.0	2.4	29.3	19.3	2.1	5.2	0.5	0.8
LSD (P=0.05)		1.8	5.3	4.1	7.5	3.0	1.3	0.3	1.5	3.2	3.2	0.6	5.2	0.5	0.8
Phosphatidylethanolamine (PE)															
Sow milk	−1 d	13.2	5.7	23.6	30.8	9.9	4.7	0.2	tr	16.7	6.9	0.4	21.7	2.1	5.5
	−7 d	14.6	5.0	23.3	29.6	5.9	3.2	0.2	tr	11.3	9.2	0.3	25.0	2.6	5.7
	−16 d	14.2	5.3	22.7	29.8	4.6	2.7	0.1	tr	10.8	8.8	0.2	27.8	2.5	4.3
Canola oil	−7 d	15.1	3.9	19.9	24.9	10.8	3.7	0.6	0.2	17.2	11.6	1.3	19.0	3.2	5.9
	−16 d	12.5	3.8	21.6	26.5	9.6	3.9	0.6	0.2	17.6	16.8	1.7	15.6	2.8	3.8
HEAR oil	−7 d	12.4	3.2	20.0	24.4	9.9	3.1	2.1	3.3	20.6	11.1	1.8	21.4	1.4	3.5
	−16 d	8.2	3.9	23.5	28.9	9.3	3.3	2.4	2.8	20.6	17.2	2.5	13.7	1.5	2.1
LSD (P=0.05)		5.9	1.2	5.3	6.3	1.5	0.8	0.9	1.2	3.7	5.7	0.7	7.9	1.4	1.7
Phosphatidylinositol (PI)															
Sow milk	−1 d	0.2	3.1	45.0	49.6	13.3	3.5	0.2	tr	18.6	5.2	0.2	20.7	0.7	2.2
	−7 d	0.2	3.4	53.7	58.9	9.1	1.9	0.1	tr	13.0	4.5	0.1	18.8	0.7	1.7
	−16 d	0.1	3.3	54.7	59.1	6.7	2.0	0.2	tr	10.3	4.4	0.1	22.8	0.5	0.9
Canola oil	−7 d	0.1	2.4	45.5	49.1	15.4	2.9	0.6	0.1	19.7	7.3	1.0	19.5	0.5	0.8
	−16 d	0.1	3.1	38.7	43.8	15.5	3.6	0.7	0.3	21.3	10.2	1.3	18.4	0.8	1.2
HEAR oil	−7 d	0.2	3.1	43.3	48.1	13.8	2.8	2.9	2.2	23.4	7.2	1.1	16.1	0.5	1.1
	−16 d	0.2	3.7	46.6	51.6	11.6	2.7	2.5	1.9	20.2	8.8	1.1	13.3	0.5	0.6
LSD (P=0.05)		0.1	2.2	7.2	6.4	4.8	0.6	0.7	0.6	5.9	3.1	0.8	5.4	0.4	0.9
Phosphatidylserine (PS)															
Sow milk	−1 d	0.4	2.5	47.4	52.0	9.5	1.8	0.5	0.5	13.6	5.3	0.8	3.9	5.2	17.9
	−7 d	0.8	4.3	51.1	58.4	7.9	0.9	0.4	0.5	11.9	4.9	0.1	5.2	5.6	13.6
	−16 d	0.6	4.0	53.6	60.8	9.1	1.1	0.4	0.7	14.6	5.1	0.1	5.1	5.8	10.5
Canola oil	−7 d	0.8	3.0	47.4	54.0	13.6	2.4	1.0	1.1	20.1	8.6	0.5	1.9	4.4	10.0
	−16 d	0.4	3.9	48.6	57.8	11.3	2.3	1.0	1.5	20.6	9.1	0.5	2.0	4.4	6.5
HEAR oil	−7 d	0.8	3.6	42.6	48.5	11.0	1.8	2.5	6.8	24.9	6.9	0.6	4.1	3.0	8.6
	−16 d	0.4	6.0	44.1	53.2	9.6	2.0	2.5	7.6	25.6	9.4	0.4	1.8	3.2	4.8
LSD (P=0.05)		0.6	2.3	8.2	9.5	5.4	0.5	1.1	2.1	9.4	3.2	0.6	3.6	2.9	5.3
Diphosphatidylglycerol (DPG)															
Sow milk	−1 d	0.2	1.8	0.6	2.7	18.6	10.8	0.1	tr	38.6	51.6	0.2	2.1	0.3	0.5
	−7 d	0.3	1.3	1.2	2.8	13.1	11.0	0.1	tr	28.5	61.4	0.4	2.3	0.2	0.4
	−16 d	0.2	1.9	1.4	3.9	9.6	8.8	0.2	tr	22.3	68.2	0.5	1.7	0.2	0.2
Canola oil	−7 d	0.3	1.2	1.1	2.8	19.2	9.8	0.3	0.1	32.4	55.4	3.5	2.1	0.3	0.5
	−16 d	0.1	1.7	2.1	4.3	16.3	9.2	0.4	0.1	27.3	60.1	4.2	1.4	0.1	0.1
HEAR oil	−7 d	0.2	0.8	0.9	2.0	15.5	7.5	1.0	0.6	28.1	60.4	2.5	1.8	0.1	0.2
	−16 d	0.1	1.8	2.5	4.9	12.1	5.3	1.6	0.7	21.6	63.1	3.8	1.1	0.1	0.1
LSD (P=0.05)		0.2	0.7	1.8	2.5	3.9	2.1	0.6	0.1	4.9	6.6	1.4	0.8	0.3	0.5
Statistics[b]															
Effect of diet															
	PC	NS	***	NS	**	***	**	***	***	***	**	***	NS	NS	NS
	PE	NS	**	NS	NS	***	*	***	***	***	*	***	**	*	**
	PI	NS	NS	**	**	**	***	***	***	**	**	**	*	NS	NS
	PS	NS	NS	*	*	NS	***	***	***	*	*	NS	NS	NS	*
	DPG	NS	NS	NS	NS	**	**	***	***	*	*	***	NS	NS	NS
Effect of age															
	PC	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	*	NS	NS	NS
	PE	NS	NS	NS	NS	*	NS	NS	NS	NS	*	NS	NS	NS	**
	PI	*	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
	PS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	*
	DPG	*	**	NS	*	*	*	NS	NS	**	*	NS	*	NS	NS
Effect of sex															
	PC	*	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
	PE	NS	*	*	**	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
	PI	**	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
	PS	NS	NS	*	*	NS	NS	NS	NS	NS	NS	*	NS	NS	NS
	DPG	NS	NS	NS	NS	NS	NS	NS	*	NS	NS	NS	NS	NS	NS

Abbreviations: PUFA, polyunsaturated fatty acids; DMA, dimethylacetals; HEAR, high erucic acid rapeseed (42.9% 22:1n-9); LSD, least significant difference at P=0.05; d, day; tr, trace (<0.05).

[a]All values are means of two piglets/diet/time.

[b]Analysis of variance; NS, not significant (P>0.05); *P<0.05; **P<0.01; ***P<0.001.

TABLE 7

Fatty Acid Composition of Cardiac Sphingomyelin (SP) of Piglets

Fatty acid	Diets[a,b]							LSD (P=0.05)	Significance		
	Sow milk			Canola oil		HEAR oil					
	1 day	7 day	16 day	7 day	16 day	7 day	16 day		Diet	Age	Sex
	(area %)										
14:0	0.1	0.1	0.3	0.2	0.6	0.2	0.5	0.3	NS	**	NS
16:0	8.0	10.0	12.3	11.0	14.6	9.0	12.9	3.6	NS	**	NS
16:1n-7	0.5	0.7	0.8	0.8	1.0	0.5	1.1	0.7	NS	NS	NS
17:0	0.6	0.3	0.3	0.4	0.5	0.3	0.3	0.1	**	NS	NS
18:0	22.6	20.0	15.9	9.0	10.3	11.7	8.3	4.2	***	NS	NS
18:1n-9	3.3	3.2	1.6	2.7	3.2	2.8	2.2	2.2	NS	NS	NS
18:1n-7	1.0	1.0	0.3	0.6	0.1	0.7	0.2	0.7	NS	NS	NS
20:0	13.4	14.3	16.8	19.5	24.7	15.1	19.6	5.4	*	*	NS
20:1n-9	0.1	0.1	0.1	0.1	0.1	0.5	0.3	0.3	*	NS	NS
22:0	7.3	8.7	11.0	11.5	13.9	10.5	9.8	3.0	*	NS	NS
22:1n-9	0.3	0.2	0.2	0.3	0.3	4.3	5.1	0.9	***	NS	NS
23:0	1.6	1.6	2.0	2.1	2.0	1.8	1.0	0.9	NS	NS	NS
23:1	0.8	0.3	0.2	0.3	0.1	0.2	tr	0.2	NS	NS	NS
24:0	7.9	9.4	11.0	8.2	6.9	6.6	2.8	4.3	*	NS	NS
24:1n-9	17.7	17.3	18.7	23.0	16.4	27.4	28.0	6.7	**	NS	NS
24:1n-7	1.5	0.8	0.6	0.6	0.2	0.6	0.3	0.6	*	NS	NS
24:2	1.5	1.8	2.1	1.1	0.4	0.9	0.5	0.7	*	NS	NS
Total saturated	61.5	64.4	69.6	61.9	73.5	55.2	55.2	10.6	**	NS	NS

[a]Abbreviations: HEAR, high erucic acid rapeseed; LSD, least significant difference at P = 0.05; tr, trace (<0.05%); NS, not significant (P>0.05); *P<0.05; **P<0.01; ***P<0.001.

[b]All values are means of two piglets/diet/time.

phospholipids after 1 or 16 weeks on diet (33). The decrease of saturated fatty acids in cardiac phospholipids of newborn piglets may be related to the inability of the newborn piglet to adequately synthesize fatty acids *de novo* (6,34), and therefore depend on the dietary oils which were very low in saturated fatty acids (Table 1).

The monounsaturated fatty acids were generally significantly higher in the cardiac phospholipids of piglets fed canola or HEAR oil than in nursing piglets. In canola oil fed piglets, 18:1n-9 was the highest, and in HEAR oil fed piglets, 20:1n-9, 22:1n-9 and 24:1n-9 significantly contributed.

The highest content of 22:1n-9 was found in SP and PS (7–8%), moderate amounts in PC, PE and PI (2–4%) and the least amount in DPG (<1%). The data in Figure 2 indicate that the incorporation of 22:1n-9 reached a maximum six days on diet, plateaued thereafter, and then increased again at 15 days on diet. There were significant differences within and between litter differences (P<0.05) indicating individual differences in ability to metabolize 22:1n-9. These results differ from those of older pigs fed HEAR oil for eight weeks (10). The selected cardiac phospholipids analyzed in that study (10) showed a high content of 22:1n-9 in PE (11%) and much smaller amounts (1.5–2.5%) in PC, DPG and SP. By comparison, three-week-old rats fed HEAR oil showed levels of 22:1n-9 similar to newborn piglets in all phospholipids except DPG, which was much higher in the rat (5–8%) (32). Since DPG is found mainly in inner mintochondrial membrane (35), a high accumulation of 22:1n-9 in rat heart DPG may have metabolic implications.

The accumulation of 20:1n-9 in piglet heart phospholipids was 2–3% except in DPG (1–1.5%) and SP (<0.5%). The concentration of 20:1n-9 in older pigs was higher in all phospholipids (3–5%) (10) which supports the evidence of an active metabolism of 22:1n-9 in the adult pig (6,7, 26,36). By comparison, rat heart phospholipids contained 1 to 2% 20:1n-9 (32) which is less than found in pigs and supports the observation of a slower metabolism of 22:1n-9 to 20:1n-9 in the rat (36).

Cardiac SP showed a significant increase of 24:1n-9 in piglets fed HEAR oil, which was accompanied by a decrease in 18:0 and 24:0 (Table 7). Older pigs showed a much smaller increase of 24:1n-9 in cardiac SP (10). On the other hand, the increase of 24:1n-9 in rat heart SP was similar to that in young piglets, but the increase was accompanied by a decrease in 20:0 and 24:0 (32). The 24:1n-9 was shown in rats to be derived from 22:1n-9 by chain elongation (37).

Dietary fatty acids did not change the characteristic patterns of PUFA in the different phospholipid classes (Table 6) such as: 18:2n-6 very high in DPG, and higher in PC and PE compared to PI and PS; 20:4n-6 higher in PE and PI compared to PC and PS; and the C_{22} PUFA higher in PE and PS than in PC and PI. However, 18:2n-6 and 18:3n-3 significantly increased in all cardiac phospholipids of piglets fed canola and HEAR oils compared to nursing piglets with the exception of 18:2n-6 in DPG. Nevertheless, despite the increase of 18:2n-6 and 18:3n-3, the PUFA derived from these fatty acids (n-6 and n-3 C_{20} and C_{22} PUFA) generally decreased, particularly in PE (Table 6). This implies some metabolic control

and/or regulation in the heart at the cellular level for elongation and desaturation, which was observed previously in rats fed different vegetable oils including canola (33).

Conclusion. The results of this study show that newborn piglets can be successfully raised on milk replacers with different vegetable oils, including oils very high in 22:1n-9. Erucic acid, at levels greater than 7% in the oil of the diet (25% oil per dry matter content), caused an increase in myocardial lipidosis beyond that observed in piglets fed milk replacer containing soybean oil. The lesion was correlated to dietary 22:1n-9 and cardiac TAG; the maximum occurred at about one week on diet. The fatty acid composition of cardiac TAG in piglets fed HEAR oil was similar to that of the dietary oil. The incorporation of 22:1n-9 into cardiac phospholipids of neonatal piglets was highest for SP and PS and least for DPG. These results indicate that the response of the neonatal piglet to 22:1n-9 was greater than that of the adult pig. Based on these results, piglets fed milk replacer with 25% oil containing 5% 22:1n-9 consumed 750 mg 22:1n-9/kg body weight/day, which showed no adverse nutritional effects, and the heart histology and lipid composition were normal.

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METHODS

Analysis of Cholesterol and Desmosterol in Cultured Cells Without Organic Solvent Extraction

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Cultured cell sterols such as cholesterol and desmosterol are usually extracted into organic solvents before they are quantified with cholesterol esterase and oxidase. A method to quantify these cultured cell sterols using cholesterol enzymes without prior organic solvent extraction is described. In this method, a suspension or monolayer of cultured L-M, U-937, or PC-12 cells is digested with 0.1% sodium dodecyl sulfate (SDS), and the digest treated with microbial cholesterol enzymes. The quantity of oxidized sterols produced by the reaction can be measured easily with high-pressure liquid chromatography, when a mixture of sterols is present, or by the production of hydrogen peroxide when only one sterol is present. This method is easier and safer to use than solvent extraction and can greatly expedite the quantitation of cultured cell sterols. Preliminary data show that other lipids such as choline phospholipids, triglycerides, and fatty acids can also be directly quantified in SDS cell digest by using specific enzymes to transform these lipids into hydrogen peroxides.
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Organic solvents are usually used to extract cultured cell sterols, such as cholesterol and desmosterol, before they are quantified with microbial cholesterol esterase and oxidase. The extraction ensures the accessibility of the sterols to the microbial enzymes and prevents the interference of cellular components in the quantitation of hydrogen peroxide produced by the action of microbial enzymes on the sterols (1–8).

Organic solvent extraction of cultured cells, however, also creates difficulties. These include the variable losses of solvent during extraction, the usage and disposal of volatile and dangerous chemicals, and the extensive expenditure of time and effort. For these reasons, a method to quantify cellular sterols with microbial cholesterol enzymes, but without prior extraction with organic solvent, was investigated.

MATERIALS AND METHODS

Standards and reagents. Standards and biochemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Cholesterol oxidase from *Streptomyces* species (No. C9406) and cholesterol esterase from *Pseudomonas Fluorescens* (No. C9406) were used. Analytical and high-pressure liquid chromatography (HPLC) grade solvents were obtained from Fisher (Springfield, NJ). Standard solutions of cholesterol, cholesterol oleate, and β-sitosterol were prepared in isopropanol and stored in light-proof containers.

Cell cultures. (L-M (CCL 1.2), U-937 (CRL 1593) and PC-12 (CRL 1721) cells were purchased from American Type Culture Collection, Rockville, MD. L-M cells are a line of mouse fibroblasts which synthesize desmosterol instead of cholesterol (9). L-M cells are cultured in Eagle minimum essential medium (No. M1018) containing Hank's salt mixture, L-glutamine (2 mM), and non-essential amino acids. The medium is supplemented with sodium bicarbonate (4.2 mM), Bacto-peptone (0.5%), penicillin (100 mg/L), and streptomycin (100 mg/L). Stock L-M cells are grown in 75 cm^2 flasks maintained in ambient atmosphere at 37°C. Cells are dislodged with trypsin (1 mg/mL phosphate-buffered saline (PBS), pH 7.4) when they reach confluency, treated with trypsin inhibitor (1 mg/mL PBS), and washed with PBS. Aliquots of suspended cells are taken and used either as suspension or to prepare monolayers in a six-well plate. For this purpose, 7.5×10^5 cells are seeded in each of the wells and allowed to recover for 24 hr. At this time, the monolayers generally reach a 75% confluency containing about 200 μg of protein and 10 nmoles of desmosterol in each well. The monolayers are washed with PBS before use in experiments. U-937 cells are human monocytes, and they are grown as a suspension in RPMI 1640 medium supplemented with 10% fetal bovine serum and 5% CO_2. PC-12 cells are rat adrenal pheochromocytoma cells and are grown as a suspension in RPMI 1640 supplemented with 10% horse serum, 5% fetal bovine serum and 5% CO_2.

Digestion of cells. Unless otherwise specified, all cells are digested with sodium dodecyl sulfate (SDS) buffer containing 0.1% SDS, 1mM (ethylenedinitrilo)tetraacetic (EDTA), and 0.1M tris(hydroxymethyl)aminomethane (Tris), pH 7.4. SDS digest for cells grown in a monolayer is prepared by adding 1 mL of SDS buffer to the monolayer in each of the wells of the six-well plate and the plate incubated at 37°C for 5 min. The gelatinous mixture in the well is then taken up and discharged through a 19-gauge needle attached to a 1 mL disposable syringe. This process is repeated (4–8 times) until a homogenous solution is obtained. SDS digest for suspension cells is prepared by adding concentrated SDS buffer to cell suspension to obtain a 0.1% concentration of SDS. The gelatinous mixture is then homogenized as described for cells in monolayer. SDS cell digest is stored at 4°C for up to 30 days, or longer when frozen. The amounts of sterol and protein in the cell digest remain unchanged when stored under these conditions.

Analysis of sterols. Sterols analyzed in this study include cholesterol, desmosterol and β-sitosterol. The latter is used as an internal standard for the HPLC analysis of cholesterol and desmosterol. There is no difference in the rate of oxidation of these sterols by the enzymes used in this study (10). The oxidized forms of these sterols are identified and quantified by a modified version of a previously described HPLC procedure (10).

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Abbreviations: EDTA, (ethylenedinitrilo)tetraacetic acid disodium salt; HPLC, high-pressure liquid chromatography; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate.

Typical incubation for HPLC analysis involves the transfer of 50 μL of isopropanol containing 2 μg of internal standard, β-sitosterol, to a screw-cap tube (1.3 × 100 cm). A 200-μL aliquot of cell digest, digestion buffer, or a mixture of both is added. This is followed by the addition of 50 μL of an enzyme mixture. For free sterols, the mixture contains 150 mM sodium phosphate, pH 7.0, 30 mM sodium taurocholate, 1.02 mM carbowax 6000, and 0.1 units of cholesterol oxidase. Cholesterol esterase (0.1 units) is added to the enzyme mixture when total sterols are to be measured. The reaction mixture is incubated for 60 min at 37°C and extracted with petroleum hydrocarbon (bp, 35–60°C) as described before (10). The organic layer is recovered, solvent is evaporated with nitrogen, and the remaining oxidized sterols are redissolved in acetonitrile for injection into HPLC.

The extraction of oxidized sterols from the aqueous mixture is necessary to prevent the aqueous mixture from damaging the column. The extraction process is tedious and an alternative was investigated. It was found, empirically, that a 1:1 (v/v) mixture of 1 M KOH and acetonitrile remains separated in two different layers, instead of forming a homogenous solution. This suggests that KOH may be used to cause acetonitrile-containing oxidized sterols to separate from an equal volume mixture of acetonitrile and aqueous reaction mixture containing oxidized sterols. This possibility was tested and the partition in the presence of KOH and acetonitrile was found to be as efficient as with petroleum hydrocarbon in extracting oxidized sterols from the aqueous reaction mixture. Since this approach is less time-consuming and safer, it was adapted for routine use in this study. For this purpose, a 0.2-mL aliquot of KOH (2.5 M) was added to the reaction mixture after incubation at 37°C for 60 min. The mixture was vortexed and 0.5 mL of acetonitrile was added. The resulting biphasic mixture was vortexed vigorously for 30 sec and centrifuged for 1 min (1,000 × *g*). An aliquot of the upper layer, acetonitrile, was taken and injected directly into the HPLC.

The conditions and the equipment used for HPLC were reported previously (10). Briefly, chromatography is performed at room temperature with an Ultrasphere-XL C18 column (3 μm, 75 × 4.6 mm) (Beckman Instruments, Fullerton, CA) equipped with a guard cartridge. The column is eluted with a 1:1 (v/v) mixture of methanol and acetonitrile at a flow rate of 1 mL/min. Eluted oxidized sterols are quantitated by ultraviolet absorption at 240 nm. Sterol peak heights are measured and ratios between cholesterol or desmosterol and β-sitosterol determined. The ratios obtained are used to calculate the amounts of cholesterol and desmosterol present in the samples. Standard curves were developed for each sterol individually.

Hydrogen peroxides produced by cholesterol oxidase are made fluorescent and quantified by fluorometry using previously established procedures (8). For this purpose, a 50-μL aliquot of water containing 0.2 unit of horseradish peroxide and 0.2 mg of *p*-hydroxyphenylacetic acid is added to the reaction mixture, prepared as described for HPLC analysis. The mixture is incubated as described for HPLC analysis. One mL of 50 mM sodium phosphate, pH 7.4, is added after the incubation. The samples are mixed and fluorescence is read at an excitation wavelength of 325 nm and an emission wavelength of 415 nm using an Aminco SPF-500 (American Instrument Co., Silver Spring, MD).

Other analyses. Aliquots of SDS cell digest were taken and protein was measured with bicinchoninic acid reagent marketed by Pierce (Rockford, IL).

Statistical methods. The results are presented as means ± SEM. The significance of difference between group means is evaluated by Student's t-test.

RESULTS AND DISCUSSION

Cultured cell sterols, such as cholesterol and desmosterol, are usually extracted into organic solvents before they are quantified with cholesterol esterase and oxidase (1–8). Organic solvent extraction of cultured cells is laborious, dangerous, and impractical when a large number of samples is involved. The extraction, however, is needed in order to allow cholesterol enzymes to gain complete access to the cellular sterols and to remove nonlipid cellular components which may interfere with the subsequent quantitation of enzymatic products, oxidized sterols and hydrogen peroxide.

Instead of extraction with organic solvents, the accessibility of cultured cell cholesterol can be ensured by digesting the cells with a detergent which is compatible with the microbial enzymes. The search for this detergent was initiated by testing the ability of several common detergents at commonly used concentrations to dissolve cultured L-M cells. Individual solutions of Triton X-100 (1%), sodium taurocholate (1%), Kyro EOB (0.5%), Carbowax 6000 (10 mM), and SDS (0.1%) were prepared in Tris buffer (0.1 M, pH 7.4) with 1 mM EDTA. One-mL aliquots of each detergent were added to a confluent monolayer of L-M cells in each well of a six-well plate. The plates were incubated for 5 min at 37°C and examined under a light microscope. Recognizable cell remnants were observed in all wells except the one treated with SDS. The ability of 0.1% SDS to digest cells was clearly superior and hence was further tested.

The optimal concentration of SDS for the digestion of cells was tested by the accessibility of cell sterol to the action of microbial enzymes. For this purpose, cholesterol oxidase and esterase were added to a suspension of L-M cells together with various concentrations of SDS. The suspensions were incubated under standard conditions and the amounts of L-M cell desmosterol measured by HPLC. The results are shown in Figure 1. Maximal amounts of desmosterol were detected with SDS at concentrations equal to or greater than 0.1%. Because of this, the concentration of SDS for cell digestion was kept at 0.1%.

The compatibility of 0.1% SDS with microbial cholesterol oxidase and esterase was tested by assaying various amounts of free cholesterol or cholesteryl oleate standards in the absence and presence of 0.1% SDS. The amounts of oxidized cholesterol recovered in incubations with and without SDS under standard incubation conditions are identical for free cholesterol and cholesteryl oleate. The rates of oxidation for each type of cholesterol are linear up to 25 nmoles in the presence of 0.1% SDS and 50 nmoles in the absence of SDS (data not shown). The difference is probably caused by the reduction in the activity of microbial enzymes by SDS. Since HPLC can detect as little as 10 pmoles of oxidized sterols (10),

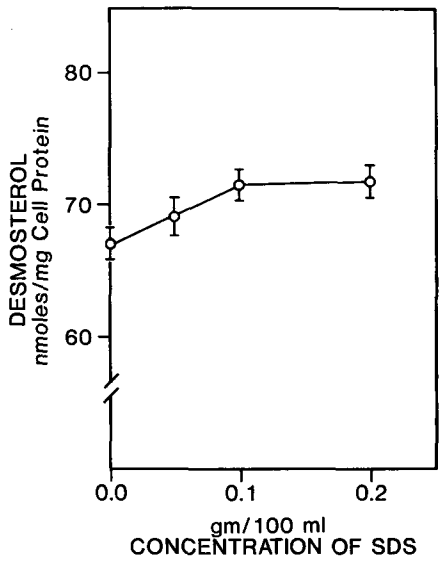

FIG. 1. The effect of SDS concentrations on the amounts of desmosterol detected in cultured L-M cell suspension (n ≥ 5).

TABLE 1

Sterol Content of Cell Digests Without and with Organic Solvent Extraction[a]

Cell lines	Without extraction		With extraction	
	FS	TS	FS	TS
L-M	65.43 +0.29	65.17 ±0.41	65.50 ±0.21	65.53 ±0.33
U-937	60.73 ±0.55	61.69 ±0.42	60.72 ±1.00	62.55 ±0.58
PC-1 2	38.66 ±0.13	39.30 ±0.18	38.53 ±0.27	38.86 ±0.05

[a]In nmoles/mg cell protein (n ≥ 4); FS, free sterol; TS, total sterol.

further attempts to extend the linearity of the assay are unnecessary.

The differences in the amounts of sterols between SDS cell digests and organic solvent extracts of cell digests were investigated next in three different cell lines. For this study, cell digests were either incubated directly with microbial enzymes or extracted with chloroform and methanol as described by Ames (11). Total lipids in the cell digest were recovered, dissolved in isopropanol, reconstituted with 0.1% SDS and microbial enzymes, and incubated as usual.

The results are given in Table 1. There is no difference between the amounts of free and total sterols measured directly in cell digests and indirectly in the extracts from the digests. This is a further indication that microbial enzymes are able to access all sterols in the SDS digest. In addition, the lack of difference in the amount of free sterol between cell digest and the extract also suggests that endogenous cellular cholesterol esterifying and hydrolyzing enzymes present in SDS cell digests are inactive during the 60 min incubation of SDS digest with microbial enzymes at 37°C.

TABLE 2

Recovery of Cholesterol Standards from SDS Digest of L-M Cells[a]

Sterols measured	Without standard	With standard
2.5 nmoles free cholesterol added		
Free cholesterol	N.D.	2.47 ± 0.04
Free desmosterol	1.61 ± 0.03	1.63 ± 0.02
3.0 nmoles cholesterol oleate added		
Esterified cholesterol	N.D.	3.15 ± 0.02
Esterified desmosterol	0.10 ± 0.12	0.06 ± 0.15

[a]Values for esterified sterols are obtained by subtracting the amount of free sterols from the amount of total sterols found in the samples. Amounts are given in nmoles/assay (n ≥ 5); N.D., not detected.

To confirm this, 50 μL of isopropanol containing β-sitosterol and either free cholesterol or cholesterol oleate standards were added and incubated with L-M cell SDS digest for 3 hr at 37°C before the usual incubation with the microbial enzymes. The results are shown in Table 2. The recovery of free cholesterol and cholesteryl oleate was quantitative after 3 hr of incubation with SDS cell digest. Thus, endogenous cellular enzymes mediating the synthesis and hydrolysis of steryl esters are inactive in cell digest during incubation with microbial enzymes. This is necessary in order to calculate the amount of esterified sterols present in cell digest which is obtained by subtracting the amount of free sterols from the amount of total sterols found in the digest.

The inactivity of endogenous cellular cholesterol enzymes suggests that endogenous cellular enzymes capable of altering the amount of hydrogen peroxide produced by exogenous microbial cholesterol enzymes may also be inactive in SDS cell digest. Their inactivity would allow hydrogen peroxide produced by microbial cholesterol enzymes to be measured directly and equated to the amount of sterols present in the cell digest. Quantifying hydrogen peroxide in SDS cell digest with fluorometry is less laborious than quantifying oxidized sterols by HPLC. The former may be advantageous when only one type of sterol is present in cultured cells.

The possible use of hydrogen peroxides to quantify cellular sterols in SDS cell digest is examined by comparing the quantities of sterols as reported by fluorometry and by HPLC. The comparison is made with the slopes of their regression line. Perfect agreement is represented by a slope of 1. The results are shown in Table 3. The

TABLE 3

Comparison Between HPLC (Y) and Fluorescence (X) Analyses of Cultured Cell Total Sterols in Cell Digest[a]

Cell lines	Equations for linear regression	r
L-M	Y = 0.36 + 0.96X	0.999
PC-12	Y = 0.38 + 0.14X	0.972
U-937	Y = 0.01 + 0.87X	0.993

[a](n ≥ 8).

amounts reported by fluorescence are equal to or less than the amounts reported by HPLC, depending on the cell types. Since the amounts reported by fluorescence strongly correlate with the amounts reported by HPLC, lesser amounts reported by fluorescence can be corrected with the regression equations between the two methods. No correction is necessary for direct fluorescence measurement of digests obtained from L-M cells.

Hydrogen peroxide is the end-product for enzymatic analysis of several lipid classes. The feasibility of quantifying hydrogen peroxide directly in SDS cell digest suggests that other cellular lipids may also be quantified directly without prior organic solvent extraction. In preliminary experiments, enzymes used to analyze choline phospholipids (12), triglycerides (13), and free fatty acids (14) are found to be compatible with SDS. Direct analysis of these lipids in SDS cell digest is currently under investigation.

The results clearly show that cultured cell sterols can be quantified directly with microbial enzymes after the digestion of cultured cells with 0.1% SDS. This method can be used in place of organic solvent extraction and thereby eliminate a significant source of error in the quantitation of cultured cell cholesterol. This, in turn, will facilitate studies on the metabolism and transport of lipids in cultured cells.

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Rapid Analysis of Fatty Acids in Plasma Lipids

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A rapid and convenient procedure for the quantitative determination of the fatty acid composition of plasma lipids is described. Human plasma was applied directly to the preadsorbent zones of thin-layer silica gel plates with added antioxidant, internal standards and carriers. The thin-layer chromatography (TLC) plates were partially developed with methanol followed by chloroform/methanol (1:1, v/v), and then they were fully developed in hexane/diethyl ether/acetic acid (80:20:1, v/v/v) to separate the major classes of lipids. Silica gel from regions containing the separated lipids was scraped into screw-capped tubes and treated with boron trifluoride-methanol prior to gas chromatography. The method of direct application to TLC plates gave yields and compositions of fatty acids very similar to the method of applying extracted plasma lipids. This relatively simple method is suitable for analyzing the fatty acids in plasma lipids from a 50 microliter finger-tip blood samples from an individual, and it may be useful in wide-scale screening of different individuals to estimate the relative amounts of ingested polyunsaturated fatty acids.
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Highly unsaturated 20-carbon fatty acids (HUFA) influence the rate of biosynthesis of prostaglandins (1) and leukotrienes which, in turn, influence the frequency and severity of some diseases and disorders (2). The tissue abundances of the 20-carbon fatty acids of the n-3 and n-6 type reflect the relative amounts of these two types of fatty acid in the diet because neither of these can be formed *de novo* in animals or humans. Recent progress in understanding the quantitative relationships between the tissue composition of the various HUFA and the dietary intakes of 18:2n-6 and 18:3n-3 (3) make it seem likely that estimates of the amounts of n-3 and n-6 acids ingested might be made from analyses of the HUFA in plasma phospholipids. In addition, such estimates may be independently checked by using data on 18:2n-6 and 18:3n-3 in plasma triglycerides (3). A careful study of the role of the n-3 and n-6 fatty acids in diet-disease relationships can alert people to the prudent limits of dietary changes related to these fatty acids. For example, a longitudinal study involving the range of dietary patterns seen in the U.S. and Japan could provide much useful information (4). One concept to be tested is whether a rapid chemical analysis of the n-3/n-6 pattern in plasma lipids would be more useful than the labor-intensive and highly variable nutrient assessment tools (*e.g.*, ref. 5) currently available.

While considering a possible large-scale survey to correlate the relative amounts of n-3 and n-6 nutrients that are ingested with the frequency or severity of disease, we examined the feasibility of devising a rapid assay of the 20- and 22-carbon highly unsaturated fatty acids (HUFA) to reflect an individual's intake of n-3 and n-6 nutrients and the potential for eicosanoid biosynthesis. We wanted a method for analyzing fatty acid composition in plasma lipids that was simple, quantitative, and did not cause structural changes or side reactions of the fatty acids. Traditional methods for the extraction and separation of lipids (*e.g.*, refs. 6–8) and the conversion to methyl esters (*e.g.*, refs. 9–11) require several steps, and many different methods have been used to extract and analyze lipids in plasma. These methods use a number of organic solvents that are removed by evaporation in the absence of oxygen (employing an inert gas, usually nitrogen). After separating lipid classes by silica gel thin-layer chromatography (TLC), the resolved lipid classes are often extracted from the silica gel with an organic solvent that is then evaporated prior to preparing methyl esters from the lipid fractions.

This paper demonstrates the feasibility of direct application of small samples of plasma to the preadsorbent layer of a silica gel TLC plate and of transmethylation of lipid fractions without extraction of the lipid from silica gel. The utility and ease of this rapid method for fatty acid composition studies makes it suited for analyzing many samples with minimal effort.

MATERIALS AND METHODS

Boron trifluoride-methanol (12% BF_3 in methanol) was obtained from Supelco, Inc. (Bellefonte, PA). Freshly opened reagent was redistributed to glass ampoules and sealed to prevent progressive deterioration during storage at 4°C. All solvents were of reagent grade. Fatty acids and their methyl esters, triglycerides, and cholesteryl esters were obtained from NuChek Prep (Elysian, MN) and phospholipids from Sigma Chemical Co. (St. Louis, MO). Vacutainers were obtained from Becton Dickinson Vacutainer Systems (Rutherford, NJ) and microhematocrit tubes and Sure-Seal tuber sealant were from American Scientific Products (McGaw Park, IL). Silica gel G preactivated thin-layer (250 μm thick) chromatography plates with preadsorbent zone were from Analtech (catalog #, Newark, DE). The DB-225 capillary gas-liquid chromatography (GLC) column was from J&W Scientific (Folsom, CA).

A solution of internal standards (ISTD) with anti-

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Abbreviations: BHT, di-(*t*-butyl)-hydroxytoluene; CE, cholesteryl ester; FID, flameionization detector; GLC, gas-liquid chromatography; HUFA, highly unsaturated fatty acids; ISTD, internal standard mixture; PC, phosphatidylcholine; TG, triacylglycerol; TLC, thin-layer chromatography.

oxidant and lipid carriers was prepared to contain the following in mg/mL: *tert*-butylhydroxytoluene (BHT), 2.00; dilauroyl phosphatidylcholine (PC) (12:0 PC), 3.700; diheptadecanoyl phosphatidylcholine (17:0 PC), 0.310; lauric acid (12:0), 3.34; heptadecenoic acid (17:1), 0.060; docosatrienoic acid (22:3), 0.022; tri-tridecanoylglycerol (13:0 TG), 2.810; triheptadecanoylglycerol (17:0 TG), 0.251; cholesteryl tridecanoate (13:0 CE), 3.84; cholesteryl heptadecanoate (17:0 CE), 0.270.

Blood samples. Blood was collected using heparinized microhematocrit tubes. The tubes were plugged by tube sealant and centrifuged at 3000 rpm for 15 min at 4°C. Volumes of plasma and red blood cells were estimated from the length of the tubes (35 mm = 25 mL). Plasma was removed with a Hamilton 50-μL syringe (Reno, NV) and stored at −40°C prior to analysis. Alternately, blood was collected using Vacutainer Tubes which were then centrifuged at 3000 rpm (1500 g) for 30 min at 4°C. Plasma was removed and stored at −40°C. In each case, packed erythrocytes are also available for analysis of fatty acid composition (*e.g.*, ref. 3).

Extraction of lipids from plasma. One-hundred μL of plasma was transferred to a Teflon-lined, screw-capped test tube, and 50 μL of ISTD was added. Then 0.7 mL of water, 2.0 mL of methanol and 1.0 mL of chloroform (containing 100, μg BHT) were added in sequence with vigorous mixing after each addition; the lipids were then extracted and concentrated under a stream of nitrogen as noted above. The residue was dissolved in 100 μL of chloroform methanol (2:1, v/v) for application to TLC plates.

Thin-layer chromatography. Fifty μL of ISTD was spotted on each 2-cm wide lane of the plate, and then either 75 μL of plasma or extracts form 75, μL plasma were applied in alternate lanes onto the plate and allowed to air dry. The TLC plate was developed first in methanol to approximately 1.5 cm above the preadsorbent border, then air dried under the hood until the protein spot was no longer translucent. After evaporating the methanol, the plate was again developed to 1.5 cm with chloroform/methanol (1:1, v/v) to complete the extraction of lipids from the plasma protein, then air dried again under the hood for five to ten min to completely evaporate the solvent. The plate was then fully developed to 15 cm above the adsorbent band using hexane/diethyl ether/acetic acid (80:20:1, v/v/v). Paper tank liners were not used at any time during the TLC procedure. The resulting lipid bands were visualized by spraying the plate with rhodamine 6G (0.02% in 95% ethanol) and viewing the plate in UV light. R_f values were noted and individual bands were then scraped into screw-cap tubes.

Transmethylation. To each tube containing silica, 1.0 mL of methanol containing 50 μg BHT was added. To the phospholipid, triglyceride and cholesteryl ester fractions, 1.41 μg of 22:3 internal standard was added to follow HUFA recovery. All sample tubes were then treated with 1.0 mL of the BF_3 reagent, tightly capped and placed in a boiling water bath for 30 min. After heating, the tubes were withdrawn from the bath and cooled. Then, either methyl docosanoate (22:0 Me) or methyl tricosanoate (23:0 Me) was added in the quantity of 3.98 μg as an internal reference for calculations of the GLC results. The fatty acid methyl esters were extracted by adding 1.0 mL of hexane (with 50, μg BHT) and 3.0 mL of water, and the tubes were centrifuged to facilitate hexane separation. After centrifugation, the hexane extracts were removed and concentrated under a stream of nitrogen. The extracted derivatives from the cholesteryl ester (CE) band contained free cholesterol that was removed by passing the 1.0 mL layer through a 2 cm (1.0 mL) column of Florisil (or alumina) in a Pasteur pipette and rinsing with petroleum ether diethyl ether (9:1, v/v), collecting the second 2 mL for analysis.

Gas-liquid chromatography (GLC). GLC was done on a Hewlett Packard Model 5890A Chromatograph (Hewlett Packard, Norwalk, CT) fitted with a Model 7673 Automatic Split injection system and a flame ionization detector (FID) using a bonded, flexible, fused silica capillary column (30 m × 0.25 mm I.D.; DB-225 coating thickness 0.25 μm). The oven temperature was programmed from 140°C to 180°C at a rate of 20°C/min and then from 180°C to 240°C at 3°C/min and held at the final temperature for 2 min. When analyzing the cholesteryl ester fractions, the final temperature (240°C) was held for 34 min to elute cholesterol and its derivatives that may have eluted from the small florisil (or alumina) columns. The hydrogen carrier flow rate was 1.3 mL/min, and the nitrogen make-up gas was 28 mL/min. The injection port and detector temperatures were 250°C and 260°C, respectively. The autosampler was programmed to rinse the 10 μL syringe, once with sample, pumped four times to remove air bubbles, and then inject 5 μL of sample with a split ratio of about 9:1. The syringe was then rinsed five times with solvent. The peak areas were integrated and reported using a Hewlett Packard Model 3393A computing integrator and stored on the hard disk of a Hewlett Packard Vectra AT. The integrator was programmed with the theoretical relative response factors (12) of fatty acids to the flame detector to convert raw data to corrected peak areas. The stored results were converted to spreadsheet format (Lotus 1-2-3) by using Hewlett Packard "FileServer" software (HP3393/0123).

RESULTS AND DISCUSSION

In preliminary attempts to avoid interference by the silica gel from the scraped TLC bands, we first extracted the lipids from the silica gel prior to transmethylation (Table 1). However, when comparing duplicate sets, we noted that the set that was scraped and the lipids extracted with chloroform/methanol (1:2, v/v) prior to transmethylation gave compositions and yields similar to the other set that was transmethylated directly without extraction from silica gel (Table 1). Thus extraction from the silica gel was not necessary for the analysis, and subsequent analyses omitted that time-consuming step. We also found that adding benzene recommended by others (10) was not beneficial with these samples (results not shown).

To further simplify the entire analysis of the methyl esters from plasma lipids, we tested the feasibility of omitting the extraction of lipids from the plasma prior to TLC. First, 50 μL of ISTD was applied to the origin

TABLE 1

Esterification of Samples from TLC[a]

Fatty acid	Extracted μmol/mL	On silica gel μmol/mL
16:0	2.10 (0.14)	2.12 (0.07)
16:1n-7	0.33 (0.08)	0.25 (0.01)
18:0	0.69 (0.02)	0.74 (0.02)
18:1n-9	2.27 (0.06)	2.31 (0.00)
18:2n-6	2.86 (0.16)	3.06 (0.16)
18:3n-6	0.05 (0.01)	0.05 (0.00)
18:3n-3	0.05 (0.01)	0.04 (0.00)
20:3n-6	0.12 (0.01)	0.14 (0.01)
20:4n-6	0.56 (0.03)	0.65 (0.05)
20:5n-3	0.08 (0.01)	0.08 (0.01)
22:6n-3	0.19 (0.03)	0.23 (0.04)

[a]Aliquots of extracts of plasma of total lipids were applied to the silica gel and then scraped off for analysis. Values for standard deviation are given in parenthesis.

of each TLC lane to provide antioxidant, carriers and internal standards. Then, lipid extracts from 75 μL of plasma were applied to the preadsorbent zone of the TLC plate, and in an adjacent zone a corresponding amount (75 μL) of plasma was applied directly (without extracting first). The brief development to 1.5 cm with methanol was used to dehydrate the applied samples, and denature the lipoprotein complexes to facilitate the chromatography. A second brief development with methanol was occasionally used prior to a 1.5-cm development with chloroform/methanol (1:1, v/v) to further condition the plate. After these solvents had been evaporated from the plate, the plate was fully developed, and the lipids were located, scraped into tubes, and transmethylated and analyzed as described in the Materials and Methods section. Other labs have examined the method of direct application (13), but the small plasma samples were sufficient only for fluorescence measurements of the separated lipids. The present method employs larger amounts of plasma with methanol to speed the dehydration of the larger aqueous samples needed for fatty acid analyses.

The gas chromatographic conditions using capillary columns described in the Materials and Methods section provided excellent resolution of all methyl esters of interest within 20 min (Fig. 1). The low initial oven temperature was programmed to permit elution of the solvent and reagent impurities and the medium chain length carrier methyl esters (12:0 and 13:0) prior

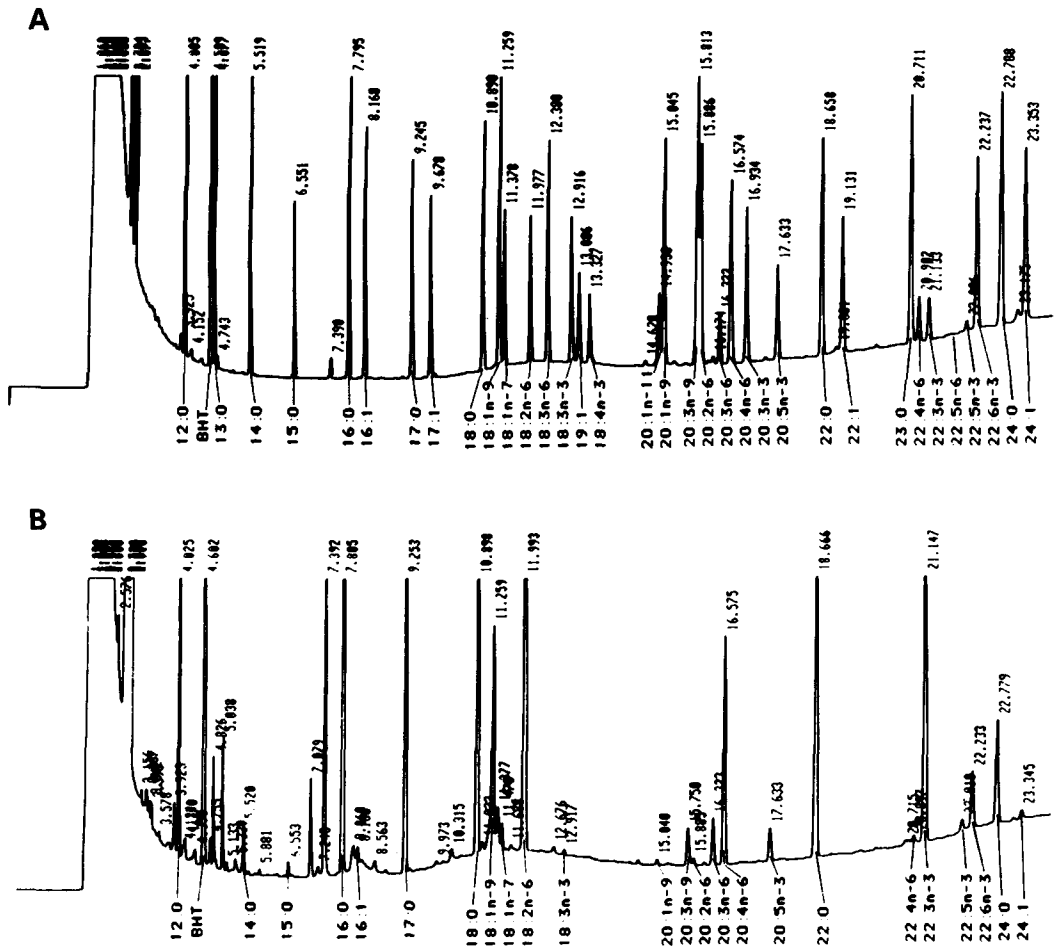

FIG. 1. Gas chromatographic separation of methyl esters and standards. Separation of components of (A) the standard reference calibration mixture; and (B) a representative sample of esters from the plasma phospholipid region which contains the accompanying standards and carrier materials.

TABLE 2

Comparison Between Direct Application and Prior Extraction[a]

	Phospholipid		Non-esterified acids	
	Direct	Extracted	Direct	Extracted
14:0	0.4 (1.1)	0.6 (0.1)	2.9 (0.6)	5.0 (0.8)
16:0	33.7 (1.1)	31.7 (0.7)	43.9 (3.6)	43.0 (4.0)
16:1	1.3 (0.2)	1.0 (0.0)	3.4 (0.2)	2.8 (0.3)
18:0	13.7 (1.4)	14.2 (1.1)	9.2 (0.3)	10.3 (0.8)
18:1	11.2 (1.0)	11.1 (0.6)	25.7 (0.8)	24.2 (2.1)
18:2n-6	21.1 (2.0)	22.1 (1.9)	11.4 (0.4)	10.2 (0.8)
18:3n-6	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.4 (0.0)
18:3n-3	0.6 (0.1)	0.5 (0.1)	0.6 (0.1)	0.9 (0.1)
20:2/20:3	0.9 (0.1)	0.8 (0.3)	0.4 (0.0)	0.6 (0.1)
20:3n-6	2.7 (0.3)	3.0 (0.3)	0.3 (0.1)	0.4 (0.1)
20:4n-6	9.1 (1.0)	9.5 (0.8)	1.1 (0.1)	1.2 (0.1)
20:5n-3	1.1 (0.3)	1.2 (0.2)	0.4 (0.0)	0.7 (0.1)
22:4n-6	1.0 (0.4)	0.5 (0.2)	0.0 (0.0)	0.0 (0.0)
22:5n-3	1.1 (0.2)	0.8 (0.2)	0.5 (0.1)	0.8 (0.0)
22:6n-3	3.3 (0.4)	3.5 (0.3)	0.7 (0.1)	1.0 (0.0)
μmoles/mL	4.3	4.6	0.7	0.8
	Triglycerides		Cholesteryl esters	
	Direct	Extracted	Direct	Extracted
14:0	2.9 (0.5)	3.0 (0.6)	1.1 (0.1)	2.2 (0.3)
16:0	34.2 (4.0)	35.4 (5.0)	14.7 (0.7)	17.2 (1.3)
16:1	4.3 (0.5)	4.5 (0.7)	2.4 (0.4)	2.9 (0.4)
18:0	4.8 (1.0)	5.1 (1.4)	1.9 (0.5)	1.2 (0.1)
18:1	33.9 (5.7)	33.1 (6.6)	17.3 (0.2)	15.8 (0.6)
18:2n-6	16.4 (3.1)	15.9 (3.6)	52.6 (0.9)	46.4 (2.5)
18:3n-6	0.0 (0.0)	0.8 (0.2)	1.3 (0.1)	1.3 (0.2)
18:3n-3	1.0 (0.4)	0.7 (0.1)	0.5 (0.1)	0.6 (0.0)
20:2/20:3	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
20:3n-6	1.2 (0.7)	0.3 (0.1)	1.2 (0.2)	4.8 (0.2)
20:4n-6	1.4 (0.2)	1.3 (0.2)	6.7 (0.1)	5.7 (0.2)
20:5n-3	0.6 (0.0)	0.9 (0.0)	1.0 (0.1)	1.1 (0.1)
22:4n-6	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
22:5n-3	0.5 (0.1)	0.5 (0.1)	0.0 (0.0)	0.0 (0.0)
22:6n-3	0.8 (0.1)	0.7 (0.1)	0.6 (0.0)	0.3 (0.1)
μmoles/mL	2.8	2.7	3.0	3.8

[a]Fatty acid composition is expressed as the mean weight percent based on eight different samples with the standard error of the mean given in parenthesis.

to the esters of the major naturally occurring fatty acids. The capillary column resolved 18:1n-9 from 18:1n-7, 20:2n-6, and 22:6n-3 from the 24-carbon derivatives, 24:0 and 24:1. Resolution of all major fatty acids was achieved in 25 min, and the standard mixture of methyl esters shown in Figure 1A was frequently used to confirm analytical performance and to recalibrate retention time assignments made by the integrating calculator. When resolution of 20:2n-6 from 20:3n-9 was not required, excellent resolution of all the other esters could be obtained in less than 20 min. The use of a robotic autosampler permitted overnight analysis of more than 30 samples, and data stored on hard disc was converted to a standard spreadsheet format for publication.

A typical chromatogram of methyl esters from plasma phospholipids (Fig. 1B) illustrates the dominance of 16:0, 18:0, 18:1 and 18:2, relative to the small amounts of HUFA that are measured. The peaks for internal standards and antioxidant provide quality control data that ensure valid interpretations. If the methyl ester sample has been evaporated (concentrated) too vigorously prior to analysis, the carrier esters, 12:0 and 13:0, will be decreased, and if autoxidation has occurred, the relative ratios of 22:3n-3 to 17:0 will be decreased. In control assays without added standard, the very small peaks in the region of 17:0 and 17:1 were negligible (<1%) relative to the amount of internal standard routinely added. To diminish the risk of spontaneous loss of solvent (hexane; bp 69°C) with concomitant autoxidation, 50 μL of decane (bp 174°C) was added, permitting samples to be kept at 10°C for weeks without loss of any of the polyunsaturated esters.

Separation of the lipid classes using the TLC conditions as described gave good resolution of PL (R_f, 0.0–0.15), cholesterol (R_f, 0.20–0.26), non-esterified acids (R_f, 0.30–0.44), TG (R_f, 0.60–0.76), and cholesteryl esters (R_f, 0.85–0.92). The similar recoveries of 17:1 (or 17:0) and 22:3n-3 in each lipid fraction (using either direct application or the extraction method) indicated that the standard HUFA was recovered intact without any autoxidative loss during the analytical procedures. In addition, the fatty acid compositions of the four

TABLE 3

Results With Different Amounts of Plasma Applied to TLC[a]

μL Applied	120	100	80	40	20	10	Mean
			Phospholipids				
16:0	29.62	29.20	29.06	27.98	29.53	33.53	29.82 (1.74[b])
16:1	2.02	1.29	1.72	1.78	1.20	1.40	1.57 (0.29)
18:0	14.65	15.02	15.12	15.98	14.88	15.68	15.22 (0.46)
18:1n-9	9.91	9.22	9.70	9.99	10.75	11.08	10.11 (0.63)
18:1n-7	1.36	1.35	1.58	1.99	1.48	1.58	1.56 (0.21)
18:2	26.96	26.98	26.48	23.55	24.16	21.98	25.02 (1.91)
18:3n-6	0.00	0.00	0.00	0.00	0.00	0.00	0.00 (0.00)
18:3n-3	0.00	0.00	0.00	0.00	0.00	0.00	0.00 (0.00)
20:3n-6	1.40	1.50	1.60	2.03	1.61	1.38	1.59 (0.22)
20:4n-6	8.30	8.68	8.70	8.62	8.48	7.16	8.32 (0.54)
20:5n-3	1.36	1.29	1.43	1.12	1.25	0.96	1.24 (0.16)
22:5n-3	0.64	0.97	0.55	0.88	1.02	0.51	0.76 (0.20)
22:6n-3	2.50	3.27	2.83	3.90	4.25	2.63	3.23 (0.65)
Total μg/uL	0.88	0.96	0.96	1.00	0.99	0.92	0.95 (0.04)
			Triglycerides				
16:0	21.16	20.84	21.14	21.67	21.82	23.09	21.62 (0.74)
16:1	2.52	2.18	2.66	2.76	2.56	4.13	2.80 (0.62)
18:0	5.57	5.80	5.90	6.88	6.79	8.05	6.50 (0.85)
18:1n-9	32.19	32.70	32.07	31.81	31.51	28.87	31.53 (1.24)
18:1n-7	2.10	2.12	2.16	2.01	2.00	2.02	2.07 (0.06)
18:2	24.79	24.92	24.27	23.19	22.70	20.23	23.35 (1.61)
18:3n-6	1.13	0.99	1.09	1.10	1.02	1.38	1.12 (0.13)
18:3n-3	1.30	1.27	1.33	1.21	1.20	1.35	1.28 (0.06)
20:3n-6	0.37	0.38	0.42	0.49	0.49	0.38	0.42 (0.05)
20:4n-6	1.42	1.54	1.50	1.40	1.35	1.35	1.43 (0.07)
20:5n-3	0.72	0.77	0.77	0.70	0.67	0.75	0.73 (0.04)
22:5n-3	0.49	0.44	0.45	0.48	0.69	0.36	0.49 (0.10)
22:6n-3	1.94	1.88	1.96	1.98	2.40	2.55	2.12 (0.26)
Total μg/μL	0.37	0.40	0.41	0.46	0.45	0.50	0.43 (0.04)

[a]Fatty acid composition is expressed as weight percent.
[b]Values are the mean with the standard deviation given in parentheses.

major lipid fractions were the same with both methods. The fatty acid compositions of the triglycerides and non-esterified fatty acids were primarily comprised of 16:0, 18:0, 18:1 and 18:2 (90%) with only 0.6 to 1% of the n-3 fatty acid, 18:3n-3, and very small amounts of the 20- and 22-carbon HUFA (Table 2). In contrast, the plasma phospholipids contained significant amounts of both n-6 HUFA (20:3n-6, 2.7%; 20:4n-6, 9.1%) and n-3 HUFA (20:5n-3, 1.1%; 22:5n-3, 1.1% 22:6n-3, 3.3%) and also higher levels of 18:2n-6 (21%). The absence of 18:2n-6 and 18:3n-3 from Table 2 illustrates how minor components may not be detected quantitatively with such small samples.

The fatty acid compositions for the major types of plasma lipids (Table 2) are similar to those reported in 1979 (14) for typical Americans, although the level of 18:2n-6 in the plasma TG of the young Chicagoans in this study (15.9%) is slightly higher than the 14.4% reported 10 years ago for semi-rural individuals (14). On the other hand, both values are higher than the 12.5% reported for Finnish individuls (15). There is good general agreement among the three studies that fatty acids in plasma phospholipids are about 45% saturated, about 35% C_{16} and C_{18} unsaturated, and about 20% HUFA. Historical increases in dietary 18:2n-6 may underlie some differences found in the literature. For example, a recent report (16) showed that total plasma fatty acids contained 35% 18:2n-6 as compared to only 27% observed in 1979 (14). Unfortunately, the recent work failed to resolve the major lipid classes, making it difficult to discern whether the high level of 18:2n-6 was attributable to greater ingestion (reflected in triglyceride 18:2n-6) or to greater contents of cholester esters (with their typical high contents of 18:2n-6). The relatively low content of 16:1 and high contents of 18:2n-6 and 20:4n-6 in the 1987 report (16) suggests that individuals in that study ingested greater amounts of n-6 nutrients as compared to the Minnesota individuals in 1973 to 1975, who were described in the 1979 report (14).

Because wide range screening of plasma fatty acids from different individuals would be easiest with a single finger-tip sample (50 μL of blood; 20 μL of plasma), we tested the analytical procedure with small amounts of plasma. Table 3 compares the results with direct application to the TLC plate of different amounts of plasma from the same individual, and it indicates that 10 μL samples may be unsatisfactory. Analyses were

performed routinely on phospholipids and triglycerides because the very low concentration of non-esterified fatty acid in plasma requires large samples (>100 μL) for reliable assay, and the cholesteryl esters require additional treatment to remove cholesterol that interferes in chromatography. Analytical results with aliquots of 5 to 150 μL of plasma were very similar (results not shown), although the triglyceride fraction scraped from the TLC plate showed a systematic rise in the calculated amount of methyl stearate (18:0) with progressively smaller samples, indicating that a slight impurity co-chromatographing with methyl stearate on gas chromatography had been present in that TLC region. All other fatty acids appeared to be measured without such appreciable interference.

The present streamlined method permits collecting blood samples from 12 to 20 individuals in the morning, resolving the lipid fractions by TLC, converting them to methyl esters in the afternoon, and analyzing overnight the fatty acid composition of the phospholipids and triglycerides with the aid of the autosampler. We believe that the demonstrated success of this combination of methods provides clinical epidemiologists with a suitable tool for large-scale screening of plasma samples from selected populations.

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A Method for the Quantitative Analysis of Molecular Species of Alkylacylglycerol and Diacylglycerol

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We describe a method for the quantitative analysis of molecular species of diacylglycerol and alkylacylglycerol as their diradylglycerobenzoate derivatives. Synthetic internal standards were used to provide quantitative determinations of the low levels of diacylglycerol and alkylacylglycerol and their individual molecular species in cultured cells. Diradylglycerols were isolated by thin-layer chromatography (TLC), converted to their benzoate derivatives and separated into subclasses by TLC. The molecular species of each subclass were analyzed by reversed-phase high performance liquid chromatography. Thirty-six species of diglyceride-type molecules were identified in Madin-Darby canine kidney cells. These cells were shown to contain 7.88 nmoles of diacylglycerol and 3.97 nmoles of alkylacylglycerol per μmole of phospholipid. Both subclasses contain predominantly monoenoic and saturated species. This technique should be valuable for studies examining the origin and metabolism of these important intracellular mediators.
Lipids 25, 748–752 (1990).

The hydrolysis of cell membrane phospholipids to produce diradylglycerols is an important link between receptor occupancy and cellular response for many hormones and neurotransmitters. Diradylglycerols serve as activators of protein kinase C during cell stimulation (1). In Madin-Darby canine kidney (MDCK) cells, protein kinase C activation has been linked to arachidonic acid release in response to both phorbol esters (2) and hormones (3,4). Recent studies have shown that both diacylglycerol (DAG) and alkylacylglycerol (AAG) are generated during stimulation of neutrophils (5,6) and MDCK cells (7). Although DAG is thought to act through its ability to activate protein kinase C, the biological role of AAG in signal transduction is less clear, as it has been reported to inhibit (8) or stimulate (9) protein kinase C, and to induce other unique cellular responses (10,11). In addition to their role in signal transduction, both DAG and AAG serve as intermediates in the synthesis of phospholipids and their content in unstimulated cells may reflect the rates of phospholipid synthesis and turnover.

Recent interest in cellular diradylglycerol metabolism has led to the development of several techniques for their analysis. Quantitation of total diradylglycerols has been performed using *E. coli* DAG kinase and [^{32}P]ATP conversion of diradylglycerols to [^{32}P]phosphatidic acid (12). The procedure has been refined for separate measurement of DAG and AAG by selective destruction of the DAG fraction with *Rhizopus* lipase (13) or alkaline hydrolysis (6). Individual subclasses of diradylglycerols have been measured after separation by thin-layer chromatography (TLC) using staining and densitometry (14). The fatty acid composition of DAG has been examined for comparison to potential precursor lipids as a means of identifying its metabolic source (15–18). Analysis of the molecular species composition of diradylglycerols is a more powerful approach than fatty acid analysis alone since it yields more precise structural information. Several procedures have been used to examine molecular species of phospholipids by high performance liquid chromatography (HPLC) or gas liquid chromatography (GLC) as derivatives of their diradylglycerol products (19–22). However, few studies have examined the species composition of native diradylglycerols, and these were limited to the diacyl fraction (23–27).

In this paper, we describe the analysis of molecular species of diradylglycerols from MDCK cells. Diradylglycerols were examined by HPLC as their benzoate derivatives using procedures adapted from those developed by Blank *et al.* (19) for the analysis of phospholipid species. The technique described here provides a sensitive means of determining quantities of the DAG and AAG subclasses and their individual molecular species. The method also allows resolution of the *sn*-1(3),2 and *sn*-1,3 isomers of the DAG species.

MATERIALS AND METHODS

Materials. Fatty acid anhydrides were purchased from Nu-Check Prep (Elysian, MN). Solvents were HPLC grade from Burdick and Jackson (Muskegon, MI). TLC plates coated with silica gel H were from Analtech (Newark, DE). All other lipids, enzymes and other reagents, unless indicated, were from Sigma Chemical Co. (St. Louis, MO). 1-[^{14}C]Palmitoyl-2-decanoyl-*sn*-glycerophosphocholine was synthesized from 1-[^{14}C]palmitoyl-glycerophosphocholine (New England Nuclear, Boston, MA) by the method of Gupta *et al.* (28) using capric anhydride. The product was converted to the diglyceride form using phospholipase C from *Bacillus cereus* as described by Mavis *et al.* (29). Unlabelled 1-palmitoyl-2-decanoyl-*sn*-glycerol was synthesized by partial acylation of 1-palmitoyl-*sn*-glycerol. The monoglyceride (10 mg) was incubated at room temperature with capric anhydride (6.5 mg) and dimethylaminopyridine (6 mg) for 3 hr in 0.2 mL chloroform/pyridine (4:1, v/v). The diglyceride was isolated from the reaction products by TLC using the solvent system hexane/diethyl ether/ammonium hydroxide (40:60:1, v/v/v). The 1,2- and 1,3-isomers were subsequently separated by TLC using hexane/diethyl ether (40:60, v/v). 1-*O*-Hexadecyl-2-decanoyl-*sn*-glycerol was synthesized in a similar manner from 1-*O*-hexadecylglycerol. 1-[^{14}C]Palmitoyl-2-decanoyl-*sn*-glycerol was diluted with the unlabelled compound to a specific radioactivity of 3.29 μCi/μmol.

Cell culture. MDCK cells (American Type Culture Collection, Rockville, MD) were cultured in Dulbecco's Modified Eagle Media with 10% heat inactivated fetal bovine serum, penicillin (100 U/mL) and streptomycin

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Abbreviations: AAG, alkylacylglycerol; DAG, diacylglycerol; GLC, gas liquid chromatography; HPLC, high performance liquid chromatography; MDCK, Madin-Darby canine kidney; TLC, thin-layer chromatography.

(100 μg/mL), and were passaged by trypsinization. Cells were harvested at, or near, confluence (1.6–1.9 × 10^5 cells/cm^2).

Isolation and derivatization of diradylglycerols. Cell monolayers were scraped into methanol after rinsing with serum-free medium. 1-[^{14}C]Palmitoyl-2-decanoyl-*sn*-glycerol (12.5 nmol) and 1-*O*-hexadecyl-2-decanoyl-*rac*-glycerol (8.5 nmol) were added as internal standards to monitor recovery of DAG and AAG, and the lipids were extracted by the method of Bligh and Dyer (30). The total diglyceride fraction was isolated by TLC using the solvent system hexane/diethyl ether (40:60, v/v) on silica gel H plates that had been impregnated by spraying with boric acid (0.25 M in ethanol/water, 50:50, v/v) before activation. The low levels of free diradylglycerols present in MDCK cells precluded their direct visualization on TLC plates and a broad band was therefore isolated that corresponded to the migration of diradylglycerol standards and which included the cholesterol fraction. Lipid fractions were visualized by spraying with dichlorofluorescein, the respective adsorbent areas were scraped off, and the lipids were extracted with hexane/ethanol/water (1:1:1, v/v/v). The diglyceride fraction was converted to the benzoate derivatives as described by Blank *et al.* (19) using 2 mg dimethylaminopyridine and 5 mg benzoic anhydride in 0.15 mL of benzene. After 2 hr shaking at room temperature, 0.5 mL ammonium hydroxide was added and the products were extracted twice with 2 mL of hexane. The diradylglycerobenzoates were separated into alkylacyl, alk-1-enylacyl, and diacylglycerol subclasses by TLC as described previously (19), using the solvent system benzene/hexane/diethyl ether (50:45:5, v/v/v). Once it was determined that alk-1-enylacylglycerol was not present in the diglyceride fraction of MDCK cells, the solvent system hexane/diethyl ether (45:5, v/v) was used (in which the alk-1-enylacyl and alkylacyl fractions would comigrate), because it produced better separation of the DAG and AAG fractions. The diradylglycerobenzoates were also separated from the cholesteryl benzoate during this step. The subclasses were extracted from the silica gel with hexane/ethanol/water (1:1:1, v/v/v).

HPLC analysis. The molecular species of the isolated subclasses were analyzed by reversed-phase HPLC using a modification of the procedures described by Blank *et al.* (19). The diacylglycerobenzoate species were resolved using an Ultrasphere-ODS column (Altex, Berkley, CA; 4.5 × 250 mm, 5 μm particle size) and a mobile phase of acetonitrile/isopropanol (70:30, v/v) at 35°C with a flow rate of 1.4 mL/min and detection at 230 nm.

Alkylacylglycerobenzoate species were resolved similarly using a mobile phase acetonitrile/isopropanol (63:37, v/v). Data collection and peak integration from HPLC was carried out with a Maxima Chromatography Workstation (Dynamic Solutions, Millipore, Milford, MA). The mass of the species of diradylglycerobenzoates was quantified by measuring the absorbance at 230 nm using 13,200 as the molar absorptivity. This value was confirmed by analysis of the diacylglycerobenzoate prepared from [^{14}C]dipalmitoyl phosphatidylcholine of known specific radioactivity. The content of AAG and DAG present in the original lipid extracts of MDCK cells was calculated from the total areas of the peaks eluting from HPLC after correcting for recovery of the internal standard of each subclass. The peaks eluting from HPLC were identified by their retention times relative to synthetic standards and by GLC analysis.

Identification of HPLC fractions by GLC. For diacylglycerobenzoates, collected fractions were subjected to methanolysis (0.6% sodium methoxide in methanol for 15 min), and the methyl ester derivatives of the acyl groups were analyzed using a DB-23 capillary column (J&W Scientific, Folsom, CA) with splitless injection in hexane. The initial oven temperature was 40°C for 0.5 min and increased to 140°C at 70°C/min, followed by an increase to 250°C at 5°C/min and then maintained at 250°C for 10 min. Following methanolysis of the alkylacylglycerobenzoate species, the alkylglycerols were converted to alkyl-2,3-trimethylsilylglycerols by reaction with *bis*(trimethylsilyl)trifluoroacetamide and 1% trimethylchlorsilane (Regis Chemical Co., Morton Grove, IL; 50 μL in 150 μL pyridine for 10 min at 50°C). The trimethylsilyl derivatives of alkylglycerols and the methyl esters derived from the acyl groups were resolved simultaneously using the conditions described for methyl ester analysis.

Phospholipid phosphorus determination and statistics. Total lipid phosphorus was measured by the method of Rouser *et al.* (31). All values are expressed as mean ± sample standard deviation with the number of replicates given in parentheses.

RESULTS AND DISCUSSION

The procedures described here were developed to determine the quantities of molecular species of DAG and AAG in cultured cells. The total diradylglycerol fraction was isolated by TLC, together with cholesterol, from lipid extracts of MDCK cells and converted to the diradylglycerobenzoate derivatives. A second TLC fractionation separated the benzoate derivatives of the diglyceride fraction into subclasses and removed cholesteryl benzoate and the benzoylation reagents. The species of DAG and AAG were resolved by reversed-phase HPLC and quantities of individual species were calculated from the absorbance at 230 nm. 1-[^{14}C]Palmitoyl-2-decanoyl-*sn*-glycerol and 1-*O*-hexadecyl-2-decanoyl-*sn*-glycerol were synthesized for use as internal standards to measure the recovery of DAG and AAG, respectively. These species were used because their benzoate derivatives are separated from all endogenous diradylglycerol species by HPLC. Radiolabelling the internal standard served as a convenient means of measuring the mass once the specific radioactivities were determined.

When mixtures of synthetic diacylglycerols (14:0-14:0, 16:0-16:0 and 18:0-18:0) were analyzed to establish recoveries, the calculated concentrations were found to be 99.5 ± 3.8% (n=9) of original concentrations. The average recovery of the benzoyl derivatives was 76.5%, and recovery was not dependent upon the type of species. We have reproducibly measured total cellular DAG or AAG from cultured cells at levels as low as 250 pmol and single species as low as 50 pmol, although the limit of sensitivity depends on detector noise and the number of diglyceride-type species present. Measurements of total DAG and AAG by HPLC were routinely performed on samples from 10–15 × 10^6 MDCK cells. Measurements of individual species of DAG and AAG required ten-fold larger amounts of cells to provide sufficient material for GLC

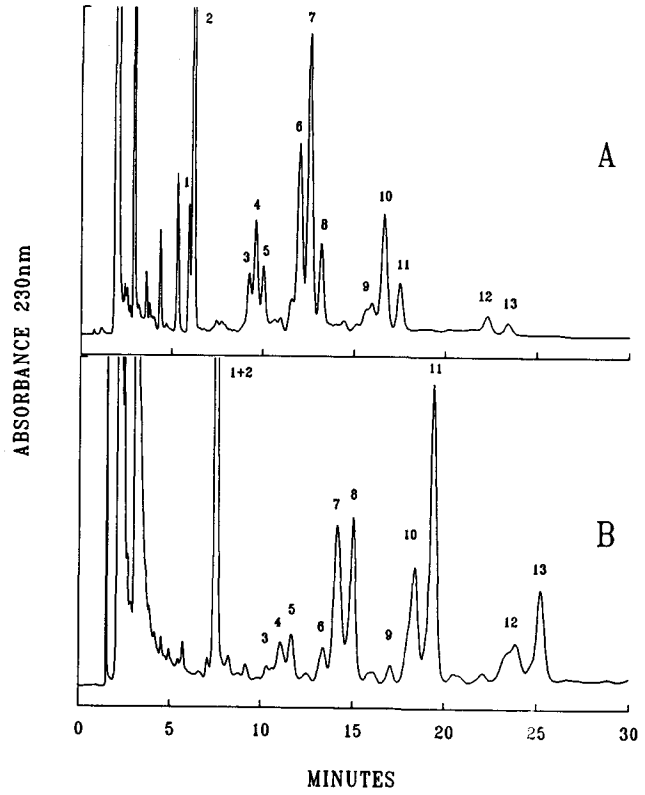

FIG. 1. HPLC separations of benzoate derivatives of molecular species of diacylglycerol (A) and alkylacylglycerol (B) from MDCK cells. Peak identification numbers refer to species shown in Table 1.

analysis of the fractions of DAG and AAG eluting from HPLC.

The HPLC elution patterns of the benzoate derivatives of DAG and AAG from MDCK cells are shown in Figure 1. Since no peaks were detected upon HPLC examination of the area on TLC corresponding to the alk-1-enylacyl fraction of the diradylglycerobenzoates (n=3), we conclude that MDCK cells do not contain a significant amount of alk-1-enylacylglycerol. We observed a significant degree of isomerization of DAG due to acyl migration during isolation from MDCK cells. The isomerization was reduced, but not eliminated, by using TLC plates impregnated with boric acid and limiting the time which samples were kept dry. Figure 2 shows the HPLC elution patterns of the benzoate derivatives of DAG from MDCK cells when the 1,2- and 1,3-isomers were isolated separately or together during the initial TLC preparation (although the isomer is referred to as 1,2-DAG, the 2,3-DAG enantiomer may also be present). The DAG fraction could be resolved into 11 separate peaks of 1,2-DAG and 11 peaks of 1,3-DAG which overlap and interdigitate in the unfractionated DAG to form 15 peaks. The similar patterns of 1,3-DAG and 1,2-DAG indicate that each species contains a similar proportion of the 1,3 isomer. We used 16:0-10:0 DAG, that was ≥98% in the 1,2 form, as internal standard to monitor and correct for the isomerization of cellular DAG during sample preparation. Since the 1,3 isomer content of the benzoate derivatives of DAG from MDCK cells was consistently similar to the 1,3 content of the internal standard derivative, we expect that the 1,3-DAG species from MDCK cells arose due to isomerization during their preparation and are not present in significant amounts *in vivo*. The positional isomers of the alkylacylglycerobenzoate species were not well resolved on reversed-phase HPLC and it was not possible to determine if the endogenous AAG fraction contains a significant amount of the 1,3 isomer.

The identities and quantities of molecular species of diradylglycerols from MDCK cells are shown in Table 1. The retention times of the species in peaks eluting from HPLC corresponded to those of synthetic standard

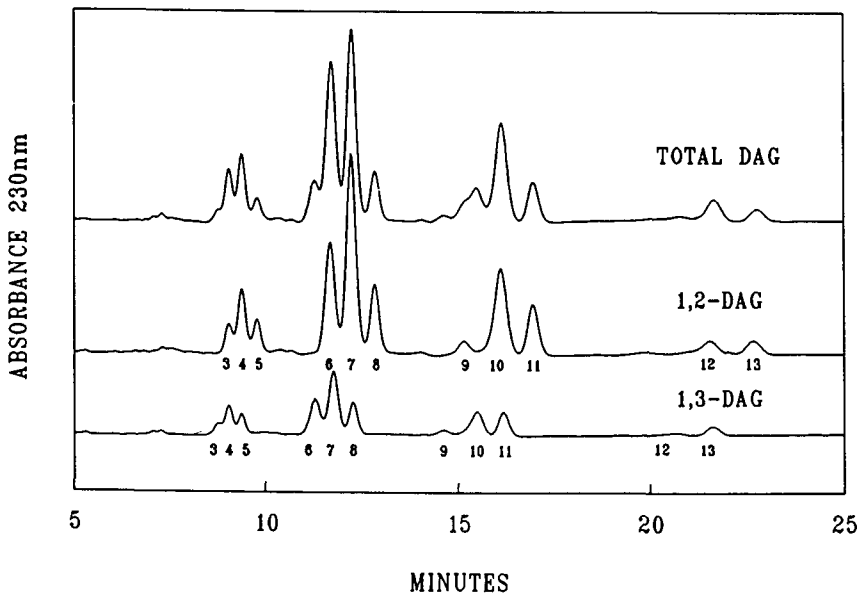

FIG. 2. HPLC separation of benzoate derivatives of diacylglycerol isolated from MDCK cells as: total diacylglycerol (upper), 1,2-diacylglycerol (middle), or 1,3-diacylglycerol (lower). Peak identification numbers refer to species given in Table 1.

TABLE 1

Identification and Quantities of Molecular Species of Diradylglycerols from MDCK Cells

Peak number[a]	Species[b]	Diacylglycerol[c] nmol/μmol phospholipid	%	Alkylacylglycerol[d] nmol/μmol phospholipid	%
1	16:0-10:0 *sn*-1,2	internal standard	—	internal standard	—
2	16:0-10:0 *sn*-1,3	internal standard	—	internal standard	—
3	16:1-18:1	0.35 ± 0.06	4.5	0.05 ± 0.02	1.2
4	14:0-18:1/16:0-16:1	0.61 ± 0.04	7.8	0.13 ± 0.02	3.4
5	14:0-16:0	0.34 ± 0.05	4.3	0.13 ± 0.02	3.3
6	18:1-18:1	1.25 ± 0.18	15.9	0.11 ± 0.01	2.8
7	16:0-18:1/18:0-16:1	1.75 ± 0.14	22.3	0.58 ± 0.06	14.6
8	16:0-16:0/14:0-18:0	0.67 ± 0.10	8.4	0.50 ± 0.08	12.5
9	18:1-20:1	0.30 ± 0.08	3.8	0.06 ± 0.01	1.6
10	18:0-18:1/20:0-16:1	0.87 ± 0.15	11.1	0.51 ± 0.05	13.0
11	16:0-18:0/14:0-20:0	0.40 ± 0.06	5.0	1.01 ± 0.15	25.4
12	18:0-20:1/20:0-18:1	0.13 ± 0.02	1.6	0.26 ± 0.04	6.6
13	18:0-18:0/16:0-20:0	0.10 ± 0.02	1.3	0.39 ± 0.05	9.7
	Other	1.10 ± 0.31	14.1	0.24 ± 0.08	6.1
	Total	7.88 ± 0.40	100	3.97 ± 0.36	100

[a] Numbers correspond to peaks in Figures 1 and 2.

[b] Denotes the length of the carbon chain and the number of double bonds in the two aliphatic groups.

[c] Values are nmol *sn*-1,2-diacylglycerol per μmol phospholipid ± sample standard deviation for five separate determinations. Peak areas were adjusted to correct for isomerization based on the amount of *sn*-1,3 internal standard generated during preparation of the samples.

[d] Values are nmol alkylacylglycerol (including *sn*-1,3 isomers, if present) per μmol phospholipid for five separate determinations.

diradylglycerols. The aliphatic groups present in species from individually collected peaks were also examined by GLC analysis in two separate preparations. The acyl groups of diglyceride benzoates were analyzed as methyl ester derivatives and, for the alkylacylglycerobenzoates, the alkylglycerol moieties were analyzed as *bis*(trimethylsilyl) derivatives. The diradylglycerol content of MDCK cells was found to be 7.88 ± 0.40 and 3.97 ± 0.36 nmoles per μmole phospholipid for DAG and AAG, respectively (n=5). The DAG of MDCK cells is composed of species containing saturated and monoenoic acyl groups, with high amounts of species containing palmitate, stearate and oleate. This is unlike the DAG from rat hepatocytes (25) and a Chinese hamster embryo fibroblast cell line (26), which contain a significant amount of species with polyunsaturated acyl groups. These differences in the degree of unsaturation of the DAG may reflect the extent to which these molecules arise through *de novo* synthesis or from the turnover of phospholipids. The fatty acid content of the serum may also influence the acyl group composition of diradylglycerol.

We determined that AAG represents 33.5% of the total diglyceride fraction of MDCK cells. Although AAG is reported to be an important intracellular mediator with biological properties which may differ from DAG, there have been few reports on the quantity of AAG in resting or stimulated cells. AAG has been reported to be approximately equal to (14) or slightly less than (13) DAG in human neutrophils and absent in Chinese hamster ovary fibroblasts (26). The analysis of the molecular species of AAG presented here is the first that has been reported. The AAG fraction of MDCK cells is composed of species with saturated and monoenoic aliphatic groups. The level of fully saturated species is particularly high, constituting 50% of the total ether-linked diradylglycerols.

Our studies show that subclasses and molecular species of diglyceride-type components of cultured cells can be quantitatively measured by HPLC as their benzoate derivatives. The method offers an improved approach to diradylglycerol analysis since it provides both quantitation and the power of molecular species analysis. Importantly, the individual DAG and AAG subclasses can be analyzed separately. The sensitivity appears to be adequate to determine the patterns of species of DAG and AAG in most systems. This technique should prove to be a useful tool in studies that examine the origin and metabolism of diradylglycerols. It may also be useful in providing information with regard to the activation of protein kinase C by different species or subclasses of diradylglycerols in stimulated cells.

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Capillary Gas-Liquid Chromatographic Separation of Oxo and Oxo-Hydroxy Bile Acid Isomers of the *allo* and Normal Series

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Capillary gas-liquid chromatographic separation was studied for 60 oxo and oxo-hydroxy bile acids of the *allo* (5α-H, A/B-*trans*) and normal (5β-H, A/B-*cis*) series, which differ from one another in the number, position, and configuration of oxo and hydroxy groups at positions C-3, C-4, C-6, C-7, and/or C-12. Good separation of the isomers was achieved by the use of an aluminum-clad fused-silica capillary column coated with bonded and cross-linked methyl polysiloxane.
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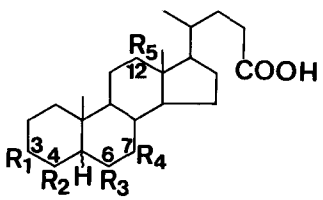
There have been a number of reports on the gas-liquid chromatographic separation, identification, and quantification of bile acids, after suitable derivatization, on non-selective and/or selective phases (1–3). However, most studies have been primarily concerned with derivatives of the hydroxylated bile acids (4,5). Similar studies on oxo and oxo-hydroxy bile acids of both the *allo* (5α-H; A/B-*trans*) and the normal (5β-H; A/B-*cis*) series, despite of their biochemical interest (6,7), have been hampered by the limited availability of these compounds.

As spin-off of a synthetic program on potential bile acid metabolites, a variety of C_{24} oxo and oxo-hydroxy bile acids related to 5α- and 5β-cholanoic acids have been made available. The compounds differ from each other in the number, position and configuration of the substituents at positions C-3, C-4, C-6, C-7, and/or C-12 (Scheme 1). In



R_1, R_2, R_3, R_4, R_5 = O, OH or H

SCHEME 1

the present paper, we report the gas-liquid chromatographic separation of sixty oxo and oxo-hydroxy bile acids of the 5α and 5β series on a non-selective capillary column. An aluminum-clad fused-silica capillary column coated with a very thin film (0.1 μm) of a highly thermostable, bonded and cross-linked methyl polysiloxane (equivalent to OV-101) was used. This column provides excellent separations and short analysis times in gas-liquid chromatography (GC) (8).

EXPERIMENTAL PROCEDURES

Samples and reagents. Almost all of the C_{24} 5α- and 5β-cholanoic acids with 1–3 oxo groups at positions C-3, C-6, C-7, and/or C-12 were from our laboratory collections. All solvents were analytical reagent grade and were used without further purification.

The silylating reagents, TMS-HT (hexamethyldisilazane and trimethylchlorosilane in anhydrous pyridine) and *N*-dimethylethylsilylimidazole (DMESI), were obtained from Tokyo Kasei Kogyo Co. (Tokyo, Japan).

GC instrument and column. A Shimadzu GC-7A gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector, capillary split injector, and data-processing system (Chromatopac C-R3A; Shimadzu) was used. The GC instrument was fitted with an aluminum-clad flexible fused-silica capillary column (25 m × 0.25 mm I.D.) with a thin film (0.1 μm) of bonded and cross-linked methyl polysiloxane (equivalent to OV-101). It was operated isothermally under the following conditions: carrier gas flow rate, 1.5 mL/min (helium); splitting ratio, 1:50; and column temperature, 260°C. The column, a HiCap CBPM1, was purchased from Shimadzu.

GC derivatization. Initially, oxo and oxo-hydroxy bile acid samples were converted into their C-24 methyl esters by treatment with diazomethane in diethyl ether (4). The oxo-hydroxy bile acid methyl esters were further converted to their methyl ester trimethylsilyl (Me-TMS) and methyl ester dimethylethylsilyl (Me-DMES) ethers using TMS-HT and DMESI, respectively (4). Aliquots of the derivatized oxo and oxo-hydroxy bile acid solutions were injected into the GC column together with an internal reference standard.

RESULTS AND DISCUSSION

Table 1 shows the relative retention times (RRT) and methylene unit (MU) values of 60 oxo and oxo-hydroxy bile acids on a non-selective capillary column (HiCap CBPM1; Shimadzu). RRT values are expressed relative to the methyl ester silyl ether derivatives of deoxycholic (3α,12α-dihydroxy-5β-cholanoic) acid (DCA), and MU values were determined relative to C_{22}-C_{38} *n*-alkanes. The differences in MU values between analogous 5α- and 5β-derivatives are expressed as $\Delta MU_{\alpha\text{-}\beta}$, and $\Delta MU_{D\text{-}T}$ gives the differences between the Me-TMS and Me-DMES values for the same compound (4).

In order to facilitate a comparison of the retention data with those of hydroxylated bile acids that have previously been reported (4,5), TMS and DMESI ethers were chosen as the hydroxyl derivatives of oxo-hydroxy bile acids. Use of this column, instead of a selective liquid phase, such as QF-1 (9) or PEG-HT (10), offered the advantage of a rapid and distinct separation of ketonic bile acid isomers

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Abbreviations: DCA, deoxycholic acid, 3α,12α-dihydroxy-5β-cholanoic acid; DMESI, *N*-dimethylethylsilylimidazole; GC, gas-liquid chromatography; Me-DMES, methyl ester dimethylethylsilyl; Me-TMS, methyl ester trimethylsilyl; MU, methylene unit; RRT, relative retention time; TMS-HT, hexamethyldisilazane and trimethylchlorosilane in anhydrous pyridine.

TABLE 1

RRT and MU Values of Oxo and Oxo-Hydroxy Bile Acids of the 5α and 5β Series on HiCap CBPM1 Column[a]

Methyl cholanoate	Me-TMS					Me-DMES					ΔMU_{D-T}	
	RRT		MU			RRT		MU				
Substituents	5α	5β	5α	5β	$\Delta MU_{\alpha-\beta}$	5α	5β	5α	5β	$\Delta MU_{\alpha-\beta}$	5α	5β
Ketone												
3-OXo	0.97	0.89	31.76	31.40	0.36	0.58	0.54					
6-Oxo	0.89	0.77	31.40	30.84	0.56	0.54	0.46					
7-Oxo	0.84	0.71	31.18	30.79	0.39	0.51	0.43					
12-Oxo	0.77	0.69	30.84	30.66	0.18	0.46	0.42					
3,6-Dioxo	1.55	1.55	33.62	33.62	0	0.93	0.93					
3,7-Dioxo	1.45	1.27	33.37	32.94	0.43	0.87	0.75					
3,12-Dioxo	1.45	1.32	33.37	33.05	0.32	0.87	0.80					
7,12-Dioxo	1.19	1.01	32.56	32.00	0.56	0.72	0.61					
3,7,12-Trioxo	2.00	1.70	34.63	34.00	0.63	1.20	1.02					
Monooxo-monohydroxy												
3-Oxo-4β-OH		1.11		32.31			0.81		33.09			
3-Oxo-6α-OH		1.36		33.12			1.08		34.22			
3-Oxo-6β-OH		1.18		32.54			0.94		33.69			
3-Oxo-7α-OH	1.10	1.23	32.26	32.70	−0.44	0.86	0.94	33.30	33.69	−0.39	1.04	0.99
3-Oxo-7β-OH	1.49	1.35	33.47	33.07	0.40	1.18	1.04	34.56	34.10	0.46	1.09	1.03
3-Oxo-12α-OH	1.21	1.11	32.63	32.31	0.32	0.92	0.81	33.58	33.09	0.49	0.95	0.78
3-Oxo-12β-OH	1.21	1.06	32.63	32.11	0.52	0.98	0.83	33.82	33.16	0.66	1.19	1.05
6-Oxo-3α-OH	1.39	1.39	33.23	33.23	0	1.13	1.11	34.39	34.31	0.08	1.16	1.08
6-Oxo-3β-OH	1.81		34.24			1.51		35.52			1.28	
7-Oxo-3α-OH		1.36		33.12			1.07		34.18			1.06
12-Oxo-3α-OH		1.36		33.12			1.07		34.18			1.06
12-Oxo-3β-OH		1.26		32.82			1.04		34.06			1.24
12-Oxo-7α-OH	0.92	0.97	31.59	31.81	−0.22	0.73	0.76	32.64	32.81	−0.17	1.05	1.00
12-Oxo-7β-OH	1.24	1.10	32.73	32.29	0.44	0.94	0.88	33.65	33.40	0.25	0.92	1.11
Monooxo-dihydroxy												
3-Oxo-7α,12α-$(OH)_2$	1.23	1.38	32.73	33.17	−0.44	1.27	1.40	34.84	35.25	−0.41	2.11	2.08
6-Oxo-3α,7β-$(OH)_2$	15.9		33.74			1.60		35.75			2.01	
7-Oxo-3α,6α-$(OH)_2$		1.52		33.56			1.54		35.62			2.06
7-Oxo-3β,6α-$(OH)_2$		1.52		33.56			1.60		35.77			2.21
7-Oxo-3α,12α-$(OH)_2$		1.60		33.76			1.57		35.70			1.94
7-Oxo-3β,12α-$(OH)_2$		1.52		33.56			1.51		35.55			1.99
12-Oxo-3α,7α-$(OH)_2$	1.63	1.68	33.84	33.94	−0.10	1.71	1.71	36.03	36.03	0	2.09	2.09
12-Oxo-3α,7β-$(OH)_2$	1.81	1.81	34.24	34.24	0	1.88	1.88	36.39	36.39	0		
12-Oxo-3β,7α-$(OH)_2$	1.67	1.49	33.94	33.48	0.46	1.73	1.53	36.08	35.58	0.50	2.14	2.10
12-Oxo-3β,7β-$(OH)_2$	2.27	1.77	35.15	34.16	0.99	2.39	1.88	37.33	36.39	0.94	2.18	2.23
Dioxo-monohydroxy												
7,12-Dioxo-3α-OH		1.89		34.42			1.54		35.61			1.19
7,12-Dioxo-3β-OH		1.75		34.11			1.46		35.39			1.28
Miscellaneous												
3-Oxo-Δ^4	(1.14)		(32.44)			(0.69)						
3-Oxo-7α-OH-Δ^4	(1.29)		(32.95)			(1.02)		(34.01)			(1.06)	
3-Oxo-12α-OH-Δ^4	(1.39)		(33.23)			(1.08)		(34.23)			(1.00)	
3-Oxo-7α,12α-$(OH)_2$-Δ^4	(1.50)		(33.52)			(1.55)		(35.65)			(2.13)	

[a]RRT values were expressed relative to the methyl ester silyl ether derivatives of deoxycholic (3α,12α-dihydroxy-5β-cholanoic) acid (DCA). The designations 5α and 5β refer to 5α- and 5β-cholanoates, respectively. $\Delta MU_{\alpha-\beta}$ and ΔMU_{D-T} values, respectively, show the difference in the MU values between 5α and 5β series (a negative value denotes that the retention time of the 5β derivatives is longer than that of the 5α) and the difference in the MU values between the Me-DMES and Me-TMS ethers. MU values for the Me-DMES ether derivatives of oxo bile acids are the same as the corresponding Me-TMS ethers.

without prior derivatization of carbonyl groups (Fig. 1). An attempt to obtain the *O*-methyloxime-TMS ethers of oxo-hydroxy bile acid methyl esters by a one-step derivatization of carbonyl and hydroxyl groups by reaction with *N*-methoxy-*N,O-bis*-(trimethylsilyl)carbamate (11) did not provide satisfactory results on this column; the products often gave multiple peaks, probably due to formation of the *syn* and *anti* isomers of *O*-methyloxime (12).

For eight monoketones differing in the position and stereochemistry of the A/B-ring fusion, the following order of mobility was observed on the HiCap CBPM1 column: (i) in each series oxo substituents in the methyl cholanoate nucleus increases the retention time in the order of C-12 < C-7 < C-6 < C-3; and (ii) the monoketones in the 5β series moved faster than the corresponding 5α analogs (positive $\Delta MU_{\alpha-\beta}$ values). The two pairs, 3- (5β) *vs* 6- (5α) and 6- (5β) *vs* 12- (5α) ketones, could not be separated with this column.

The dioxo compounds likewise resemble the monoketones in that the relative mobilities of analogous

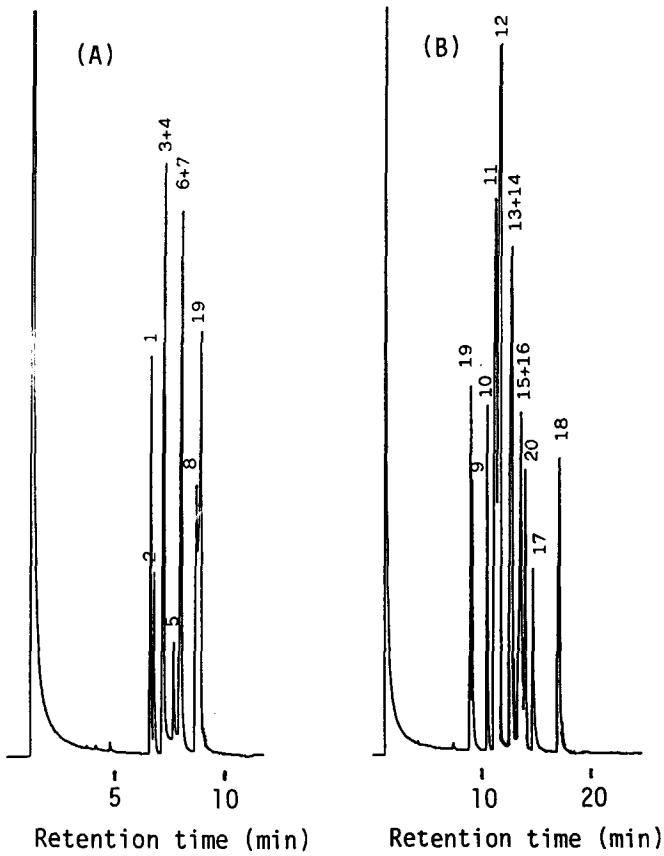

FIG. 1. Capillary GC of a mixture of (A) mono- and (B) di- and trioxo bile acid methyl esters on HiCap CBPM1. Peak identification and position of oxo groups: 1, 12 (5β); 2, 7 (5β); 3, 6 (5β); 4, 12 (5α); 5, 7 (5α); 6, 3 (5β); 7, 6 (5α); 8, 3 (5α); 9, 7,12 (5β); 10, 7,12 (5α); 11, 3,7 (5β); 12, 3,12 (5β); 13, 3,7 (5α); 14, 3,12 (5α); 15, 3,6 (5α); 16, 3,6 (5β); 17, 3,7,12 (5β); 18, 3,7,12 (5α); 19, Me-TMS of DCA; and 20, Me-DMES of DCA.

derivatives of the two C-5 series follow a similar order, *i.e.*, 7,12- < 3,7- < 3,12- < 3,6-diketones, and each of the C-5 epimeric pairs usually gave positive $\Delta MU_{\alpha-\beta}$ values. However, the C-5 epimeric 3,6-diketones had identical retention times, indicating the possibility that GC may cause rearrangement of 5β-3,6-diketone to its more stable 5α-epimer on the column (13).

For monooxo-monohydroxy, monooxo-dihydroxy, and dioxo-monohydroxy compounds, the effect of hydroxyl groups on the retention times was more pronounced with this phase than the effect of oxo groups. The equatorially hydroxylated 3α-epimers in the 5β series, regardless of the number and position of oxo groups, were usually eluted after the corresponding axial 3β-epimers, while the reverse (*axial* 3α < *equatorial* 3β) was true within the 5α series, because conformationally the C-3 hydroxyl groups are reversed in the 5α- and 5β-compounds. In addition, the presence of an *equatorial* 7β-hydroxyl group in both series causes a more pronounced retarding effect than that of the *axial* 7α ones.

Although 3-keto-Δ^4 derivatives often give two GC peaks on a QF-1 column, probably due to the formation of a degradation product (*e.g.*, enol TMS ethers) (3,14), these compounds gave a single peak with HiCap CBPM1 column.

Of the two sets of 21 oxo and oxo-hydroxy compounds of the 5α and 5β series examined, most of the pairs were well separated on this column as either Me-TMS or Me-DMES ethers ($\Delta MU_{\alpha-\beta} \neq 0$), and usually gave positive $\Delta MU_{\alpha-\beta}$ values with a few exceptions [*i.e.*, C-5 epimeric 3-oxo-7α-OH, 12-oxo-7α-OH, and 3-oxo-7α,12α-$(OH)_2$]. A comparison of the MU values between the Me-TMS and Me-DMES derivatives shows that in general they are quite similar, suggesting that the structure of the silyloxy group has little effect on the degree of separation. In analogy with hydroxylated bile acids reported previously (4), nearly consistent increases in the ΔMU_{D-T} values were observed for the oxo-hydroxy compounds; the values observed were in the range of 0.78–1.28 for monooxo-monohydroxy and dioxo-monohydroxy derivatives and 1.94–2.23 for monooxo-dihydroxy derivatives, respectively. The increment of approximately 1 unit separating the two classes of compounds, regardless of other structural characteristics, is useful for estimating the number of hydroxyl groups present in the molecule of an unknown bile acid.

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Separation and Quantification of Heart and Liver Phospholipid Classes by High-Performance Liquid Chromatography Using a New Light-Scattering Detector

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This work describes a one-step separation of rat tissue phospholipid classes by high-performance liquid chromatography (HPLC) using a silica column and a new light-scattering detector (LSD). Complete separation of phosphatidylcholine, phosphatidylethanolamine, diphosphatidylglycerol, phosphatidylinositol, phosphatidylserine, sphingomyelin, lysophosphatidylethanolamine, and lysophosphatidylcholine was obtained. Direct quantification was achieved after detector calibration for each phospholipid class. The detector response was shown to be linear within the ranges used. The LSD results agreed well with those obtained by phospholipid phosphorus assay. The present method was applied to rat heart and rat liver phospholipid analysis.
***Lipids 25,* 756-759 (1990).**

A technique to separate organ phospholipid classes was recently developed in our laboratory (1) using a two-step high-performance liquid chromatography (HPLC) procedure. This separation required a gradient elution system and an ultraviolet (UV) detector. The ultraviolet detector is generally the most widely-used (2,3); flame-ionization detectors are used to a lesser extent because they are commercially less readily available (4). There are disadvantages in using ultraviolet detectors, an important one being the inability to use chloroform as solvent. In addition, since quantitation (5) depends upon the fatty acid composition of the phospholipids, and because natural phospholipids usually contain a variety of fatty acids, direct quantitation can be difficult. Hence, phospholipid classes are most often quantified by measuring phospholipid phosphorus.

The development of a new type of detector, the light-scattering detector (LSD), which is insensitive to solvents and allows direct quantitation, has led to a marked improvement in the analysis of lipid classes (6-9). The principle of the LSD has been described in several papers (10-15). Using a light-scattering detector (ACS 750/14), Christie (8,9) reported complete separation and quantitation of various lipid classes from animal tissues.

The present paper describes a modified procedure (8) for the separation and the direct quantitation of phospholipid classes from rat liver and rat heart tissue using a 5 μm silica column and a 4-solvent elution gradient (chloroform, hexane, 2-propanol, water). Quantitations based on a Cunow LSD are being compared with those obtained by phospholipid phosphorus determinations.

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Abbreviations: DPG, diphosphatidylglycerol; HPLC, high-performance liquid chromatography; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LSD, light-scattering detector; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SD, standard deviation; SM, sphingomyelin; UV, ultraviolet.

EXPERIMENTAL

Standards and samples. Cholesterol, diphosphatidylglycerol (bovine heart), phosphatidylcholine (bovine heart), phosphatidylethanolamine (bovine liver), phosphatidylinositol (soybean), phosphatidylserine (bovine brain), sphingomyelin (bovine erythrocyte), lysophosphatidylethanolamine (egg yolk), lysophosphatidylcholine (egg yolk), phosphatidic acid (egg yolk) and phosphatidylglycerol (egg yolk) were obtained from Sigma Chemical Co. (La Verpillière, France). Their purity (≈99%) was checked by HPLC. Tissue samples were obtained from Wistar rat liver and heart. Lipids were extracted according to the method of Folch *et al.* (16). Phospholipids were separated from other lipids using the technique described by Juanéda and Rocquelin (17). Solvents of HPLC grade (SDS, Marseille, France) were vacuum filtered on a 0.2 μm millipore filter.

Materials. The HPLC instrument was a Varian model Vista 54 liquid chromatograph (Les Ulis, France) using a ternary solvent mixture, with a 100 μL Valco compressed-air injector fitted with a loop and a programmer-integrator. A Gilson Model 201 fraction collector was used for semi-preparative analyses. The detector was a Cunow LSD Model 10, developed by Lafosse *et al.* (18) at Orléans University (France) and commercialized by Cunow (Cergy, France). The detector was connected to filtered compressed air. The nebulized air pressure was set at 30 psi and the "additional air" at 7.5 psi. The heated pipe temperature was 40°C. The LSD was fitted with a splitter for collecting phospholipid fractions. The same column (25 cm × 7.5 mm i.d.) was used for both analytical (direct quantitation) and semi-preparative work (quantitation and collected lipid classes). The column was packed in our laboratory according to the method of Coq *et al.* (19), using Lichrosorb Si60 silica, 5 μm (N = 104000 plates/m).

Methods. The solvent composition of the three vessels was: A, hexane; B, 2-propanol/chloroform (4:1, v/v); C, 2-propanol/water (1:1, v/v). Separation was achieved using a ternary gradient. Injection of phospholipids alone (and not of total lipids) as well as column characteristics required the selection of specific gradients (Table 1). The flow rate was 2.5 mL/min. It was necessary to re-equilibrate the column for 10 min prior to subsequent injections. Chloroform/methanol (2:1, v/v) was used as injection solvent.

RESULTS AND DISCUSSION

The separation obtained with a standard phospholipid mixture is shown in Figure 1. In this system, diphospha-

TABLE 1

Percentage Composition of the Ternary Gradient Used for the Separation of Phospholipid Classes[a]

Time	Vessel A	Vessel B	Vessel C
0	42	52	6
25	32	52	16
65	32	52	16
65.1	42	52	6

[a]Time is expressed in minutes. The solvent composition of the three vessels was: A, hexane; B, 2-propanol/chloroform (4:1, v/v); C, 2-propanol/water (1:1, v/v).

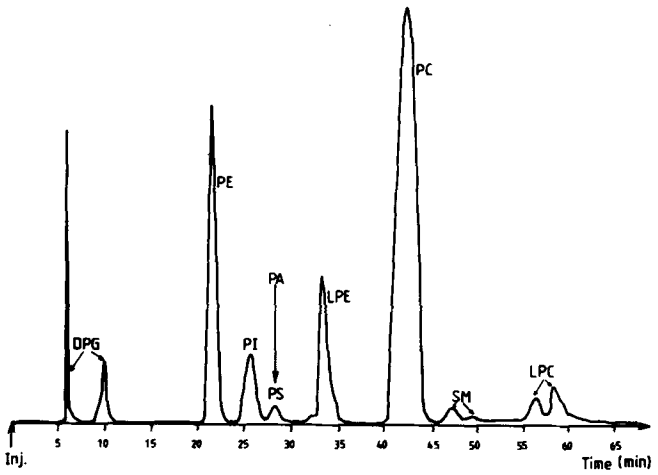

FIG. 1. Separation of standard phospholipids (0.32 mg). Elution conditions are described in Experimental Section.

tidylglycerol (DPG) was eluted as two fractions. When the solvent gradient described by Christie (8) was applied, only one peak was observed. Lysophosphatidylethanolamine (LPE), sphingomyelin (SM) and lysophosphatidylcholine (LPC) were also eluted as two fractions. Phosphatidic acid (PA) and phosphatidylserine (PS) were eluted together. Phosphatidylglycerol (PG) was eluted just ahead of phosphatidylethanolamine (PE).

Calibration curves. Solutions in chloroform/methanol (2:1, v/v) of given amounts of each phospholipid class were prepared. A known and constant amount of cholesterol (500 μg/mL) was added as internal standard to each solution. Under our conditions, cholesterol was eluted ahead of DPG. Concentration ranges were chosen to resemble the natural abundance of each phospholipid class in the respective organ. Five replicate analyses for each range point were performed, and peak areas were integrated. Calibration curves were established from the ratios of phospholipid area to cholesterol area (Y), and from the ratios of phospholipid weight to cholesterol weight (X). Figure 2 shows that the response was linear ($Y = a + bX$) within the ranges chosen. A significant but small deviation from linearity was observed with phosphatidylinositol, but the deviation can be considered negligible. Table 2 shows the slope (b) and the intercept (a) with standard deviations (SD) as well as the correlation coefficients (r) for each lipid class. It was shown that each

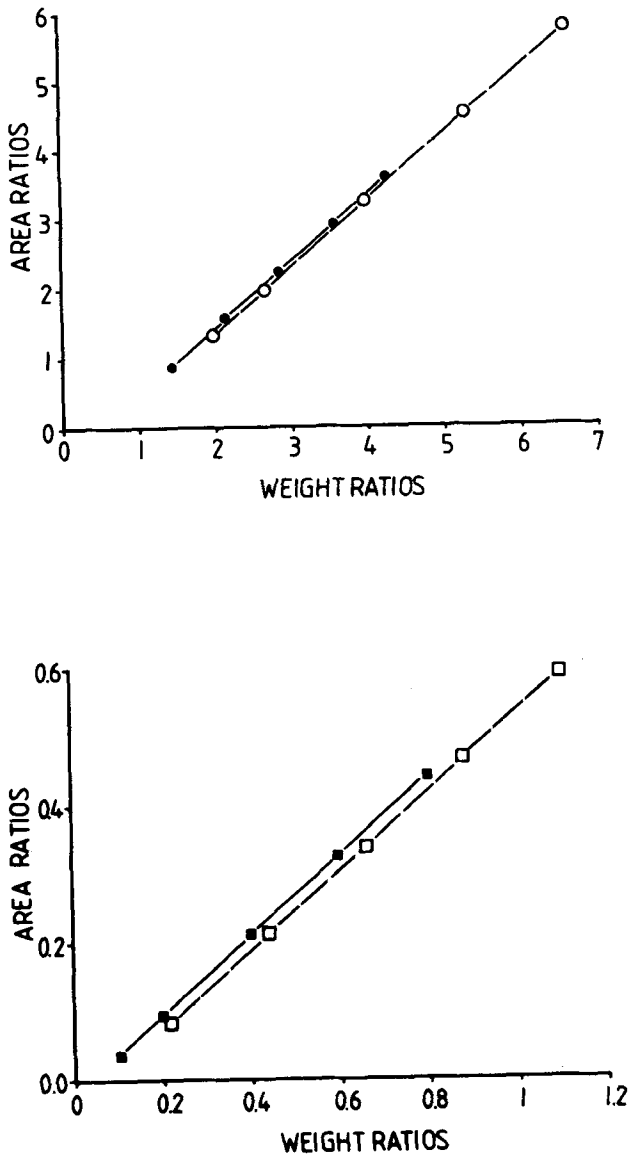

FIG. 2. Calibration lines of four phospholipid classes: phosphatidylcholine (○), phosphatidylethanolamine (●), phosphatidylinositol (□), phosphatidylserine (■). The calibration lines are determined for the X-axis by the ratios of phospholipid weight *versus* cholesterol weight and for the Y-axis by the ratios of phospholipid area *versus* cholesterol area. Each point represents the mean of 5 determinations.

TABLE 2

Linear Calibration of LSD for Each Phospholipid Class[a]

	b ± SD	a ± SD	r
PC	0.953 ± 0.0382	−0.568 ± 0.1692	0.982
PE	0.939 ± 0.0275	−0.441 ± 0.0828	0.990
DPG	0.971 ± 0.0227	−0.237 ± 0.0311	0.999
PI	0.575 ± 0.0125	−0.044 ± 0.0091	0.995
PS	0.577 ± 0.0128	−0.021 ± 0.0063	0.994
SM	0.481 ± 0.0194	0.000 ± 0.0126	0.982
LPE	0.566 ± 0.0181	−0.012 ± 0.0060	0.989
LPC	0.274 ± 0.0072	−0.005 ± 0.0024	0.992

[a]The following symbols are used: b, slope; a, intercept; r, correlation coefficient. For other abbreviations, see footnotes to title page.

TABLE 3

Comparison Between Phosphorus Assay and LSD Method for the Determination of Rat Liver Phospholipid Class Composition[a]

	Phosphorus			LSD			Comparison	
	Mean	SD	SD%	Mean	SD	SD%	Means	Variances
DPG	2.5	0.26	10.5	3.1	0.51	16.5	*	NS
PE	20.3	0.81	4.0	18.5	0.97	5.2	**	NS
PI	10.6	0.36	3.4	10.0	0.56	5.6	*	NS
PS	3.2	0.49	15.5	2.9	0.27	9.3	NS	NS
LPE	0.41	0.021	4.9	0.51	0.079	15.7	**	NS
PC	59.1	0.79	1.3	60.8	0.96	1.6	*	NS
SM	3.0	0.28	9.2	3.5	0.37	10.5	*	NS
LPC	0.76	0.072	9.2	0.67	0.081	11.9	*	NS

[a]For both methods, results are expressed as the mean percentage (mean), the standard deviation (SD) and the percentage standard deviation (SD%) of each phospholipid class. Each analysis was repeated 8 times.

*P<0.05, **P<0.01, NS = not significant.

TABLE 4

Phospholipid Distribution (% of Total Phospholipids) of Rat Heart and Liver[a]

	Heart			Liver		
	Mean	SD	SD%	Mean	SD	SD%
DPG	10.8	0.21	1.93	5.6	0.27	4.75
PE	37.0	0.13	0.59	23.0	0.28	1.24
PI	4.8	0.26	5.54	8.9	0.41	4.59
PS	2.5	0.17	6.76	2.6	0.19	7.46
LPE	0.59	0.033	5.53	0.56	0.047	8.32
PC	41.9	0.47	1.13	55.3	0.65	1.17
SM	1.6	0.14	8.84	3.1	0.18	5.79
LPC	0.91	0.031	3.43	0.90	0.076	8.49

[a]Four determinations were made for heart phospholipids and 12 for liver phospholipids.

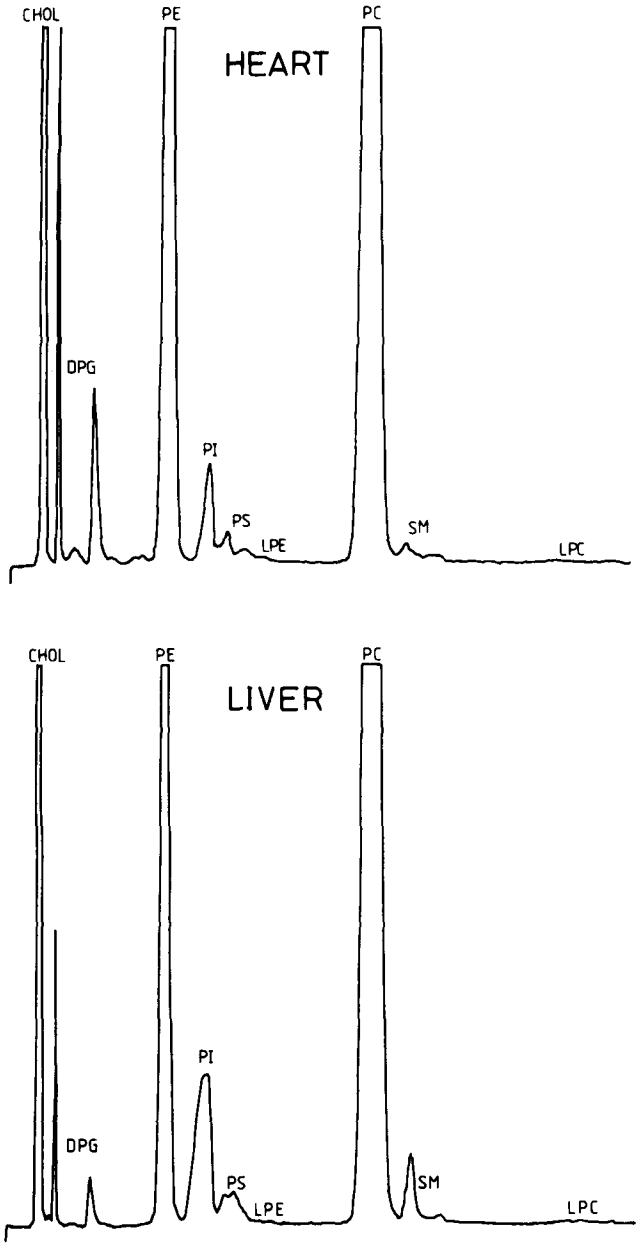

FIG. 3. Separation of rat heart and liver phospholipids (0.45 mg). Elutoin conditions are described in Experimental Section.

phospholipid class requires its own calibration curve. The nature of the acyl group (unsaturation and/or chain length) had no influence.

Comparison of the phosphorus assay and the LSD assay. For this purpose, eight 5/mg injections of rat liver phospholipids were made, and part of the effluent (10%) emerging from the column was diverted to the detector while the remainder was collected. The collected fractions were adjusted to a fixed volume and aliquots were used for phosphorus assay (20). For each assay method, results were expressed as % of total phospholipid. The statistical tests involved comparison of means and variation.

Table 3 shows that for all lipid classes the variances did not differ from one method to another, indicating that the assay in a reproducible fashion did not depend on the detection method used. By contrast, the means were significantly different except in case of PS. However, observed differences were small. One can conclude that there was a satisfactory agreement between the two methods.

Quantitation of rat heart and liver phospholipids. For this purpose, 400 to 500 μg of phospholipid was injected. All of the effluent was directed towards the detector. Figure 3 shows the chromatograms obtained and Table 4 the phospholipid compositions. The results are similar to those that were previously obtained in our laboratory (21,22) and by others (8,23–25). The coefficients of variation (SD%) were very satisfactory especially for minor lipid classes (LPE, LPC).

In conclusion, LSD allows the direct quantitation of phospholipid classes after HPLC and is much easier and faster than are phospholipid phosphorus measurements. The LSD is insensitive to organic solvents, particularly chloroform, and allows the direct quantitation of tissue phospholipids.

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COMMUNICATIONS

Is Intestinal Villus Phospholipase A_2/Lysophospholipase Bound Pancreatic Carboxylester Lipase?

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Similarities in substrate specificity, localization and molecular weight between villus membrane phospholipase A_2/lysophospholipase and carboxylester lipase of pancreatic origin suggested their possible identity. To test this, a preparation of the phospholipase A_2/lysophospholipase released from brush border vesicles by papain was compared to authentic, pancreatic carboxylester lipase. Susceptibility of both activities to the inhibitor, diisopropylfluorophosphate, was consistent with their identity, but inconclusive. It also indicated that two populations of phospholipase A_2 species may be present in the papain-released preparation. However, comparison of binding of the activities to Sepharose-coupled, anti-carboxylester-lipase IgG indicates that they are immunologically distinct.
Lipids 25, 760–762 (1990).

Phospholipase activity associated with the intestinal brush border has been reported for both the rat (1) and guinea pig (2). Fractionation and solubilization studies (3–5) have shown that these activities are on the outer surface of brush border vesicles and can be released by papain treatment. The detergent-solubilized enzyme from rat has a molecular weight of 170 kDa, as determined by SDS-PAGE under non-reducing conditions. However, if it is released by papain, 120 kDa and 35 kDa species are found by gel filtration chromatography (5). These molecular weights are much higher than those for pancreatic and venom phospholipases A_2. Also markedly different from typical phospholipases are the catalytic characteristics of the proteins. The rat and guinea pig phospholipase A_2/lysophospholipase both catalyze the hydrolysis of the *sn*-2 positions of diacyl phospholipids, the *sn*-1 position of lysophospholipids and have no requirement for Ca^{2+} (3,4).

From the above characteristics, it has been concluded that this type of enzyme is not a pancreatic-type phospholipase A_2, but rather a stalked, brush border protein, like sucrose-isomaltase or leucine aminopeptidase (5). However, the descriptions are similar in many respects to those of another pancreatic protein, carboxylester lipase (6). These proteins are also of high molecular weight. In the pig, monomers of 74 kDa and dimers of 167 kDa have been purified to homogeneity, and recently a 69 kDa form from rat pancreas has been cloned and expressed (7). Moreover, as suggested earlier (8), the latter enzyme is identical to rat pancreatic lysophospholipase (9). Although carboxylester lipases are usually associated with the hydrolysis of neutral glycerides, vitamin esters and cholesteryl esters, they do hydrolyze diacyl phosphatidylcholines (10). The specific activity of the human pancreatic enzyme toward medium-chain phosphatidylcholines is as high as 197 μmol/min/mg, although activity toward long-chain phosphatidylcholines was much less. A value of 22.8 μmol/min/mg was obtained for the phospholipase A_2/lysophospholipase purified from guinea pig brush border (3). Additionally, carboxylester lipase (cholesterol esterase) activity is associated with the intestinal brush border (11), the pancreatic enzyme has been immunologically identified there (12) and the pancreatic enzyme binds to intestinal cells (13). The latter attachment is through a heparin binding site on the enzyme and could conceivably be released by papain treatment.

These similarities prompted us to investigate the relationship between the intestinal phospholipase A_2/lysophospholipase and pancreatic carboxylester lipase using catalytic and immunological methods. The results indicate that although they share common properties, they are distinct classes of proteins.

MATERIALS AND METHODS

Reagents. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine was from Avanti Polar Lipids (Pelham, AL) and the [1-^{14}C]oleoyl analog was from NEN-Dupont (Wilmington, DE). Oleic acid was from NuChek Prep (Elysian, MN). CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate detergent), HEPES(*N*[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]) buffer and diisopropylfluorophosphate were from Calbiochem (San Diego, CA). Pierce Chemical Company (Rockford, IL) supplied BRIJ-35 detergent and the Immunopore Protein-A-Agarose kit. L-Cysteine hydrochloride monohydrate, *p*-nitrophenylbutyrate and CNBr-activated agarose were from Sigma Chemical Company (St. Louis, MO), and papain was from Boehringer-Mannheim Biochemicals (Indianapolis, IN).

Enzymes: Phospholipase A_2/lysophospholipase. Male Sprague-Dawley rats (3–4, 250–300 g) were killed by decapitation and brush border vesicles prepared as previously described (1). These were stored frozen for up to one week before being treated with papain for 1 hr at 37°C (5) to solubilize phospholipase A_2/lysophospholipase activity. Monomeric, carboxylester lipase was purified to homogeneity from porcine pancreas as previously described (14). Using the assays described below, it had a specific activity of 1,500 μmol/min/mg towards *p*-nitrophenylbutyrate.

Phospholipase A_2 activity was determined using the release of [1-^{14}C]oleic acid from 1-palmitoyl-2-[1′-^{14}C]oleoyl-*sn*-glycero-3-phosphocholine (0.052 μmol, 0.58 mCi/mmol). The incubation procedure was that of Pind and Kuksis (4), with slight modifications. Specifically, total volume was reduced to 0.15 mL and the reaction was stopped by addition of 5 μL of 12 N HCl.

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In all cases, hydrolysis was less than 20% of added phospholipid. Unreacted substrate and product oleic acid were separated by direct application of ~75 μL of the mixture and 15 μL of 1.6 mM oleic acid carrier in hexane onto a Whatman Linear-K TLC silica gel plate followed by development with $CHCl_3$/methanol/water (65:25:1, v/v/v). Radioactivity was quantitated using a Berthold Linear Analyzer (Berthold Analytical, Nashua, NH). Carboxylester lipase activity was measured spectrophotometrically by monitoring the appearance of the *p*-nitrophenolate ion (15). A unit of either activity is defined as 1 μmol of substrate consumed or product released/min. Protein was determined by the BioRad dye binding procedure (16) using bovine serum albumin as a standard.

Enzyme modification. Each reaction mixture contained 0.3 mL of carboxylester lipase [3.25 units in 50 mM potassium phosphate buffer, 100 mM NaCl, 0.1% (w/v) BRIJ-35, pH 7.25], 0.3 mL of papain-released phospholipase A_2 (0.0109 units) or 0.3 mL containing 3.25 units of carboxylester lipase and 0.0109 phospholipase A_2 units of papain-released phospholipase A_2, to which 0.01 of 30 mM diisopropylfluorophosphate in isopropanol was added. After 30 min incubation with shaking at 24°C, the mixtures were cooled to 4°C and remaining enzyme activities determined.

Antibody binding. The preparation of rabbit antiserum to monomeric, porcine carboxylester lipase has been described (8). Isolation of the IgG fraction from immune and control sera using Protein A Agarose and their coupling to CNBr-Sepharose were performed essentially as earlier reported (8), except that in the coupling step 5 mg of dialyzed IgG was used per mL of swollen CNBr-Sepharose. For studies of enzyme binding to control and anti-carboxylester-lipase Sepharose, the gels were equilibrated in 50 mM phosphate buffer, pH 7.25, containing 100 mM NaCl and 0.1% (w/v) BRIJ-35. Each incubation mixture contained 0.04 mL of settled control or anti-carboxylesterase-lipase Sepharose plus carboxylester lipase (4.6 units in 0.2 mL of gel equilibration buffer), papain-released phospholipase A_2 (0.0037 units in 0.2 mL) or both. After incubation at room temperature for 1 hr, the samples were centrifuged at 1,500 × *g* for 15 min and the supernatants assayed for remaining enzyme activity.

RESULTS AND DISCUSSION

Following the procedure of Pind and Kuksis (5), intestinal brush border vesicles were prepared from 250–300 g male Sprague-Dawley rats and treated with papain to release the activity. A summary of the purification is shown in Table 1. Specific activity in the initial homogenate was 0.0085 μmol/min/mg as compared to the value of 0.0063 reported by Pind and Kuksis (4). Those authors did not report yield figures for the papain-released material, but their brush border vesicle preparation had a specific activity of 0.126 μmol/min/mg (4) *vs* 0.308 for the preparation shown in Table 1. Also reported in Table 1 are activities measured using *p*-nitrophenylbutyrate, a good substrate for carboxylester lipase (*e.g.*, 17). Based on this assay, specific activity decreased ~100-fold during purification. This trend is opposite to that observed for phospholipase A_2, suggesting that the proteins are different. However, the result is inconclusive because the expression of phospholipase activity by carboxylester lipase depends strongly on the phospholipid and the assay conditions (10). Conceivably, the expression of phospholipase A_2 activity could require association with the receptor on the brush border. Because of such uncertainties, further use of catalytic specificity to differentiate species was not attempted.

A potentially useful property of carboxylester lipases is their high sensitivity to covalent modification and inhibition by diisopropylfluorophosphate (*e.g.*, 14). Table 2 shows the results of treatment of the papain-released enzyme preparation and authentic carboxylester lipase with the inhibitor. As expected, inhibition of the authentic carboxylester lipase was complete both in the presence and absence of the solubilized phospholipase preparation. More than 85% of the phospholipase A_2 activity was eliminated by the inhibitor, suggesting that part of the activity could be from a carboxylester lipase-like enzyme. However, with another enzyme preparation inhibition was only 60%. Also, in a separate experiment a preparation of brush border membranes (*i.e.*, before papain treatment) was treated with diisopropylfluorophosphate. This resulted in an 88% loss of phospholipase activity. Thus, more than one phospholipase A_2 activity may be present in the preparation. Both could have been present on the luminal surface of the intestinal brush border, or at least one could have arisen from contamination of the vesicle preparation with other particulate fractions known to possess phospholipase A_2 activity (18), or from pancreatic phospholipase A_2 carried through the purification. It is also possible that receptor or its fragments in the brush border membrane or papain-released enzyme preparation protected the enzyme from inhibi-

TABLE 1

Purification of Phospholipase A_2 from Rat Intestinal Brush Border

Sample	Vol	Activity units PNPB[a]	Activity units PLA_2[a]	Protein mg	Specific activity units/mg PNPB	Specific activity units/mg PLA_2
Homogenate	200	847.6	1.746	206	4.12	0.00847
Brush border vesicles	11	0.28	0.217	0.704	0.398	0.308
Post-papain supernatant	1.82	0.00375	0.0336	.100	0.0375	0.336

[a]PNPB, hydrolysis of *p*-nitrophenylbutyrate; PLA_2, hydrolysis of 1-palmitoyl-2-[1′-^{14}C]oleoyl-*sn*-glycero-3-phosphocholine. See Materials and Methods for conditions and procedure.

TABLE 2

Inhibition of Carboxylester Lipase and Papain-Released Phospholipase A_2 by Diisopropylfluorophosphate

Sample[b]	Inhibitor present	Activity remaining[a] $10^3 \times$ units/mL (%) PNPB	PLA$_2$
CEL	+	0	n.d.
CEL + PRPA$_2$	+	0	6.1 (13)
CEL + PRPA$_2$	−	10,800 (99.3)	47.8 (102)
PRPA$_2$	+	n.d.	6.1 (12)

[a]Percent activity remaining is expressed as a percentage as compared to that of a control mixture. CEL + PRPA$_2$ kept at 4°C.

[b]CEL, carboxylester lipase; PRPA$_2$ papain-released phospholipase A_2.

TABLE 3

Immunoadsorption of Carboxylester Lipase and Phospholipase A_2/Lysophospholipase

Sample[a]	Bound IgG	% Activity in supernatant PNPB	PLA$_2$
CEL	Immune	25	—
CEL	Control	79	—
PRPA$_2$	Immune	—	86
PRPA$_2$	Control	—	82
CEL + PRPA$_2$	Immune	28	92
CEL + PRPA$_2$	Control	101	106

[a]CEL, carboxylester lipase; PRPA$_2$ papain-released phospholipase A_2.

tion or that inhibition is dependent on the extent of papain digestion. With regard to the latter, papain digestion releases two proteins of 120 and 35 kDa, which have both lysophospholipase and phospholipase A_2 activities (5).

In an effort to provide a more definite comparison of the activities, an immunological approach was used. Previously, it has been shown that antiserum from rabbits immunized with porcine carboxylester lipase will bind rat pancreatic carboxylester lipase. Also, in immobilized form, the porcine IgG fraction from the serum binds a similar enzyme in rat liver homogenates (8). The IgG fraction from the same batch of serum as well as a sample of non-immune IgG were each immobilized on Sepharose 4B. Aliquots of each were mixed with enzyme samples which were matched with respect to their *p*-nitrophenylbutyrate hydrolyzing activity. The results (Table 3) show that only 25% of carboxylester lipase activity remains unbound as compared to 80% with control IgG. In contrast, the amount of phospholipase A_2 activity bound is comparable to the control. When both activities were present in the same sample, the results for each were essentially identical. Thus, the data clearly demonstrate that little, if any, of the enzyme(s) responsible for the phospholipase A_2 activity is bound. It should be noted that the antibody preparation is polyclonal. This should prevent the bound receptor fragment, if any, from completely blocking enzyme-antibody interaction. Also, the anti-carboxylester-lipase Sepharose was able to bind authentic carboxylester lipase in the presence of the phospholipase A_2/lysophospholipase preparation. This shows that the antibodies were not being neutralized by other proteins in the phospholipase A_2/lysophospholipase preparation. Addition of carboxylester lipase to a brush border membrane preparation prior to papain treatment resulted in a complete inactivation of its activity toward *p*-nitrophenylbutyrate. While this may reflect a species difference, it is consistent with the activities residing in distinct proteins.

Overall, the data indicate that the intestinal phospholipase A_2/lysophospholipase is distinct from pancreatic carboxylester lipase. Moreover, they show that the bulk of the phospholipase A_2 activity is inhibited by the covalent serine esterase inhibitor, diisopropylfluorophosphate, distinguishing it from classical phospholipase A_2. Whether the lack of total inhibition is indicative of the presence of two enzymes or of related, proteolytic fragments will require additional study.

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Availability of Linoleic Acid from Cereal-Pulse Diets

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Cereals and pulses alone provide nearly two-thirds of the daily linoleic acid requirement in habitual Indian diets. Two-thirds of the lipids present in cereals is in bound form. To investigate to what extent the essential fatty acids (EFA) present in cereals and pulses are biologically available, weanling rats were fed rice-pulse based diets either without supplementation or supplemented with one of three vegetable oils—coconut, palmolein or groundnut oil. Plasma phospholipid fatty acid composition was used to assess the EFA status, with ratios of eicosatrienoic/arachidonic acids (20:3n-9/20:4n-6) above 0.2, indicating linoleic acid deficiency. In the unsupplemented group, the levels of linoleic and arachidonic acids were low as compared to the groundnut oil fed group. However, the ratio of 20:3n-9/20:4n-6 was less than 0.2, indicating that there was no linoleic acid deficiency. This shows that the linoleic acid present in rice and pulse may be readily available.
Lipids 25, 763-766 (1990).

Lipids present in cereals exist both in free and bound form. The free form consists largely of triglycerides and is ether extractable, whereas a more vigorous approach is required to extract the more complex lipids ("bound fat") (1). Cereals and millets contain about 3% "fat" in which linoleic acid is one of the major fatty acids (2,3). In habitual Indian diets, the staple is cereals and millets (4,5). The daily intake of cereals, millets and pulses provide about 16 g "fat" (6% as fat calories), which can meet nearly two-thirds of the daily linoleic acid requirement of an adult man consuming 2400 calories (6). However, two-thirds of the lipids present in cereals and millets are in the bound form and it is not well known to what extent the "bound fat" is biologically available. The present study was undertaken to investigate whether the linoleic acid present in rice and pulse is available to meet the linoleic acid requirement in rats.

MATERIALS AND METHODS

Polished rice, pulse and oils were purchased at a supermarket. The composition of the diets is given in Table 1. Two experiments were carried out to determine the adequacy of rice-pulse based diets in terms of linoleic acid requirements. In the first experiment, the effects of feeding rice-pulse based diet alone were compared with diets providing groundnut oil. In the second experiment, the effects of oils containing low levels of linoleic acid (coconut and palmolein) were compared with the effects of groundnut oil.

In the first experiment, fourteen weanling male Wistar/NIN rats (inbred strain of National Institute of Nutrition, Hyderabad) were divided into two groups. Five percent groundnut oil was added to the diet of group I, and 5% D-glucose was added to that of group II. At the end of three months of feeding, blood was collected by puncture of the ophthalmic venous plexus with heparin coated glass capillary tubes with minimum possible stress to the animal. Food had been removed from the cages 16 hr prior to blood collection. In the second experiment, three groups of rats (seven in each group) were fed a rice-pulse based diet containing different vegetable oils, and after five weeks of feeding, blood was collected as described above.

TABLE 1

Composition of the Diets (g/100 g Diet)

	Experiment 1		Experiment 2		
Groups	I	II	III	IV	V
Rice (polished) (Oryza sativa)	74	74	74	74	74
Pulse[a] (Cajanus cajan)	15	15	15	15	15
Casein	1	1	1	1	1
Vegetable oil	5 (GNO)[b]	Nil	5 (GNO)	5 (PO)[c]	5 (CO)[d]
Vitamin mix[e]	1	1	1	1	1
Salt mix[f]	4	4	4	4	4
D-Glucose	Nil	5	Nil	Nil	Nil
Kcals/100 g	350	330	350	350	350

[a] Legume with seedcoat removed.
[b] Ground nut oil.
[c] Palmolein.
[d] Coconut oil.
[e] Reference 7.
[f] Reference 8.

Diet analysis. Diet (200 mg) and heptadecanoic acid (1 mg) were hydrolyzed with 1 mL 4 N HCl for 30 min at 60°C and then for 30 min at 90°C. The lipids were extracted with chloroform/methanol (2:1, v/v), saponified, and the fatty acid methyl esters were prepared as described earlier (6). The fatty acid methyl esters were analyzed using Silar 10C column fitted in Varian 3700 gas chromatograph (Varian Associates, Palo Alto, CA) equipped with a flame ionization detector as described earlier (6).

The individual fatty acids were identified by frequent comparisons with authentic standards and calculated on a Varian 4270 integrator. The amount of each fatty acid was calculated from the area of the internal standard (17:0).

All standard fatty acid methyl esters and heptadecanoic acid were purchased from NuChek Prep (Elysian, MN). Solvents used were of analytical grade and contained 25 mg butylated hydroxytoluene per liter. The total fat content was calculated by dividing the sum of the total fatty acids by the conversion factor of 0.85, as suggested for rice by Paul *et al.* (9). The recovery of linoleic and α-linolenic acids was 88%. Protein was estimated by the macrokjeldhal method.

Blood analysis. The blood samples were centrifuged for 20 min and plasma was separated while taking care not to disturb the buffy coat containing the platelets. Plasma was analyzed for total cholesterol (10) and triglycerides

Abbreviations: EFA, essential fatty acids; HDL, high density lipoprotein; LDL, low density lipoprotein; NIN, National Institute of Nutrition; TLC, thin-layer chromatography; VLDL, very low density lipoprotein.

TABLE 2

Estimated Nutrients in the Diets (g/100 g Diet)

Groups	Experiment 1		Experiment 2		
	I	II	III	IV	V
Saturated fatty acids	1.3	0.44	1.5	2.7	5.0
Monoenes	2.1	0.35	2.2	2.7	0.8
Linoleic acid	2.3 (5.8 en%)	0.48 (1.2 en%)	2.3 (5.8 en%)	0.9 (2.3 en%)	0.54 (1.4 en%)
α-Linolenic acid	0.03	0.02	0.04	0.03	0.01
Total fat	6.1	1.5	6.4	6.8	6.8
Protein	10.6	10.7	10.5	10.6	10.7

TABLE 3

Plasma Fatty Acid Composition of Rats Fed Rice-Pulse Diets (nmole %)[a]

Groups	Experiment 1			
	Phospholipids		Total lipids	
	I (7)	II (7)	I (5)	II (5)
18:2n-6	8.2 ± 0.5	5.6 ± 0.4[b]	20.0 ± 0.8	10.3 ± 1.2[b]
20:4n-6	22.4 ± 1.2	16.6 ± 0.7[c]	17.3 ± 2.3	9.5 ± 0.65[c]
20:3n-9	0.6 ± 0.1	1.2 ± 0.1[c]	0.2 ± 0.04	0.7 ± 0.2[c]
20:3n-9/20:4n-6 ratio	0.03 ± 0.004	0.07 ± 0.009[c]	0.01 ± 0.004	0.06 ± 0.02[c]
Total saturated fatty acids	47 ± 1.3	51.5 ± 1.4	26.3 ± 4.3	33.4 ± 1.9
Total monoenes	14.5 ± 1.0[d]	16.3 ± 0.5[d]	32.3 ± 2.2[e]	37.6 ± 1.8[e]
Total n-6	35.5 ± 1.4	27.6 ± 1[b]	38.0 ± 2.7	20.5 ± 1.5[b]
Total n-3	2.2 ± 0.4	2.4 ± 0.2	0.5 ± 0.1	0.7 ± 0.1

[a] Values are mean ± SE. Number in parentheses indicates the number of animals in each group. Statistical Analysis by Students' *t*-test.

[b] $P < 0.001$.

[c] $P < 0.05$.

[d] 18:1 plus 20:1.

[e] 18:1.

TABLE 4

Plasma Phospholipid Fatty Acid Composition of Rats Fed Rice-Pulse Diets with Different Vegetable Oils (nmole %)[a]

Group	Experiment 2		
	III (7)	IV (7)	V (7)
18:2n-6	8.7 ± 0.4	8.0 ± 0.7	8.2 ± 0.9
20:4n-6	18.8 ± 1.1	18.8 ± 1.4	11.9 ± 1.1[b]
20:3n-9	1.1 ± 0.2	1.1 ± 0.2	1.5 ± 0.2
20:3n-9/20:4n-6 ratio	0.06 ± 0.01	0.06 ± 0.01	0.14 ± 0.02[b]
Total saturated fatty acids	50 ± 2.7	51.5 ± 3.1	54.5 ± 3.1
Total monoenes[c]	10.3 ± 0.6	11.5 ± 0.7	11.5 ± 0.8
Total n-6	33.4 ± 1.9	30.7 ± 2.2	24.5 ± 2.5[d]
Total n-3	2.5 ± 0.6	2.3 ± 0.5	2.6 ± 0.3

[a] Values are mean ± SE. Number in parentheses indicates number of animals studied. Statistical analysis by one way analysis of variance and difference between groups done by Students' *t*-test.

[b] $P < 0.01$.

[c] 18:1.

[d] $P < 0.02$.

(11). Plasma very low density lipoproteins (VLDL) and low density lipoproteins (LDL) were precipitated by heparin manganese chloride (12) and high density lipoprotein (HDL) cholesterol was estimated in the supernatant. Plasma lipids were extracted and fatty acid methyl esters of total lipids were prepared as described earlier (13). Plasma phospholipids were separated by thin-layer chromatography (TLC) and their fatty acid methyl esters were prepared. The fatty acid methyl esters were analyzed by gas chromatography.

RESULTS

Analyses of the diets (Table 2) showed that the rice-pulse based diets provided 10.7% protein and 1.5% fat. The data on fatty acid composition of the diets indicate that group I rats received 2.3 g (5.8 en%) and group II rats 0.48 g (1.2 en%) linoleic acid per 100 g diet. In the second experiment, the coconut, palmolein and groundnut oil groups received 0.5 g (1.4 en%), 0.9 g (2.3 en%) and 2.3 g (5.8 en%) linoleic acid, respectively.

Daily food intake, gain in body weight, plasma total cholesterol and triglyceride levels were similar in groups I and II (data not given). The data on plasma total lipid and phospholipid fatty acid composition (Table 3) show that the levels of linoleic, arachidonic and total n-6 fatty acids were lower in group II as compared to group I (Table 3). The total n-3 fatty acids were similar in both the groups. Although the levels of eicosatrienoic acid (20:3n-9) and the 20:3n-9/20:4n-6 ratio were higher in group II as compared to group I, the ratios in both groups were well below 0.2.

In the second experiment, plasma levels of triglycerides, cholesterol and HDL cholesterol were similar in the three groups (data not given). The results on plasma phospholipid fatty acid composition presented in Table 4 show that the mean levels of linoleic acid, eicosatrienoic acid, total saturated fatty acids, total monoenes, and total n-3 fatty acids were similar in the three groups. The mean levels of arachidonic acid, total n-6 fatty acids, and the ratio of 20:3n-9/20:4n-6 were similar in palmolein (group IV) and groundnut oil (group III) fed rats. In rats fed coconut oil, the levels of arachidonic acid were low and the 20:3n-9/20:4n-6 ratio was high as compared to the other two groups, but was still below 0.2.

DISCUSSION

Plasma and tissue lipid fatty acid composition and the various indices calculated from it are used widely to assess the essential fatty acid status. In linoleic acid deficiency, the levels of eicosatrienoic acid increase and those of arachidonic acid decrease. The ratio of 20:3n-9/20:4n-6 is a useful index of linoleic acid status, with a ratio above 0.2 indicating poor linoleic acid status (14,15). The ratio could be even reduced to 0.02 by feeding 2% of total energy as γ-linolenic acid (18:3n-6) to linoleic acid deficient rats. Further, the lowering of the ratio was associated with a continuous improvement in weight gain and feed efficiency (16).

In our earlier study in rats fed casein based diet (17), linoleic acid levels in plasma total lipids were shown to be proportional to the levels of dietary linoleic acid. With an increase in plasma linoleic acid, the levels of arachidonic acid increased, but those of eicosatrienoic acid and the ratio of 20:3n-9/20:4n-6 decreased. In the safflower oil and groundnut oil groups, which received 9.4 and 4.2 en% linoleic acid, the ratio of 20:3n-9/20:4n-6 was 0.02 and 0.06, respectively (17). In the coconut oil group, which received 0.18 en% linoleic acid, the ratio was 0.96, indicating severe linoleic acid deficiency. In the palmolein group, which received 1.3 en% linoleic acid, the linoleic acid status was found to be marginally adequate (20:3n-9/20:4n-6 = 0.32). In the present study, where the rats were fed rice-pulse based diet, 1.3 en% linoleic acid provided from rice and pulse alone, was able to keep the ratio of 20:3n-9/20:4n-6 below 0.2 both in plasma total lipids as well as in phospholipids. Since rice and pulse were the only source of dietary linoleic acid throughout the post-weaning growth period up to three months of age, these findings suggest that the linoleic acid of rice and pulse must be available for maintaining the linoleic acid status.

In the second experiment, while the linoleic acid from rice and pulse was similar in the three groups, the vegetable oils furnished different levels of linoleic acid. Despite differences in the intakes of linoleic acid, the mean levels of arachidonic acid and the ratio of 20:3n-9/20:4n-6 were similar in palmolein and groundnut oil fed rats. This shows that the requirements of linoleic acid are fully met even when palmolein—which contains less linoleic acid (10%)—is included in rice-pulse based diets. The observation that the linoleic acid status is normal, when palmolein is included in rice-pulse based diets, whereas it is only marginally adequate in rats fed casein based diets (17) confirms the availability of linoleic acid from cereal-pulse based diets.

The requirement of linoleic acid is known to be high when diets contain higher levels of saturated fatty acids (14). The relatively lower levels of arachidonic acid and the higher ratio of 20:3n-9/20:4n-6 in the coconut oil group could probably be due to the high intake of saturated fatty acids. However, the ratio was below 0.2 as compared to the ratio of 0.96 when coconut oil was used in a casein based diet (17). This observation also shows that the linoleic acid present in cereals and pulses is readily available.

ACKNOWLEDGEMENTS

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REVIEW

The Interaction of Prostaglandin E_1 with Serum Lipoproteins. Possible Role in Cholesterol Homeostasis

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Prostaglandin (PG) E_1 significantly stimulates the rate of cholesterol esterification in plasma. This effect could be attributed to an enhancement by PGE_1 of the interlipoprotein transfer of phosphatidylcholine and cholesteryl esters, *i.e.*, the substrate and product of lecithin-cholesterol acyltransferase (LCAT). The enhancement effect appears to be due to a rearrangement of the lipoprotein surface induced by specific interaction of PGE_1 with some apolipoproteins, although the binding capacity of serum lipoproteins for PGE_1 was found to be rather weak. To explain these findings, an hypothetical non-equilibrium model was put forward. The purpose of the present article is to summarize available data on the PGE_1-lipoprotein interaction.
***Lipids* 25, 767-774 (1990).**

Prostaglandin (PG) E_1 is a potent vasodilator (1). It is known to lower arterial blood pressure, to increase peripheral blood flow, to inhibit smooth muscle cell proliferation and platelet aggregation and to rescue heart tissue from ischemic damage [reviewed in (1) and the literature cited therein]. The beneficial effects of PGE_1 have been seen in the treatment of peripheral vascular diseases (2,3). In this context it is noteworthy that PGE_1 lowers the cholesterol content in rabbit (4) and human (5) aorta smooth muscle cells; however, the underlying mechanism is not clear. Since aberrations in cholesteryl ester metabolism are believed to be a factor that predisposes to atherosclerosis, we thought it would be of interest to investigate the influence of prostaglandin E_1 on cholesterol esterification in plasma. Our first experiments revealed that in whole human plasma small amounts of PGE_1 significantly stimulate the rate of cholesterol esterification. Subsequent studies demonstrated that this effect could be attributed to an enhancement by PGE_1 of the interlipoprotein transfer of phosphatidylcholine and cholesteryl esters, *i.e.*, the substrate and product of lecithin-cholesterol acyltransferase (LCAT). The enhancement, in turn, appeared to be due to a rearrangement of the lipoprotein surface induced by specific interaction of PGE_1 with some apolipoproteins. However, the binding capacity of serum lipoproteins for PGE_1 was found to be rather weak. To explain these findings, an hypothetical non-equilibrium model was put forward. The purpose of the present article is to summarize available data on the PGE_1-lipoprotein interaction and to challenge other investigators to test the validity of our hypothesis.

Effect of PGE_1 on cholesterol esterification. When added to whole human plasma, small amounts of PGE_1 markedly enhanced the rate of cholesterol esterification in a concentration dependent manner (Table 1 and Fig. 1)

TABLE 1

Influence of Prostaglandins on the Esterification of Cholesterol in Human Plasma (6)[a]

Prostaglandin (10^{-9} M)	Relative rate of cholesterol esterification (percent of control)
E_1	163
E_2	108
$F_{2\alpha}$	100

[a]Cholesterol esterification assay was carried out by incubation (37°C, 1 hr) of [^{3}H]cholesterol (3.23 μM/mL, radioactivity 5-10 μCi/mL) with plasma (0.3 mL). [^{3}H]Cholesteryl ester formation was determined by thin-layer chromatography of the lipid extracts on silica gel plates (using petroleum ether/diethyl ether/acetic acid, 90:10:1, v/v/v) as developing solvent) and counting the radioactivity of the cholesteryl ester fractions. Cholesteryl oleate was used as standard.

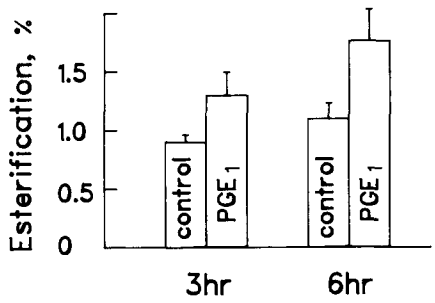


FIG. 1. Influence of PGE (10^{-8}M) on the rate of LDL-cholesterol esterification in the presence of lipid depleted plasma and unlabeled HDL_3 (7). [1,2-^{3}H]Cholesterol was incorporated into LDL by coincubation of an ethanolic cholesterol solution with LDL suspension for 4 hr at 36°C. Cholesteryl esters were determined as indicated in Table 1.

(6,7). This effect appeared to be highly specific because in the same concentration range other prostaglandins of related structure were inactive. For example, $PGE_{2\alpha}$, even at relatively high concentrations, did not influence the rate of cholesterol esterification. Prostaglandin E_2 produced some enhancement of cholesterol esterification, but to a much smaller degree than did PGE_1. Moreover,

Abbreviations: APC, arthrylvinyl-labeled phosphatidylcholine; apo-A_1, apolipoprotein A_1; apo-B, apolipoprotein B; ASM, arthrylvinyl-labeled sphingomyelin; HDL, high density lipoprotein; LCAT, lecithin-cholesterol acyltransferase; LDL, low density lipoprotein; LEP, lipid exchange protein; PG, prostaglandin; PGA_2, prostaglandin A_2; PGE_1, prostaglandin E_1, PGE_2, prostaglandin E_2; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$.

in the concentration range from 10^{-10} M to 10^{-8} M, the effect of PGE_2 was concentration independent and thus probably nonspecific.

At first glance, the effect of PGE_1 on the rate of cholesterol esterification seemed to involve some catalytic step, because significant enhancement of the reaction was observed even at PG concentrations as low as 10^{-10} M, which corresponded to one prostaglandin molecule for more than 10^3 lipoprotein particles.

In principle, such influence of PGE_1 on the esterification of cholesterol could be attributed to interaction of the prostaglandin either directly with LCAT or with high density lipoprotein (HDL) apolipoprotein A_1 (apo-A_1) which is known to be a cofactor of the LCAT reaction. However, when lipoprotein-depleted plasma was incubated with phosphatidylcholine-cholesterol liposomes or with HDL recombinants obtained from purified apo-A_1, egg lecithin and cholesterol, no enhancing effect of PGE_1 was seen (7). Neither could we observe any stimulation of the LCAT reaction by PGE_1 when the prostaglandin was incubated with lipid-depleted plasma and either HDL or low density lipoprotein (LDL). Only when both HDL and LDL were present in the incubation mixture, was PGE_1 able to stimulate the esterification of LDL-cholesterol (7). We presumed, therefore, that the effect of PGE_1 on cholesterol esterification in plasma could be caused by prostaglandin-induced enhancement of interlipoprotein transfer of the substrates and (or) products of LCAT. As is well known, cholesteryl esters synthesized within high density lipoproteins may be transferred to less dense lipoproteins by an exchange reaction which is catalyzed by a cholesteryl ester transfer protein (8–10). Specifically, the cholesteryl esters of LDL probably are acquired from HDL *via* transfer involving plasma lipid exchange proteins (11). Because LDL is taken up by specific receptors in the liver, the HDL-to-LDL transfer of cholesteryl esters potentially provides an important step in cholesterol catabolism.

Taking these considerations into account, we undertook a study aimed at clarifying the influence of PGE_1 on the spontaneous and protein-mediated transfer of [^{3}H]cholesteryl esters and [^{3}H]phosphatidylcholine from HDL_3 to LDL (7). HDL_3 was chosen as donor because upon Mn^{2+} precipitation, HDL_3 is more completely separated from LDL than other HDL subfractions.

The results obtained demonstrated that among a number of different prostaglandins tested, only PGE_1 was able to stimulate interlipoprotein lipid transfer. PGE_1 considerably facilitated the spontaneous transfer of both [^{3}H]dipalmitoyl phosphatidylcholine and [^{3}H]cholesteryl esters from HDL_3 to LDL, and this effect was aided by the presence of partially purified lipid exchange proteins from human plasma (Fig. 2). During incubation, the PGE_1-stimulated spontaneous and protein-mediated lipid transfer continued to increase steadily; no retardation of the transfer process was observed.

The stimulation of lipid transfer by PGE_1 was concentration dependent and saturable (Fig. 3). The action of PGE_1 appeared to be strikingly efficient—even at 10^{-10} M, PGE_1 increased the spontaneous transfer of cholesteryl esters significantly both in the absence and in the presence of partially purified plasma lipid exchange protein(s). As already mentioned, such low concentration

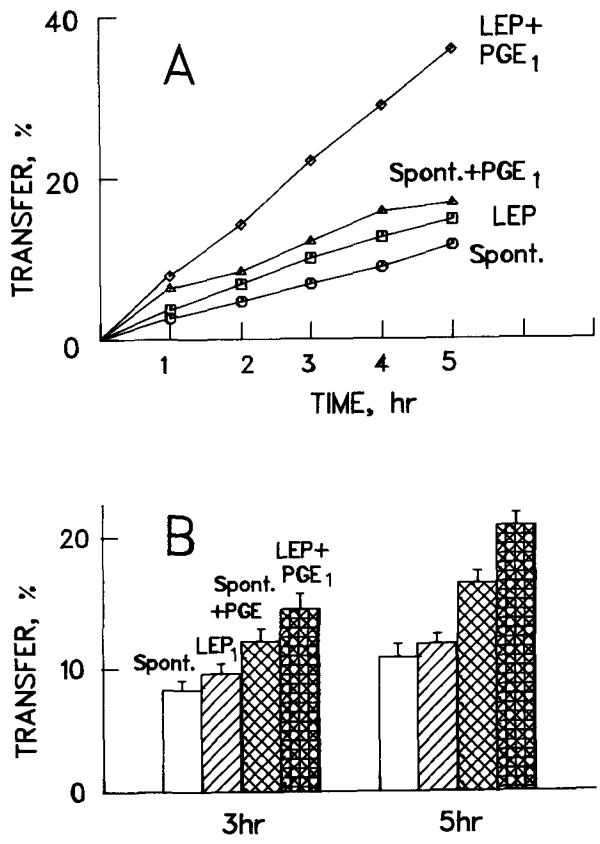


FIG. 2. Influence of PGE_1 (10^{-8}M) on the rate of spontaneous and of lipid exchange protein (LEP)-mediated lipid transfer from HDL_3 to LDL (0.3 and 0.6 mg protein/mL, respectively). A, [^{3}H]-Dipalmitoyl phosphatidylcholine; B, [^{3}H]cholesteryl esters. Partly purified LEP was isolated from the d >1.21 g/mL infranatant of human plasma by sequential chromatography on phenyl-Sepharose and DEAE-cellulose, and assay of the lipid transfer activity was carried out as described (10). Samples were coincubated at 36°C and the donor and acceptor lipoproteins were separated by Mn^{2+} sedimentation in phosphate buffer solution (from ref. 7).

corresponded to one prostaglandin molecule per 10^3–10^4 lipoprotein particles.

Existing data indicate that the spontaneous and the protein-mediated interlipoprotein transfer occur by different mechanisms. The kinetics of spontaneous transfer of phospholipids between lipoproteins are consistent with a mechanism involving a rate limiting dissociation of monomers from the lipoprotein surface, which is then followed by rapid diffusion of the lipid monomers through the aqueous phase. In contrast, kinetic studies of protein-mediated interlipoprotein cholesteryl ester transfer suggest that the transfer takes place *via* formation of a ternary collison complex involving donor and acceptor lipoproteins and lipid transfer proteins (11).

Since PGE_1 enhanced both the spontaneous and protein-mediated interlipoprotein lipid transfer in a similar way, it seemed likely that enhancement by prostaglandin was caused by its interaction with the donor and (or) acceptor lipoproteins rather than by prostaglandin-induced modification of the lipid exchange protein(s). Therefore, we postulated that PGE_1 induces a structural rearrangement of the lipoprotein surface. Our further studies were designed to test this idea.

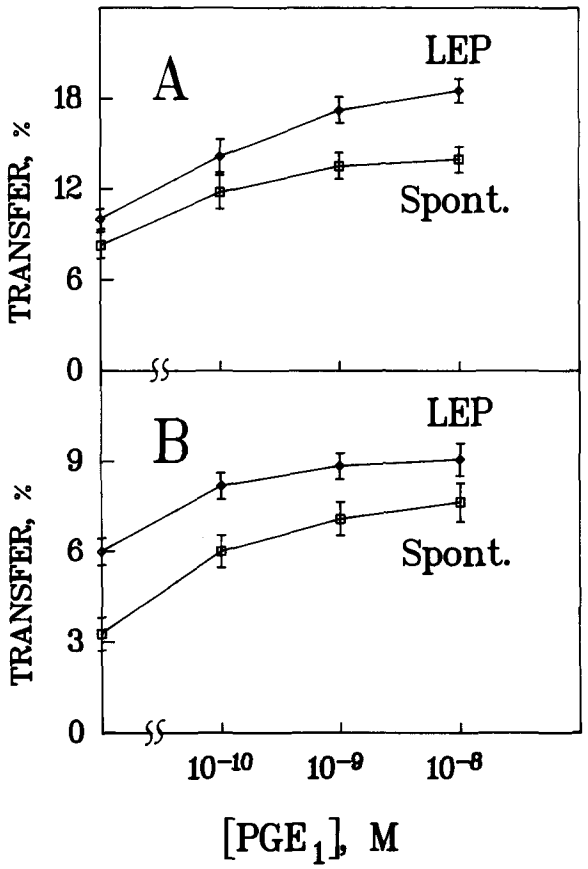
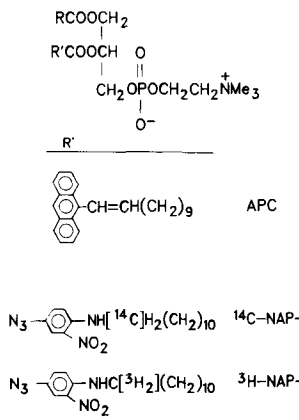


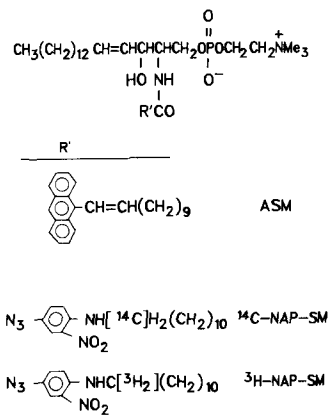
FIG. 3. Dependence of the rate of HDL_3-to-LDL lipid transfer on the PGE_1 concentration in the medium. A, Transfer of [^{3}H]-dipalmitoyl phosphatidylcholine; B, transfer of [^{3}H]cholesteryl esters. Samples were incubated for 5 hr at 36°C. Concentrations of HDL_3 and LDL and other experimental conditions are as in Figure 2. Under the separation conditions used, some coprecipitation of HDL_3 with LDL did occur. This value was taken as "percent of transfer at time zero" (from ref. 7).

phosphatidylcholines



SCHEME 1

sphingomyelins



SCHEME 2

Prostaglandins as modulators of the lipoprotein surface structure. In order to visualize changes in the molecular organization of lipids at the lipoprotein surface, we employed two independent approaches based on the use of fluorescent or photoactivatable analogs of two major lipoprotein phospholipids, phosphatidylcholine and sphingomyelin.

In the fluorescent studies we incorporated anthrylvinyl-labeled phosphatidylcholine (APC) or sphingomyelin (ASM) (Schemes 1 and 2) into the lipoprotein surface and then registered changes in their fluorescence parameters brought about by addition of prostaglandins (12-15).

The fluorescence parameters measured in our experiments were the steady state fluorescence polarization (P), which depends reversely on the fluidity of the probe's microenvironment and the tryptophane-to-probe resonance excitation energy transfer, which decreases sharply with increasing distance (r) between the donor and acceptor fluorophore according to the equation:

$$E = R_0^6/(R_0^6 + r^6)$$

where E is the efficiency of energy transfer and R_0 a characteristic constant, the so called Foerster radius.

APC and ASM are new fluorescent probes which were synthesized in our laboratory several years ago (16,17). In our experience, the anthrylvinyl group proved quite suitable for fluorescence polarization and energy transfer measurements because the lifetime of that fluorophore in the excited state is sufficiently short and its fluorescence excitation maximum overlaps with the emission maximum of tryptophane (18). In preliminary studies on model systems, anthrylvinyl-labeled phospholipids showed at least three important advantages in comparison with several other membrane probes (18): (i) being nonpolar, flat and non-bulky, the anthrylvinyl group induces relatively small perturbations of the lipid bilayer, (ii) when attached to the end of one of the hydrocarbon chains of a phospholipid, the anthrylvinyl group localizes uniformly in the center of the bilayer; and (iii) anthrylvinyl-labeled lipids mimic the behavior of their corresponding natural prototypes in artificial and biological membranes.

In multicomponent systems in which phase separation occurs, anthrylvinyl-labeled lipid probes remain associated with their natural counterparts (18). This makes anthrylvinyl-labeled lipids powerful tools in studies of the lipid molecular organization in biological membranes, as

well as in the surface layer of serum lipoproteins.

Although both the phosphatidylcholine probe APC and the sphingomyelin probe ASM incorporate spontaneously into the lipoprotein surface with the same efficiency and similar rates, they appear to reside in different surface domains of the lipoprotein globulae (19).

This conclusion is based on three observations: (i) in homogeneous environments, the two phospholipid probes show similar fluorescence polarization (17); however, when both are incorporated into HDL or LDL, much greater differences are seen in their fluorescence polarization values; (ii) when inserted into the LDL surface, the two phospholipid probes show different phase behavior of their corresponding host lipids, as can be inferred from the plots of their fluorescence polarization against temperature (Fig. 4); and (iii) in HDL_2 and HDL_3, there is an efficient energy transfer from apo-A_1 tryptophanyls to ASM but not to APC (19).

If the fluorescent labeled phospholipid probes APC and ASM reflect the behavior of phosphatidylcholine and sphingomyelin in lipoprotein particles, the above observations suggest that at the lipoprotein surface these two phospholipids distribute nonrandomly forming different domains. The domains probably consist of phosphatidylcholine- or sphingomyelin-enriched lipid mixtures in a state of dynamic equilibrium. Their formation may be caused by different types of interaction between phosphatidylcholine or sphingomyelin and apolipoproteins.

Using ASM- or APC-labeled high density (HDL_2, HDL_3, apo-A_1 recombinants) and low density lipoproteins, we observed that in all these lipoproteins both the fluorescence polarization (*P*) and the efficiency of the tryptophane-to-probe energy transfer (*E*) were sensitive to the presence of small amounts of PGE_1 (Fig. 5) (13-15). The effects of PGE_1 appeared to be caused by its interaction with protein rather than with lipid at the lipoprotein surface because no changes in *P* and *E* were observed upon addition of the prostaglandin to APC- or ASM-labeled liposomes with a lipid composition resembling that of the lipoprotein surface (14,15). On the other hand, with HDL-recombinants prepared from the same lipids and apo-A_1, the PGE_1-induced changes of *P*and *E* were similar to those observed with HDL_2 or HDL_3 (14).

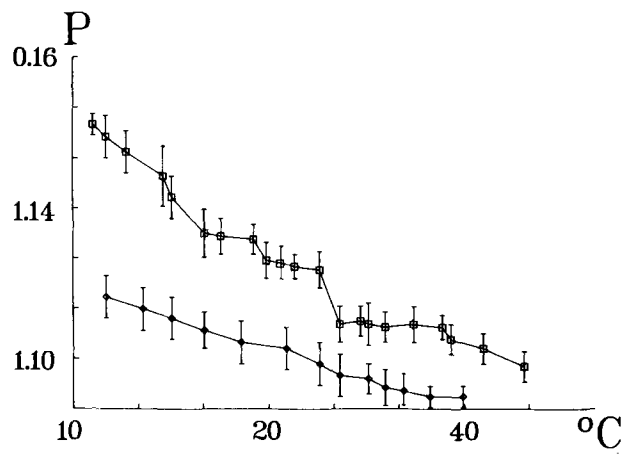


FIG. 4. Temperature dependence of the fluorescence polarization P in LDL labeled with ASM (□) or APC (◇) (from ref. 15).

The changes in the *P*and *E* values induced by PGE_1 proved to specifically depend on the prostaglandin structure because the closely related PGE_2 produced no measurable changes in the fluorescence parameters of ASM- or APC-labeled HDL and LDL and did not interfere with the action of PGE_1 (14,15).

A high structural specificity of the prostaglandin-lipoprotein interaction was also seen with prostaglandins of the F-series. Thus, $PGF_{2\alpha}$ induced an increase in the fluorescence polarization of ASM-labeled low density lipoproteins, whereas $PGF_{1\alpha}$ did not influence the *P*-values of these lipoproteins (Fig. 6) (15). At the same time, a given prostaglandin appeared to affect different phospholipids on the lipoprotein surface to a different

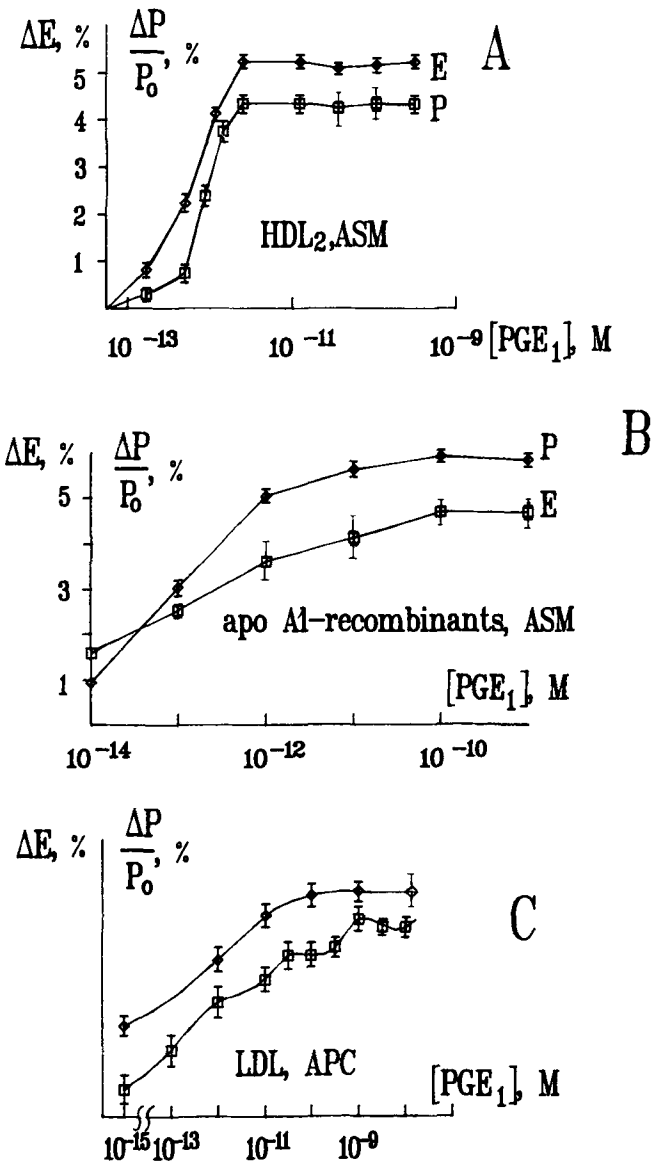


FIG. 5. Influence of PGE_1 on the tryptophane-to-probe energy transfer, E, and fluorescence polarization, P, in ASM-labeled HDL_2 (A), apo-A_1 recombinants (B), and in APC-labeled LDL (C). Lipoprotein concentration, 1 mg protein/mL. Before measurement, each sample was incubated with PGE_1 for 30 min at 36.5°C. ΔP=P−Po, where P and Po are the fluorescence polarization values in the presence and absence of PGE_1 (from refs. 14 and 15).

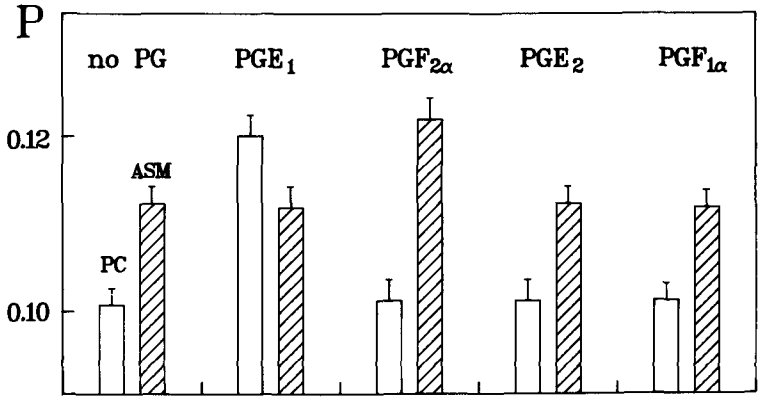


FIG. 6. Influence of prostaglandins on the fluorescence polarization of LDL labeled with fluorescent phosphatidylcholine (empty bars) or sphimgomyelin (hatched bars). The lipoprotein (1 mL protein/mL) was labeled with APC or ASM and incubated with PG (10^{-9}M) for 30 min at 36.5°C (from ref. 14).

degree. For example, in LDL PGE_1 caused an increase of P_{APC} but did not influence the value of P_{ASM}, whereas $PGF_{2\alpha}$ induced a large change of P_{ASM}, but not of P_{APC} (15). One may deduce from these data that PGE_1 and $PGF_{2\alpha}$ may cause different types of lipid domain rearrangement on the LDL surface, and that such structural reorganization may be mediated by interaction of the prostaglandins with apolipoproteins.

In an attempt to confirm this conclusion in an independent fashion, we studied the influence of prostaglandins on LDL labeled with the photoreactive analogs of phosphatidylcholine and sphingomyelin, namely NAP-PC and NAP-SM. Upon photolysis, these photoactivatable lipids generate highly reactive nitrene radicals which covalently bind to protein sites that are in contact with the probe. Thus, one can identify changes in the relative position of hydrophobic protein segments and surrounding lipids (21,22). Recently a photoactivatable analog of phosphatidylethanolamine labeled in the polar head group moiety was shown to cross-link efficiently to apolipoprotein B (apo-B) of LDL and to apo-A_1 of HDL (23).

After illumination of LDL labeled with [^{14}C]NAP-PC or [^{3}H]NAP-SM, a minor part of the photoactivatable probes was covalently bound to apolipoprotein B. In the presence of low amounts of PGE_1 ($\sim 10^{-9}$ M), the binding efficiency of NAP-SM increased significantly, whereas that of NAP-PC remained almost unchanged (Table 2) (15). By contrast, addition of $PGF_{2\alpha}$ selectively enhanced the binding efficiency of NAP-PC to apo-B, but did not influence cross-linking of NAP-SM (15). These photolabeling experiments demonstrated that addition of PGE_1 or $PGF_{2\alpha}$ to LDL caused different changes in the average distance between certain apo-B segments and photoactivatable analogs of phosphatidylcholine and sphingomyelin.

The reversibility of PGE_1-induced changes of lipoprotein surface organization. The PGE_1-induced changes in fluorescent polarization of fluorescent labeled HDL or LDL were partly reversible. When the prostaglandin was removed by dialysis (14), charcoal treatment (15) or by adding excess albumin (14), the fluorescence polarization values decreased approaching (but never achieving) the initial level. They could be raised again by adding fresh PGE_1. Upon repetition, the degree of reversibility decreased gradually, and after several (5-6) cycles, the samples became turbid and irresponsive towards the prostaglandin. A possible explanation of this behavior may relate to the fact that the aggregation of both HDL_2 and LDL is somewhat enhanced in the presence of PGE_1.

After removal of the prostaglandin, the time required for relaxation was relatively long (Table 3) (14). Interestingly, the relaxation of the fluorescence polarization *P* occurred much slower than that of the tryptophane-to-probe energy transfer *E*. This difference is understandable because changes in *E* are related to conformational transition of few apolipoprotein molecules, whereas

TABLE 2

Influence of PGE_1 (10^{-9} M) on the Binding of Photoactivatable Isotopically Labeled Sphingomyelin NAP-SM and Phosphatidylcholine NAP-PC to Apo-B in LDL Samples Obtained from Different Donors

	$(A_{PG} - Ao)/Ao$[a]	
	NAP-SM	NAP-PC
Donor 1	17	−1
Donor 2	49	−2

[a] A_{PG} and Ao are the binding efficiencies of photoactivatable lipid in the presence and absence of PGE_1. The binding efficiency is the percentage of total added photoactivatable lipid covalently attached to apo-B (from ref. 15).

TABLE 3

Influence of Excess Human Serum Albumin (HSA) on PGE_1-induced Changes of the Fluorescence Polarization (P) and Tryptophane-to-Probe Energy Transfer (E) in ASM-labeled HDL_2[a]

	Half-time of saturation after adding PGE_1	Half-time of relaxation after adding HSA
P	160 sec	600 sec
E	145 sec	≅30 sec

[a] Concentrations of PGE_1, HSA and HDL were 10^{-9} M, 10^{-6} M, and 0.6-1.0 mg protein/mL, respectively.

changes in P reflect packing changes of many lipid molecules organized in a cooperative manner.

The low binding capacity of lipoproteins for prostaglandin E_1. In the course of our investigations, we attempted to isolate a prostaglandin-lipoprotein complex after incubation of HDL_2 or LDL with various amounts of tritium-labeled PGE_1. The isolation procedures employed involved centrifugation, gel-filtration and ultrafiltration. In no case was it possible to detect any radioactivity beyond background noise in the lipoproteins separated after incubation (14,15).

The binding of [^{3}H]PGE_1 to HDL_2 or LDL also was examined by equilibrium dialysis in buffer solution. The maximal binding capacity of the lipoproteins for PGE_1 was very low (approximately 1 PGE_1 molecule per 10^3–10^4 lipoprotein particles) (14,15). Hence, if a PGE_1-lipoprotein complex exists, its lifetime must be quite short. Nevertheless, such unstable binding seems to produce a rearrangement of the lipoprotein surface which may have important physiological consequences.

A non-equilibrium model of prostaglandin-lipoprotein interaction. The fact that the PGE_1-induced changes in fluorescence polarization and energy transfer values in fluorescent labeled lipoproteins are already saturated at extremely low prostaglandin concentration could be explained by assuming that either only a small fraction of the lipoprotein globula is sensitive towards the prostaglandin or that the latter induces a chain reaction (*e.g.*, aggregation) among the lipoprotein particles. However, the first explanation is incompatible with the comparatively large change of fluorescence polarization induced by the prostaglandin and the second one seems unlikely because the PGE_1-induced changes are reversible. Furthermore, the aggregation of lipoproteins, although somewhat enhanced by PGE_1, appeared to be slow in comparison to the changes of the fluorescent parameters. We were therefore forced to look for some other mechanisms which would allow a single PGE_1 molecule to induce structural changes in a large number of discrete lipoprotein targets. We proposed that in an open nonequilibrium system characterized by a short lifetime of the ligand-receptor complex, a high diffusion constant of the ligand in the medium and long relaxation times exceeding the interval between two effective ligand-receptor collisions, high sensitivity can be achieved through accumulation of the system's responses to the effector (24). A kinetic model of such a system is shown in Figure 7. The model postulates that the ligand, L, binds to the receptor, R, only in the resting state and that the lipid environment of the receptor "remembers" the ligand-receptor interaction for a longer time than the receptor itself. In this way, the system RLip could be transferred into an altered state, RLip*, which is far removed from equilibrium.

The PGE_1-lipoprotein system seems to satisfy these conditions because the PGE_1-induced changes are reversible (or partly reversible), the lifetime of the PGE_1-lipoprotein complex is short, and the diffusion constant of PGE_1 molecules in water should be in the order of 10^{-10} m^2/sec, whereas the relaxation times after removal of PGE_1 are in the range of 10^1–10^2 seconds. As mentioned above, after removal of PGE_1 the relaxation times of the fluorescence polarization and energy transfer are different (Table 3). By contrast, the times required for saturation of these two parameters are similar.

This can be rationalized by assuming that the relaxation rates of P and E depend on the behavior of the lipids and the PGE_1-binding apolipoprotein, respectively, whereas their saturation rates are determined by a common factor, the frequency of effective PGE_1-HDL collisions. If so, the time required for saturation should depend inversely on the concentration of the prostaglandin in the medium. This proved to be true for the PGE_1-induced changes of P in HDL_2 and LDL (Fig. 8).

In conclusion, our results demonstrate that the binding capacity of HDL or LDL for prostaglandin E_1 is very small. This suggests that serum lipoproteins probably do not serve as vehicles for prostaglandin transport in the blood-stream.

Formally, the interaction of prostaglandin E_1 with HDL or LDL resembles the interaction of a ligand with a specific receptor because it is time and concentration dependent, reversible, and saturable. However, it differs from classical ligand-receptor interactions because a

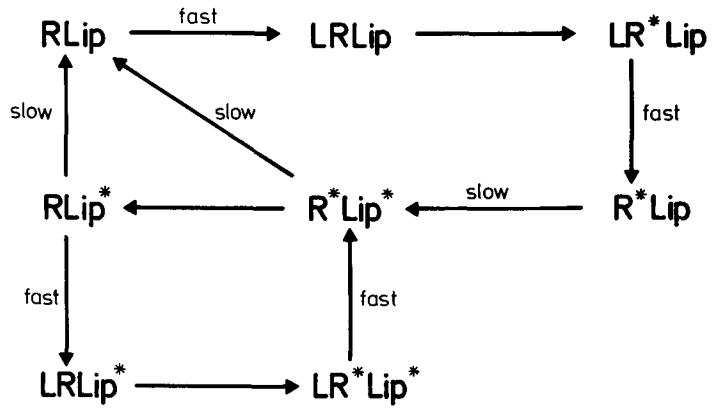


FIG. 7. Kinetic non-equilibrium model of the interaction of prostaglandin E_1 (L) with a lipoprotein particle. The model postulates that the prostaglandin binds to the "receptor" apolipoprotein R only for a short time and that the lipid environment Lip of the receptor "remembers" the ligand-receptor collision for a longer time than the receptor itself. As a result, the system RLip is transferred into an altered state, RLip*, despite the absence of stable binding of the prostaglandin.

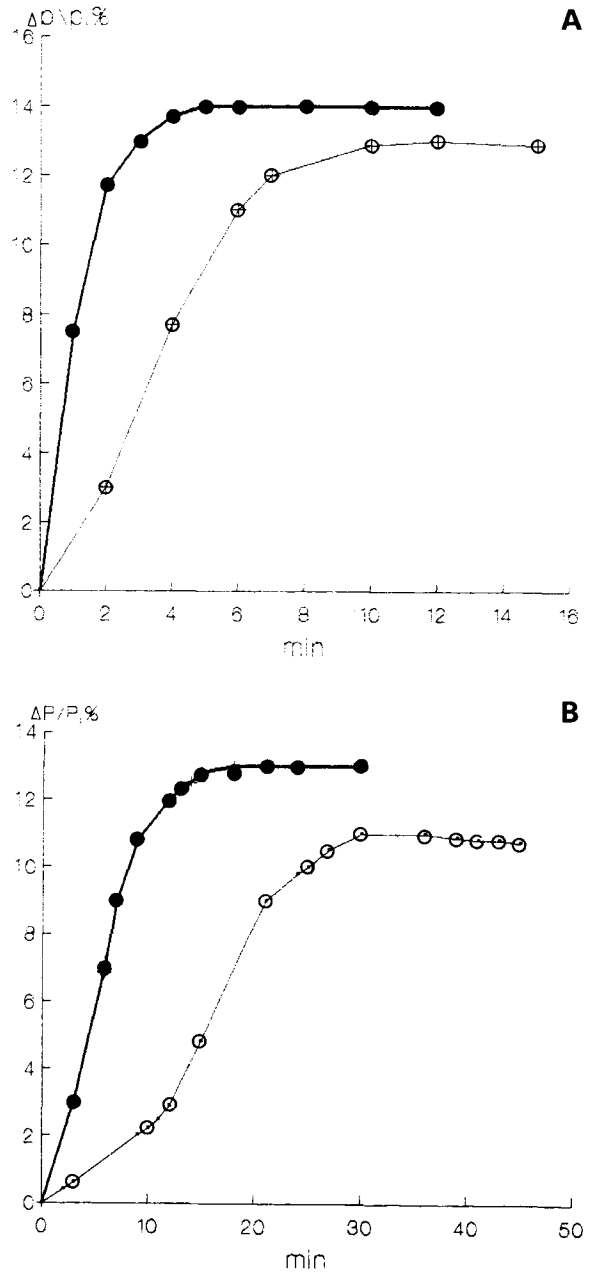


FIG. 8. Time course of PGE_1-induced changes in the fluorescence polarization of fluorescent labeled HDL_2 and LDL (0.4-0.6 protein/mL). Empty circles, 10^{-10}M PGE_1; black circles, 10^{-9} M PGE_1.

single ligand molecule (PGE_1) is able to change the state of a large number of lipoprotein target particles. In order to explain this unusual fact we propose a "non-equilibrium" model, where sufficiently slow relaxation of the lipids surrounding the receptor can result in accumulation of responses to transient ligand-receptor interactions. We assume that in the lipoprotein surface layer, slow relaxation processes are related to changes in the lipid domain organization. If this assumption is correct, we can envision the following chain of events induced by the presence of PGE_1 in the medium: Contact of the prostaglandin with a specific site of one of the apolipoproteins results in a fast conformational change of the local binding site, but other parts of the polypeptide chain and the surrounding lipids remain unchanged. This situation appears to induce some sort of strain. Relief from this strain requires transition of many lipid molecules organized in a cooperative manner and, therefore, can only occur slowly. When the prostaglandin molecule dissociates off, the original conformation of the local binding site is restored quickly and the lipoprotein particle starts to relax and to return to its initial state. However before this relaxation process is completed, a new prostaglandin molecule binds to the receptor maintaining the changed state of the lipoprotein surface. In this way, the lipoprotein particle may be transferred into a new steady state which is far from equilibrium. The question whether the non-equilibrium model is restricted to the interaction of HDL and LDL with PGE_1 or may be applied to other ligand-receptor systems as well remains to be answered. A general point of concern is that according to the proposed model the ligand, although binding poorly, is able to induce a structural change of the lipoprotein surface. Such a situation could arise if the ligand-induced conformational change of the "receptor" promotes dissociation of the ligand, thus resulting in a short "on-off" time.

The physiological consequences of our observations also remain to be defined. The results of our studies suggest that *in vitro* the HDL-to-LDL transfer of cholesteryl esters is markedly stimulated by PGE_1, which modulates lipoprotein surface organization through interaction with some of the apolipoproteins. In the organism, interlipoprotein transfer of cholesteryl esters is a necessary step in the conversion of HDL_3 to HDL_2 and has been postulated as essential for the removal of cholesterol from peripheral cells (25). This then suggests the possibility that the direct interaction of PGE_1 with serum lipoproteins may be a factor involved in the regulation of cholesterol homeostasis. Indirectly, such view is corroborated by the fact that among the prostaglandins tested, only the antiatherogenic PGE_1 stimulates interlipoprotein cholesteryl ester transfer, whereas PGE_2, $PGF_{2\alpha}$, $PGF_{1\alpha}$ and PGA_2 have no influence on the rate of transfer. However, the presence of appreciable amounts of PGE_1 in serum is still a subject of controversy. Moreover, we must, of course, keep in mind that in the organism where other prostaglandins—including prostacylin and thromboxane as well as many other atherogenic and antiatherogenic factors—are acting simultaneously, the situation is much more complex. Nevertheless, we believe that further investigation in this field offers promise of a more complete understanding of the pathogenesis of atherosclerosis.

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Properties of an Acid Cholesteryl Ester Hydrolase Inhibitor from Rat Serum

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The inhibitory effect of a protein isolated from rat serum on lysosomal acid cholesteryl ester hydrolase (acid CEH; EC.3.1.1.13) activity was studied. An inhibitor was purified from rat serum following ultracentrifugation and heat treatment using column chromatography on Sephacryl S-200 and ultrafiltration. The purified inhibitor appeared as a single protein band in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. The molecular weight of the inhibitor was 28,000 Daltons as judged by gel filtration on Sephacryl S-200 and SDS-polyacrylamide gel electrophoresis. The purified inhibitor was shown to be apolipoprotein A-I (apo A-I), the major apolipoprotein of high-density lipoprotein (HDL), using immunoprecipitation with rat anti-apo A-I immunoglobulin (Ig)G. Inhibition of acid CEH activity by apo A-I was dependent on the concentration of apo A-I. The values of V_{max} obtained were similar with or without apo A-I. Apo A-I of various other mammalian species, including human, bovine and rabbit, also inhibited acid CEH activity. Other apolipoproteins, such as apo A-II and apo B, also showed inhibiting activity. On the other hand, apo A-I had no effect on the activity of other enzymes found in lysosomes, such as cathepsin D, β-glucuronidase and acid phosphatase. The results suggest that apolipoproteins may play a role in the regulation of hydrolysis of cholesteryl esters in lipoproteins, that have been transferred to the liver, and that the inhibition of acid CEH activity by apo A-I may be a characteristic of the lipid-binding protein or be due to changes of the lipid/water interface.
***Lipids 25,* 775–778 (1990).**

Evidence exists that lysosomal acid cholestryl ester hydrolase (CEH) catalyzes the hydrolysis of cholesteryl esters that enter the cell in the form of lipoproteins (1), and that variations in acid CEH activity may play a role in the metabolism of cholesteryl esters in tissues, including liver, spleen and aorta (2). Acid CEH isolated from liver has been well-characterized (3–8).

Much less is known about the regulation of acid CEH activity in the liver under physiological conditions. Therefore, identification of factors that regulate acid CEH activity is of major importance.

An inhibitor of acid lipase has previously been isolated from rat serum by Gorin *et al.* (9), and from gall bladder mucosa by Neiderhiser (10). In addition, we have previously reported the presence of a cytosolic protein that inhibits the activity of acid CEH (11). In the present study, we found in serum a protein that has an inhibitory effect on acid CEH. This report describes the properties of this acid CEH inhibitor from rat serum.

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Abbreviations: Apo A-I, apolipoprotein A-I; CEH, cholesteryl ester hydrolase; EDTA, ethylenediaminetetraacetate; HDL, high-density lipoprotein; IG, immunoglobulin; LDL, low-density lipoprotein; SDS, sodium dodecyl sulfate; VLDL, very low-density lipoprotein.

MATERIALS AND METHODS

Chemicals and radiochemicals. Cholesteryl [1-^{14}C]oleate (specific activity 58.6 mCi/mmol) was purchased from New England Nuclear Corp. (Boston, MA). Trypsin, chymotrypsin, trypsin inhibitor, phenolphthalein glucuronide, phenylphosphate, hemoglobin, human apo A-I, apo A-II, apo B, bovine apo A-I and rabbit apo A-I were purchased from Sigma Chemical Co. (St. Louis, MO). Sephacryl S-200, Polybuffer Exchanger 94, Polybuffer 74 and standard proteins for electrophoresis were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Centriflo (Type CF-25) was purchased from Amicon Co. (Danvers, MA). Rat anti-apo A-I IgG was a gift from Tsumura Co. (Tsukuba, Chiba, Japan).

Preparation of lysosomes. Young male Sprague-Dawley rats weighing 180–200 g were killed by decapitation and the livers were perfused with ice-cold 1.15% KCl solution at 4°C. The tissues were homogenized in 8 vol of ice-cold 0.25 M sucrose/1 mM ethylenediaminetetraacetate (EDTA)/0.01 M Tris-HCl buffer (pH 7.5). The homogenate was fractionated by the method of Brecher *et al.* (12) to obtain the lysosomal fraction.

Purification of acid CEH. All preparation steps were carried out at 4°C. Acid CEH was prepared as described previously (8).

Acid cholesteryl ester hydrolase assay. Acid CEH activity was measured by the method of Brecher *et al.* (12). The substrate was prepared as described previously (11). The sensitive microassay was carried out in 0.1 M acetate buffer (pH 5.0) in a final volume of 0.3 mL. All assays were done using quantities of protein (usually less than 1 μg) and incubation times (10 min) that ensured nearly linear rates of substrate hydrolysis. One unit of acid CEH inhibitor was defined as the amount of protein inhibiting 50% of acid CEH activities under assay conditions. Specific inhibitory activity was expressed as units per mg protein.

Assay of lysosomal marker enzymes. Cathepsin D was assayed by the method of Hirado *et al.* (13), using hemoglobin as the substrate. The amount of reaction product was assayed by the method of Lowry *et al.* (14). β-Glucuronidase was assayed using phenolphthalein glucuronide as the substrate. Phenolphthalein liberated was measured by the method of Gianetto and De Duve (15). Acid phosphatase was measured using phenylphosphate as the substrate essentially as described previously (16).

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis in 7% gel was carried out by the method of Laemmli (17). The purified acid CEH inhibitor (50 μg) and marker protein (20 μg each) were heated at 100°C for 3 min in 0.1 M sodium phosphate buffer, pH 7.4, containing 2% SDS, 5 M urea and 5% 2-mercaptoethanol before electrophoresis. β-Galactosidase, phosphorylase B, bovine serum albumin, carbonic anhydrase and soybean trypsin inhibitor were used as molecular weight standards.

Protein determination. Protein was determined by the method of Lowry *et al.* (14) using crystalline bovine serum albumin as a standard. Eluates from columns were determined by measuring the absorbance at 280 nm.

Acid CEH inhibitor localization in lipoprotein fractions. Lipoproteins were separated from sera of male Sprague-Dawley rats (250 g body weight) as described previously (18). The animals were fasted for 24 hr before bleeding. Density criteria for the various lipoprotein fractions were as follows: d < 1.006 for very low-density lipoprotein (VLDL), d = 1.019–1.040 for low-density lipoprotein (LDL) and d = 1.063–1.21 for high-density lipoprotein (HDL) (unless specified otherwise).

Purification of acid CEH inhibitor from rat serum. HDL was separated by ultracentrifugation using a Hitachi RP 65 rotor (18). After dialysis against 0.15 M NaCl, the HDL fraction was boiled for 5 min and centrifuged at 5,000 rpm for 20 min. The supernatant was chromatographed on a Sephacryl S-200 column (3 × 60 cm) equilibrated with 20 mM Tris-HCl buffer (pH 7.8) containing 0.15 M NaCl. The fractions of eluate containing the inhibitor were pooled and dialyzed against 50 vol of 0.01 M Tris-HCl buffer (pH 7.4). The dialysate was concentrated by ultrafiltration on Centriflo (Type CF-25). The concentrated sample was rechromatographed using the same column for further purification.

RESULTS

As shown in Figure 1, acid CEH inhibitor activity was associated with all lipoprotein fractions, as well as the lipoprotein-free serum fraction. The specific activity was especially high in the HDL fraction.

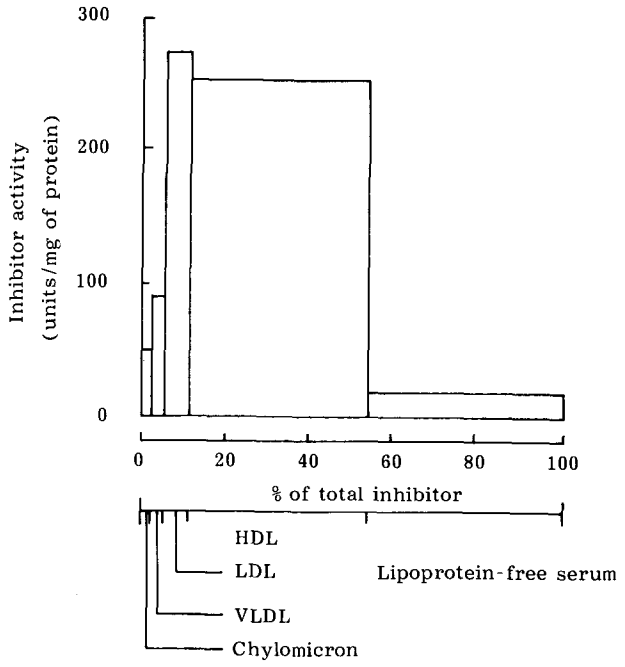

FIG. 1. Distribution of acid CEH inhibitor in rat serum. Results are expressed as specific inhibitory activity cumulative percentages of total recovered inhibitory activities. Total recovery of inhibitory activity was 90%.

The acid CEH inhibitor was purified from rat serum as described in Materials and Methods. Table 1 summarizes the results of the individual purification steps by which the acid CEH inhibitor was purified 97-fold from rat serum. The purified inhibitor showed an apparently single protein band corresponding to a molecular weight of 28,000 as judged by SDS-polyacrylamide gel electrophoresis (data not shown).

As shown in Figure 2, incubation of the purified inhibitor with increasing amounts of anti-apo A-I at 20°C for 30 min produced a progressive decrease in the inhibitory activity of the purified inhibitor on acid CEH activity. These results confirm that the purified inhibitor was apo A-I.

Acid CEH activity was markedly inhibited by addition of purified apo A-I. The inhibitory effect of apo A-I protein on acid CEH activity was concentration dependent, with addition of about 0.5 μg of apo A-I causing approximately half-maximal inhibition of acid CEH activity (Fig. 3).

The kinetics of inhibition of acid CEH, which is illustrated in Figure 4 as Lineweaver-Burk plots, showed that apo A-I inhibited acid CEH competitively. The apparent Km values were 14.3 μM and 8.6 μM in the presence and absence of apo A-I, respectively.

TABLE 1

Purification of Serum Inhibitor on Acid CEH

Purification	Total protein (mg)	Specific activity (units/mg of protein)	Purification (fold)
Serum	1043.8	28.0	1.0
HDL fraction	27.4	216.0	7.7
Boiled HDL fraction	27.4	763.4	27.3
Sephacryl S-200	6.4	1123.6	40.1
Ultrafiltration	1.8	2717.4	97.1

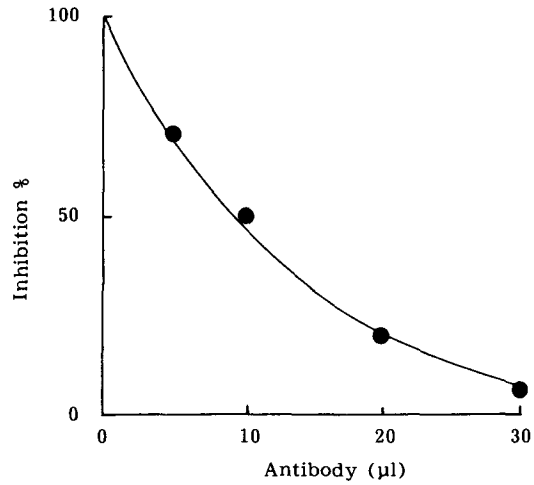

FIG. 2. Reversion of apo A-I induced acid CEH inhibition by an anti-apo A-I IgG. Dilutions (1:20) of rat anti-apo A-I IgG with 10 mM sodium phosphate, pH 7.4, were incubated with 2 μg purified inhibitor for 30 min at 20°C. Following the incubation, the samples were subjected to standard assay conditions.

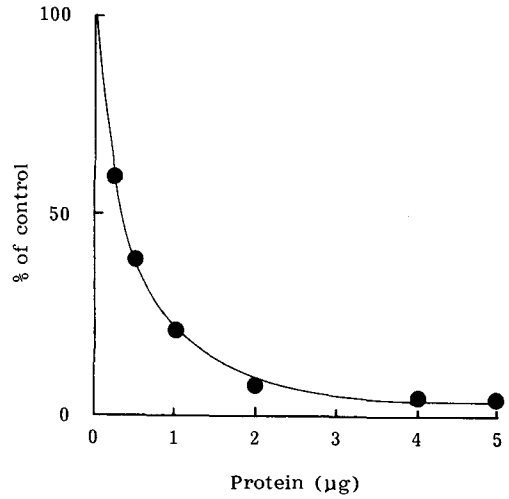

FIG. 3. Effect of apo A-I on acid CEH activity. The 100% value corresponds to the activity under standard assay conditions.

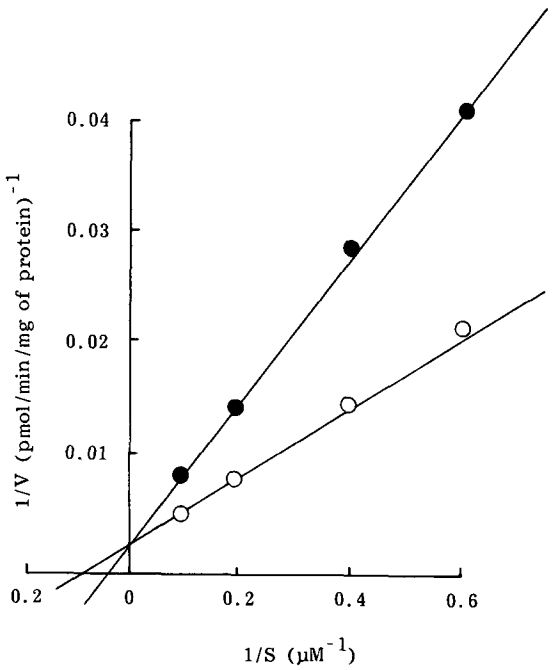

FIG. 4. Lineweaver-Burk plots of acid CEH in the presence (●) or absence (○) of apo A-I (0.5 μg). Standard assay conditions are as described in Materials and Methods.

Next, we tested the inhibition of acid CEH activity by various apo A-I types, such as those from human, bovine and rabbit. We found that there was no significant difference in the inhibitory effects of the different apo A-I types on acid CEH activity (data not shown).

In order to determine whether the inhibitory effect of the apoprotein on acid CEH activity was specific for apo A-I, we also evaluated other apolipoproteins, such as human apo A-II and apo B (data not shown). We found that under the conditions chosen for human apo A-I, human apo A-II or apo B also inhibited acid CEH activity to similar extents. However, apo A-I did not inhibit three other lysosomal enzymes, namely cathepsin D, acid phosphatase and β-glucuronidase, but slight inhibition of neutral CEH and steapsin was observed (data not shown). The concentrations of apo A-I used in these experiments were the same as those in Figure 3.

DISCUSSION

In the present study, we found an inhibitor of lysosomal acid CEH which is particularly prominent in the HDL fraction of rat serum. The purified acid CEH inhibitor gave a single band on SDS-polyacrylamide gel electrophoresis and showed a molecular weight of 28,000 daltons.

The inhibitor was found mainly in the HDL and d = 1.21 bottom fractions. The HDL fraction contains several proteins, most of which have been classified as apolipoprotein A-I. Apo A-I has previously been reported to have a molecular weight of 28,000 daltons and an unusually high content of the α-helical conformation (19–23). As shown in Figure 2, immunoprecipitation of the purified protein, using rat anti-apo A-I IgG, completely removed acid CEH inhibitory activity. These results confirmed that the purified inhibitor is apo A-I.

On the other hand, apolipoprotein is an important structural and functional component of serum lipoproteins which function as receptor ligands and enzyme cofactors. Kubo *et al.* (24) reported that human plasma apo A-I inhibited the hydrolysis of triglyceride catalyzed by hepatic triglyceride lipase. Furthermore, Kinnunen and Ehnholm (25) reported that apo C inhibited the hydrolysis of triglyceride catalyzed by hepatic triglyceride lipase. The inhibitory effect of apo A-I on acid CEH activity in this study is very similar to that of apo A-I reported by Kubo *et al.* (24) and apo C reported by Kinnunen and Ehnholm (25).

On the other hand, as for the inhibition of acid lipase, Gorin *et al.* (26) reported that an inhibitor of acid lipase in cultured fibroblasts is present in human and calf serum. This inhibitor was found mainly in Cohns fraction IV of these sera, and was shown to have a molecular weight of 50,000 daltons and to be heat-labile. In the present work, apo A-I had an apparent molecular weight of 28,000 daltons as estimated by SDS-polyacrylamide gel electrophoresis and was heat-stable. These results suggest that apo A-I is different from the acid lipase inhibitor in human serum as described by Gorin *et al.* (26).

Moreover, we had reported previously that a cytosolic protein in rat liver has an inhibitory effect on acid CEH activity (11). However, it seems that this cytosolic inhibitor and apo A-I are not the same, because the former is heat-labile and has a higher molecular weight than apo A-I.

In the present study, acid CEH inhibitor was also found in the d = 1.21 bottom fraction. The inhibitory effect of the bottom fraction may be due to an apo A-I or to other apolipoproteins released from their respective lipoprotein particles during the separation process by ultracentrifugation. However, it is not clear at present whether apo A-I and the inhibitor from the bottom fraction are the same.

Two cholesteryl ester hydrolase activities with differing pH optima are known to be present as neutral and acid CEH in various tissues. It seems likely that the

hydrolysis of cholesteryl esters in lipoproteins in intact cells is catalyzed mainly by a lysosomal enzyme (1). This enzyme can be inhibited by apo A-I and other apolipoproteins. If apo A-I is related to the regulation of cholesteryl ester utilization in intact cells, its introduction into the intracellular environment, probably the lysosomes, may be an essential process. However, it is not clear at present whether or not apo A-I can be taken up by lysosomes in intact cells.

The surface properties of apolipoproteins at the lipid/water interface have been described in earlier studies. Ibdah *et al.* (27) reported apo A-II to have a higher affinity for the lipid/water interface than does apo A-I. In the present study, we demonstrated that the inhibitory effects of apo A-I and apo A-II were not significantly different, and that apo B also inhibited acid CEH activity with the same potency as did apo A-I. Moreover, apo A-I caused competitive inhibition of the hydrolytic activity of acid CEH on cholesteryl esters. However, it is not clear at present whether the inhibition of acid CEH activity by apo A-I is due to its properties as a lipid-binding protein or to changes at the lipid/water interface.

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Influence of Dietary Fats on Butyrylcholinesterase and Esterase-1 (ES-1) Activity in Plasma of Rats

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We studied the effects of dietary fats, especially fish oil, on the activities of esterase-1 (ES-1) and butyrylcholinesterase in the plasma of rats. The identification of nutritional determinants of these enzymes could provide clues as to their physiological function. Fish oil, when compared with corn oil, consistently caused increased activities of both enzymes. Plasma ES-1 activity, but not butyrylcholinesterase activity, was increased after isocaloric replacement of carbohydrates by coconut fat. Dietary medium-chain triglycerides, when compared with corn oil, produced decreased and increased activities of butyrylcholinesterase and ES-1, respectively. Various plant fats, such as corn oil, linseed oil, coconut fat, palm oil, palm kernel oil, soybean oil and rapeseed oil, did not differentially influence butyrylcholinesterase activities. Plasma triglyceride concentrations were lowered by fish oil and increased by coconut fat and palm kernel oil. For individual rats in 5 out of 6 experiments, weak, negative correlation coefficients of the order of 0.3 were found between the changes in plasma butyrylcholinesterase activities and in plasma triglyceride concentrations.
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The plasma of vertebrate animals contains various enzymes that can catalyze the hydrolysis of artificial fatty acid esters of aromatic alcohols, such as α-naphthylacetate and *p*-nitrophenylacetate (1). Although the physiological function of these esterases is still obscure, there is evidence that they are involved in lipid metabolism (2–4).

We have recently described (4) that the replacement of isocaloric amounts of carbohydrates by fat in the diet of rats caused a slight increase in plasma total esterase activity as measured with *p*-nitrophenylacetate as substrate. This increase was associated with a pronounced increase of the activity of the so-called esterase-1 (ES-1) isozyme, an anodal, fast-moving plasma esterase zone in polyacrylamide gel electrophoresis. The fat-induced increase in ES-1 activity was similar for coconut fat and corn oil (4), suggesting that the amount of fat in the diet, rather than the type of fat, influences ES-1 activity. However, since only two fat types were compared, final conclusions appeared premature.

Van Houwelingen *et al.* (5) reported that a dietary supplement of fish paste, when compared with meat paste, lowered pseudo(butyryl)cholinesterase activity in the plasma of healthy volunteers. This might imply that n-3 polyunsaturated fatty acids in fish oil decrease the activity of butyrylcholinesterase, an esterase whose physiological function is presently not known. The well-known triglyceride-lowering action of dietary fish oil (6) would substantiate this implication because there is some evidence that decreased plasma butyrylcholinesterase activities are associated with decreased concentrations of plasma triglycerides (7).

In the course of six studies with rats on the effects of dietary fat types on plasma lipids, we also had the opportunity to address the questions: i) Do plasma ES-1 and butyrylcholinesterase share common characteristics concerning their response to changes in the amount and type of fat in the diet? The information obtained could provide clues as to the physiological function of plasma esterases. ii) Can plasma ES-1 activity be influenced by the type of fat in the diet, especially by fats rich in n-3 fatty acids, such as fish oil and linseed oil? iii) Does plasma butyrylcholinesterase activity in rats respond to increased fish oil intake, as would be anticipated on the basis of a study carried out in humans (5)? iv) Is there a relationship between diet-induced changes in plasma butyrylcholinesterase activity and plasma triglyceride concentrations? A direct relationship has been suggested to exist, at least in humans with various types of hyperlipoproteinemia (7). This communication attempts to answer these four questions.

MATERIALS AND METHODS

Animals. Outbred Wistar rats (Cpb:WU, Wageningen, The Netherlands), aged 3 to 8 weeks, were used throughout. They had been fed *ad libitum* a commercial, pelleted diet (RMH-B®, Hope Farms BV, Woerden, The Netherlands). Male rats were used, except for Experiment 6 in which females were used. The rats of Experiments 1, 3, 4 and 6 were kept three in a cage in wire-topped Makrolon-3 cages (UNO BV. Zevenaar, The Netherlands) with a layer of sawdust as bedding. The animals of Experiments 2 and 5 were housed individually in stainless-steel cages with wire-mesh bases exactly as described previously (8). The cages were located in a room with controlled lighting (12 hr/day; light 0700–1900 hr) at constant temperature (20–22°C) and relative humidity (50–70%).

On day 0 of each experiment, the rats were divided into 2 to 5 dietary groups of 6 to 18 animals each. Within each experiment, the groups had similar distributions of body weight. The mean values were: Experiment 1, 59 g; Experiment 2, 265 g; Experiments 3 and 6, 62 g; Experiment 4, 64 g and Experiment 5, 78 g.

Diets. During the experimental period, the rats were fed purified diets and had access to tap water *ad libitum.* In Experiment 6, the diets were in pelleted form; in the other experiments they were in meal form. All diets contained at least 2.1% of energy as corn oil (Mazola®, Knorr Caterplan Gmbh, Heilbronn/Neckar, F.R.G.), so as to provide sufficient linoleic acid. Separate batches of diet base

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Abbreviation: ES-1, esterase-1.

mixtures (without the variable fat sources; Experiments 1-4) or complete diets (including the variable fat sources; Experiments 5 and 6) were made for each experiment. The diet base mixtures or complete diets were stored at 4°C until feeding. Table 1 shows the ingredient composition of the diets. Crude fat concentration and fatty acid composition of the diets can be found in Table 2.

Experiment 1: effect of isoenergetic amounts of fish oil versus corn oil in diets with a low or high background of coconut fat. To 88 g of the low-coconut fat base mixture, either 12 g of corn oil or fish oil was added. The oils were stored at 4°C until use. Fish oil was kept under an atmosphere of nitrogen to avoid autooxidation. The diets were balanced for cholesterol in the fish (menhaden) oil (Unilever Research Laboratory, Vlaardingen, The Netherlands), which was found to contain 349 mg of cholesterol/100 g. The high-coconut fat base mixtures were formulated by isoenergetically replacing 40.5 g of sucrose in the low-coconut fat base mixture with 18 g of coconut fat (Croklaan NV, Wormerveer, The Netherlands). To 65.5 g of the high-coconut fat base mixtures either 12 g of corn oil or fish oil was added. The four different meal diets obtained after addition of either corn or fish oil were fed to the rats. Fresh diets were prepared every two days.

Experiment 2: effect of fish oil versus corn oil. To 86 g of the low- and high-cholesterol base diets, 8.5 g of fish oil and corn oil were added, respectively. Fresh diets were prepared every two days. The diets were balanced for cholesterol in the fish oil (144 mg/100 g).

Experiment 3: effect of fish oil versus corn oil in diets with either glucose or sucrose as carbohydrate source. To 88 g of the cholesterol-free and high-cholesterol base mixtures containing either glucose or sucrose, 12 g of fish oil and corn oil were added, respectively. The four meal diets thus obtained were fed to the rats. Fresh diets were prepared every two days. The diets were balanced for cholesterol in the fish oil (599 mg/100 g).

Experiment 4: effect of linseed oil versus corn oil in diets with either glucose or sucrose as carbohydrate source. For this experiment, the diet base mixtures containing either glucose or sucrose had the same composition as the cholesterol-free base mixtures of Experiment 3. To 88 g of the glucose and sucrose base mixtures, either 12 g of linseed oil (Unilever Research Laboratory) or corn oil was added. The four diets thus obtained were fed to the animals. Fresh diets were prepared every two days.

Experiment 5: effect of medium-chain triglycerides versus corn oil. Complete diets containing either corn oil or medium-chain triglycerides (Ceres®, Vanden Bergh & Jurgens, Rotterdam, The Netherlands) were made for this experiment.

Experiment 6: effect of various plant fats. For this experiment complete diets were made. The variable fat sources were either coconut fat, palm oil, palm kernel oil, soybean oil or rapeseed oil. The latter four fat sources were purchased from Unilever Research Laboratory.

Blood sampling. At the beginning (days −5 to 0), and at the end of each experiment (Experiments 1-4, day 14; Experiment 5, day 30; and Experiment 6, day 21), blood samples of the non-starved rats were taken by orbital puncture while under light diethyl ether anaesthesia. Plasma was collected by low-speed centrifugation and kept at −20°C until analysis.

Analyses. Crude fat concentrations and fatty acid composition of the diets were determined according to Folch *et al.* (10) and Metcalfe *et al.* (11), respectively. Cholesterol in the fish oil was determined by gas-liquid chromatography (12). Plasma triglycerides were measured enzymatically according to Sullivan *et al.* (13).

Plasma esterase patterns were determined by vertical, 4.5-12.0% (w/v) polyacrylamide gradient gel electrophoresis at pH 9.0 according to Beynen *et al.* (14). This system gives better separation of esterases than the one used previously (4). Ten μL of a mixture containing 75% (v/v) of plasma and 10% (w/w) of glycerol in distilled water was added to the slots. On each slab gel a plasma ES-1 standard was run. This standard consisted of pooled rat plasma. The plasma ES-1 standard was stored in small portions at −20°C until use. The same standard was used throughout the experiments. After electrophoresis, the gels were stained as described (4), except that α-naphthylbutyrate was used as substrate instead of a mixture of α-naphthylacetate and α-naphthylpropionate because the former substrate had been found to be hydrolyzed at higher rates. The intensity of the ES-1 band was measured by densitometric scanning of the stained gels at 530 nm and was expressed relative to the intensity of the ES-1 standard. In essence, this method of reporting ES-1 activities does not differ from that used earlier (4). Unfortunately, in Experiment 6 we had not sufficient plasma to determine ES-1 activity.

Plasma butyrylcholinesterase activity was determined by the method of Ellman *et al.* (15) using butyrylthiocholine as substrate.

Statistics. Student's 1-sample t-test for paired data was used to evaluate within the dietary groups the significance of changes that occurred during the experimental period. For comparing group means, data were analyzed with Student's t-test or Scheffé's test. Two-way analysis of variance (with or without a co-variate) was performed for factorial experiments. For each experiment, Pearson correlation coefficients were calculated for the change of plasma butyrylcholinesterase activity and that of plasma triglyceride concentration; their significance was assessed by a two-tailed test. Statistical analyses were carried out using a SPSSX computer program (16).

RESULTS

Experiment 1. Dietary fish oil, when compared with corn oil, reduced body weight gain, irrespective of the amount of fat in the background diet (Table 3). The reduced weight gain in the rats fed fish oil was associated with diminished feed intake (results not shown), possibly due to reduced palatability. It is unlikely that decreased palatability would be caused by autooxidation of the fish oil diet, because the amount of n-3 fatty acids in the fish oil diet (Table 2) was not lower than would be expected. There was no effect of fat amount in the diet on weight gain. The diets with a high background of coconut fat produced a greater increase in plasma ES-1 activity than did the diets with low background of coconut fat. Dietary fish oil induced higher plasma ES-1 activities than corn oil. The change in plasma butyrylcholinesterase activity was not affected by the amount of fat in the diet. Fish oil produced significantly higher butyrylcholinesterase activities than did corn oil. Dietary fish oil lowered plasma

TABLE 1

Composition of the Diets

	Diets								
	Experiment 1		Experiment 2	Experiment 3		Experiment 4		Experiment 5	Experiment 6
	Low-coconut fat background	High-coconut fat background		Glucose background	Sucrose background	Glucose background	Sucrose background		
Ingredients (g)									
Casein	15.1	15.1	16.0	15.1	15.1	15.1	15.1	16.0	15.1
Corn oil	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Coconut fat	0	18.0	0	0	0	0	0	0	0
Variable fat source[a]	12.0	12.0	8.5	12.0	12.0	12.0	12.0	15.0	15.0
Cholesterol	0.042 (0)[b]	0.042 (0)[b]	0.08 (0.0678)[b]	0.072 (0)[b]	0.072 (0)[b]	0	0	0	1.0
Sucrose	62.898 (62.94)[b]	22.398 (22.44)[b]	0	0	62.868 (62.94)[b]	0	62.94	0	0
Glucose	0	0	0	62.868 (62.94)[b]	0	62.94	0	0	0
Dextrose	0	0	18.09 (18.1022)[b]	0	0	0	0	9.835	27.47
Corn starch	0	0	20.0	0	0	0	0	9.835	27.47
Molasses	0	0	10.0	0	0	0	0	10.0	5.0
Cellulose	3.0	3.0	15.0	3.0	3.0	3.0	3.0	15.0	2.0
$CaCO_3$	1.24	1.24	0.62	1.24	1.24	1.24	1.24	0.62	1.24
$CaHPO_4$	0	0	0.61	0	0	0	0	0.61	0
$MgCO_3$	0.14	0.14	0.07	0.14	0.14	0.14	0.14	0.07	0.14
MgO	0	0	0.03	0	0	0	0	0.03	0
$KHCO_3$	0.77	0.77	1.8	0.77	0.77	0.77	0.77	1.8	0.77
KCl	0.1	0.1	0	0.1	0.1	0.1	0.1	0	0.1
$NaH_2PO_4.2H_2O$	1.51	1.51	0	1.51	1.51	1.51	1.51	0	1.51
NaCI	0	0	0.5	0	0	0	0	0.5	0
Vitamin premix[c]	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2
Mineral premix[c]	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Total (g)	100.0	77.5	94.5	100.0	100.0	100.0	100.0	82.5	100.0

[a]The variable fat sources were corn oil or fish oil (Experiments 1-3), corn oil or linseed oil (Experiment 4), corn oil or medium-chain triglycerides (Experiment 5) and coconut fat, palm oil, palm kernel oil, soybean oil or rapeseed oil (Experiment 6). Separate batches of diet base mixtures (without the variable fat sources: Experiments 1-4) or complete diets (including the variable fat sources: Experiments 5 and 6) were made for each experiment.

[b]Values in parentheses refer to the amount of ingredient if fish oil was used as variable fat source.

[c]The composition of the mineral and vitamin premixes has been described elsewhere (9).

TABLE 2

Analyzed Amount of Crude Fat and Fatty Acid Composition of the Diets

	Diets																				
	Experiment 1				Experiment 2		Experiment 3				Experiment 4				Experiment 5		Experiment 6				
	Low-coconut fat background		High-coconut fat background				Glucose background		Sucrose background		Glucose background		Sucrose background								
	Corn oil	Fish oil	Corn oil	Fish oil	Corn oil	Fish oil	Corn oil	Fish oil	Corn oil	Fish oil	Corn oil	Linseed oil	Corn oil	Linseed oil	Corn oil	MCT[a]	Coconut fat	Palm oil	Palm kernel oil	Soybean oil	Rapeseed oil
Chemical analysis (g/100 g diet)																					
Crude fat	13.0	13.0	40.3	39.5	10.5	10.4	13.4	12.6	13.0	13.1	13.9	13.0	13.0	12.8	19.0	18.5	17.0	17.1	17.1	16.6	16.9
Fatty acids[b] (g/100 g fatty acids)																					
8:0	0	0	3.6	3.5	0	0	0	0	0	0	0	0	0	0	0	52.7	6.6	0	3.3	0	0
10:0	0	0	2.5	2.6	0	0	0	0	0	0	0	0	0	0	0	37.5	4.8	0	2.9	0	0
12:0	0	0.2	22.5	23.7	0	0.2	0	0.1	0	0.1	0	0	0	0	0	1.4	40.6	0.4	41.7	0.3	0.1
14:0	0	6.7	10.3	13.8	0	6.2	0.1	7.6	0.1	7.5	0	0.1	0.1	0	0	0.1	17.3	1.0	15.4	0.2	0.1
14:1	0	0	0	0.1	0	0.1	0	0.1	0	0	0	0	0	0	0	0	0	0	0	0	0
15:0	0	0.5	0	0.2	0	0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
16:0	10.2	18.1	10.7	14.3	10.3	17.7	10.1	18.2	10.1	18.1	10.2	5.9	10.3	5.8	10.3	0.9	10.4	38.3	9.5	10.5	5.9
16:1	0.1	8.3	0	3.7	0.2	7.5	0.2	10.0	0.2	9.9	0.1	0.1	0.2	0	0	0	0	0.2	0	0.2	0.2
17:0	0	0.4	0	0.2	0	0.4	0.1	0.5	0.1	0.5	0	0	0.1	0	0	0	0	0.1	0	0	0
18:0	1.9	3.2	3.2	3.8	1.9	3.0	1.8	3.4	1.8	3.4	1.8	3.4	2.0	3.3	1.8	0.1	3.9	4.2	3.1	3.9	2.2
18:1	30.2	14.9	18.5	11.4	29.5	15.0	29.7	15.2	29.7	15.2	30.1	18.3	29.9	18.2	30.2	2.5	9.7	40.6	17.2	23.5	53.1
18:2	55.3	6.0	27.4	3.9	55.8	7.6	55.3	5.9	55.0	6.1	55.3	19.0	55.2	18.9	55.5	4.2	6.3	13.7	6.4	53.0	28.4
18:3n-6	0	0.2	0	0.1	0	0.2	0	0.3	0	0.3	0	0	0	0	0	0	0	0	0	0	0
18:3n-3	0.8	0.9	0.4	0.4	0.8	0.8	0.8	0.8	0.8	0.9	0.9	51.6	0.9	51.7	0.8	0	0	0.2	0	6.2	5.5
18:4n-6	0	2.5	0	1.0	0	2.1	0	0.3	0	2.4	0	0	0	0	0	0	0	0	0	0	0
20:0	0.4	0.3	0.3	0.2	0.5	0.3	0.4	0.2	0.5	0.2	0.5	0.2	0.4	0.2	0.4	0	0.1	0.4	0	0.4	0.6
20:1n-9	0.3	2.7	0.2	1.3	0.3	2.9	0.4	1.7	0.4	1.7	0.4	0.2	0.4	0.3	0.3	0	0	0.2	0	0.3	1.3
20:2n-6	0	0	0	0.1	0	0.2	0	2.1	0	0.2	0	0	0	0	0	0	0	0	0	0	0.1
20:3n-3	0	1.1	0	0.5	0	1.0	0	0.6	0	0.6	0	0.1	0	0	0	0	0	0	0	0	0
20:4n-6	0	0	0	0.1	0	0.1	0	0.1	0	0.1	0	0	0	0	0	0	0	0	0	0	0
20:5n-3	0	14.1	0	6.1	0	13.0	0	13.1	0	13.6	0	0	0	0	0	0	0	0	0	0	0
22:0	0.2	0	0.1	0	0.2	0.1	0.2	0.1	0.1	0.1	0.1	0.1	0.2	0.1	0.2	0	0	0.1	0	0.5	0.4
22:1n-9	0.1	1.6	0.2	0.8	0	2.2	0	0.3	0.1	0.4	0	0	0.1	0	0	0	0	0	0	0	0.4
22:3n-3	0	0.2	0	0.1	0	0.2	0	0.1	0	0.1	0	0	0	0	0	0	0	0	0	0	0
22:5n-3	0	2.2	0	1.0	0	2.1	0	2.1	0	2.1	0	0	0	0	0	0	0	0	0	0	0
22:6n-3	0	8.3	0	3.6	0	8.4	0	8.4	0	8.4	0	0	0	0	0	0	0	0	0	0	0
24:0	0.2	0	0.2	0	0.2	0.1	0.2	0.1	0.2	0.1	0.2	0.1	0.2	0.1	0.2	0	0	0.1	0	0.3	0.2
24:1	0.1	0.4	0	0.2	0	0.6	0.1	0.5	0	0.4	0	0	0	0	0	0	0	0	0	0	0.2
Sat total[c]	12.9	29.4	53.4	62.6	13.1	28.5	12.9	30.2	12.9	30.0	12.8	9.8	13.3	9.5	12.9	92.7	83.7	44.6	75.9	16.1	9.5
Mono total	30.8	27.9	18.7	17.5	30.0	28.3	30.4	27.7	30.4	27.6	30.6	18.6	30.6	18.5	30.5	2.5	9.7	41.0	17.2	24.0	55.2
Poly total	56.1	35.5	27.8	16.9	56.6	35.7	56.1	33.8	55.8	34.9	56.2	70.7	56.1	70.7	56.3	4.2	6.3	13.9	6.4	59.2	34.0

[a]MCT, medium-chain triglycerides.

[b]Fatty acids in shorthand notation: the number before and after the colon represents the number of carbon atoms and of double bands, respectively.

[c]Sat, saturated; Mono, monounsaturated; Poly, polyunsaturated.

TABLE 3

Effect of Isoenergetic Amounts of Fish Oil *Versus* Corn Oil in Diets with a Low or High Background of Coconut Fat (Experiment 1)[a]

	Dietary variables				
	Low-coconut fat background		High-coconut fat background		
Measure	Corn oil	Fish oil	Corn oil	Fish oil	Sign.[b]
Body weight (g)					
Day 0	59 ± 1	59 ± 1	59 ± 1	59 ± 1	
Day 14	110 ± 4	97 ± 2	105 ± 3	96 ± 2	F
Gain	51 ± 4	38 ± 2	46 ± 3	37 ± 2	F
Plasma esterase activity					
ES-1 (relative to ES-1 standard)					
Day 0	142 ± 14	170 ± 10	166 ± 14	170 ± 10	
Day 14	245 ± 29	394 ± 38	399 ± 37	461 ± 52	A,F
Change	+103 ± 22[c]	+224 ± 33[c]	+233 ± 33[c]	+291 ± 53[c]	A,F
Butyrylcholinesterase (nmol/min/mL)					
Day 0	141 ± 5	139 ± 7	128 ± 8	133 ± 6	
Day 14	129 ± 6	164 ± 14	115 ± 5	149 ± 7	A,F
Change	−12 ± 3[c]	+25 ± 8[c]	−13 ± 5[c]	+16 ± 9[c]	F
Plasma triglyceride concentration (mM)					
Day 0	1.4 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	
Day 14	1.6 ± 0.1	1.1 ± 0.1	1.9 ± 0.2	1.1 ± 0.1	F
Change	+0.2 ± 0.1[c]	−0.3 ± 0.2	+0.5 ± 0.2	−0.3 ± 0.1[c]	F

[a] Results, expressed as means ± SEM for 12 animals per group, except for the ES-1 activities which are given for 10–12 animals per group because some individuals were homozygous for the ES-1[b] genotype, resulting in the absence of esterase ES-1.

[b] Significance ($p < 0.05$) was calculated by two-way analysis of variance: A, effect of amount of background fat; F, effect of type of fat (fish oil *versus* corn oil). For statistical analysis of final values and changes of plasma parameters, respectively, body weight and body weight gain were used as co-variate.

[c] Plasma values significantly different from zero ($p < 0.05$).

triglyceride concentrations when compared with corn oil. For individual rats, there was a statistically significant correlation between the change in plasma butyrylcholinesterase activity and plasma triglyceride concentration ($r = -0.30$; $n = 48$; $p < 0.05$).

Experiment 2. Final body weight and body weight gain (Table 4) and feed intake (results not shown) were not influenced significantly by the type of dietary fat (Table 4). Thus the effects of dietary fish oil on feed intake and body weight gain seen in Experiment 1 seem to be age-dependent, because in Experiment 2 older rats were used. Fish oil caused an increase in plasma ES-1 activity and butyrylcholinesterase activity, when compared with corn oil. Fish oil markedly decreased plasma triglyceride concentrations. For individual animals, there was a negative, not statistically significant, association between the change in butyrylcholinesterase activity and that in plasma triglyceride level ($r = -0.22$; $n = 36$; $p > 0.1$).

Experiment 3. Rats grew somewhat faster on the diets containing glucose when compared with sucrose. In keeping with Experiment 1, fish oil reduced weight gain (Table 5) and feed intake (results not shown) when compared with corn oil. Rats fed the glucose diets had higher plasma ES-1 activities than their counterparts fed the sucrose diets. Dietary fish oil produced higher plasma ES-1 activities than did dietary corn oil. Fish oil also increased plasma butyrylcholinesterase activity. The type of dietary carbohydrate did not affect plasma butyrylcholinesterase activity. Fish oil decreased triglyceride concentrations when compared with corn oil, irrespective of the type of carbohydrate in the diet. The changes in butyrylcholinesterase *versus* those in triglycerides correlated statistically significant for individual rats ($r = -0.31$; $n = 48$; $p < 0.05$).

Experiment 4. Body weight gain was greater on the glucose than sucrose diets. The type of dietary fat did not influence weight gain (Table 6). Plasma ES-1 was not significantly influenced by the type of fat, although linseed oil tended to increase ES-1 activity, when compared with corn oil. Dietary glucose, when compared with sucrose, elevated final plasma ES-1 activities but did not influence the change in ES-1 activity during the experimental period. Neither the type of carbohydrate nor that of fat significantly influenced plasma butyrylcholinesterase activities and triglyceride concentrations. There was no correlation between the changes in plasma triglyceride concentrations and butyrylcholinesterase activities ($r = 0.07$; $n = 48$; $p > 0.1$).

Experiment 5. Fat type did not affect body weight gain (Table 4). The diet containing medium-chain triglycerides, when compared with the corn oil diet, lowered plasma butyrylcholinesterase activity, but tended to increase the plasma ES-1 activity. Medium-chain triglycerides increased triglyceride concentrations when compared with

TABLE 4

Effect of Fish Oil or Medium-Chain Triglycerides *Versus* Corn Oil (Experiments 2 and 5)[a]

Measure	Dietary variables			
	Experiment 2		Experiment 5	
	Corn oil	Fish oil	Corn oil	Medium-chain triglycerides
Body weight (g)				
Day −4 or −5[b]	264 ± 3	266 ± 3	76 ± 6	80 ± 5
Day 14 or 30[c]	322 ± 5	324 ± 5	321 ± 15	336 ± 15
Gain	58 ± 2	58 ± 2	245 ± 10	256 ± 11
Plasma esterase activity				
ES-1 (relative to ES-1 standard)				
Day −4 or −5	204 ± 13	219 ± 17	279 ± 32	298 ± 45
Day 14 or 30	198 ± 14	288 ± 17[d]	565 ± 35	700 ± 50
Change	−6 ± 9	+69 ± 26[d,e]	+286 ± 49[e]	+402 ± 63[e]
Butyrylcholinesterase nmol/min/mL)				
Day −4 or −5	114 ± 5	110 ± 5	110 ± 4	110 ± 3
Day 14 or 30	78 ± 3	83 ± 3	80 ± 6	65 ± 2[d]
Change	−36 ± 4	−27 ± 3[f,e]	−30 ± 4	−45 ± 4[d,e]
Plasma triglyceride concentration (mM)				
Day −4 or −5	2.1 ± 0.1	1.9 ± 0.1	0.7 ± 0.1	0.7 ± 0.1
Day 14	2.4 ± 0.2	0.8 ± 0.1[d]	1.2 ± 0.2	2.2 ± 0.2[d]
Change	+0.3 ± 0.2	−1.1 ± 0.1[d,e]	+0.5 ± 0.2	+1.5 ± 0.2[d,e]

[a]Results, expressed as means ± SEM for 16–18 (Experiment 2) or 6 (Experiment 5) animals per group.

[b]Initial values of Experiments 2 and 5 refer to day −4 and −5, respectively.

[c]Final values of Experiments 2 and 5 refer to day 14 and 30, respectively.

[d]Significantly different from the group fed the corn oil diet ($p < 0.05$; two-tailed Student's t-test).

[e]See Table 3, footnote c.

[f]Significantly different from the group fed the corn oil diet ($p < 0.05$; one-tailed Student's t-test).

corn oil. There tended to be a negative association between the change of plasma butyrylcholinesterase activities and that of triglyceride levels ($r = -0.33$; $n = 12$; $p > 0.1$).

Experiment 6. Between the five dietary groups there were no significant differences in body weight gain and plasma butyrylcholinesterase activities (Table 7). Coconut fat and palm kernel oil increased plasma triglyceride concentrations, when compared with the other plant fats. The changes in plasma triglyceride concentrations and butyrylcholinesterase activities were negatively associated ($r = -0.28$; $n = 60$; $p < 0.05$).

DISCUSSION

In keeping with our previous study (4), plasma ES-1 activities were increased after the feeding of a high-coconut fat diet (Table 3). In contrast, plasma butyrylcholinesterase activity did not respond to the amount of fat in the diet. Other observations also suggest that plasma ES-1 and butyrylcholinesterase react differentially to dietary fats. Dietary medium-chain triglycerides lowered butyrylcholinesterase activities, when compared with corn oil, whereas this fat preparation induced increased activities of ES-1 (Table 4).

Although it is clear from this study, that dietary fats may induce different effects concerning plasma ES-1 and butyrylcholinesterase activities, it is also evident that fish oil, when compared with corn oil, consistently produced increased activities of both enzymes (Tables 3–5). Since diets with different background compositions were used, it would follow that the effect of fish oil is neither masked nor enhanced by other components of the diet. Since fish oils are rich in n-3 polyunsaturated fatty acids, and corn oil is rich in the n-6 polyunsaturated fatty acid, linoleic acid (Table 2), it could be suggested that n-3 fatty acids specifically activate the esterases. However, this suggestion may not be tenable because linseed oil, which is rich in the n-3 fatty acid, α-linolenic acid (Table 2), did not significantly influence the esterases when compared with corn oil (Table 6). That butyrylcholinesterase is specifically influenced by dietary fish oil would also follow from comparison of various plant fats (Table 7). The five plant fats, tested, produced similar plasma butyrylcholinesterase activities.

Apart from the amount and type of fat in the diet, plasma ES-1 activities also appeared to be sensitive to the type of dietary carbohydrate. Glucose induced higher activities than sucrose (Tables 5 and 6). This may suggest that ES-1 is not uniquely involved in lipid metabolism.

TABLE 5

Effect of Isoenergetic Amounts of Fish Oil *Versus* Corn Oil in Diets with Either Glucose or Sucrose as Carbohydrate Source (Experiment 3)[a]

	Dietary variables				
	Glucose background		Sucrose background		
Measure	Corn oil	Fish oil	Corn oil	Fish oil	Sign.[b]
Body weight (g)					
Day 1	62 ± 1	63 ± 1	62 ± 1	62 ± 1	
Day 14	124 ± 5	110 ± 5	119 ± 4	94 ± 3	F,C
Gain	62 ± 4	47 ± 4	57 ± 4	32 ± 3	F,C
Plasma esterase activity					
ES-1 (%, relative to ES-1 standard)					
Day 0	146 ± 17	192 ± 14	162 ± 15	174 ± 18	
Day 14	363 ± 41	532 ± 38	315 ± 32	412 ± 33	F,C
Change	+217 ± 32[c]	+340 ± 36[c]	+153 ± 38[c]	+238 ± 36[c]	C
Butyrylcholinesterase (nmol/min/mL)					
Day 0	142 ± 7	133 ± 6	133 ± 7	133 ± 7	
Day 14	127 ± 7	142 ± 6	120 ± 4	151 ± 7	
Change	−15 ± 4[c]	+9 ± 5	−13 ± 6[c]	+18 ± 5[c]	F
Plasma *triglyceride concentration* (mM)					
Day 0	1.5 ± 0.1	1.6 ± 0.1	1.6 ± 0.1	1.5 ± 0.1	
Day 14	1.5 ± 0.2	0.8 ± 0.1	1.7 ± 0.2	0.8 ± 0.1	F
Change	−0.0 ± 0.2	−0.8 ± 0.1[c]	+0.1 ± 0.3	−0.7 ± 0.1[c]	F

[a,b,c]See legend to Table 3. C, effect of type of carbohydrate.

TABLE 6

Effect of Linseed Oil *Versus* Corn Oil in Diets with Either Glucose or Sucrose as Carbohydrate Source (Experiment 4)[a]

	Dietary variables				
	Glucose background		Sucrose background		
Measure	Corn oil	Linseed oil	Corn oil	Linseed oil	Sign.[b]
Body weight (g)					
Day 0	64 ± 1	64 ± 1	64 ± 1	64 ± 1	
Day 14	130 ± 4	126 ± 4	115 ± 4	117 ± 4	C
Gain	66 ± 4	62 ± 4	51 ± 3	53 ± 4	C
Plasma esterase activity					
ES-1 (%, relative to ES-1 standard)					
Day 0	197 ± 19	226 ± 20	209 ± 13	196 ± 24	
Day 14	212 ± 22	266 ± 31	188 ± 24	239 ± 27	C
Change	+15 ± 20	+40 ± 21	−21 ± 20	+43 ± 14[c]	
Butyrylcholinesterase (nmol/min/mL)					
Day 0	137 ± 5	140 ± 8	137 ± 10	138 ± 3	
Day 14	111 ± 5	108 ± 7	114 ± 6	125 ± 4	
Change	−26 ± 5[c]	−32 ± 5[c]	−23 ± 5[c]	−13 ± 4[c]	
Plasma triglyceride concentration (mM)					
Day 0	1.5 ± 0.1	1.6 ± 0.1	1.6 ± 0.1	1.5 ± 0.1	
Day 14	1.1 ± 0.1	0.8 ± 0.1	1.3 ± 0.1	1.1 ± 0.1	F
Change	−0.4 ± 0.2[c]	−0.8 ± 0.2[c]	−0.3 ± 0.2	−0.4 ± 0.1[c]	

[a,b,c]See legend to Table 3. C, effect of type of carbohydrate.

TABLE 7

Effect of Various Plant Fats (Experiment 6)[a]

Measure	Dietary variables					
	Coconut fat	Palm oil	Palm kernel oil	Soybean oil	Rapeseed oil	Sign.[b]
Body weight (g)						
Day 0	61 ± 1[c]	61 ± 2[c]	62 ± 2[c]	61 ± 2[c]	62 ± 2[c]	
Day 21	139 ± 3[c]	143 ± 3[c]	136 ± 3[c]	141 ± 3[c]	134 ± 3[c]	
Gain	78 ± 3[c]	82 ± 2[c]	74 ± 3[c]	80 ± 2[c]	72 ± 3[c]	
Plasma butyrylcholinesterase activity (nmol/min/mL)						
Day 0	139 ± 6[c]	141 ± 8[c]	143 ± 4[c]	139 ± 6[c]	139 ± 5[c]	
Day 21	150 ± 15[c]	158 ± 6[c]	148 ± 11[c]	156 ± 12[c]	165 ± 10[c]	
Change	+11 ± 10[c]	+17 ± 10[c]	+5 ± 9[c]	+17 ± 11[c]	+26 ± 11[c,*]	
Plasma triglyceride concentration (mM)						
Day 0	1.1 ± 0.1[c]	1.3 ± 0.2[c]	1.0 ± 0.1[c]	1.1 ± 0.1[c]	1.0 ± 0.1[c]	
Day 21	3.3 ± 0.5[c]	1.5 ± 0.2[d,e]	2.3 ± 0.3[c,d]	0.7 ± 0.1[e]	0.7 ± 0.1[e]	F
Change	+2.2 ± 0.4[c,*]	+0.2 ± 0.3[d,e]	+1.3 ± 0.4[c,d,*]	−0.4 ± 0.1[e,*]	−0.3 ± 0.1[e,*]	F

[a]Results, expressed as means ± SEM for 12 animals per group.

[b]Significance ($p < 0.05$) was calculated by one-way analysis of variance. F, effect of type of fat.

[c–e]Groups not sharing a common lower-case superscript (c–e) are significantly different ($p < 0.05$, Scheffé's test).

*See Table 3, footnote c.

Since plasma butyrylcholinesterase was not influenced by the type of dietary carbohydrate, it implies that ES-1 and butyrylcholinesterase do not share similar characteristics as to their regulation by diet.

In human volunteers, consumption of a fish paste caused a decrease in plasma butyrylcholinesterase activity when compared with a meat paste (5). In rats, fish oil produced increased activities of butyrylcholinesterase. This implies that either rats and man respond differently to fish oil or that the observed effects of the fish paste (5) are not related to its oil content.

It has been suggested (7) that increased plasma butyrylcholinesterase activities are associated with increased concentrations of plasma triglycerides, at least in hyperlipoproteinemic subjects. Fish oil very effectively lowered plasma triglyceride concentrations when compared with corn oil (Tables 3–5), but it increased plasma butyrylcholinesterase activities in rats. As would be expected (17), dietary medium-chain triglycerides increased plasma triglyceride concentrations. However, this fat preparation decreased butyrylcholinesterase activities (Table 4). Coconut fat and palm kernel oil markedly elevated plasma triglyceride concentrations but did not significantly affect butyrylcholinesterase (Table 7). These observations speak against a positive relationship between butyrylcholinesterase and plasma triglycerides. In fact, for individual rats in 5 out of 6 experiments, negative correlation coefficients of the order of 0.3 were computed between the change of butyrylcholinesterase activities and that of plasma triglycerides. The correlations, which were rather weak indeed, did not always reach statistical significance, and their physiological relevance is unknown. In any event, the suggested (7) direct relationship between butyrylcholinesterase and triglycerides is not borne out in the present study.

Briefly, we have shown that plasma ES-1 and butyrylcholinesterase activities can be modified by the composition of the diet. Fish oil, when compared with corn oil, consistently increased the activities of both enzymes. The physiological relevance of this observation is not clear.

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Modulation of Fatty Acid Incorporation and Desaturation by Trifluoperazine in Fungi

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The effects of trifluoperazine (TFP) on [1-^{14}C]fatty acid incorporation into the lipids of *Mortierella ramanniana* var. *angulispora* were studied. TFP decreased [1-^{14}C]-fatty acid incorporation into phosphatidylcholine, phosphatidylethanolamine and triacylglycerol, but greatly increased ^{14}C-labeling in phosphatidic acid. These changes in [1-^{14}C]fatty acid incorporation induced by TFP were accompanied by a decrease in desaturation of some [1-^{14}C]fatty acids taken up by the fungal cells. When [1-^{14}C]lioleic acid (LA) was incubated with the fungal cells, total γ-linolenic acid (GLA) formation from incorporated [1-^{14}C]LA decreased, but the ^{14}C-labeled GLA content in individual lipid classes was essentially unchanged. This suggests that the site of the TFP effect on GLA formation from [1-^{14}C]LA taken up from the medium is not the desaturase acting on LA linked to complex lipids. On the other hand, GLA formation from [1-^{14}C]oleic acid was much less susceptible to TFP, which suggests that in this fungus Δ6 desaturation to GLA has at least two different pathways with different degrees of susceptibility to TFP.
Lipids 25, 787-792 (1990).

Although some fungi have long been known to contain polyunsaturated fatty acids (1,2), the mechanisms of their biosynthesis have remained unclear. Aerobic desaturation has been well characterized in oleic acid formation, where thioester derivatives are used as substrates. On the other hand, it has been shown that the direct desaturation of fatty acid bound to complex lipids occurs in eukaryotic microorganisms (3) and plants (4). However, only a few desaturases using acyl-CoA or acyl carrier protein (ACP) derivatives as substrates (5-7) and no desaturases using complex-lipid linked fatty acids have been purified to homogeneity, presumably because of the instability of the enzymes. Thus, the elucidation of the molecular nature of the desaturases awaits further investigation. A recent genetic approach may become a powerful tool to clarify the diversity of the desaturases (8-10).

Mortierella fungi have a high lipid content and contain polyunsaturated fatty acids such as γ-linolenic acid (GLA) (11,12), arachidonic acid (13,14) and eicosapentaenoic acid (15), depending on the species. In previous studies (16,17) we investigated regulatory mechanisms which may determine the GLA composition in individual lipid classes in *Mortierella ramanniana* var. *angulispora* and reported the differential synthesis of GLA between neutral lipids and phospholipids in the fungus. In the present study we tried to modulate lipid metabolism in this fungus to further clarify the relationship between fatty acid incorporation into lipid classes and its desaturation thereafter. For this purpose, we used trifluoperazine (TFP), which belongs to the class of the antipsychotic agents, phenothiazines, because TFP and its related phenothiazine drug, chlorpromazine, have been reported to modify glycerolipid metabolism in mammalian cells (18). In addition, Δ6 desaturation of exogenous [1-^{14}C]linoleic acid (LA) or [1-^{14}C]oleic acid (OA) was compared in regard to its susceptibility to TFP.

MATERIALS AND METHODS

Materials. [1-^{14}C]Stearic acid (59 mCi/mmol), [1-^{14}C]OA (59 mCi/mmol) and [1-^{14}C]LA (59 mCi/mmol) were obtained from New England Nuclear (Boston, MA). Unlabeled stearic acid, OA, LA, GLA, TFP, chlorpromazine, cerulenin, ouabain, cytochalasin B and colchicine were purchased from Sigma (St. Louis, MO). Silica gel G thin-layer chromatographic (TLC) plates were obtained from Merck (Darmstadt, Federal Republic of Germany), and KC18 (reversed phase) TLC plates were acquired from Whatman (Maidstone, U.K.). All solvents were of reagent grade.

Microorganisms and culture conditions. Mortierella ramanniana var. *angulispora* (IFO 8187) was obtained from the culture collection of the Institute of Fermentation (Osaka, Japan). The fungi were maintained on a yeast-extract/malt-extract agar medium. The liquid medium contained glucose, inorganic salts and vitamins, as described previously (16).

Incorporation of ^{14}C-labeled fatty acids into fungal lipids and extraction of lipids. ^{14}C-Labeled fatty acids were incorporated as described previously (17), except that several drugs were added. One mL of fungal cell culture grown in rotary shakers (180 rpm) at 30°C for one day, when cells were at the exponential growth phase, were incubated with 3.4 μM (0.2 μCi/mL) [1-^{14}C]fatty acids at 30°C for 0.5-4 hr in the presence or absence of TFP or other drugs. Since these drugs were dissolved in ethanol, control experiments were performed at a final ethanol concentration of 1% (v/v). After incubation, the fungal cells were cooled on ice and washed with 1 mL of 0.1 M phosphate buffer (pH 6.0), followed by centrifugation (1000 g, 5 min) to remove ^{14}C-labeled fatty acids not taken up by the fungal cells. Lipids were extracted from the fungal cell pellets with 3 mL of chloroform/methanol (1:2, v/v). After 1 hr, 1 mL of chloroform and 1 mL of 0.1 M phosphate buffer were added. The upper aqueous layer was washed twice with 1 mL of chloroform, and the lower chloroform layers wre collected.

Lipid analysis. Lipids were analyzed as described previously (17). For fatty acid analysis, extracted lipids were transmethylated and the resultant fatty acid methylesters were separated by reversed phase TLC on KC18 plates. Neutral lipid classes and polar lipid classes were separated by TLC on Silica gel 60 plates. When

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Abbreviations: ACP, acyl carrier protein; DG, diacylglycerol; FFA, free fatty acid; GL, glycolipid; GLA, γ-linolenic acid; LA, linoleic acid; OA, oleic acid; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; TFP, trifluoperazine; TG, triacylglycerol; TLC, thin-layer chromatography.

necessary, two-dimensional TLC was performed for checking ^{14}C-labeled TLC fractions of polar lipids. ^{14}C-Labeled spots were detected by autoradiography and scraped into scintillation vials. Radioactivity was determined with a Beckman liquid scintillation system (model LS1701; Beckman, Fullerton, CA) with automatic quenching correction.

Other methods. The dry cell weight and total lipid content were measured by weight as described previously (16). Lactate dehydrogenase was assayed as described by Kornberg (18).

RESULTS

Effects of TFP on [1-^{14}C]LA incorporation and desaturation. TFP changed the [1-^{14}C]LA incorporation into individual lipids in this fungus as shown in Table 1. Though TFP inhibited cell proliferation of the fungus, the fungal cells kept their cell integrity as judged by the retention of the cytosolic marker enzyme, lactate dehydrogenase, up to 3×10^{-4} M TFP. When the fungal cells, having been treated with TFP, were washed to remove TFP and were incubated in normal medium, they resumed proliferation. Modification of the [1-^{14}C]LA incorporation profile by TFP was similar to that described in rat hepatocytes (19). ^{14}C Incorporation into triacylglycerol (TG), phosphatidylcholine (PC) and phosphatidylethanolamine (PE) was decreased by TFP, whereas ^{14}C incorporation into phosphatidic acid (PA) was increased. These results were quite evident from the dose dependence of the TFP effects on the ^{14}C incorporation into individual lipids (Fig. 1). Figure 1 also shows the order in which ^{14}C incorporation into individual lipids was decreased in response to an increase in TFP concentration. ^{14}C Incorporation into PC or PE was most susceptible to TFP; ^{14}C incorporation into TG, phosphatidylserine (PS), PA and diacylglycerol (DG) was decreased by TFP in this order. TFP also caused accumulation of [^{14}C]FFA (free fatty acids which meant that exogenous [1-^{14}C]LA was not utilized for incorporation into individual complex lipids. Since TFP did not affect *in vitro* acyl-CoA synthetase activity (data not shown), the increase in [^{14}C]-FFA may not be caused by direct inhibition of utilization of fatty acids, but by inhibition of the transport of exogenous fatty acids in the fungal cells. Table 2 shows the time course of ^{14}C incorporation into individual lipids with or without 10^{-4} M TFP. TFP affected ^{14}C incorporation into PC, PS and PA at the early stages of incubation irrespective of whether it increased or decreased

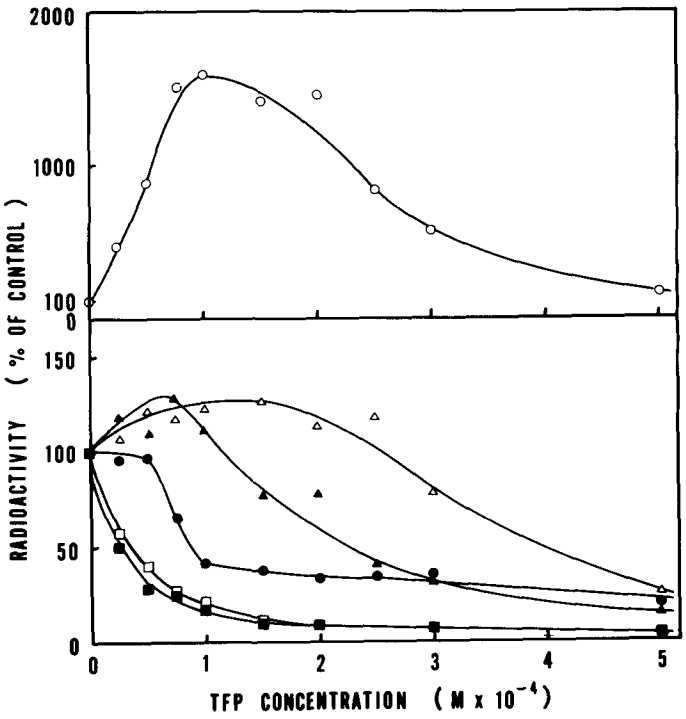

FIG. 1. Dose dependence of TFP effects on [1-^{14}C]LA incorporation into major lipid classes. [1-^{14}C]LA was incubated with fungal cells for 1 hr. [1-^{14}C]LA incorporation into TG (●), DG (△), PC (■), PE (□), PS (▲), and PA (○) was changed by raising the TFP concentration. Values are expressed as percentages of radioactivities incorporated in the absence of TFP (means of duplicates).

TABLE 1

Effects of TFP on [1-^{14}C]LA Incorporation into Various Lipid Classes[a]

	^{14}C Incorporation (DPM $\times 10^{-3}$/mg DCW[c])		
Lipids[b]	Control	10^{-4}M TFP	5×10^{-4}M TFP
TG	54.2	26.3	5.6
DG	3.3	4.2	0.6
FFA	65.0	84.2	203.9
PC	46.7	17.3	2.8
PE	21.8	11.0	1.7
PS	10.9	15.2	3.8
PA	0.9	10.7	2.3
GL	4.2	1.3	0.2
PI	3.0	2.0	0.5
Total	221.0	201.0	234.5

[a]Values are means of triplicates. [1-^{14}C]LA were incubated with fungal cells for 1 hr.

[b]There were some unidentified spots in which ^{14}C incorporation was increased by TFP. TFP also modified [1-^{14}C]LA incorporation into sterol esters, which caused a slight change of the Rf value of sterol esters.

[c]DCW, dry cell weight.

TABLE 2

Effects of TFP on Time Course of [1-^{14}C]LA Incorporation[a]

	^{14}C Incorporation (DPM $\times 10^{-3}$/mg DCW[b])					
	Control			10^{-4} M TFP		
Lipids	0.5 hr	1 hr	4 hr	0.5 hr	1 hr	4 hr
TG	22.3	55.3	118.9	19.5	22.2	30.3
DG	2.1	2.6	3.1	1.3	2.1	4.4
FFA	117.4	115.7	88.3	121.4	155.5	123.7
PC	23.4	36.2	34.4	14.8	16.3	13.1
PE	10.2	15.7	18.1	88.8	10.3	9.0
PS	6.9	6.7	4.5	13.2	10.4	6.4
PA	0.7	0.7	0.8	9.5	8.6	8.0
GL	1.9	2.5	4.5	1.1	1.2	4.5
PI	1.3	2.3	1.9	1.5	1.6	1.5
Total	118.6	241.3	278.9	193.5	231.2	200.9

[a]Values are means of duplicates.

[b]DCW, dry cell weight

incorporation. On the other hand, ^{14}C incorporation into TG, DG and PE was greatly affected at the later incubation times. Since ^{14}C incorporation into PA initially increased so rapidly and then decreased after longer incubations, we suggest that the initial ^{14}C-labeled PA was not derived from the degradation of ^{14}C-labeled TG, PC and PE, but accumulated because of inhibition of *de novo* lipid synthesis via PA.

When Δ6 desaturation of [1-^{14}C]LA to [^{14}C]GLA in the total lipids of the fungus was observed by a similar experiment, TFP lowered GLA formation from LA, as is shown in Table 3. The dose dependent decrease in GLA formation from LA caused by TFP is also shown in Figure 2. Chlorpromazine, a phenothiazine drug, caused similar inhibitory effect on Δ6 desaturation, although cerulenin, an inhibitor of fatty acid synthetase, did not affect GLA formation. Drugs which were assumed to interact with the plasma membrane or cytoskeleton did not affect GLA formation.

TABLE 3

Effects of Various Drugs on GLA Formation from [1-^{14}C]LA[a]

Drugs		GLA formation[b] (%)
Control		14.5 ± 1.5 (100%)
Trifluoperazine	10^{-4} M	4.8 ± 0.2 (33%)
	5×10^{-4} M	1.4 ± 0.3 (10%)
Chlorpromazine	10^{-4} M	11.5 ± 0.9 (79%)
	5×10^{-4} M	2.6 ± 0.4 (17%)
Cerulenin	10^{-4} M	13.4 ± 0.1 (92%)
	10^{-3} M	13.8 ± 1.3 (95%)
Ouabain	10^{-4} M	14.0 ± 0.5 (96%)
	10^{-3} M	13.7 ± 0.1 (95%)
Cytochalasin B	10^{-4} M	16.3 ± 0.8 (112%)
	10^{-3} M	14.0 ± 0.3 (97%)
Colchicine	10^{-4} M	14.3 ± 0.6 (99%)
	10^{-3} M	13.8 ± 1.1 (95%)

[a][1-^{14}C]LA was incubated with fungal cells for 1 hr.

[b]Values represent the [^{14}C]GLA content in total lipids after each drug treatment (means of triplicates ± S.D.).

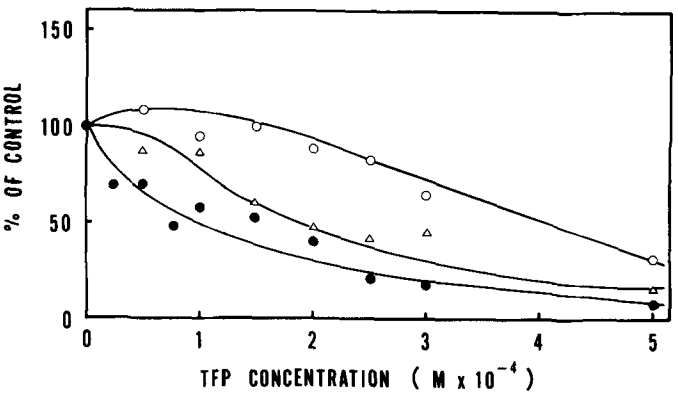

FIG. 2. Dose dependence of TFP effects on the desaturation from [1-^{14}C]LA or [1-^{14}C]OA. After [1-^{14}C]LA or [1-^{14}C]OA was incubated with fungal cells for 1 hr, the extracted lipids were transmethylated and radioactivities of the resultant methyl esters of OA, LA and GLA were determined. Effects of TFP on the conversion from [1-^{14}C]LA to [^{14}C]GLA (●), from [1-^{14}C]OA to [^{14}C]GLA (○), and from [1-^{14}C]OA to [^{14}C]LA (△) were evaluated as percentages of that in the absence of TFP. Values are means of duplicates.

To examine how the decrease in GLA formation in total lipids influenced the distribution of ^{14}C-labeled GLA in individual lipids, we measured the relative amount of ^{14}C-labeled GLA in the total radioactivity incorporated into each lipid (Table 4). Though slight changes of ^{14}C-labeled GLA content in individual lipids were observed with changes in TFP concentration, these could not account for the total decrease in GLA formation caused by TFP. The results suggest that the site of the TFP effect on Δ6 desaturation was not desaturation which acted on LA incorporated into individual lipids, but desaturation which acted on LA prior to being incorporated into complex lipids.

Effects of TFP on [1-^{14}C]stearic acid or [1-^{14}C]OA incorporation. To examine whether the decrease in LA desaturation due to TFP was also observed with other fatty acids, the effects of TFP on [1-^{14}C]stearic acid and [1-^{14}C]OA incorporation were also analyzed (Table 5). Table 5 shows that the desaturation of [1-^{14}C]stearic acid taken up into fungal cells was decresed by 10^{-4} M TFP, whereas the desaturation of [1-^{14}C]OA taken up was not affected by 10^{-4} M TFP. In particular, GLA formation from [1-^{14}C]OA added to the medium was unaffected by 10^{-4} M TFP, which was different from GLA formation from [1-^{14}C]LA added to the medium. Therefore, Δ6 desaturation and GLA formation from [1-^{14}C]OA may have a pathway different from that of added [1-^{14}C]LA.

Figure 2 also shows the dose dependency of the effects of TFP on the desaturation from [1-^{14}C]OA. Compared with the Δ6 desaturation from exogenous [1-^{14}C]LA, Δ6 desaturation from exogenous [1-^{14}C]OA was much less susceptible to TFP. At a concentration of 2×10^{-4} M TFP, Δ6 desaturation from added [1-^{14}C]OA was not decreased. TFP dependence of Δ12 desaturation of added [1-^{14}C]OA was quite similar to that of Δ6 desaturation of added [1-^{14}C]LA.

The effects of TFP on [1-^{14}C]OA incorporation into various lipids are shown in Table 6. The effects of TFP were similar to those on [1-^{14}C]LA incorporation (Table 2). ^{14}C Incorporation into TG, PC and PE was

TABLE 4

[^{14}C]GLA Content of Major Lipid Classes After TFP Treatment for One Hour

	[^{14}C]GLA content[a] (%)			
		TFP		
Lipids	Control	10^{-4}M	2×10^{-4}M	5×10^{-4}M
TG	10.9 ± 1.5 (100%)	11.9 ± 0.5 (109%)	11.2 ± 1.4 (103%)	12.6 ± 1.5 (116%)
PC	30.2 ± 1.2 (100%)	33.6 ± 1.4 (111%)	32.1 ± 7.6 (106%)	27.3 ± 3.2 (90%)
PE	45.4 ± 1.6 (100%)	42.1 ± 1.0 (93%)	39.0 ± 0.5 (86%)	41.7 ± 3.5 (92%)
PS	40.9 ± 2.2 (100%)	38.6 ± 2.3 (94%)	32.4 ± 7.9 (79%)	37.5 ± 6.2 (92%)
PA	—[b]	38.7 ± 2.7	33.2 ± 5.1	30.1 ± 2.3

[a]Values represent the [^{14}C]GLA content in each lipid class (means of triplicates ± S.D.). Values in parentheses represent percent of control in each lipid class.

[b]Not tested because ^{14}C-labeled PA was trace.

TABLE 5

Effects of TFP on the Desaturation of [1-^{14}C]Stearic Acid or [1-^{14}C]Oleic Acid[a]

Incorporated fatty acid	Addition	Time (hr)	[^{14}C]Fatty acid (%)				Total ^{14}C (DPM × 10^{-4})
			18:0	18:1	18:2	18:3[b]	
[1-^{14}C]Stearic acid	none	1	89.4	7.4	2.0	1.2	19.8
		4	84.3	10.7	3.1	1.9	20.2
	10^{-4}M TFP	1	91.9	4.8	2.0	1.3	15.6
		4	92.1	4.8	1.9	1.2	18.3
[1-^{14}C]Oleic acid	none	1	1.0	86.2	5.7	7.1	19.5
		4	1.5	79.5	7.5	11.5	18.8
	10^{-4}M TFP	1	0.9	84.8	5.2	9.1	15.0
		4	1.3	80.0	6.6	12.0	15.9

[a]Values are means of duplicates. [b]GLA.

TABLE 6

Effects of TFP on [1-^{14}C]OA Incorporation Into Various Lipid Classes[a]

Lipids	^{14}C Incorporation (DPM × 10^{-3}/mg DCW[b])		
	Control	10^{-4}M TFP	5 × 10^{-4}M TFP
TG	37.9	24.0	2.0
DG	3.1	4.1	0.3
FFA	119.3	123.2	157.3
PC	11.5	12.3	0.2
PE	10.6	3.0	0.3
PS	4.6	4.7	0.6
PA	0.4	6.8	1.4
GL	1.1	0.6	0.3
PI	1.1	0.6	0.1
Total	196.7	189.0	175.2

[a]Values are means of duplicates. [1-^{14}C]OA was incubated with fungal cells for 1 hr.
[b]DCW, dry cell weight.

decreased, whereas ^{14}C incorporation into PA, PS and DG was increased.

DISCUSSION

The present study shows that in fungi the amphiphilic, cationic drug TFP modulates fatty acid incorporation into individual lipid classes as well as fatty acid desaturation. TFP decreased ^{14}C-labeled fatty acid incorporation into PC, PE and TG, but increased ^{14}C incorporation into PA. This was similar to the effect of phenothiazine drugs observed in mammalian cells (19-21). Another phenothiazine drug, chlorpromazine, showed similar tendencies, as did TFP (data not shown). One explanation for the modulation of fatty acid incorporation by amphiphilic phenothiazine drugs has been the inhibition of phosphatidate phosphohydrolase (20). In rat hepatocytes, chlorpromazine has been suggested to block the association of this enzyme with the membrane, thus preventing its transition to an active form (20). Other explanations which focussed on the inhibition of PC synthesis were proposed for HeLa cells (22) and GH_3 pituitary cells (23). In HeLa cells, CTP:phosphocholine cytidylyltransferase was shown to be inhibited by TFP and chlorpromazine, which would account for the decrease in PC synthesis. In GH_3 pituitary cells, TFP was shown to stimulate degradation of PC and sphingomyelin, which may cause an apparent decrease of [^{3}H]choline incorporation into these lipids.

From the dose dependence of the TFP effect on lipid metabolism in *Mortierella* fungi, differences in [^{14}C]fatty acid incorporation into individual lipid classes were distinct at a concentration of less than 2-3 × 10^{-4} M TFP. At higher levels, TFP gradually casued non-specific cell damage accompanied by a decrease in [^{14}C]fatty acid incorporation into all lipid classes (Fig. 1). Thus, we focused on the TFP effect at concentrations of less than 2-3 × 10^{-4} M. In these concentration ranges, [^{14}C]fatty acid incorporation into PC and PE was more susceptible to TFP than incorporation into TG (Fig. 1). Moreover, a slight increase in [^{14}C]fatty acids incorporation into DG by TFP was observed. These results suggest that inhibition of phosphatidate phosphohydrolase mainly occurs in this fungus because of the large accumulation of ^{14}C-label in PA, while factors other than reduced DG availability for PC, PE and TG synthesis may be involved in the decreased synthesis of these lipids. Increased degradation of PC, PE and TG due to TFP appears less likely, because increased ^{14}C incorporation into DG due to TFP was apparent at longer incubation times, whereas the decrease of ^{14}C-incorporation, especially into PC and PE, occurred early in the experiments (Table 2).

Another aspect of the phenothiazine effect reported for mammalian cells was accumulation of labeled acidic phospholipids such as phosphatidylinositol (PI) and phosphatidylglycerol (PG) upon labeled fatty acid incorporation (19,24). In the fungus, a slight accumulation of ^{14}C-label in PS was observed at the expense of PI and PG. Though little is known about the lipid metabolism in *Mortierella* fungi, it may be similar to that known for the lower eukaryote, *S. cerevisiae*, which has been extensively studied. In *S. cerevisiae*, the synthesis of phospholipids may be similar to that in higher eukaryotes, except for the synthesis of PS (25). PS is synthesized from CDP-DG and serine in *S. cerevisiae* (26), whereas in mammalian cells (27) PS is synthesized by a base exchange reaction involving PE. Thus, the differences in PS biosynthetic pathways between the mammalian cells and the

eukaryotic microorganisms probably contributed to the difference in ^{14}C accumulation in acidic phospholipids induced by amphiphilic cationic drugs.

Recently, TFP was reported to modify acyltransfer to phospholipids and cholesterol in fibroblasts (28). In this case, TFP enhanced [^{14}C]fatty acids incorporation into total phospholipids, which was not observed in the fungus. However, [^{14}C]fatty acid incorporation into sterol esters was modified by TFP in the fungus.

There have been several reports on inhibitors of desaturases. Substituted pyridazinones are well known to inhibit desaturase activities in higher plants (29,30) and in animals (31). The agents have been reported to directly affect the enzymes and to selectively act on certain desaturases, *e.g.*, the Δ15 desaturase in higher plants (32). Though TFP is known to interfere with lipid metabolism as mentioned above, a lowering of the desaturase activity by TFP has not previously been reported.

TFP caused no significant changes in the ^{14}C-labeled GLA content of individual lipid classes when the fungal cells were incubated with [1-^{14}C]LA (Table 4). This suggests that the site of action of TFP on the total Δ6 desaturation activity from exogenous [1-^{14}C]LA is not the Δ6 desaturase. An increase or decrease in the ^{14}C-labeled GLA content would occur in certain lipid classes if this type of Δ6 desaturase exists in the fungus, as it does in higher plants (33), and is inhibited by TFP. Δ6 Desaturation of exogenous LA, with a CoA derivative as substrate, may occur in the fungus. To confirm this possibility, one would need to follow changes in acyl-CoA labelling in further experiments. On the other hand, we have shown that ^{14}C-labeled GLA produced from [1-^{14}C]-LA exists in esterified rather than free form (16). This suggests that if Δ6 desaturation uses a CoA derivative as substrate, specific acyl transfer may be followed by Δ6 desaturation to incorporate GLA into specific lipids as obsessed in Δ12 desaturation (34). Thus, TFP may act on fatty acid transfer and Δ6 desaturation.

GLA formation from [1-^{14}C]OA was not changed at higher TFP concentrations, which suggests that sequential desaturation uses a different pool. Varying responses of different pathways of desaturation at the same chain position have been suggested from the differential effects of the compound, BASF 13-338 in *Arabidopsis* (30), in which α-linolenic acid in monogalactosyldiacylglycerol and in PC were produced under different controls. This raises the possibility that different pathways may exist for Δ6 desaturation in this fungus, *i.e.*, one which uses an LA derivative not linked to complex lipids as a substrate is responsible for the Δ6 desaturation from LA taken up from the medium, and the other which uses LA linked to complex lipids as a substrate is responsible for the Δ6 desaturation from LA derived from OA within the fungal cells. Though there is evidence which supports the existence of desaturases acting on complex lipid-linked fatty acids in microorganisms (35,36), animals (37) and plants (33,38-42), it is still unknown whether these desaturase systems work in *Mortierella* fungi.

It has been proposed that in higher plants OA in PC is desaturated to LA or GLA, which are channeled into the acyl-CoA pool by the reverse reaction of an acyl-CoA:lysophosphatidylcholine acyltransferase. The acyl-CoA thus generated is utilized in TG synthesis (43). In borage seeds, this mechanism may regulate the GLA composition of TG (44). In the fungus, we have shown that LA, which has been esterified into phospholipids such as PC, PE and PS, is readily desaturated to GLA, which is then transferred to TG (17). These results appear consistent with the proposed mechanism in higher plants, although there is no *in vitro* evidence as yet which suggests that this mechanism is also operative in this fungus. Thus, TFP might exert its effect on Δ6 desaturation by blocking some steps in the above scheme. If TFP modulates fatty acid specificity of lysophospholipid acyltransferase(s) so that exogenous [1-^{14}C]OA is preferentially utilized for desaturation as compared to exogenous [1-^{14}C]LA, the difference in TFP susceptibility between exogenous [1-^{14}C]OA desaturation and exogenous [1-^{14}C]LA desaturation will become interpretable. In addition to the possibilities described above, TFP may directly act on the desaturase enzyme(s).

TFP is widely known to block the action of Ca^{2+}-calmodulin (45). However, it remains to be seen whether the TFP effect on lipid metabolism is connected to the action of Ca^{2+}-calmodulin. It is presently not known whether acyltransferases or fatty acid desaturases are affected by Ca^{2+}-calmodulin.

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Effects of High α-Linolenate and Linoleate Diets on Erythrocyte Deformability and Hematological Indices in Rats

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Rats were fed either a high α-linolenate diet or a high linoleate diet from weaning to 4 mon of age. Soybean oil was used as a control. Phospholipid compositions of erythrocytes from the three dietary groups were not significantly different. However, the difference in the α-linolenate (18:3n-3)/linoleate (18:2n-6) ratio of the diets was reflected in the n-3/n-6 ratios of the 20 and 22 carbon highly unsaturated fatty acids except for docosahexaenoic acid (22:6n-3) in the phospholipids. Despite the significant differences in the fatty acid compositions of phospholipids, no measurable differences were detectable in erythrocyte deformability, whole blood viscosity and hematological indices of the three dietary groups. These results indicate that the beneficial effects of the high α-linolenate diet, as compared with the high linoleate diet, are exerted without significant changes in these parameters.

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Starting from epidemiological studies of Greenland Eskimos and Danes (1–3), the effects of fish oil on the prevention of thrombotic diseases have been extensively studied in animals and in humans (4–9). The precise mechanisms for the suppression of platelet aggregability, the major risk factor for thrombosis, by fish oil eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) still remain to be clarified. However, these n-3 highly unsaturated fatty acids appear to decrease platelet aggregability i) by decreasing the availability of arachidonate (20:4n-6) for eicosanoid synthesis by competitively inhibiting the incorporation of arachidonate into phospholipid *via* the acyltransferase systems (10–12); ii) by competitively inhibiting the eicosanoid synthesis at the cyclooxygenase step (13–16); and iii) by differential physiological activities of eicosanoids derived from arachidonate compared to those from EPA (2,17). Furthermore, fish oils have hypolipidemic activities (5,18), and feeding fish oil to humans has been reported to increase erythrocyte deformability and decrease blood viscosity (19,20), which is interpreted to be beneficial for keeping a smooth flow of blood through capillary veins resulting in the prevention of thrombosis.

α-Linolenic acid is a precursor of EPA and DHA. Therefore, perilla oil, which contains more than 50% α-linolenic acid and has long been consumed as a vegetable oil in northern parts of Asia, is expected to have effects similar to those of fish oils. In fact, such beneficial effects have been shown in animal models. These include suppression of platelet aggregability (21), allergic responses (22), tumor metastasis (23) and tumorigenesis (24), as well as an increase of mean survival time (25). Other effects include improved learning ability (26–28) and retinal function (29). Linoleate and α-linolenate are converted to eicosanoids with very different physiological activities. Yet, relatively small differences would be expected in the physicochemical properties of their elongated and desaturated products, *i.e.* arachidonate (20:4n-6) and EPA (20:5n-3), or 22:4n-6 and 22:5n-3. In the current experiments, we have compared the effects of relatively long-term feeding of perilla oil and safflower oil, both of which contain similar proportions of saturated and monoenoic acids but differ markedly in the proportions of α-linolenic and linoleic acids, on the physicochemical properties of red blood cells (RBC) from rats.

MATERIALS AND METHODS

Animals and diets. Conventional laboratory chow (Nihon Clea Co., Tokyo, Japan) was treated with hexane according to a method used in the preparation of vegetable oils. To this low-fat diet were added 2% vitamin mixture (Nihon Clea Co.) (26) and 5% safflower oil, perilla oil or soybean oil. The perilla oil was prepared from the seeds of *Perilla frutescens* (beafsteak plant) (Ohta Oil Co., Okazaki, Japan). Each oil contained 0.1% vitamin E and the diet contained a total of 25 mg vitamin E/100 g. The fatty acid compositions of the diets are shown in Table 1. Only the diets with peroxide values below 30 meq/kg were used in feeding studies.

TABLE 1

Fatty Acid Composition of the Diets[a]

Fatty acid	High linoleate diet (safflower oil) (%)	Control diet (soybean oil) (%)	High α-linolenate diet (perilla oil) (%)
16:0	10.2	13.8	10.6
16:1	0.6	0.6	0.7
18:0	2.4	3.6	1.9
18:1n-9	12.5	21.0	14.6
18:2n-6	70.5	52.0	19.0
18:3n-3	1.6	8.1	52.5
20:4n-6	0.1	tr	0.1
20:5n-3	0.4	0.4	0.4
22:5n-3	0.6	tr	tr
22:6n-3	0.2	tr	0.1
n-3/n-6 ratio	0.04	0.16	2.77

[a]The diets contained 5% oils. Fatty acids are expressed by the carbon chain: the number of double bond, and the first double bond numbered from the methyl terminus is designated as n-9, n-6 or n-3.

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Abbreviations: DHA, docosahexaenoic acid; DI, deformation index; DMA, dimethylacetal; EDTA, ethylenediaminetetraacetate; EPA, eicosapentaenoic acid; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; PC, phosphatidylcholine; PE, phosphatidylethanolamine; RBC, red blood cell.

Male Sprague-Dawley rats were obtained from Shizuoka Laboratory Animal Corp. (Shizuoka, Japan). At 4 wk of age, the animals were fed the experimental diets for 7 wk prior to mating and then throughout pregnancy and lactation. The male pups (F1) were weaned to the same diets consumed by the dams. The F1 rats were used at 5 mon of age. No difference was observed in the growth rates and appearances of the three dietary groups.

Lipid analysis. Blood samples were obtained from rats fed *ad libitum* by heart puncture and heparinized. After removal of plasma and buffy coat by centrifugation at 3,000 rpm for 5 min, the erythrocytes were washed three times with 20 vol of isotonic 50 mM Hepes-buffered saline (pH 7.4 at 315 mosM) at 4°C. Lipids were extracted from the erythrocyte preparations with chloroform/methanol (2:1, v/v) at room temperature. Phospholipids were separated by two-dimensional thin-layer chromatography on Silica Gel (Merck 60) with solvent systems consisting of chloroform/methanol/28% NH_4OH (30:20:3, v/v/v) and chloroform/acetone/methanol/acetic acid/water (10:4:2:3:1, by vol) for the first and second dimension, respectively. Lipids were located by spraying with 0.03% Rhodamine in ethanol and the corresponding bands were scraped off the plate. Lipids were extracted with chloroform/methanol (2:1, v/v) and were quantitated by gas chromatography (GC) as fatty acid methyl esters using heptadecanoic acid as an internal standard. Cholesterol was quantitated by GC as its trimethylsilyl ether with ergosterol as an internal standard.

Determinations of hematological indices. Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were calculated from hematocrit values measured with a microhematocrit centrifuge (Kubota Manufacturing Co., Model KH120, Tokyo, Japan) (30). Cell numbers were measured with an automatic counter (Toa Medical Electronic Co., Model CC-110, Kobe, Japan), and hemoglobin concentrations were determined by the CN-methemoglobin method found in reference 41.

Whole blood viscosity. Rats (F1) at 11 mon of age were anesthetized with nembutal (50 μg/g body weight). Blood samples from rats fed *ad libitum* were taken from abdominal aorta and ethylenediaminetetraacetate (EDTA) was added. The blood was immediately diluted with a saline solution, and the relative viscosity was measured by an Ostwald viscometer at 25°C.

Erythrocyte deformability. Erythrocyte deformability was measured with a cone-plate rheoscope constructed by attaching a cone-plate viscometer (Tokyo Keiki Co., Model B with 0.8° transparent cone) to an inverted microscope—Olympus, Model MT—(31,32). Flash photographs of deformed cells under high or low-shear stress were taken on Kodak Tri-X film. The degree of deformation was expressed by the "deformation index," DI, defined by DI = (L − B)/(L + B), where L and B are the lengths of long and short axes of ellipsoidally deformed cells (33). Erythrocytes were suspended in isotonic dextran solution to make a hematocrit of 0.2% to observe cell deformation in a diluted suspension. The shear rates were changed from 75.2 to 526.4 s^{-1}. The dextran concentration was 20% (18.6 cP).

RESULTS

Phospholipid and cholesterol contents of washed erythrocytes from rats fed the high linoleate, control or high α-linolenate diet are shown in Table 2. The content and composition of the different phospholipids in erythrocytes were not affected by these diets. The levels of plasmalogens were also similar among the three dietary groups (see dimethylacetal (DMA) values in Table 3). Cholesterol content was slightly less in the control group than in the high linoleate group, and the cholesterol/phospholipid ratio tended to be lower in the control group than in the other two dietary groups.

Fatty acid compositions of total lipids are shown in Table 3.

The difference in the 18:3n-3/18:2n-6 ratios of the diets was reflected in the proportions of 20 and 22 carbon highly unsaturated fatty acids except 22:6n-3. Despite a significant difference in 18:3n-3/18:2n-6 ratio of the diets, very little difference was observed in the proportions of these acids in the phospholipids. In the high α-linolenate group, the proportion of α-linolenic acid increased in plasma and hepatic neutral lipids (data not shown), but not significantly in erythrocytes.

The composition of polyunsaturated fatty acids in phosphatidylcholine (PC), a major phospholipid, is shown

TABLE 2

Phospholipid and Cholesterol Content of the Washed Erythrocytes[a]

Dietary group	Total PL[b]	PC	PE	SM	PS	PI	lysoPC	Cho	Cho/PL
High linoleate (n=4)	89.4 ±7.7	46.3 ±3.9	24.9 ±2.3	9.2 ±0.8	4.3 ±0.5	1.4 ±0.5	3.4 ±1.0	140.5* ±9.1	1.59 ±0.10
Control (n=3)	96.5 ±8.3	47.5 ±3.9	25.8 ±6.5	9.7 ±1.5	5.7 ±0.9	4.3 ±2.2	3.6 ±0.5	104.7* ±9.7	1.14 ±0.21
High α-linolenate (n=4)	81.6 ±5.7	42.1 ±2.8	20.5 ±2.4	11.2 ±1.5	4.3 ±0.6	1.2 ±0.2	2.2 ±0.1	129.3 ±2.3	1.48 ±0.10

[a]Averages of determinations (±SE) for 3 to 4 rats.

[b]The contents of phospholipids (PL), phosphatidylcholine (PC), phosphatidylethanolamine (PE), sphingomyelin (SM), phosphatidylserine (PS), phosphatidylinositol (PI) and lysophosphatidylcholine (lysoPC) are expressed as μg fatty acid/10^9 cells, and the cholesterol (Cho) content as μg/10^9 cells.

*Significantly different between the high linoleate and the control groups ($p<0.05$) in Student's t-test.

TABLE 3

Chain Composition of Rat Erythrocyte Total Lipids[a]

Aliphatic chain	High linoleate (%)	Control (%)	High α-linolenate (%)
16DMA	3.4 ± 0.1	3.1 ± 0.1	3.4 ± 0.2
16:0	34.2 ± 1.7	33.1 ± 2.4	33.5 ± 1.0
18DMA	3.7 ± 0.1	3.7 ± 0.1	3.8 ± 0.3
18:0	20.1 ± 1.0	21.1 ± 1.0	20.2 ± 0.9
18:1n-9	4.2 ± 0.3	5.0 ± 0.5	5.1 ± 0.3
18:2n-6	5.9 ± 0.5	5.6 ± 0.6	5.9 ± 0.4
18:3n-3	0.1 ± 0.1	0.2 ± 0.2	0.6 ± 0.3
20:4n-6	12.6 ± 1.4 b	10.5 ± 2.8	7.9 ± 0.5 b
20:5n-3	0 ± 0	0.2 ± 0.1 a,bb	2.1 ± 0.1 bb,cc
22:4n-6	2.1 ± 0.3 bb	1.6 ± 0.6 c	0.2 ± 0.1 bb,c
22:5n-6	0.7 ± 0.1 a,bb	0.2 ± 0.1 a	0 ± 0 bb
22:5n-3	1.1 ± 0.1 bb	1.0 ± 0.5 cc	3.0 ± 0.3 bb,cc
22:6n-3	1.9 ± 0.6	0.7 ± 0.4	1.3 ± 0
24:1	4.5 ± 0.2	6.5 ± 0.1	4.7 ± 0.5
n-3/n-6 ratio	0.15	0.12	0.50

[a]Figures represent mean ±SE (n=3 or 4). The figures with the same symbols denote statistically significant differences; aa($p<0.01$) and a($p<0.05$) for the high-linoleate group *vs* the control group; bb($p<0.01$) and b($p<0.05$) for the high-linoleate group *vs* the high α-linolenate group; cc($p<0.01$) and c($p<0.05$) for the control group *vs* the high α-linolenate group in Student's t-test.

Abbreviation: DMA, dimethylacetal.

in Figure 1A. The proportions of saturated and monoenoic fatty acids were very similar in the three dietary groups. The differences in the diets, however, were mainly reflected in the proportions of 20:5n-3 and 20:4n-6. The proportions of 22 carbon highly unsaturated fatty acids were relatively minor components in PC. In phosphatidylethanolamine (PE), another major phospholipid, the dietary n-3/n-6 ratios were reflected in the proportions of 20:4n-6, 22:4n-6, 22:5n-6, 20:5n-3 and 22:5n-3, but the proportions of 22:6n-3 were not significantly different within the three dietary groups (Fig. 1B). As a whole, the total 20 carbon unsaturated fatty acids and the total 22 carbon unsaturated fatty acids were kept relatively constant despite the differences in diets being fed.

Hematological indices were compared among the three dietary groups. MCVs were 61.1 to 61.5 (± 0.7) μm^3, hemoglobin concentrations were 5.09 to 5.19 (± 0.08) mmol/L cells, hematocrit values were 45.2 to 48.0 (± 3.2)%, MCH amounts were 20.2 to 20.5 (± 0.3)pg, MCHC were 32.8 to 33.5 (± 0.5)%, and RBC numbers were 7.37 to 7.85 (± 0.10)× 10^6 cells/mm^3. There were no statistically significant differences in any of these parameters for the three dietary groups.

Deformability of erythrocytes was measured by a rheoscope at two shearing rates (Table 4). As expected, the DI values were lower at a lower shearing rate, but the DI values were not significantly different among the three dietary groups. Erythrocytes with unusual shapes were not observed in any of the three dietary groups.

The relative viscosity of whole blood sample was measured with an Ostwald viscometer (Table 4). No difference was observed in the relative viscosity values of the three dietary groups.

DISCUSSION

Oral administration of EPA or fish oils rich in EPA and DHA has been reported to increase the deformability and decrease the viscosity of human and animal erythrocytes (19,20,34–37), and EPA content of erythrocytes has been positively correlated with RBC deformability (20,34–36). The extent of dietary-induced changes in fatty acid compositions (Fig. 1) was comparable to those seen in human studies (20,34–36), but no differences were observed in erythrocyte deformability and whole blood viscosity (Table 4). The deformability measured in the present study by using a rheoscope (Table 4) is known to be affected by such factors as intracellular viscosity (a function of hemoglobin content), surface areas/volume ratio (or the lipid content/volume), membrane fluidity, and morphology. In fact, we have shown that erythrocyte deformability measured with a rheoscope is affected by vitamin E content (38,39), by calcium (40,41), by cholesterol content, and by membrane-protein crosslinking (42,43).

Both fish oil and high α-linolenate perilla oil were shown to have beneficial effects in common in respect to some chronic diseases in animal models. However, the presence of the 22 carbon fatty acid DHA in fish oil appears to induce, *e.g.* changes in the proliferation of peroxisomes (44) and in mitochondrial enzyme activities (45). The three major n-3 fatty acids—18:3n-3, 20:5n-3 and 22:6n-3—differ in metabolic pathways in animal cells: 18:3n-3 is incorporated through the *de novo* phosphatidate synthetic pathway; 20:5n-3 is incorporated *via* the acylCoA:lysophospholipid acyltransferase pathway; and 22:6n-3 uses the diacylglycerol:CDP-ethanolamine ethanolaminephosphotransferase pathway (11,12). Although 1-acyl-glycerophosphate acyltransferase (46) and 1-acyl-glycerophosphocholine acyltransferase (47) in erythrocytes may utilize free fatty acids from plasma, the latter enzyme is usually selective for the elongation-desaturation products (12). On the other hand, the former is relatively selective for 18 carbon unsaturated fatty acids, but does not appear to be operative because the phospholipid *de novo*

TABLE 4

Effects of Dietary Oils on the Deformability of Red Blood Cells and on Relative Viscosity of Whole Blood[a]

Dietary group	Deformation index		Relative viscosity at dilution of		
	$\dot{r} = 75.2\ s^{-1}$	$\dot{r} = 526.4\ s^{-1}$	1/2	1/3	1/6
High linoleate	0.23 ± 0.02	0.46 ± 0.01	2.16 ± 0.14	1.61 ± 0.06	1.26 ± 0.01
Control	0.24 ± 0.01	0.44 ± 0.10	2.20 ± 0.04	1.66 ± 0.01	1.26 ± 0.01
High α-linolenate	0.22 ± 0	0.46 ± 0.01	2.12 ± 0.06	1.64 ± 0.06	1.24 ± 0.01

[a]Deformation index (DI) values were measured at shear rates ($\dot{r}$) of 75.2 and 526.4 s^{-1} for the cells suspended in 20% dextran (18.6 cp). Whole blood samples were diluted with saline and the relative viscosity was measured by an Ostwald viscometer at 25°C. Figures represent mean values ± SE for 4 rats in each group.

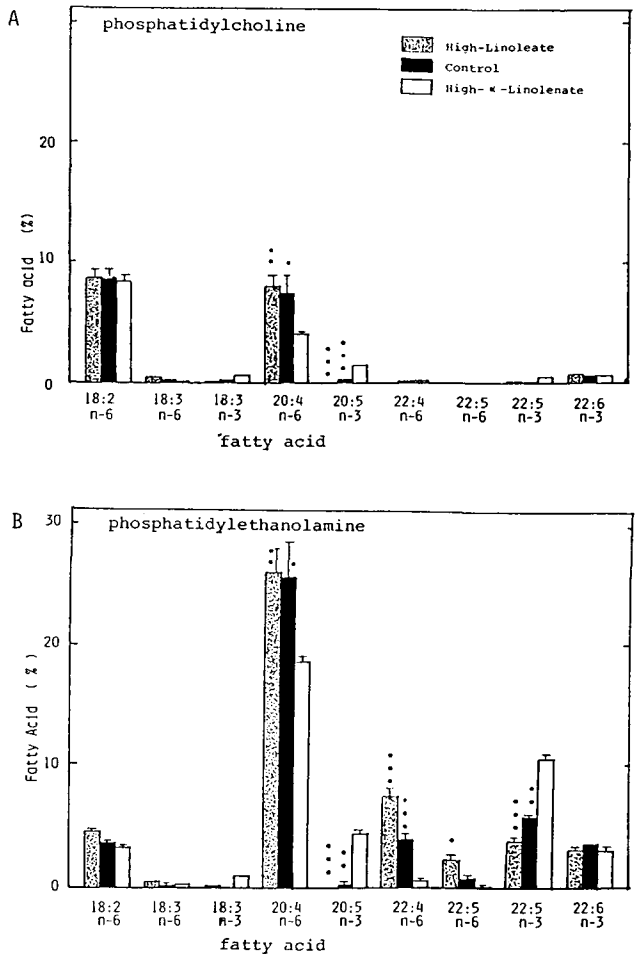

FIG. 1. Fatty acid compositions of erythrocyte phosphatidylcholine (A) and phosphatidylethanolamine (B). Figures represent means of determinations for 3 to 4 rats in each group. Statistical significance between the high α-linolenate group and other groups is shown as * ($p<0.001$), ** ($p<0.01$) or * ($p<0.05$).**

synthetic pathway is not expressed in mature erythrocytes. The differential physiological effects of fish oil and perilla oil may, at least in part, be due to such differences. The difference in hypolipidemic activities may also account for the observed difference. In any case, the results obtained here indicate that the beneficial effects of a high α-linolenate diet as compared to a high linoleate diet (21-29) are exerted without significant changes in erythrocyte deformability, whole blood viscosities, and hematological indices.

Parsons *et al.* (48) found that a diet-induced decrease in 18:2n-6 content of the erythrocytes from cystic fibrosis patients was accompanied by a dramatic increase in the proportion of cells as echinocytes. No such shape changes were induced by a long-term feeding of perilla oil which is rich in 18:3n-3 but relatively poor in 18:2n-6. Apparently, the 18:2n-6 content of the high α-linolenate diet (19%) is enough to meet the requirement of 18:2n-6. In fact, very little difference was observed in the proportions of 18:2n-6 in erythrocyte phospholipids by feeding high linoleate, control, and high α-linolenate diets (Fig. 1). Thus, these data point out differences between 18 and 20 or 22 carbon fatty acids which require further study and need to be more clearly defined.

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Reduced Adipose 18:3ω3 with Weight Loss by Very Low Calorie Dieting[1]

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The human undergoing rapid and sustained weight loss by very low calorie dieting (VLCD) derives the majority of daily energy needs from adipose fatty acids. To evaluate the rates of metabolic utilization of individual fatty acids in humans, two groups of adult women outpatients were studied during major weight loss by VLCD. The diets used were either food or formula, providing the recommended dietary allowance for minerals and vitamins, with fat contents of 2–20 g/d. Group 1 consisted of 10 subjects [initial body mass index (BMI) 32.7, 157% of ideal body weight (IBW)] with a mean loss of 17.7 kg in 3–5 months. Group 2 consisted of 14 subjects (initial BMI 36.7, 167% of IBW) with a mean loss of 25.6 kg in 4–5 months. Adipose tissue biopsies were obtained by needle aspiration from Group 1 before and after weight loss and from Group 2 before, at the midpoint, and after weight loss. With weight loss in Group 1, the adipose tissue content of 18:1ω9, 18:2ω6, and 20:4ω6 did not change, but 18:3ω3 fell (0.67 to 0.56 wt%, $p<0.0001$) as did 20:5ω3 (0.08 to 0.05, $p<0.01$). Adipose tissue 22:6ω3 rose from 0.03 to 0.07 ($p<0.01$). In Group 2, only 18:3ω3 showed a change, falling from 0.71 to 0.69 to 0.59 wt% across weight loss ($p = 0.03$ by analysis of variance). We conclude that the major fatty acids are oxidized in proportion to their composition in adipose triglyceride. The significant reduction in the concentration of 18:3ω3 during weight loss is unique among fatty acids. Its accelerated removal from adipose tissue indicates either a preferential step in β-oxidation or a defined need during supplemented fasting which exceeds its rate of provision from adipose stores.
Lipids 25, 798–806 (1990).

Adipose tissue triglycerides (TG) contain a complex mixture of long chain fatty acids which can be mobilized during periods of dietary privation for essential fatty acid (EFA) and energy needs. The adipose fatty acid composition depends upon the composition of the dietary lipids ingested over an extended period of time (1,2). Several investigators have noted the relative homogeneity of the fatty acid composition of adipose tissue among people of diverse ethnic backgrounds and diets (3,4), suggesting that its composition is metabolically controlled to some degree. Most consistent among the constituent fatty acids of adipose tissue is oleic acid (18:1ω9), which is obtained both from the diet and by endogenous synthesis; whereas linoleic acid (18:2ω6) is variable within a population and also over time (5).

The greater variability of 18:2ω6 than 18:1ω9 is likely due to the dietary essentiality of the former. As the North American diet has provided an increasing dose of 18:2ω6, its content in adipose tissue appears to have about doubled in the last 25 years (6). This change in the content of the principal polyunsaturated fatty acid (PUFA) in adipose tissue has been attributed by some to be causally associated with the declining incidence of coronary atherosclerosis over this time period (7).

α-Linolenic acid (18:3ω3) is also found in human adipose tissue (5,7), albeit in considerably lower quantity than 18:2ω6. As with linoleate, 18:3ω3 cannot be synthesized endogenously and is thus present only as it is available from the diet. α-Linolenic acid is the metabolic precursor for the elongation and desaturation products eicosapentaenoic acid (20:5ω3) and docosahexaenoic acid (22:6ω3), which have also been associated with reduced risk for atherosclerosis (8–10). While the essentiality of this family of fatty acids remains a topic of debate, there are two case reports of childhood human deficiency with symptomatic improvement concurrent with ω3 fatty acid supplementation (11,12), and both of these reports concluded that the minimal ω3 fatty acid intake should be 0.5% of calories. If 18:3ω3 and its products are necessary for adult human well-being, the specific requirements remain undefined (13). The optimal intake of ω3 fatty acids may vary with the dietary content of ω6 fatty acid (14), as the products derived from 18:3ω3 in tissue lipids are suppressed by high levels of dietary 18:2ω6 and *vice versa* (15,16).

Although the relationship between dietary EFA intake and adipose tissue content continues to be addressed in the literature, the effect of weight loss on adipose tissue composition has received little attention since the pioneering work of Hirsch over 30 years ago. Given a 2.5 MJ (600 kcal) per day fat-free diet for up to 150 days, obese humans showed no change in the proportion of palmitic (16:0), oleic, and linoleic acids in adipose tissue despite over 30 kg of weight loss (17). The response of the lesser components (such as 20:4ω6, 18:3ω3, 20:5ω3, and 22:6ω3) to weight loss was not reported in those studies. However, the metabolism and conservation of these minor components of adi-

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Abbreviations: ANOVA, analysis of variance; BMI, body mass index (weight in kg divided by the square of height in meters); CE, cholesteryl esters; DHA, docosahexaenoic acid (22:6ω3); EFA, essential fatty acid; EPA, eicosapentaenoic acid (20:5ω3); FAME, fatty acid methyl esters; FFA, free (non-esterified) fatty acids; FFM, fat free mass; IBW, ideal body weight; MFP, meat/fish/poultry very low calorie diet; OPTI, Optifast formula diet (Sandoz Nutrition); PL, phospholipids; PUFA, polyunsaturated fatty acids; RDA, recommended dietary allowance; TG, triglyceride; VLCD, very low calorie diet.

pose tissue are of increasing interest in view of their important roles in membrane structure, where they exist in greater concentration, and also as substrates for eicosanoid synthesis.

Despite the rush to clinical trials of fish oil in a number of illnesses, the basics of human storage, pool size, and metabolism of the ω3 fatty acid family remains relatively unexplored. In view of the emerging importance of the ω3 fatty acids and the lack of knowledge about their conservation by humans during major weight loss, we performed a study of the composition of human adipose tissue in two groups of women undergoing weight loss by very low calorie dieting.

METHODS

This project was conducted with two separate groups of subjects. Subject Group 1 was studied in a multidisciplinary weight management clinic at the University of Minnesota. Subject Group 2 was studied under similar circumstances at the University of California at Davis. In both locations, the subjects were randomized to receive one of two very low calorie diets providing between 1.8-2.5 MJ (420 and 600 kcal) daily with different protein, carbohydrate, and fat content. The study protocols were approved by the University of Minnesota Human Subjects Committee and by the Human Subjects Review Committee at the University of California at Davis. Written informed consent was obtained from each subject prior to participation.

Subjects: Group 1. Twelve obese female volunteers, ages 22 to 39, were recruited for the study in response to posted notices. All were at least 20 kg in excess of their ideal body weight [IBW—taken as the median value of the medium frame range from the Metropolitan Life Insurance Tables (18)]. Preadmission studies included physical exam, electrocardiogram, and routine hematological and serum biochemical studies for cardiac, hepatic, renal, thyroid, or pancreatic β-cell dysfunction. All subjects were weight stable in a 3 kg range for the month prior to beginning the study. A diet history taken at the time of admission to the study indicated that none of the subjects restricted animal products or vegetable fats in their habitual diets. The subjects were assigned to one of two different VLCD's. Two subjects dropped out prior to completing the final tests. The characteristics of the 10 subjects completing the protocol are shown in Table 1.

Subjects: Group 2. Twenty-one obese female volunteers, ages 22 to 42, were recruited in response to newspaper advertisements. All were at least 25 kg in excess of IBW. Pre-selection screening was identical to that for Group 1 subjects above. All subjects ate an unrestricted omnivorous diet. The subjects were randomly assigned to one of two VLCDs. Seven subjects dropped out before completing the final testing. The characteristics of the 14 subjects completing the protocol are shown in Table 2.

Diets. During the first two weeks of the outpatient protocol, all subjects were prescribed a 4.2 MJ/day (1000 kcal/day diet) containing 90 g protein. Following the two week adaptation period, subjects were assigned to one of two VLCDs.

Diet No. 1 (MFP) consisted of three daily portions of lean meat, low fat fish or poultry providing 1.5 g protein per kg IBW in 1.9-2.5 MJ (450-600 kcal) daily. The lipid content of this diet consisted of those fats inherent in the foods selected, and was judged by diet

TABLE 1

Characteristics of Subjects in Group No.1

Subject	Age (years)	Height (cm)	Initial weight (kg)	%IBW[a]	BMI[b]	Weight loss (kg)	Weight loss rate (kg/wk)
Food VLCD (Diet No. 1)							
1	37	172	105.0	168	35.5	17.5	1.03
2	28	172	93.2	152	31.5	15.2	0.58
3	33	167	99.3	172	35.6	14.9	0.82
4	34	166	99.7	178	36.2	21.8	1.98
5	33	177	89.5	133	28.6	16.4	0.78
Mean	33	171	97.3	161	33.5	17.2	1.04
SEM	1	2	2.7	8	1.5	1.2	0.25
Formula VLCD (Diet No. 2)							
1	24	169	107.6	177	37.7	20.6	2.06
2	22	170	87.8	149	30.4	22.4	1.72
3	32	157	82.0	155	33.7	19.0	1.46
4	35	162	73.4	135	28.0	17.2	1.20
5	28	168	84.0	142	29.8	11.4	0.50
Mean	28	165	87.0	152	31.9	18.1	1.39
SEM	2	2	5.7	7	1.7	1.9	0.26
Total Mean	31	168	92.2	156	32.7	17.6	1.21
SEM	2	2	3.4	5	1.1	1.1	0.18

[a]IBW, ideal body weight, taken as mid-range of medium frame value.
[b]BMI, body mass index (weight in kilograms divided by the square of the height in meters).

TABLE 2

Characteristics of Subjects in Group No. 2

Subject	Age (years)	Height (cm)	Initial weight (kg)	%IBW[a]	BMI[b]	Weight loss (kg)	Weight loss rate (kg/wk)
Food VLCD (Diet No. 1)							
1	38	164	85.9	146	32.0	24.7	1.45
2	23	169	106.0	170	37.2	25.5	1.36
3	40	156	99.0	185	40.6	26.7	1.34
4	22	165	102.5	171	37.6	22.8	1.28
5	27	164	83.2	141	31.0	23.7	1.10
6	42	161	82.3	145	31.6	23.6	1.25
Mean	32	163	93.2	160	35.0	24.5	1.30
SEM	4	2	4.3	7	1.6	0.6	0.05
Formula VLCD (Diet No. 2)							
1	30	152	87.8	171	37.8	28.0	1.57
2	39	169	100.7	161	35.3	28.5	1.92
3	32	160	129.1	231	50.4	27.2	1.68
4	30	168	100.6	163	35.8	25.7	1.71
5	40	166	114.9	189	41.5	29.6	1.67
6	39	168	112.4	183	40.0	28.5	1.90
7	40	170	87.1	137	30.1	22.2	0.97
8	32	168	101.7	165	36.2	24.1	1.59
Mean	35	165	104.3	175	38.4	26.7	1.63
SEM	2	2	5.0	10	2.1	0.9	0.10
Total Mean	34	164	99.5	168	36.9	25.8	1.48
SEM	2	1	3.6	7	1.4	0.6	0.08

[a]IBW, ideal body weight, taken as mid-range of medium frame value.
[b]BMI, body mass index (weight in kilograms divided by the square of the height in meters).

history to range from 15–20 g/d. Based upon the diet histories of the subjects and published tables for the fatty acid contents of these animal fats, approximately 1% of this dietary fat was 18:3ω3 (for an estimated daily intake of <200 mg for this fatty acid). This diet was supplemented daily with 25 mEq of potassium bicarbonate (K-Lyte, Bristol Laboratories, Evansville, IN), 4 tablets of calcium/ magnesium antacid (Calcitrel, Sterling, New York, NY) providing 800 mg and 200 mg, respectively, of these minerals and a folate-containing multivitamin with minerals (Centrum, Lederle, Wayne, NJ). In total, the food portions plus supplements provided at least 100% of the National Research Council Recommended Dietary Allowance (19) for the major and trace minerals and vitamins.

Diet No. 2 (OPTI) was a commercial formula (Optifast, Sandoz, Minneapolis, MN) providing a daily intake of 1.8 MJ (420 kcal) with 70 g protein and 30 g carbohydrate. The protein source for this formula was pasteurized egg albumin and casein. The formula also provided 2 g/d of fat as partially hydrogenated soybean oil. By gas chromatographic analysis (see Methods, below), the composition of this lipid source was predominantly saturated (29%) and monounsaturated (37%) fatty acids. The linoleic acid content was 16% and α-linolenic acid 1.4% providing, respectively, 320 mg/d and 30 mg/d of these two fatty acids. The mineral and vitamin content of the formula met or exceeded the RDA for minerals, trace minerals and vitamins. To prevent symptoms of weakness or fatigue, subjects from both diet groups were supplemented with up to 3 g of sodium daily with boullion.

It should be emphasized that the amount of formula (and hence calories) given all subjects on Diet No. 2 was fixed, whereas the protein and calories provided by Diet No. 1 was variable depending upon stature. The range of energy intakes between groups during the VLCD varied from 420 kcal/d for Diet No. 2 to 450–550 kcal/d for Diet No. 1. Thus, the diets were similar in energy content but were neither precisely isocaloric nor isonitrogenous. While these diets differed in fat composition, neither provided more than 0.2 g/day of ω3 fatty acids and thus had little effect on the ω3 pool if its oxidative flux was in the 1.0–1.5 g/d (0.5% total calories) range projected (11,12).

During the diet phase, the subjects participated in weekly outpatient group sessions as well as in individual sessions for support and education in weight management topics. Subjects in Group No. 1 and Group No. 2 were encouraged to continue with the VLCD for a total weight loss of 40 lbs (18 kg) and 60 lbs (27.3 kg), respectively. Upon completion of the weight loss, subjects underwent a gradual refeeding process (six weeks) and were then assigned to maintenance groups where they received continued weekly support and advice on following a high carbohydrate, low fat maintenance diet.

Monitoring. Body weight, resting pulse, blood pressure, and breath acetone (by gas chromatography, Caldetect Inc., Richmond, CA) were measured weekly. Resting electrocardiograms and fasting blood by venipuncture were obtained monthly. Blood analyses by routine semi-automated techniques included complete blood count, glucose, serum electrolytes, calcium, phospho-

rus, magnesium, urea nitrogen, creatinine, uric acid, albumin, aspartate aminotransferase, and alkaline phosphatase. The weekly weights and breath ketones effectively monitored for subject adherance during the outpatient protocol, while the blood tests and ECG monitored for side effects of the diets or supplements.

Body composition analysis. Group No. 2 subjects had body composition determined by hydrostatic weighing at three different time points: before dieting, at approximately 30 lbs weight loss, and following 50–60 lbs weight loss. Subjects were fully submerged while a continuous weight reading was obtained for a 3–5 second interval following maximal exhalation. The residual lung volume was measured at the time of analysis by nitrogen washout. This procedure was repeated until three consistent weights were obtained. Body density and percent body fat were calculated using the Siri formula (20) and Brozek equations, respectively (21). Fat free mass (FFM) was calculated as body weight minus total body fat.

Adipose tissue sampling and analysis. Subcutaneous adipose tissue was obtained by biopsy with a 14 gauge needle following local anesthesia. Group 1 subjects were biopsied from the gluteal fat before and after approximately 40 lbs of weight loss. Group 2 subjects were biopsied from the lateral thigh before, after 30 lbs of weight loss, and finally after 50–60 lbs lost. After careful washing in saline solution and blotting, the adipose tissue was extracted in a ground glass homogenizer by the method of Folch *et al.* (22), the triglyceride and phospholipid fractions were separated by thin-layer chromatography, and their fatty acid composition was determined by capillary gas chromatography (23). Specifically, 8–22 mg of adipose tissue was extracted with 5 mL of chloroform/methanol (2:1, v/v), after which 5 mL of water was added to extract the water solubles. After flushing with nitrogen and vortexing, the water/methanol layer was removed and discarded. The chloroform layer was blown to dryness with nitrogen. The residual lipids were dissolved in a minimal amount of chloroform and applied to a silicic acid thin-layer chromatography plate under nitrogen and developed in petroleum ether (b.p. 30–60°C)/diethyl ether/acetic acid (80:20:1, v/v/v) to separate the phospholipids (PL), free fatty acids (FFA), TG, and cholesteryl esters (CE). The TG and PL fractions were scraped separately from the plate, esterified with 5% HCl in methanol, and the fatty acid methyl esters (FAME) extracted in petroleum ether (b.p. 30–60°C). The sample was blown down with nitrogen, and heptane added prior to capillary gas chromatographic analysis.

A Packard 428 gas chromatograph (Packard Instruments, Norwalk, CT) equipped with a 50-meter by 0.25 mm bonded 007 FFAP fused silica capillary column (Quadrex, New Haven, CT) was employed to separate the FAME. The column temperature was programmed to rise from 190 to 220°C at 2°C/min with a final hold, separating 12:0 to 22:6ω3. The detector temperature was 270°C and the injector temperature 250°C. Helium was used as carrier gas at a flow rate of 1.4 mL/min and a split ratio of 1:65. FAME were identified by comparison with authentic standards, and peak areas were integrated as relative weight (wt%) using a microprocessor.

Due to the overwhelming predominance of triglyceride in adipose tissue and the close similarity of the fatty acid profile in the adipose tissue vs the isolated triglyceride fraction of the adipose tissue, the lipids of adipose tissue from eight of the subjects in Group No. 2 were not separated by TLC. In a pilot study comparing the fatty acid profiles of adipose tissue either subjected to thin-layer chromatography to remove the phospholipid fraction or not (A.B. Tang, unpublished results, 1990) the variation between repeat analyses by the same method was as great as the variation between the two methods performed on the same sample. For fatty acids making up less than 1% by weight of adipose tissue, the results of repeated analyses varied as much as 10%.

The total lipids from the tissue sample were extracted, processed to form methyl esters as noted above, and analyzed on a Hewlett-Packard (Palo Alto, CA) 5980 gas chromatograph also equipped with a 50-meter by 0.25 mm bonded 007 FFAP fused silica capillary column (Quadrex) to separate the FAME (12:0 to 22:6ω3). The column temperature was programmed to rise from 190 to 215°C at 2°C/min with a final five-min hold, followed by 0.3°C/min rise in temperature to 224°C. All other parameters were identical to those described above.

Statistical analysis. Data analysis was performed using the PC-SAS (24) package on a microcomputer. Changes in the level of each specific fatty acid were assessed separately in Group 1 and in Group 2 by analysis of variance (ANOVA) for both time and diet effects. *Post hoc* tests for individual differences between time points following the ANOVA were done using the least significant differences test. Due to the number of individual tests done with each diet group, the threshold for significance was $p<0.01$.

RESULTS

Of 33 subjects recruited for the study groups, a total of 24 (10 from Group No. 1 and 14 from Group No. 2) completed the two protocols. The rates of weight loss for the Group No. 1 subjects are shown in Table 1. Eight of the 10 subjects lost 0.8 kg or more per week, indicating excellent adherence to the diets. The rates of weight loss for the Group No. 2 subjects are shown graphically in Figure 1. The data are calculated as percent loss from initial weight, and this Figure illustrates the highly consistent responses within and between dietary groups. These results, plus the rapid weight loss of the majority of Group No. 1 subjects, indicate excellent diet adherence generally and specifically indicate that little if any additional fat calories were consumed during the VLCD by our subjects.

In addition to the rates of weight loss indicating the quality of adherence to the VLCD, additional monitoring parameters included breath acetone determinations. Eight of ten subjects in Group No. 1 and all of Group No. 2 subjects achieved and sustained values exceeding the equivalent of 0.4 mM β-hydroxybutyrate

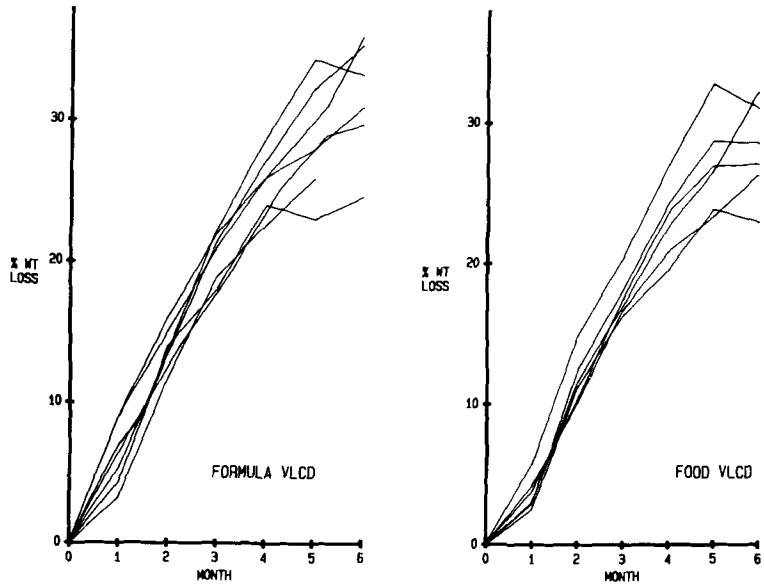

FIG. 1. Rates of weight loss for Group No. 2 subjects, calculated as percent of initial weight, and indicating uniform adherence to the VLCD.

during the VLCD. This increase over baseline values (less than 0.05 mM in the pre-weight loss phase) indicated consistent avoidance of unprescribed dietary carbohydrate. Serum analyses indicated routinely normal electrolytes and liver function tests. The only parameter found consistently to exceed the normal range was serum uric acid in the MFP VLCD subjects. This value rose transiently and resolved to normal while the subjects were still taking the VLCD.

Body compositional data for Group No. 2 subjects determined before, during, and after the VLCD weight loss are shown in Table 3. Total adipose weights are provided at each time point, plus total change for these parameters and fat free mass across weight loss. The changes in body composition were similar for both diets. There were no significant differences between the diets for total weight loss, fat loss, or loss of FFM.

The fatty acid contents of adipose tissue from the gluteal regions of Group No. 1 subjects before and after weight loss are shown in Table 4. The only statistically significant changes were in the ω3 fatty acids. α-Linolenic acid declined by 16% ($p<0.001$). To illustrate the uniformity of this change among subjects, the data for individual subjects are shown graphically in Figure 2, along with the values for linoleic and γ-linolenic (18:3ω6) acids. Neither of these latter two fatty acids (one an isomer of 18:3ω3) showed a similar pattern of decline with weight loss.

The highly unsaturated products of 18:3ω3 metabolism, 20:5ω3, and 22:6ω3 are also of interest. While present in only trace amounts in the adipose tissue of our subjects, 20:5ω3 declined and 22:6ω3 rose with weight loss in Group No. 1. Comparing the diet groups separately (MFP *vs* OPTI), there were no differences in the responses of the two diet groups.

The fatty acid contents of adipose tissue taken from the lateral thigh of the Group No. 2 subjects before, during, and after weight loss are shown in Table 5. Analyzed by ANOVA, the only significant change with weight loss occurred in 18:3ω3, which fell from 0.71 to 0.59 wt% (17%, $P<0.01$). To illustrate the net effects of weight loss, the individual data for 18:3ω3, 18:2ω6, and 18:3ω6 are shown in Figure 3. Of 14 subjects, 12 showed a decline in 18:3ω3, whereas there was no consistent pattern in the data for 18:2ω6 and 18:3ω6.

In neither group was there an effect of the type of VLCD used on the adipose fatty acid composition over time. This is consistent with the observation that adipose composition equilibrates with dietary intake with a half life of 680 days (25), and also with the fact that our subjects' dietary fat intake was inconsequential (in the range of 1-14%) as compared to daily net oxidation (140 g/d by body composition change).

DISCUSSION

The primary finding of this study is that very low calorie diets employed for rapid weight loss in humans reduce the content of α-linolenic acid in the remaining body adipose pool. This was a consistent finding in two separate groups of subjects experiencing major weight loss and was independent of the type of VLCD (food or formula) employed. There was also close agreement between the two groups in the extent of the reduction. In Group No. 1, 18:3ω3 fell from 0.67 to 0.56 wt% (a 16% decline with 17 kg of weight loss) and in Group No. 2 it fell from 0.71 to 0.59 (an 18% decline with 26 kg of weight loss). There were no other consistent changes in adipose fatty acid composition across both diet groups. The reduction in 20:5ω3 seen in Group No. 1 did not occur in Group No. 2, and the rise in 22:6ω3 in Group No. 2 did not achieve statistical significance as it did in Group No. 1.

By studying the composition of adipose tissue dur-

TABLE 3

Body Composition, Group No. 2 Subjects

Subject	Before		During		After		Change with VLCD		
	Total wt[a]	Adipose wt	Total wt	Adipose wt	Total wt	Adipose wt	Total wt	FFM[b] wt	Adipose wt
Food VLCD (Diet No. 1)									
1	85.9	38.4	74.8	29.5	61.2	18.3	24.7	4.6	20.1
2	106.0	48.2	92.9	37.7	80.5	25.2	25.5	2.5	23.0
3	99.0	49.8	83.4	38.8	72.3	28.0	26.7	4.9	21.8
4	102.5	54.0	88.7	42.4	79.7	32.4	22.8	1.2	21.6
5	83.2	40.6	69.8	28.4	59.5	22.1	23.7	5.2	18.5
6	82.3	37.5	68.9	26.8	58.7	19.3	23.6	5.4	18.2
Mean	93.2	44.8	79.8	33.9	68.7	24.2	24.5	4.0	20.5
SEM	4.3	2.8	4.1	2.7	4.1	2.2	0.6	0.7	0.8
Formula VLCD (Diet No. 2)									
1	87.7	34.1	73.9	25.0	59.7	13.7	28.0	7.6	20.4
2	100.7	47.1	84.8	34.8	72.2	23.8	28.5	5.2	23.3
3	129.1	71.1	113.4	59.6	101.9	52.8	27.2	8.9	18.3
4	100.6	46.0	84.2	35.5	74.9	25.3	25.7	5.0	20.7
5	114.9	55.2	95.4	40.6	85.3	33.1	29.6	7.5	22.1
6	112.4	45.5	95.5	31.0	83.9	23.2	28.5	6.2	22.3
7	87.1	38.8	73.7	24.8	64.9	17.4	22.2	0.8	21.4
8	101.7	48.5	87.1	37.6	77.6	30.0	24.1	5.6	18.5
Mean	104.3	48.3	88.5	36.1	77.6	27.4	26.7	5.9	20.9
SEM	5.0	4.0	4.6	3.9	4.6	4.2	0.9	0.9	0.6
Total Mean	99.6	46.8	84.8	35.2	73.7	26.0	25.8	5.1	20.8
SEM	3.6	2.5	3.3	2.4	3.3	2.6	0.6	0.6	0.5

[a]wt, Weight in kilograms.
[b]FFM, fat free mass.

ing major weight loss in subjects adhering to a very low calorie (and thus very low fat) diet, we have indirectly assessed the metabolic demand for specific fatty acids in a period when dietary fat intake provided at most a minor contribution to flux through the metabolic pool. This method of study has allowed us to confirm that the major fatty acid constituents of adipose tissue were mobilized in the same proportions that they existed in the tissue. In this observation we are in agreement with the prior observations of Hirsch (17). With the aid of capillary gas chromatography, however, we have also determined the minor fatty acid (less than 1% by weight) constituents of adipose triglyceride. Thus, we were able to find that adipose 18:3ω6 and 20:4ω6 did not change with weight loss, while the 18:3ω3 fell as noted above. This observation may be due in part to the relatively copious availability of the 18:2ω6 precursor in adipose tissue, while 18:3ω3 and its metabolic products (20:5ω3 and 22:6ω3) were present in much more limited quantities.

TABLE 4

Adipose Fatty Acids (Weight %) from Group No. 1 Subjects Before and After Weight Loss

Fatty acids	Before		After	
	Mean	SEM	Mean	SEM
14:0	2.66	0.15	2.91	0.01
16:0	18.59	0.49	19.39	0.47
18:0	2.92	0.23	3.15	0.24
16:1[a]	7.20	0.45	6.97	0.41
18:1[b]	46.88	0.47	46.40	0.59
20:1ω9	0.00	0.00	0.07	0.03
18:2ω6	16.00	0.69	15.59	0.60
18:3ω6	0.13	0.01	0.12	0.02
20:2ω6	0.21	0.02	0.22	0.02
20:3ω6	0.22	0.02	0.25	0.02
20:4ω6	0.34	0.03	0.35	0.03
22:4ω6	0.12	0.02	0.13	0.02
18:3ω3	0.67[c]	0.03	0.56[c]	0.03
20:5ω3	0.08[d]	0.01	0.05[d]	0.01
22:5ω3	0.09	0.02	0.11	0.02
22:6ω3	0.03[d]	0.01	0.07[d]	0.01

[a]Sum of 16:1ω9 and 16:1ω7.
[b]Sum of 18:1ω9 and 18:1ω7.
[c]Values differ at $p < 0.01$.
[d]Values differ at $p < 0.001$.

In human feeding studies to determine whole body oxidation of specific 18-carbon fatty acids, Jones *et al.* (26) observed preferential oxidation of ^{13}C-labeled dietary 18:2ω6 as compared to 18:1ω9 which, in turn, was oxidized in preference to 18:0. In a subsequent study of substrate oxidation by indirect calorimetry, Jones and Schoeller (27) reported significant effects on respiratory quotient and thermic effect of food by diets differing in polyunsaturated fat contents. Our results from the present study indicate no metabolic preference for 18:2ω6 over 18:1ω9 or 18:0 when these substrates are delivered from endogenous stores. This suggests that the difference in results from the above studies in contrast to ours may be due to gastrointestinal partitioning of dietary fats that does not occur when adipose triglyceride fatty acids are mobilized.

With the body composition studies available from

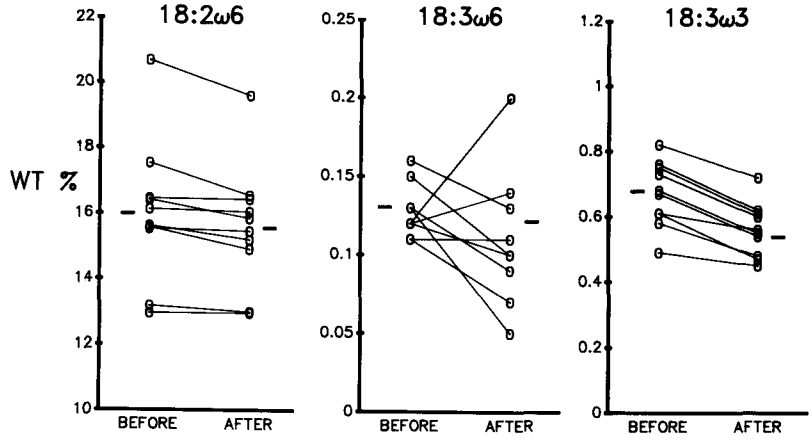

FIG. 2. Individual Group No. 1 values for adipose tissue 18:2ω6, 18:3ω6, and 18:3ω3 before and after 17 kg of weight loss.

TABLE 5

Adipose Fatty Acids (Weight %) from Group No. 2 Before, During, and After Weight Loss

Fatty acids	Before		During		After	
	Mean	SEM	Mean	SEM	Mean	SEM
14:0	2.44	0.12	2.45	0.12	2.36	0.10
16:0	19.81	0.53	19.92	0.57	19.76	0.53
18:0	3.00	0.17	3.02	0.16	3.22	0.19
16:1[a]	7.01	0.32	6.70	0.23	6.23	0.27
18:1[b]	45.08	0.46	45.10	0.43	45.81	0.45
20:1ω9	0.65	0.03	0.64	0.02	0.71	0.02
18:2ω6	16.04	0.49	16.34	0.53	16.06	0.38
18:3ω6	0.09	0.01	0.10	0.01	0.10	0.01
20:2ω6	0.29	0.03	0.28	0.02	0.30	0.02
20:3ω6	0.27	0.02	0.31	0.02	0.31	0.02
20:4ω6	0.43	0.02	0.45	0.02	0.48	0.04
22:4ω6	0.17	0.01	0.21	0.02	0.23	0.02
18:3ω3	0.71[c]	0.04	0.69	0.03	0.59[c]	0.03
20:5ω3	0.03	0.01	0.03	0.00	0.03	0.00
22:5ω3	0.13	0.01	0.13	0.00	0.15	0.02
22:6ω3	0.07	0.01	0.07	0.01	0.09	0.01

[a]Sum of 16:1ω9 and 16:1ω7.
[b]Sum of 18:1ω9 and 18:1ω7.
[c]Values differ at $p < 0.01$.

Group No. 2, we determined that the rate of adipose triglyceride oxidation averaged 140 g/d over a mean duration of 140 days of dieting. With the adipose tissue content of 16% 18:2ω6, this fatty acid would be oxidized at a rate of 22 g/d, providing more than 10% of total energy needs. This net flux from triglyceride stores to oxidation is 5- to 20-fold in excess of the minimum amount necessary to avoid essential fatty acid deficiency in rats (28) and humans (29-31). While this flux of EFA from adipose stores to muscle and liver sites of oxidation does not insure availability for "essential purposes," Mascioli *et al.* (32) have shown that interruptions of continuous feeding will mobilize adipose EFA stores and prevent the occurrence of biochemical signs of EFA deficiency.

Adipose fatty acids are mobilized from adipose stores at a greater rate than they are oxidized (33), so that net oxidation (over the 4-6 month duration of this study) is the result of the balance between lipolytic release and re-esterification for each fatty acid. Thus, the rates of "exit" and "re-entry" of adipose fatty acids would allow for a net removal of a specific fatty acid from adipose stores should it experience an increased metabolic utilization in other tissues.

Viewed from a simpler perspective, however, the mobilization of α-linolenic acid during the VLCD can be thought of as consisting of two components: i) that contained in the mobilized triglyceride (*e.g.*, 20.8 kg in Group No. 2); and ii) that removed from the remaining adipose tissue. These amounts during 140 days of dieting for the Group No. 2 subjects were 142 g and 29 g, respectively. Adding the two together, this translates into 1.2 g/d, or about 0.5% of daily energy expenditure. This value is very close to the estimates of human need determined in two case studied describing deficiency of this conditionally required nutrient (11,12). Thus, unlike the 5- to 20-fold excess of 18:2ω6 provision above minimal need, the direct mobilization of 18:3ω3 during the VLCD (supplemented starvation) may not have met minimal endogenous need. In other words, despite our subjects' obesity prior to treatment, their adipose 18:3ω3 content was at best marginal to provide ≥0.5% of metabolic energy need. It is possible that the reduction in adipose tissue 18:3ω3 (*i.e.*, the 29 g removed from the remaining adipose tissue at the end of the weight loss) was the result of its accelerated recovery to meet a requirement not met by direct adipose mobilization (the 142 g component) plus dietary intake. This perspective suggests that very low calorie diets might need to be supplemented with ω3 fatty acids to avoid tissue reduction of this nutrient.

The tissue site and mechanism for the metabolic utilization of 18:3ω3 are suggested by a recent paper by Clouet *et al.* (34). The authors report preferential

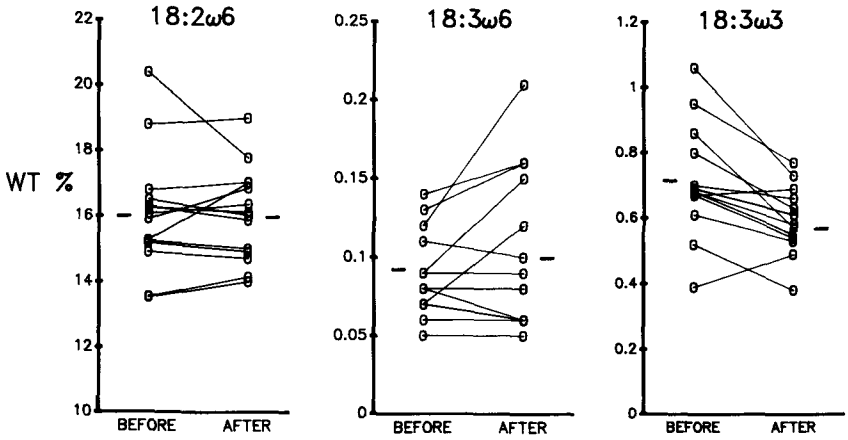

FIG. 3. Individual Group No. 2 values for adipose tissue 18:2ω6, 18:3ω, and 18:3ω3 before, at the midpoint, and after 25 kg of weight loss.

oxidation of α-linolenate (relative to other 18-carbon fatty acids) when provided to the mitochondria in the non-esterified form, but this was not the case for α-linolenate provided as the CoA ester. In our subjects undergoing supplemented fasting, the adipose-derived fatty acids would be delivered to muscle and liver in the free fatty acid form, potentially predisposing the 18:3ω3 to preferential β-oxidation in the mitochondria.

The health significance of the reduction in adipose 18:3ω3 with rapid weight loss remains to be determined. In this context, the extent to which α-linolenic acid undergoes endogenous anabolic (desaturation and elongation) conversion to EPA and DHA is important. While there is some concern that this conversion is so slow as to be inconsequential (35), a number of studies indicate that anabolic conversion occurs at a meaningful rate in humans. These include the observation that dietary 18:3ω3 supplementation increases phospholipid 20:5ω3 content (16,36-38) and the observation that vegetarians do not suffer depletion of EPA and DHA in their serum phospholipid fraction (39).

The other concern in interpreting the physiologic importance of the decline in adipose 18:3ω3 is the extent to which it interrelates with other tissue fatty acid pools. In view of the greater rate of adipose fatty acid turnover than oxidation, it is likely that other tissues are not perfectly isolated from changes in adipose α-linolenic acid content. That is, if there is a sustained reduction in adipose 18:3ω3, eventually this will affect ω3 fatty acid enrichment of other tissues.

There are a number of potential consequences of reduced body ω3 fatty acid stores. The highly unsaturated metabolic products of 18:3ω3 modulate cellular response to injury and infection (40,41), and reduce the rate of atherosclerosis in an animal model (10) and in humans (42). This participation of ω3 fatty acids in the pathogenesis of coronary occlusion offers a potential explanation of the findings of Hamm *et al.* (43), who observed that repeated weight loss in adults predisposed them to early atherosclerosis. If weight loss involving low fat intake reduces body ω3 stores, this may not be subsequently replaced when dietary ω3 intake is below 1% of total calories. Thus, a subtle but chronic risk state could be established. In view of the observation that as few as three fish meals per week (providing a mean of 0.5 g/d of ω3 fatty acids) can reduce atherosclerosis risk (8), this possible explanation for the increased coronary risk with repeated weight loss is not beyond reason.

In conclusion, we have observed a consistent decline in adipose 18:3ω3 content with major weight loss induced by very low calorie dieting. This change occurred independent of the type of VLCD (food or formula) used. Both the mechanism through which this fatty acid is selectively removed from adipose tissue and the health implications of this finding deserve further study.

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Squalene in Grapefruit Wax as a Possible Natural Protectant Against Chilling Injury[1]

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The influence of temperature conditioning on stored grapefruit against chilling injury (CI) as related to the fruit's neutral lipids in the peel was investigated. Squalene, a highly unsaturated C_{30} isoprene hydrocarbon, was found to be present in the epicuticular wax of grapefruit. The optimal temperature for biosynthesis of squalene in grapefruit was 15°C; this is also the temperature reported previously as the optimum temperature for conditioning grapefruit against chilling injury. Control and temperature-conditioned grapefruit were stored monthly over three seasons from 1986 to 1989. Fruits were rated for chilling injury and the levels of squalene were determined. An inverse relationship found between CI and squalene level suggested that squalene may protect grapefruit from CI.
Lipids 25, 807-810 (1990).

Chilling injury (CI) in grapefruit (brown stains or pits on the peel) occurs when the fruit is stored at 5°C for more than 3 weeks. Storing the fruit for 7 days at 15°C prior to the 5°C storage is the most successful means of reducing the incidence of CI in grapefruit (1-3). One approach to the CI problem is to determine compounds (lipids) which may be preferentially biosynthesized at 15°C over this 7-day period. This approach is suggested from past studies where conditioning or "cold hardening" of citrus plants protects against freeze injury (4-7). In those instances where plants were placed in controlled-atmosphere chambers at gradually lower temperatures, the level of highly unsaturated triacylglycerols (TAG) greatly increased in the leaves. TAG is generally a trace component. Hibiscus (8) and avocado plants (Nordby, H. E., unpublished data) also show this TAG increase, indicating that the phenomenon may be characteristic of subtropical plants. In a more recent study (9), grapefruit were "conditioned" *in vivo* on five 6-yr-old potted plants placed in chambers under cold-hardening regimes. Only a slight reduction in CI and a slight increase in flavedo TAG occurred during this conditioning period, suggesting that lipids other than TAG might be protecting grapefruit from CI. The present study elucidates one of these compounds, squalene, as being preferentially biosynthesized under the optimal regimes for conditioning grapefruit against CI, 15°C for 7 days.

[1]Mention of a trademark, warranty, proprietary product, or vendor does not constitute a guarantee by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

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Abbreviations: CI, chilling injury; Con, conditioned; ECL, equivalent chain length; FID, flame ionization detector; GC-MS, gas chromatograph-mass spectrograph; GLC, gas-liquid chromatography; MS, mass spectra; NCon, nonconditioned; R_f, retardation factor; TAG, triacylglycerols; TLC, thin-layer chromatography.

MATERIALS AND METHODS

Marsh grapefruit (*Citrus paradisi* Macf.) were picked monthly during the 1986-87, 1987-88 and 1988-89 seasons from a grove near Merritt Island on the east coast of Florida. Fruit were washed and dried under citrus packinghouse procedures, except waxes and fungicides were not applied. Samples were taken for preconditioning lipid analyses. Fruits were loosely packed (25 to 35/box) in nonwaxed, cardboard citrus boxes. Depending on the study being conducted, boxes were stored in 1°, 5°, 10°, 15°, and 21°C constant temperature storage rooms without light, and at 70% relative humidity, for 1 to 14 wk. After conditioning, samples for lipid analyses were removed and all boxes were stored at 5°C for 3 to 15 wk. Fruits were examined for frequency of CI (pitting or brown staining) after 3-wk storage. The boxes of fruit were examined in a 21°C examining room with 90% relative humidity and then returned to their respective storage rooms within 30 min. In exploratory studies, lipids were extracted (9) from 3-g samples of flavedo from nonconditioned (NCon) fruit that were stored 1 wk at 5°C and from fruit conditioned (Con) 1 wk at 15°C. Aliquots of the lipid extracts were analyzed by thin-layer chromatography (TLC) for their neutral lipid content. The absence of any major differences between the NCon and Con extracts, except in the hydrocarbon area of the plate, prompted the next step which was to determine whether the increased concentration of hydrocarbon in the Con fruit flavedo came from the epicuticular wax. Thus, the NCon and Con fruit were dipped in chloroform and the respective extracts were compared for their neutral lipid content by TLC as above (9).

The presence of a greater concentration of hydrocarbon in the Con fruit prompted the use of gas-liquid chromatography (GLC) to determine if any of the epicuticular wax hydrocarbons were preferentially increasing during the conditioning treatment. In these exploratory studies, hydrocarbons were isolated from other wax components by TLC (10), placed in hexane and analyzed by GLC on a polar SP1000 packed column (10) and on a 30 m × 0.53 mm megabore nonpolar DB-1 column. For the latter, helium flow was 18 mL/min, injection and flame ionization detector (FID) were at 270°C. The oven was kept at 210°C for 2 min, then temperature was increased 3°/min to 250°C, and then held for 10 min. For confirmation of a major "C_{28}" on the nonpolar phase and a "C_{31}" peak on the polar phase being squalene, these peaks were compared by GLC with standard squalene and squalane prior and after hydrogenation, respectively (11). For TLC comparisons, solvents used on silica gel plates were chloroform (Solvent A) and mixtures of diethyl ether/hexane (v/v) (9:1; Solvent B) (19:1; Solvent C) (99.5:0.5; Solvent D). Argentation TLC was done using Solvent B. Mass spectra of the isolated squalene before and after hydrogenation, as well as squalene and squalane standards, were obtained on a Hewlett Packard 5971 gas chromatograph-mass spectrograph (GC-MS) (Hewlett Packard,

Avondale, PA) with a 12 m × 0.2 mm × 0.3 μm HP-1 column operated isothermally at 230°C. Mass spectra (MS) conditions were: 70 eV; 154°C; helium flow, 1.0 mL/min; 48 torr. Spectra were compared with an NBS43K PBM library.

The optimized method devised for analyzing squalene in epicuticular wax of grapefruit was as follows: Five fruit, each weighing 300 to 450 g, were weighed and sequentially placed in a 14-cm porcelain crucible with 100 mL $CHCl_3$. Each fruit was bathed in the solvent for 5 min. An additional 10 mL chloroform was added before the next fruit was bathed and the combined wax extract was poured into a 300-mL round bottom flask. The crucible was rinsed with 10 mL chloroform and the extract reduced to near dryness on a roto-evaporator (Brinkman, Westbury, NY) at 30°C under reduced pressure. The wax residue was transferred to a 5-mL volumetric flask with chloroform containing 0.1 mg/mL chlorestane as internal standard. A Florisil Sep-Pak (Waters Assoc., Milford, MA) was washed free from any hydrocarbons by passing 10 mL hexane through the column. A 1-mL aliquot of the extract solution was placed on the Sep-Pak. Hexane was passed through column until 4 mL of eluent was collected. The sample was transferred to a tared vial and the solvent evaporated under nitrogen at room temperature. Hexane was added at a ratio of 100 μL/0.5 mg sample. Primary analyses of squalene and the C_{23}–C_{33} alkanes were run on a 30 m × 0.53 mm megabore DB-1, 1.5 m column isothermally at 265°C with injection and FID at 300°C and a helium flow of 18 mL/min. To correct for an unknown not resolved from squalene on the DB-1 column, the samples were also run on a 30 m × 0.75 mm Supelcowax, 1.0 m column isothermally at 245°C with injection and FID at 270°C and a helium flow of 18 mL/min. During the 1988–89 season, each analyses consisted of three pooled extracts (15 fruit) with duplicate determinations from each of the pools. Squalene and C_{23}–C_{33} alkanes were quantified as μg/100 g fruit. In the 1986–87 and 1987–88 season studies, an analysis consisted of five fruits individually extracted that were run through Sep-Paks and analyzed by GLC.

RESULTS AND DISCUSSION

In exploratory TLC analysis on the lipids from the flavedo of the Con and NCon grapefruit, major differences in the hydrocarbon area were evident. We previously observed that hydrocarbons were a major component of citrus epicuticular wax which could be removed from the fruit by simple dipping into chloroform (10,11). By dipping samples of Con and NCon grapefruit, we found that the material that increased with conditioning was extracted with the epicuticular wax. Again, the area of the TLC plate showing the greatest difference between Con and NCon fruit was in the retardation factor (R_f) 0.70 hydrocarbon band. The fraction from the conditioned fruit, isolated from the remainder of the wax, gave a C_{23} to C_{33} alkane profile quite similar to profiles reported for citrus (10,11) except for one major peak. This peak, accounting for 40% of the total alkanes, was unusual in that it corresponded to C_{28} whereas in most wax alkanes over 90% of the alkanes are odd-numbered (12). Also unusual was the fact that on a polar phase, this compound behaved as a C_{31} indicating that it was highly unsaturated. In previous studies (10,11), we found linear monoenes and branched saturated compounds were present in the hydrocarbons of citrus peel wax at levels not greater than 15%. Moreover, these hydrocarbons had equivalent chain length (ECL) values within 0.4 units of the linear saturated alkanes. Upon hydrogenation of the Con fruit hydrocarbon sample, the compound gave ECL values of 26.15 and 26.66 on nonpolar and polar phases, respectively, indicating that five to six double bonds were present. Squalene and squalane standards injected onto two columns gave ECL values identical to those obtained with the unknown before and after hydrogenation, respectively. On silica gel TLC plates, the wax squalene and the standard squalene were not resolved from the linear alkanes with the solvent systems A, B, or C. However, with Solvent D, squalene at R_f 0.48 was well resolved from terpenes and sesquiterpenes at R_f 0.63 and from linear saturated hydrocarbons at R_f 0.75. With $AgNO_3$-TLC, squalene gave an R_f of 0.17, quite resolved from compounds with zero to three double bonds. Mass spectra of the isolated squalene agreed with the spectra of standard squalene run under the same conditions having the major ion at *m/z* 69 (100%), the M^+ ion at 410 (0.4%), an M − 43 ion at 367 (0.7%), and an M − 69 ion at 341 (1.2%). Other ions were *m/z* 81 (50%) and 41 (28%). The relatively low intensities of the *m/z* 300 to 410 ions are characteristic of long-chain hydrocarbons as opposed to sterols which have M^+ ions in the 10 to 30% abundance range.

We did not detect squalene previously in the epicuticular wax of citrus during extensive chemotaxonomic studies on hydrocarbons from citrus peel (10,11), leaves (13), and juice sacs (14). Other investigators likewise failed to detect squalene (15–19). The 1 to 3% squalene in the hydrocarbons of epicuticular wax from fresh grapefruit was undoubtedly overlooked, degraded during the isolation steps, or not resolved from C_{28}. Squalene with six double bonds is subject to oxidation and has not previously been found in wax coatings of edible fruit.

Chloroform proved to be the best solvent, although modifications in the 3-min extraction procedure used earlier (10,11,13) were needed in order to obtain the most reliable levels of squalene and C_{23}–C_{33} alkanes in the epicuticular wax of the fruit. Thus, time-extraction studies were conducted with grapefruit dipped for successive times in chloroform. A 30-sec dip removed 94% of the squalene but less than 50% of the saturated alkanes; the shorter alkanes were extracted to a greater extent than the longer alkanes. Increasing the cumulative extraction time to 7.5 min gave complete recovery of squalene and a 95% recovery of the other hydrocarbons based on total hydrocarbons extracted over a cumulative 10-min period. Based on the above results, we selected a 5-min extraction time for our squalene-CI studies.

To separate squalene from the other wax components, a rapid chromatographic method was needed to minimize degradation of squalene. Using a Florisil Sep-Pak, it was determined that 4 mL hexane eluted the alkanes and squalene leaving the long-chain aldehydes and more polar wax components on the Sep-Pak.

To verify that squalene was preferentially synthesized at 15°C throughout the season, storage tests were run on six different dates using five prestorage "conditioning temperatures" prior to storage at 5°C (Fig. 1). On each

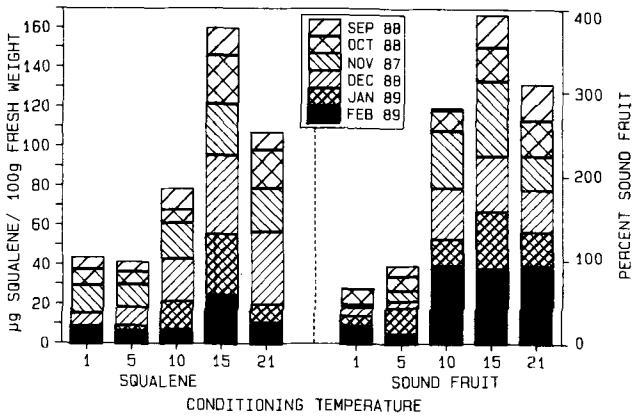

FIG. 1. Squalene in epicuticular wax of October 1988 grapefruit temperature conditioned at 15° for 1 to 21 days and for grapefruit "conditioned" 7 days at 1, 5, 10, and 21°C.

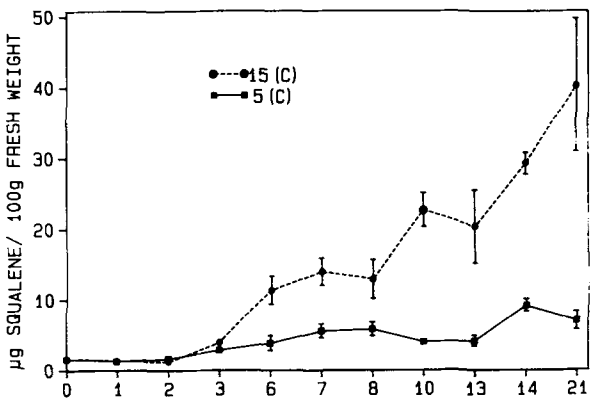

FIG. 3. Cumulative profiles of squalene and of percent sound fruit from six temperature conditioning studies throughout the grapefruit season.

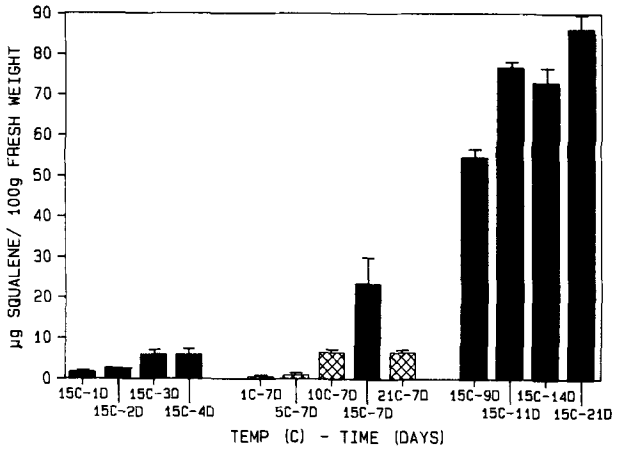

FIG. 2. Squalene in epicuticular wax of February 1989 grapefruit stored for 1 to 21 days at 5°C (nonconditioned) and for fruit stored 1 to 21 days at 15°C (conditioned).

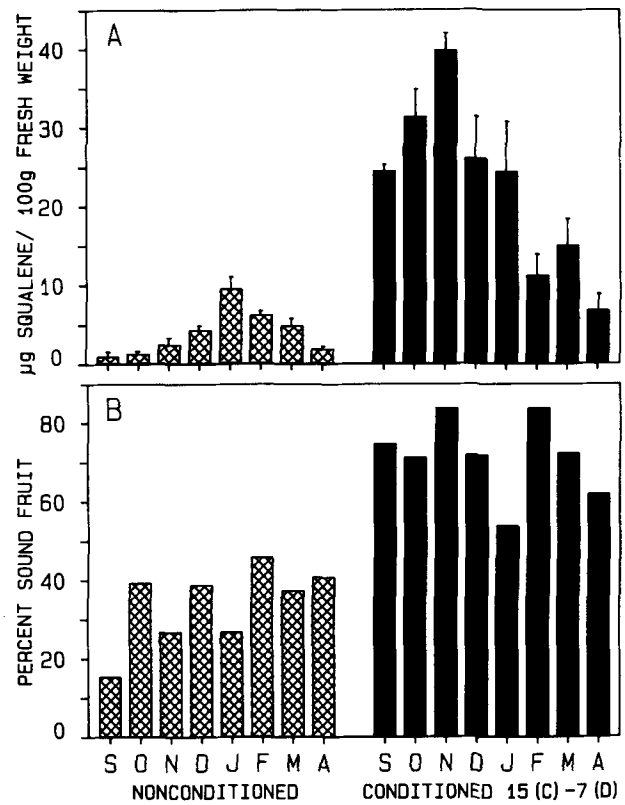

FIG. 4. (A) Monthly mean and standard error levels of epicuticular wax squalene after 7-day storage at 5°C (nonconditioned) and at 15°C (conditioned)—1 3-yr study. (B) Monthly mean percent sound fruit after 6-wk storage at 5°C for nonconditioned and conditioned grapefruit.

date, conditioning at 15°C produced the greatest amount of squalene. The mean values for the six dates were: 15° (26.7 μg), 21°C (17.9 μg), 10°C (13.2 μg), 1°C (7.2 μg), and 5°C (7.0 μg). For each of these studies the reciprocals of percent chill injury (percent sound fruit) were: 15°C (65.6%), 21°C (51.8%), 10°C (47.1%), 5°C (15.5%), and 1° (11.1%). These values along with the squalene values, depicted in a cumulative mode in Figure 1, were highly correlated, r 0.926.

Figure 2 depicts the synthesis of squalene in the epicuticular wax of early season October 1988 grapefruit placed in the optimal 15°C chamber for 21 days. For comparison purposes, squalene values along with standard errors for grapefruit kept at 1, 5, 10 and 21°C for 7 days (Fig. 1) are also depicted. Synthesis of squalene began around the third day and increased at a more rapid and steady rate from sometime after the 4th day on. Early season fruit is the most susceptible to CI, and 7-day conditioning at 15°C is the most "profitable" commercial, time-temperature conditioning regime for grapefruit (2). Thus a greater than 13-fold increase in squalene during this regime makes squalene suspect of preventing CI. A squalene synthesis time study for mid-late season grapefruit (February 1989) is shown in Figure 3. Storing fruit at 5°C for 21 days increased the squalene level by only 3 to 6 μg. From the 6th day on, squalene values for 15°C stored fruit were significantly higher (95% Con level) than values for control or 5°C fruit. The rate of squalene increase for these mid-late season fruit at 15°C, however, was less than that observed for early season fruit.

Figure 4A portrays the levels of squalene naturally present in grapefruit during the 8-mon season. Rather large standard error markers represent, in most part, the great variance observed between individual fruits and the variance in levels from fruits of three seasons. The bell-shaped trend, however, agrees with the CI susceptibility tread of grapefruit, *e.g.*, early- and late-season fruit are more susceptible to CI (2). The trend also agrees, in part, with the percent sound fruit data for these three seasons, the exception being the failure of late-season fruit to decrease in percent soundness (Fig. 4B). Temperature conditioning against CI was quite effective on early-season grapefruit, the effectiveness reaching a climax in the month of November raising the level of nonchill-injured fruit from 27% to 84% (Fig. 4B). November also was the month in which conditioning raised the level of squalene to the greatest extent, *e.g.*, from 2.5 μg to 39.8 μg (Fig. 4A). The great decline in squalene levels combined with only a slight decline in percent sound fruit from November to April indicates that perhaps other compounds are synthesized in grapefruit in the mid and late seasons that substitute for squalene as a CI protectant for early-season fruit (Fig. 4A,B). We are presently having very positive results confirming this postulate as well as with squalene application studies in which we show that squalene does reduce CI. We are also having very positive results determining the mode of protection squalene affords to the grapefruit. These reports will be published shortly. A patent (U.S. #4,921,715) has been obtained for the potential use of squalene in reducing CI.

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Influence of Dietary Fish Oil on the Relative Synthesis of Triacylglycerol and Phospholipids in Rat Liver *In Vivo*[1]

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The influence of dietary fish oil containing n-3 polyunsaturated fatty acids on the biosynthesis of triacylglycerol relative to total individual phospholipids was studied in rat liver *in vivo*. The dietary lipid (10% by weight of diet) was either sunflower oil enriched in linoleic acid (SO group) or MaxEPA fish oil/sunflower oil, 9:1 by weight (FO group) enriched in eicosapentaenoic acid (EPA, 20:5n-3) plus docosahexaenoic acid (DHA, 22:6n-3). After a 3-week feeding period, the triacylglycerol content (in μmol/g liver) was 44% lower in the FO group relative to the SO animals. The *in vivo* incorporation of [^{3}H]glycerol into individual hepatic lipids resulted in triacylglycerol/total phospholipid radioactivity ratios of 2.1 and 0.9 for the SO and FO groups, respectively. These results indicate an inhibitory effect of dietary EPA/DHA on triacylglycerol relative to phospholipid synthesis from intermediary 1,2-diacylglycerol in rat liver *in vivo*. This metabolic alteration was accompanied by a substantially lower amount (in μmol/g liver) of arachidonic acid and higher levels of EPA plus DHA in the triacylglycerol, choline glycerophospholipid (CGP), and ethanolamine glycerophospholipid (EGP) of the FO group. A moderately higher labelling of the EGP from [^{3}H]glycerol was observed in the FO as compared to the SO group (as evidenced by CGP/EGP radioactivity ratios of 1.3:1 and 1.8:1, respectively). The present study provides *in vivo* evidence for a dampening effect of dietary fish oil on the synthesis of liver triacylglycerol relative to phospholipid and a moderate alteration of *de novo* synthesis of individual phospholipids.

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The ingestion of fish oils containing n-3 polyunsaturated fatty acids as eicosapentaenoic acid (EPA, 20:5n-3) plus docosahexaenoic acid (DHA, 22:6n-3) has been found to significantly depress plasma triacylglycerol levels in human subjects (1,2) and experimental animals, including the rat (3,4). These dietary fatty acids are capable of both inhibiting the formation/secretion of very low density lipoproteins by the liver (5,6) and promoting the clearance of such lipoproteins from the circulation (7,8). The reduction in triacylglycerol production by the liver by dietary fish oil may reflect a decrease in lipogenic enzyme activities (3), increased fatty acid oxidation (5,9), and decreased phosphatidate phosphohydrolase activity responsible for 1,2-diacylglycerol availability (4,6).

A reduced synthesis of triacylglycerol has been reported from *in vitro* studies with EPA and/or DHA using rat hepatocytes (10-13) or Hep G2 cells (14). However, little *in vivo* information is available on this topic. The main purpose of the present study was to evaluate the influence of dietary fish oil enriched in EPA plus DHA on the relative synthesis of hepatic triacylglycerol and total/individual phospholipids *in vivo*. The levels and fatty acyl compositions of triacylglycerol, choline glycerophospholipid (CGP), and ethanolamine glycerophospholipid (EGP) were also studied in rats consuming diets rich in linoleic acid (18:2n-6) or EPA/DHA.

MATERIALS AND METHODS

Animals and diets. Sixteen male Sprague-Dawley rats (Charles River Canada, St. Constant, P.Q) having an average body weight of 60 g were individually housed in stainless steel cages with a 12 hr light-dark cycle and a constant room temperature of 25°C. Animals were randomly assigned to either an n-6 or n-3 enriched dietary group. The complete compositions of these two semipurified diets, which were given *ad libitum* for a three-week period, have been reported previously (15). The dietary lipid source (at 10% by weight of diets) was either sunflower oil enriched in linoleic acid (SO group) or MaxEPA fish oil supplemented with sunflower oil (FO group) to satisfy the nutritional requirement for linoleic acid. The MaxEPA fish oil concentrate was kindly provided by Mr. D. Hutchinson of R.P. Scherer Canada Inc. (Windsor, Ontario). The fatty acid compositions of the SO and FO diets are given in Table 1.

Metabolic studies in vivo *with [^{3}H]glycerol.* Following three weeks on the assigned diets, each rat was injected intraperitoneally with 0.3 mCi of [1(3)-^{3}H]glycerol (specific activity of 2.9 Ci/mmol) obtained from Amersham Canada Ltd. (Oakville, Ontario). Animals were sacrificed 15 min following injection based on a previous time study in gerbils (16), and confirmed for the rat herein

TABLE 1

Fatty Acid Composition of the Sunflower Oil and Fish Oil Diets

Fatty acid[a]	Diet	
	Sunflower oil mol %	Fish oil[b] mol %
14:0	tr	7.2
16:0	6.8	18.2
16:1	tr	9.0
18:0	5.1	3.7
18:1	15.1	13.2
18:2n-6	70.7	10.3
18:4n-3	tr	3.4
20:5n-3	tr	15.1
22:6n-3	tr	9.5

[a]Other minor fatty acids have been excluded.

[b]Includes MaxEPA fish oil/sunflower oil (9:1 ratio by weight).

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Abbreviations: CGP, choline glycerophospholipids; DG, diacylglycerol; EGP, ethanolamine glycerophospholipids; IGP, inositol glycerophospholipids; PL, phospholipids; SGP, serine glycerophospholipids; TG, triacylglycerol.

by a preliminary study, indicating a progressive increase in the labelling of total hepatic triacylglycerol and phospholipid up to 20 min following injection. The animals were anesthetized with methoxyflurane (Pitman-Moore Ltd., Mississauga, Ontario) and the livers were rapidly excised, rinsed in 0.9% saline solution, weighed, and the lipids immediately extracted by the method of Folch *et al.* (17) following homogenization in chloroform/methanol (2:1, v/v) using a Polytron homogenizer.

Lipid separations and analyses. The individual lipid classes were separated by thin-layer chromatography (TLC) on precoated silica gel 60 plates (E. Merck, Darmstadt, Federal Republic of Germany) after application of aliquots of the liver lipid concentrates (each representing 1/30th of the total liver lipid extract). For separation of the neutral lipids (including triacylglycerol and total phospholipid), TLC plates were developed in heptane/isopropyl ether/acetic acid (60:40:3, v/v/v) as the developing solvent (18). The individual phospholipids were separated by TLC using chloroform/methanol/acetic acid/water (50:37.5:3.5:2, v/v/v/v) as the developing solvent (19). Visualization of the lipid bands was performed under ultraviolet light after spraying with 6-*p*-toluidino-2-naphthalene sulfonic acid (Eastman Kodak Co., Rochester, NY). The gel scrapings containing the various lipids were dispersed in scintillation vials with 1.5 mL water, to which 13.5 mL Aquasol-2 (New England Nuclear Corp., Boston, MA) were added for liquid scintillation counting (Beckman Model LS 7800, Beckman Instruments Inc., Fullerton, CA).

The mass (in μmol/g liver) of the total triacylglycerol, CGP, and EGP, as well as their fatty acid compositions, were determined by gas-liquid chromatographic procedures (15,19) using known amounts of monopentadecanoin (NuChek Prep, Elysian, MN) as an internal standard. For this purpose, aliquots representing 1/300th and 1/200th of the total lipid extracts were taken for TLC isolation of the triacylglycerol (neutral lipid plate) and CGP and EGP (phospholipid plate), respectively.

Statistics. The experimental data were subjected to statistical analyses by a one-way analysis of variance and Student's *t*-test (20).

RESULTS AND DISCUSSION

The final body weights after three weeks on the experimental diets indicated no significant difference between the two groups; neither were there any significant differences in the final liver weights (Table 2). Lipid analyses revealed the amount of triacylglycerol content (in μmol/g liver) to be considerably lower (by 44%) in the FO as compared to the SO animals. Lower levels of hepatic triacylglycerols have been reported previously in rats consuming fish oil (5). A moderately higher level ($P<0.05$) of CGP and EGP was found in the FO group (Table 2), although the CGP/EGP mass ratio were not different (2.08 and 2.03 for SO and FO, respectively).

Fatty acid analyses (Table 3) of the triacylglycerol, CGP, and EGP indicated striking differences between the two dietary groups. All three lipid classes in the FO animals exhibited a marked accumulation of the major n-3 polyunsaturated acids (EPA and DHA) plus other n-3 members (20:4 and 22:5) together with lower levels (mol %) of the n-6 series, including arachidonic acid (AA, 20:4n-6), linoleic acid, and lesser members including 22:4n-6, 22:5n-6, and 20:2n-6. The level (mol%) of 22:5n-6 in the CGP and EGP of the FO animals was <4% that for the SO group (whereas AA levels were approximately 25% that for the SO group). The replacement n-3 for n-6 polyunsaturated acids in rat liver phospholipid and triacylglycerol has been reported upon dietary fish oil consumption (21,22). The relative availabilities and competition of n-3 *vs* n-6 fatty acid-containing precursors for lipid esterification can influence the resulting fatty acid compositions of hepatic phospholipids. Also, a dampening of microsomal desaturase activity mediating linoleic acid conversion to arachidonic acid has been observed in the livers of rats fed dietary fish oil (23). Table 3 also gives the absolute amounts of AA, EPA, and DHA in the hepatic lipids (in μmol/g liver). Whereas the pool of AA in the triacylglycerol is considerably less than that for the CGP or EGP (both diet groups), the pool of EPA in the triacylglycerol is intermediary between that of the CGP and EGP (FO group).

TABLE 2

Final Body Weights, Liver Weights, and Amounts of Triacylglycerol, Choline Glycerophospholipid, and Ethanolamine Glycerophospholipid in Livers from Animals Fed either Sunflower Oil or Fish Oil Diets[a]

Parameter	Diet	
	Sunflower oil	Fish oil
Body weight, g	251.3 ± 4.6	256.1 ± 3.5
Liver weight, g	13.1 ± 0.3	14.1 ± 0.5
Triacylglycerol (μmol/g liver wt.)	8.1 ± 1.4	4.5 ± 0.2[b]
Choline glycerophospholipid (μmol/g liver wt.)	7.2 ± 0.8	9.6 ± 0.7[b]
Ethanolamine glycerophospholipid (μmol/g liver wt.)	3.5 ± 0.2	4.8 ± 0.4[b]

[a]Values represent means ± S.E. of six animals for triacylglycerol and seven animals for choline and ethanolamine glycerophospholipids, respectively.

[b]Indicates significant differences from the sunflower oil group, $P<0.05$.

Table 4 gives the distribution of the newly-incorporated [^{3}H]glycerol amongst the various hepatic lipid classes of the two dietary groups. Most (79%) of the overall lower level of radioactivity (as dpm) incorporated into the total liver lipids of the FO animals (relative to the SO controls), ($7.50 \times 10^6 - 3.85 \times 10^6 = 3.65 \times 10^6$), can be accounted for by the reduced labelling of the triacylglycerol ($4.42 \times 10^6 - 1.53 \times 10^6 = 2.89 \times 10^6$). Whereas the dpm incorporated into triacylglycerol and 1,2-diacylglycerol were lower by 65% and 48%, respectively, in the FO groups; no significant differences ($P<0.05$) were found in the case of total phospholipid (where lower radioactivity levels of only 15% were observed, 1.80×10^6 *vs* 2.11×10^6 dpm). The incorporation of radioactive glycerol into the triacylglycerol relative to total phospholipid (evidenced by labelling ratios of 2:1 and 0.9:1 for the SO and FO groups, respectively, seen in Table 4) provides *in vivo* evidence for a suppressed *de novo* synthesis of triacylglycerol relative to phospholipid from intermediary 1,2-diacylglycerol with dietary FO.

The [^{3}H]glycerol used herein enters glycerolipids *via* established *de novo* biosynthetic pathways (24). Such

TABLE 3

Fatty Acid Composition of Triacylglycerol, Choline Glycerophospholipids, and Ethanolamine Glycerophospholipids in Livers of Animals Fed Either Sunflower Oil or Fish Oil Diets[a]

Fatty acid[b]	Triacylglycerol		Choline glycerophospholipid		Ethanolamine glycerophospholipid	
	Sunflower oil	Fish oil	Sunflower oil	Fish oil	Sunflower oil	Fish oil
			mol %			
14:0	0.9 ± 0.1	1.4 ± 0.3	0.2 ± 0.01	0.3 ± 0.03	0.1 ± 0.02	0.1 ± 0.03
16:0	22.2 ± 1.6	28.8 ± 0.7[c]	16.5 ± 0.7	28.1 ± 0.6[d]	14.2 ± 0.3	21.4 ± 0.5[d]
16:1	2.5 ± 0.3	7.4 ± 0.4[d]	0.6 ± 0.1	2.3 ± 0.1[d]	0.3 ± 0.1	0.7 ± 0.04[d]
18:0	3.8 ± 0.2	3.7 ± 0.2	25.9 ± 0.5	20.8 ± 0.8[d]	26.5 ± 0.4	24.4 ± 0.4[c]
18:1	20.8 ± 0.7	21.6 ± 0.8	4.7 ± 0.2	8.0 ± 0.2[d]	4.5 ± 0.4	3.8 ± 0.2
18:2n-6	37.0 ± 0.7	7.2 ± 0.2[d]	10.0 ± 0.2	5.8 ± 0.2[d]	5.3 ± 0.1	1.8 ± 0.1[d]
18:4n-3	0.2 ± 0.1	0.9 ± 0.1[d]	tr	0.2 ± 0.02[d]	0.1 ± 0.1	0.1 ± 0.02
20:2n-6	1.1 ± 0.1	0.2 ± 0.02[d]	1.0 ± 0.1	0.2 ± 0.02[d]	0.8 ± 0.05	0.1 ± 0.03[d]
20:4n-6	5.3 ± 0.9	0.8 ± 0.04[d]	31.3 ± 0.7	7.6 ± 0.3[d]	27.7 ± 0.5	7.4 ± 0.2[d]
20:4n-3	tr	0.5 ± 0.05[d]	tr	0.5 ± 0.1[d]	tr	0.2 ± 0.04[d]
20:5n-3	tr	9.1 ± 0.3[d]	tr	10.1 ± 0.5[d]	tr	8.6 ± 0.2[d]
22:4n-6	2.5 ± 0.5	0.1 ± 0.02[d]	0.8 ± 0.1	tr[d]	2.4 ± 0.1	tr[d]
22:5n-6	1.7 ± 0.4	0.2 ± 0.02[d]	6.1 ± 0.3	0.2 ± 0.04[d]	13.1 ± 0.6	0.3 ± 0.1[d]
22:5n-3	tr	5.3 ± 0.3[d]	0.1 ± 0.01	2.2 ± 0.1[d]	0.1 ± 0.04	4.3 ± 0.2[d]
22:6n-3	tr	10.6 ± 0.4[d]	1.5 ± 0.1	12.1 ± 0.7[d]	3.9 ± 0.2	25.8 ± 0.5[d]
			μmol/g liver[e]			
20:4n-6	1.29	0.11	4.49	1.45	1.91	0.71
20:5n-3	tr	1.22	tr	1.93	tr	0.83
22:6n-3	tr	1.42	0.22	2.31	0.27	2.48

[a] Values represent means ± S.E. of six animals for triacylglycerol and seven animals for choline and ethanolamine glycerophospholipids, respectively.

[b] Other minor fattys have been excluded.

[c] Indicates significant differences from the sunflower oil group, $P<0.05$.

[d] Indicates significant differences from the sunflower oil group, $P<0.001$.

[e] Values are calculated from Tables 2 and 3.

TABLE 4

Incorporation of [1(3)-^{3}H]Glycerol among Liver Lipids of Animals Fed Either the Sunflower Oil or Fish Oil Diets[a]

Lipid class	Sunflower oil	Fish oil
	% of Total incorporation	
Triacylglycerol	62.4 ± 2.4	42.7 ± 1.8[d]
	(4.42 ± 0.46)[b]	(1.53 ± 0.23)[d]
1,2-Diacylglycerol	5.9 ± 0.4	6.1 ± 0.7
	(0.41 ± 0.04)	(0.21 ± 0.03)[c]
Total phospholipids	30.9 ± 2.3	50.8 ± 1.4[d]
	(2.11 ± 0.14)	(1.80 ± 0.22)
Ratio (triacylglycerol/phospholipids)	2.1 ± 0.2	0.9 ± 0.1[d]
Choline glycerophospholipid	16.9 ± 1.3	22.2 ± 0.4[c]
	(1.15 ± 0.09)	(0.74 ± 0.11)[c]
Ethanolamine glycerophospholipid	9.5 ± 0.7	16.9 ± 0.7[d]
	(0.65 ± 0.04)	(0.54 ± 0.06)
Serine glycerophospholipid	0.8 ± 0.1	1.5 ± 0.2[c]
	(0.05 ± 0.01)	(0.05 ± 0.01)
Inositol glycerophospholipid	1.4 ± 0.1	1.8 ± 0.2
	(0.09 ± 0.01)	(0.06 ± 0.01)[c]
Ratio (choline glycerophospholipid/ ethanolamine glycerophospholipid)	1.82 ± 0.09	1.33 ± 0.05[d]
Total liver lipids	(7.50 ± 0.58)	(3.85 ± 0.51)[d]

[a] 0.3 mCi of [1(3)-^{3}H]glycerol was intraperitoneally injected to each animal with 15 min duration before sacrificing; values represent means ± S.E. of eight animals for the sunflower oil and fish oil groups, respectively.

[b] Parentheses represent means ± S.E. of the actual counting as disintegrations per min (dpm) after multiplying the actual values by 10^{-6}.

[c] Indicates significant differences from the sunflower oil group, $P<0.05$.

[d] Indicates significant differences from the sunflower oil group, $P<0.001$.

findings may underlie the reduced mass of hepatic triacylglycerol (but not phospholipid) resulting from FO consumption observed here (Table 2). The radioactivity data in Table 4 suggests a significant suppression in 1,2-diacylglycerol conversion to triacylglycerol *via* acyl-CoA:1,2-diacylglycerol acyltransferase in rat liver *in vivo* upon FO consumption. *In vitro* studies have observed an inhibitory effect of eicosapentaenoic acid on the liver acyltransferase (13); this and other factors may be operative *in vivo*. In addition, a diminution in lipogenic enzyme activities (3,25), including the gene expression of liver fatty acid synthetase (26), increased fatty acid oxidation (5,9), and decreased phosphatidate phosphohydrolase activity (4,6) may also contribute to the hepatic triacylglycerol lowering.

Based on the values for percentage of total incorporation, the FO animals exhibited a moderately higher labelling of the EGP relative to the CGP (CGP/EGP ratios of 1.8:1 and 1.3:1 in the SO and FO groups, respectively) and of the serine glycerophospholipid (Table 4). These findings may reflect a preferential utilization of 1-acyl 2-DHA species by the microsomal CDP-ethanolamine: 1,2-diacylglycerol ethanolaminephosphotransferase, but not the cholinephosphotransferase, in rat liver (27). The shift towards a greater proportional labelling of serine glycerophospholipid (1.5% in FO group *vs* 0.8% in SO group), presumably *via* base exchange (28) from EGP, is also in keeping with the order for percentage of total incorporation for the latter (16.9% and 9.5% for FO and SO groups, respectively).

In conclusion, the present work provides *in vivo* evidence that dietary fish oil containing n-3 fatty acids can alter *de novo* biosyntheses of triacylglycerol relative to phospholipid in rat liver as well as modifying the formation of individual phospholipids.

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Influence of Dietary Fatty Acid Composition on Cholesterol Synthesis and Esterification in Hamsters

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To investigate the effects of dietary fat quality on synthesis and esterification of cholesterol, Syrian hamsters were fed diets containing corn, olive, coconut or menhaden oils (10% w/w) with added cholesterol (0.1% w/w). After 3 weeks, animals were sacrificed 90 min following IP injection of ^3H_2O. Synthesis of free cholesterol and movement of free cholesterol into ester pools were measured from ^3H-uptake rate in liver and duodenum. Plasma total cholesterol and triglycerides levels were highest in coconut oil-fed animals, whereas hepatic total cholesterol and ester levels were elevated in olive oil-fed animals, as compared with all other groups. No diet-related differences were seen in duodenal cholesterol or total fatty acid content. In duodenum, uptake of ^3H per g tissue into cholesterol was greater compared with liver; however, within each tissue, ^3H-uptake into cholesterol was similar across groups. Notably, ^3H-uptake into cholesterol ester in liver was highest in menhaden oil-fed animals. These data suggest that menhaden fish oil consumption results in enhanced movement of newly synthesized cholesterol into ester as compared with other fat types.
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Dietary fatty acid composition has been identified as influencing plasma cholesterol levels in humans. Saturated dietary fatty acids are categorically most hypercholesterolemic (1–3). Conversely, monounsaturated fatty acids (MUFA) (3,4) and polyunsaturated fatty acids (PUFA) (1–3) lower plasma cholesterol levels when substituted for saturated fatty acids, although considerable variability between individuals has been reported in the actions of these fatty acids (5). For PUFA, linoleic acid appears to be the major cholesterol lowering fatty acid, affecting plasma cholesterol levels in a manner which extends beyond that achieved by simple replacement of dietary saturated fats. Similarly, dietary fish oils containing eicosapentaenoic acid (EPA, 20:5ω3) and docosahexaenoic acid (DHA; 22:6ω3) exhibit variable effects on plasma cholesterol content (6–10), but reduce triglyceride levels (9,10).

Although the influence of dietary fat type on lipid levels has been identified, the underlying mechanisms responsible remain to be established. Several mechanisms have been proposed. Data from animal experiments suggest that ω6 PUFA reduce plasma cholesterol concentrations by altering hepatic low density lipoprotein (LDL) metabolism, redistributing cholesterol from blood to tissue pools (11). Alternatively, ω6 PUFA may increase hepatic cholesterol esterification, which reduces the free cholesterol pool size and elevates LDL receptor synthesis (12). Conversely, fish oil fatty acids are reported to inhibit release of very low density lipoprotein (VLDL) from liver (13).

Such findings emphasize the need to study the influence of qualitative fat intake on cholesterol turnover to better understand the role of fatty acid intake in controlling plasma cholesterol levels. The purpose of the present study was to examine specific effects of feeding common dietary fats on organ cholesterol synthesis and esterification and plasma and organ lipid composition in hamsters.

MATERIALS AND METHODS

Animals and diets. Male Syrian hamsters weighing 60 g (Charles River Laboratories, Montreal, Quebec) were maintained in a windowless room artificially illuminated from 21:00 to 09:00 hr and fed laboratory diet (Rodent Laboratory Diet 5001, Purina Mills Inc., St. Louis, MO) and water *ad libitum* for two weeks. The gross diet composition was (w/w): protein, 23%; fat, 4.5%; fiber, 5.8%; ash, 7.3%; with the remainder as carbohydrate, vitamin and mineral mixes and moisture. Cholesterol content of the laboratory diet was 0.03%. Animals were weighed, randomly divided into four groups and individually housed in stainless steel, wire mesh cages. For three weeks each group was fed one of four diets containing laboratory diet with 10% (w/w) of corn (i), olive (ii), coconut (iii) or menhaden fish oil (iv). Added fats were obtained from local sources, except for menhaden oil, which was purchased from ICN Biochemicals (Cleveland, OH). Cholesterol levels of each diet containing added oil were then measured and made up to 0.1% (w/w) by the addition of free cholesterol. Diets were prepared every two weeks and stored at −10°C. Fatty acid composition of each diet was determined (Table 1) by gas-liquid chromatography (GLC) after extraction with chloroform/methanol (2:1, v/v) and methylation using boron trifluoride (14).

On day 21 of study, food cups were removed at the beginning of the dark cycle and 3 to 4 hr later animals received an IP injection of approximately 30 mCi ^3H_2O. Ninety minutes after injection animals were weighed and sacrificed by heart puncture following light anesthesia. Blood was taken for measurement of plasma lipid levels and ^3H-activity (15). Liver and duodenum were removed, flushed with saline solution and immediately frozen in liquid nitrogen. Animals were cared for in accordance with the principles of the "Guide to

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Abbreviations: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; GLC, gas-liquid chromatography; HDL, high density lipoprotein; LDL, low density lipoprotein; MCFA, medium chain fatty acids, MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; VLDL, very low density lipoprotein.

TABLE 1

Fatty Acid Composition of Diets (wt % of Total Fatty Acids)[a]

Fatty acid	Diet fat consumed			
	Corn oil	Olive oil	Coconut oil	Menhaden oil
12:0	—	—	35.0	—
14:0	1.2	1.2	16.1	7.6
16:0	13.2	14.6	13.8	19.8
16:1ω7	1.6	2.4	1.6	11.6
18:0	2.8	5.7	5.7	7.6
18:1ω9	27.3	56.9	15.0	19.9
18:2ω6	46.2	13.9	7.3	7.7
20:0	0.9	0.9	0.6	1.1
20:1ω9	0.5	0.3	0.1	1.0
18:3ω3	2.2	2.0	1.5	2.0
20:5ω3	1.1	0.7	0.8	10.5
22:5ω3	—	—	—	1.9
22:6ω3	—	—	—	5.8

[a]Fatty acids are designated by chain length, number of double bonds and position of the first double bond from the fatty acid methyl terminus. Only major dietary fatty acids are reported.

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Measurement of tissue lipid synthesis and esterification. Rates of synthesis and esterification of cholesterol were determined by the uptake rate of ^3H from ^3H_2O (16,17). Measurement of synthesis by ^3H-uptake has been previously validated (16) and yields a measure of absolute synthetic rate when the specific activity of the substrate water pool is known (17). The rate of appearance of ^3H-labeled cholesterol as ester was taken to reflect to movement of newly synthesized sterol from the free to ester form. Tissue samples were extracted in duplicate using hexane/chloroform (4:1, v/v) and water after the addition of a ^{14}C-cholesterol internal standard. Hexane/chloroform phases were combined and solvent removed under N_2. Free and esterified cholesterol bands were separated from extracts by thin-layer chromatography with double development for 1 hr followed by 45 min using petroleum ether/diethyl ether/acetic acid (85:15:1.5, v/v/v). Bands were eluted from silica scrapings using chloroform. To remove ^3H-containing fatty acids, cholesterol esters were hydrolyzed with KOH, rechromatographed and the resulting free cholesterol band was scraped from plates and eluted. Samples were aliquoted for analyses of ^3H-uptake and lipid content and composition. Tritium activity was measured by liquid scintillation counting (Isocap, Picker). Results were expressed as nmol ^3H incorporated per g tissue per hr. Aliquots for fatty acid analysis were methylated as previously described (14).

Analytical methods. Plasma, liver and duodenal cholesterol levels were determined enzymatically (18). High density lipoprotein (HDL) cholesterol was measured using a modified dextran sulfate precipitation method (19). Triglyceride levels in plasma were also determined enzymatically (20). Fatty acid composition of dietary fats was analyzed using a gas-liquid chromatograph (GLC) (model 6000, Varian Instruments, Palo Alto, CA) utilizing a 30 m SP-2330 (10%) capillary column and helium carrier gas (2 mL/min). Compositional analysis of tissue fatty acid methyl esters was carried out by GLC (model 5750, Hewlett-Packard, Hewlett-Packard, Norwalk, CT) using a 2.5 m by 3 mm steel column packed with SP-2330 (10% on 100/120 Chromosorb) and nitrogen carrier gas (10 mL/min). Chromatograph peaks were identified by comparison of their retention data with those of authentic fatty acid methyl ester standards (Supelco Inc., Bellefonte, PA). Peaks were quantified by ratio integration using a 17:0 fatty acid methyl ester standard.

Statistical analysis. Data were analyzed using one-way ANOVA (analysis of variance) procedures with Tukey's *post hoc* tests performed for inter-group comparisons. A value of $p < 0.05$ was used as a level of statistical significance.

RESULTS

Mean body weight, liver weight, liver weight to body weight ratio and food intake of hamsters fed diets differing in fat composition were similar across all groups.

Lipid contents of plasma, liver and duodenum for animals consuming different dietary fats are shown in Table 2. Plasma total cholesterol values were higher in animals fed the coconut oil diet as compared with all other groups. Corn oil-fed animals had lower plasma cholesterol levels in comparison to olive and coconut oil-fed groups. For HDL cholesterol, the group fed olive oil showed higher values compared with that consuming menhaden oil. Plasma triglyceride levels were elevated in coconut fed animals in relation to other all groups. With the exception of greater hepatic total cholesterol and cholesterol ester levels of olive oil-fed animals, no significant diet treatment effect was observed in cholesterol or fatty acid content of liver or duodenum. A white color, likely cholesterol, was noted in livers of olive oil-fed animals at sacrifice. No significant differences were observed in hepatic or duodenal fatty acid content across treatment groups.

Liver and duodenal total lipid extract fatty acid composition are shown in Tables 3 and 4, respectively, for animals fed the different diets. Overall, liver contained a greater proportion of ω3 and fewer monoenoic fatty acids as compared with duodenum, independent of dietary fat intake. Corn oil-fed animals showed highest levels of ω6 fatty acids in both tissues as compared with groups consuming other diets. Similarly, in each tissue, groups fed menhaden and olive oil diets displayed the highest level of ω3 PUFA and MUFA, respectively. Notably, whereas the duodenum responded to the coconut oil diet by increasing proportions of medium chain saturated fatty acids (MCFA) compared with other groups, liver did not. Also, liver contained a greater proportion of fatty acids of chain length greater than 18 carbons, compared with duodenum.

Uptake of ^3H into free cholesterol in liver and duodenum and esterified cholesterol in liver in animals consuming different dietary fats is illustrated in Figure 1. Incorporation rates into free cholesterol were lower in liver as compared with duodenum. In liver, no effect of diet fat type was observed in free cholesterol ^3H-uptake. Uptake into cholesterol ester was greater in groups fed menhaden oil over those fed other

TABLE 2

Plasma, Liver and Duodenal Lipid Contents[a]

	Diet fat consumed			
	Corn oil	Olive oil	Coconut oil	Menhaden oil
Plasma level (mg/100 mL)				
Cholesterol total	190.0[a]	226.1[b]	378.9[c]	221.5[a,b]
	12.0	8.7	37.0	22.0
	(n=9)	(n=9)	(n=9)	(n=9)
HDL	105.1	134.6[a]	130.9	76.6[b]
	8.6	9.0	18.8	8.2
	(n=7)	(n=7)	(n=7)	(n=7)
Triglyceride	106[a]	95.7[a]	230.6[b]	131.8[a]
	9.5	6.4	38.2	22.6
	(n=8)	(n=9)	(n=8)	(n=8)
Liver (mg/100 g tissue)				
Cholesterol total	104[a]	212[b]	82.5[a]	78.6[a]
	23	31	14	8.0
	(n=9)	(n=8)	(n=8)	(n=9)
Free	60.7	76.6	67.0	64.2
	8.0	5.8	6.5	3.1
	(n=9)	(n=8)	(n=8)	(n=9)
Esters	43.5[a]	135.6[b]	21.4[a]	14.4[a]
	16.1	27.6	6.3	5.7
	(n=9)	(n=8)	(n=8)	(n=9)
Fatty acids	3292	2969	2709	3363
	270	235	267	290
	(n=6)	(n=6)	(n=6)	(n=6)
Duodenum (mg/100 g tissue)				
Cholesterol total	49.3	58.8	66.5	52.2
	5.8	8.3	3.8	4.1
	(n=9)	(n=8)	(n=9)	(n=9)
Free	44.9	51.7	61.9	47.3
	5.7	5.8	3.7	4.0
	(n=9)	(n=8)	(n=9)	(n=9)
Esters	4.4	7.1	4.6	4.9
	0.8	2.0	0.8	0.6
	(n=9)	(n=8)	(n=9)	(n=9)
Fatty acids	4500	7168	7308	4360
	215	514	820	294
	(n=6)	(n=6)	(n=6)	(n=6)

[a,b,c]Values are means ± SEM. Any two values in the same row followed by different letters differ significantly ($p<0.05$). Letters are omitted where no differences exist.

fat types. In the duodenum, no effect of diet was observed on incorporation rate into free cholesterol.

DISCUSSION

The present study was undertaken to investigate how fats differing in fatty acid composition influence the formation and esterification rates of cholesterol, and tissue levels of specific lipids. Hamsters were selected for study as cholesterol metabolism in this species more closely resembles that of humans in comparison with other animals (21).

A tissue specific difference in cholesterol ^{3}H-uptake was observed independent of dietary treatment. Although ^{3}H-uptake per gram of wet tissue in duodenum was higher than liver, total organ synthesis was greater in liver due to its larger overall mass. The elevated per gram synthesis in duodenum over liver, in the face of comparable free sterol levels, nevertheless suggests a significant duodenal contribution to whole body synthesis.

The response of lipid metabolism to qualitative dietary fat intake was distinct for each of fats tested. Corn oil-feeding resulted in plasma cholesterol levels below those observed with olive and coconut oil-feeding, yet no significant differences were observed in hepatic or duodenal synthesis of free cholesterol compared with other groups. Fernandez *et al.* (22) reported a similar absence of difference in cholesterol synthetic rate, as measured by sterol balance, between groups of guinea pigs fed corn oil, olive oil or lard at 7.5% for five weeks. Presently, levels of ω6 PUFA were elevated in livers of corn oil-fed animals compared with those fed other diets, consistent with the notion that ω6 class fatty acids are preferred substrates in the esterification process for cholesterol (23). However, cholesterol esterification was not elevated with corn oil-feeding. Thus, our data are inconsistent with the proposed notion of PUFA enhancing esterification, thereby reducing plasma sterol levels by reducing the size of the free cellular regulatory pool (12).

Olive oil feeding, in comparison with saturated

TABLE 3

Fatty Acid Composition of Liver Whole Lipid Extract (wt % of Total Fatty Acids)[a]

Fatty acid	Diet fat consumed			
	Corn oil	Olive oil	Coconut oil	Menhaden oil
12:0	1.0 ± 0.2	0.7 ± 0.1	1.1 ± 0.3	0.9 ± 0.2
14:0	0.5 ± 0.1	0.4 ± 0.1	1.1 ± 0.2	0.9 ± 0.2
14:1	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.5 ± 0.2
16:0	20.5 ± 0.9	19.0 ± 0.5	24.0 ± 1.0	23.8 ± 2.8
16:1	1.8 ± 0.2	1.9 ± 0.1	2.6 ± 0.1	2.4 ± 0.2
18:0	16.0 ± 0.6	15.1 ± 0.6	17.1 ± 0.8	17.1 ± 1.5
18:1	15.9 ± 1.0	20.9 ± 0.6	16.6 ± 1.3	15.9 ± 2.6
18:2ω6	27.2 ± 1.6	19.7 ± 0.8	15.1 ± 1.5	14.0 ± 2.5
20:0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0
18:3ω3	0.3 ± 0.0	0.2 ± 0.0	0.4 ± 0.1	0.3 ± 0.1
20:1	0.5 ± 0.1	1.4 ± 0.5	0.4 ± 0.1	0.2 ± 0.0
20:4ω6	7.0 ± 0.5	7.8 ± 0.8	6.8 ± 0.4	6.8 ± 0.3
20:5ω3	0.8 ± 0.1	2.3 ± 0.4	3.0 ± 0.4	3.9 ± 0.6
24:1	0.2 ± 0.0	0.3 ± 0.1	0.4 ± 0.1	0.6 ± 0.1
22:5ω3	0.9 ± 0.1	1.4 ± 0.2	2.1 ± 0.2	2.1 ± 0.2
22:6ω3	7.0 ± 0.7	8.7 ± 0.5	9.1 ± 0.6	10.5 ± 0.9
Tot. sat.	37.8 ± 1.7	34.9 ± 0.8	43.1 ± 1.6	42.4 ± 4.0
Tot. mon.	18.5 ± 1.2	24.5 ± 1.1	20.1 ± 1.3	19.4 ± 2.9
Tot. ω6	34.2 ± 2.0	27.5 ± 1.5	21.9 ± 1.9	20.8 ± 2.8
Tot. ω3	9.1 ± 0.9	12.5 ± 1.0	14.6 ± 0.9	16.8 ± 1.6

[a]Values are means ± SEM. Fatty acids are designated by chain length, number of double bonds and position of the first double bond from the fatty acid methyl terminus. Only major dietary fatty acids are reported.

TABLE 4

Fatty Acid Composition of Duodenal Whole Lipid Extract (wt % of Total Fatty Acids)[a]

Fatty acid	Diet fat consumed			
	Corn oil	Olive oil	Coconut oil	Menhaden oil
12:0	1.7 ± 0.3	1.2 ± 0.2	14.9 ± 2.4	1.0 ± 0.1
14:0	1.0 ± 0.1	1.2 ± 0.1	9.1 ± 1.4	3.2 ± 0.4
14:1	0.6 ± 0.1	0.5 ± 0.0	0.7 ± 0.1	0.8 ± 0.1
16:0	20.7 ± 1.0	18.8 ± 1.3	18.0 ± 2.6	23.8 ± 1.6
16:1	1.8 ± 0.1	3.0 ±0.5	2.5 ± 0.3	5.8 ± 0.7
18:0	14.8 ± 1.0	9.1 ± 0.6	9.2 ± 1.2	11.7 ± 0.6
18:1	20.1 ± 1.1	41.7 ± 4.6	17.6 ± 2.4	24.4 ± 2.7
18:2ω6	33.0 ± 2.0	18.8 ± 0.8	20.1 ± 3.1	17.7 ± 1.5
20:0	0.6 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.5 ± 0.0
18:3ω3	0.5 ± 0.1	0.8 ± 0.1	1.1 ± 0.1	1.0 ± 0.1
20:1	0.6 ± 0.1	0.6 ± 0.0	0.8 ± 0.1	0.9 ± 0.1
20:4ω6	2.4 ± 0.2	1.5 ± 0.2	2.2 ± 0.3	2.3 ± 0.1
20:5ω3	0.4 ± 0.1	0.5 ± 0.1	0.8 ± 0.2	3.2 ° 0.4
24:1	0.3 ± 0.1	0.9 ± 0.6	0.6 ± 0.1	0.3 ± 0.0
22:5ω3	0.5 ± 0.1	0.2 ± 0.0	0.6 ± 0.2	0.9 ± 0.1
22:6ω3	1.1 ± 0.1	1.0 ± 0.3	1.2 ± 0.3	2.5 ± 0.2
Tot. sat.	38.2 ± 3.6	30.6 ± 1.2	51.7 ± 8.0	39.5 ± 1.5
Tot. mon.	23.4 ± 1.1	46.7 ± 4.9	22.2 ± 2.9	32.3 ± 3.4
Tot. ω6	35.4 ± 2.0	20.3 ± 0.7	22.3 ± 3.4	19.6 ± 1.8
Tot. ω3	3.0 ± 0.3	2.8 ± 0.3	4.1 ± 0.9	8.0 ± 0.5

[a]Values are means ± SEM. Fatty acids are designated by chain length, number of double bonds and position of the first double bond from the fatty acid methyl terminus. Only major dietary fatty acids are reported.

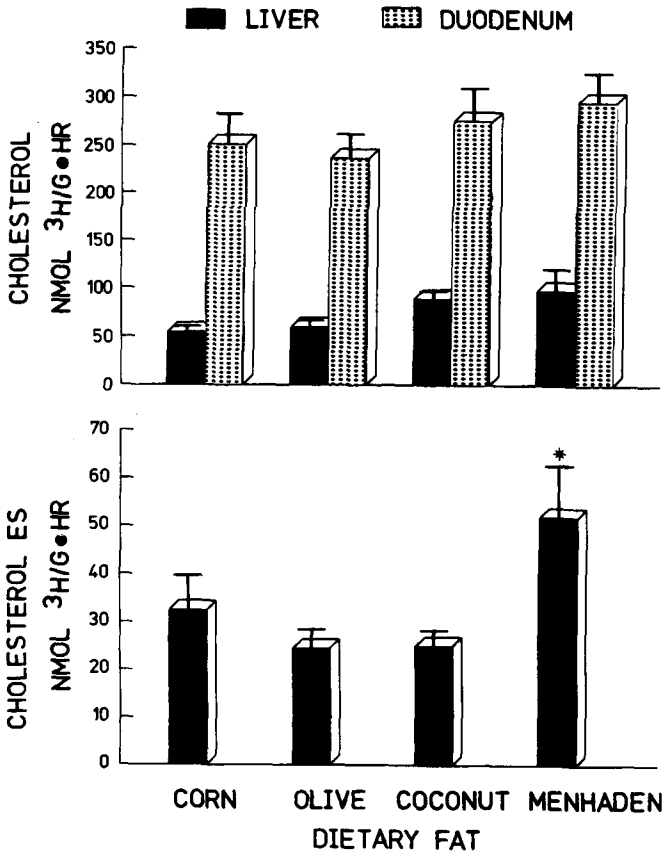

FIG. 1. Uptake of ^3H into cholesterol and cholesterol ester of liver and duodenum in hamsters consuming diets differing in dietary fat content. Values are means ± SEM. Group sizes for cholesterol were n=9, n=8, n=8, and n=8 and for cholesterol ester were n=8, n=8, n=7, and n=9 for corn, olive, coconut and menhaden oil, respectively. An asterix denotes a statistically significant difference ($p<0.05$) compared with other groups.

coconut oil, decreased plasma cholesterol levels in accordance with results observed in man (3,4). Accumulation of ester in liver, as reported previously for olive oil-fed hamsters (12), apparently is not the result of movement of new cholesterol into the ester pool. Possibly, cholesterol esterified with oleate is a poor substrate for incorporation into VLDL, however, isolated rat liver responded to perfusion with oleic, compared with linoleic, acid by increasing formation and secretion of VLDL (24). Conversely, enhanced hepatic uptake of cholesterol ester would explain the noted accumulation; however, hepatic LDL clearance rates were not significantly different across groups of hamsters fed 20% safflower, olive or coconut oil (20). The reason for ester accumulation with olive oil feeding therefore remains to be determined.

Feeding coconut oil high in MCFA resulted in an elevation of plasma total cholesterol and triglyceride levels, in the absence of an identifiable increase in tissue lipid levels or cholesterol synthesis or esterification rates. This elevation in plasma cholesterol level is consistent with the hypercholesterolemic effect of saturated fat seen in humans (5) and animals (12), although lower plasma lipids have also been reported subsequent to MCFA feeding in rats (25).

With menhaden fish oil feeding, total plasma cholesterol and triglyceride levels were reduced as compared with coconut oil feeding, but were not different from animals fed olive or corn oil diets. Animal and human studies are equivocal in the cholesterol modulating effect of feeding fish oils (6-10,26-28). The present results are consistent with findings of reduced plasma cholesterol levels following fish oil feeding compared with saturated fat (26), and approximately equal cholesterol concentrations in comparison to feeding of ω6 and ω3 fatty acid rich diets (10,27). Both liver and duodenal fatty acid composition responded to the fish oil diet by an elevation in total ω3 fatty acid levels. However, unlike previous data in rats where fish oil feeding reduced both plasma and hepatic cholesterol levels (29), no changes in free or ester levels were observed in liver or intestinal tissues of our menhaden oil-fed hamsters. Higher esterification rates for cholesterol in liver of menhaden oil-fed animals in comparison with those fed corn or olive oil suggest that longer chain ω3 PUFA are preferentially selected over other fatty acids as substrates for ester formation. Alternatively, physicochemical membrane properties may be altered through changes in cellular fatty acid composition induced by fish oil feeding (29).

In summary, the present data demonstrate that the nature of dietary fat alters plasma and organ lipid metabolism in hamsters. Specifically, dietary fat related differences in esterification of newly synthesized cholesterol are consistent with the notion of differential substrate availability for cholesterol ester formation of specific common dietary fatty acids.

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Synthesis of ω9-Tetracosynoic and ω9-Octacosynoic Acids as Entries into Tritiated Metabolic Precursors of *cis*-9-Tricosene and *cis*-9-Heptacosene in the Housefly[1]

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The syntheses of 15-tetracosynoic acid (ω9-tetracosynoic acid) and 19-octacosynoic acid (ω9-octacosynoic acid) are described. These alkynoic acids are to be tritiated to the corresponding alkenoic acids, which will be used as metabolic precursors of housefly pheromone components. The final step in each synthesis involved the coupling of 1-decyne to the lithio-salt of the appropriate ω-bromoacid. Homologation of dibromoalkanes was accomplished with triphasic catalytic displacement of bromide by cyanide ion. Oxidation of a bromo-alcohol to a bromoacid was performed in benzene with $KMnO_4$ and 18-crown-6 ether.
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The hypothesis that insect alkenes are immediate biosynthetic products of long-chain unsaturated fatty acids can be tested by determining the metabolic fate of tritiated long-chain alkenoic acids. For this purpose we are reporting the synthesis of 15-tetracosynoic (**10**) and 19-octacosynoic (**18**) acid. These two acids are to be reductively tritiated to yield the target compounds 15,16[$^3H_2$]-*cis*-15-tetracosenoic (**1**) acid and 19,20[$^3H_2$]-*cis*-19-octacosenoic (**2**) acid (see Schemes 1 and 2 in the Results and Discussion section). Acyl-CoA intermediates of the tritiated acids will be synthesized for treating the houseflies or housefly microsomal preparations.

The syntheses of many mid- to long-chain alkynoic acids have been reported previously. For example, the syntheses of the sixteen octadecynoic acids (1) as well as 2-octynoic, 2-nonynoic, and 2-eicosynoic acids (2) were described for biochemical as well as for chemical studies. Additionally, all the ynoic acids of chain length C_{10} to C_{14} were synthesized for inhibition studies of prostaglandin synthetase (3). Isomerization to the terminal position of internally located triple bonds in the synthetic acetylenic alcohols of 24, 26, 28, and 30 carbons have been reported. These were oxidized to the corresponding acids and derivatized to the methyl ester (4). To our knowledge, the final products reported here have not been previously described in the literature.

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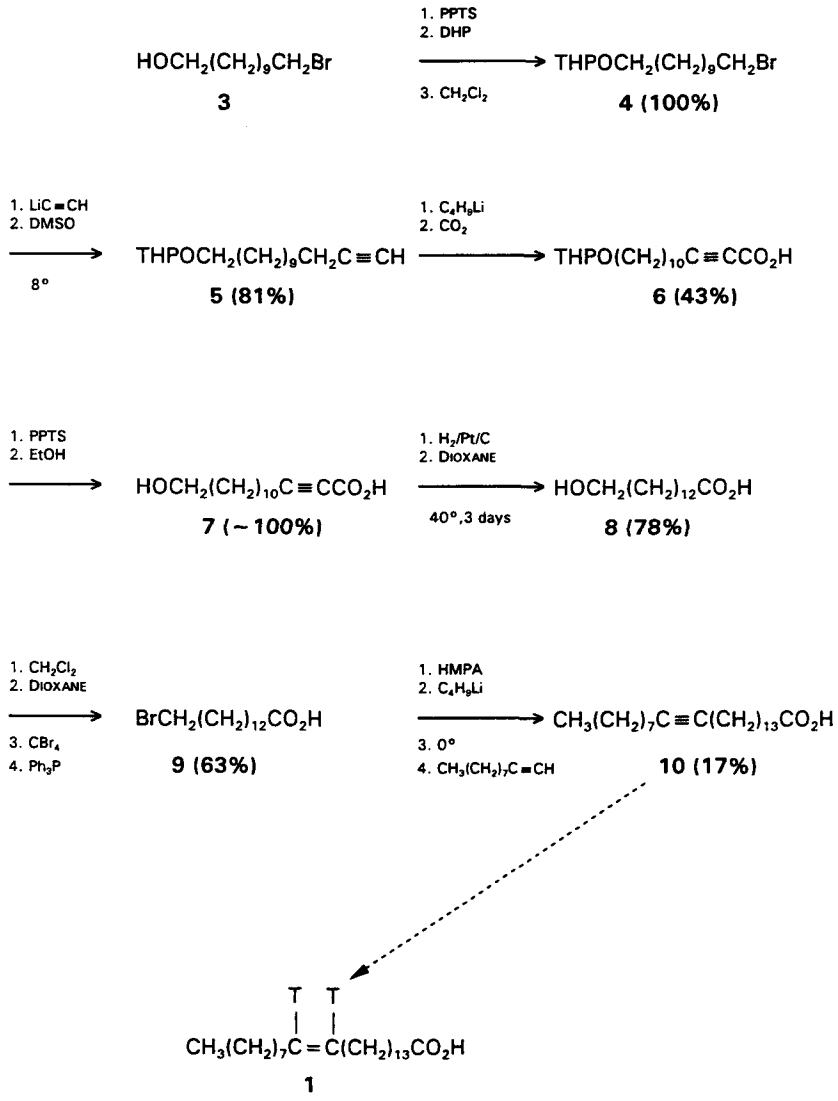
Abbreviations: ^{13}C NMR, ^{13}C nuclear magnetic resonance; CGC, capillary gas chromatography; DHP, dihydropyran; DMSO, dimethylsulfoxide; ^1H NMR, proton nuclear magnetic resonance; HMPA, hexamethyl phosphoramide; IR, infrared; MS, mass spectrum; PPTS, pyridinium *p*-toluenesulfonate; THF, tetrahydrofuran; THP, tetrahydropyran; TMS, trimethylsilyl.

RESULTS AND DISCUSSION

The synthesis of ω9-tetracosynoic acid (**10**) is shown in Scheme 1 and of ω9-octacosynoic acid (**18**) in Scheme 2. The strategy called for functional groups at both ends of the precursor molecules so that construction proceeded in a peripheral manner from a locus using commonly available reagents. Lithium acetylide was used for homologation. Once the acetylenic moiety was incorporated into the molecule, it was available for subsequent carboxylation or chain coupling reactions. This approach was required to introduce an acetylenic bond between the ninth and tenth carbon from the alkyl end of the final carboxylic acids **10** and **18**. Biochemical decarboxylation by the housefly of the tritiated alkenoic acids would then provide the labelled *cis*-9-tricosene and *cis*-9-heptacosene. In the housefly, the pheromone (*cis*-9-tricosene) is produced by the female and by males with ovary transplants, whereas the male or ovariectomized females produce *cis*-9-heptacosene (5–7). Fatty chain elongation reactions followed by decarboxylation (8) or decarbonylation of the corresponding aldehyde with retention of the carbonyl hydrogen (9) have been shown to be the intermediate steps in the biosynthesis of insect or plant alkanes and methylalkanes, and are proposed as intermediate steps for alkenes (8).

The hydroxyl group of the bromoalcohol **3** was protected as tetrahydropyran (THP) ether by using the mild catalyst pyridium *p*-toluenesulfonate (PPTS) (10) and dihydropyran to form **4**. Yields are shown in parenthesis in the Schemes. The isolated product which would decompose upon distillation was characterized by infrared (IR) and was used directly in the next step without further purification. Compound **5** was synthesized by reaction of lithium acetylide-ethylene diamine complex (11) with **4**. Carbonation of the lithium salt of **5** gave **6**. The THP ether moiety was hydrolyzed by an ethanol solution of PPTS (10) (the reagent is suitable for both the etherification and hydrolysis reactions) to provide **7** from **5**. The MS for the *bis*-trimethylsilyl (TMS) derivative of **7** was consistent with the proposed structure having a weak molecular ion (*m/z* 384), an ion at M − 15 (loss of methyl, 83% of base peak) plus diagnostic rearrangement ions of *bis*-TMS derivatives (12). A second derivative (methyl ester-TMS) of **7** provided a mass spectrum consistent with the structure of unsaturated methyl esters having a hydroxyl group derivatized as a TMS ether (13). Compound **8** was obtained by hydrogenation of **7** over platinum/carbon catalyst. Compound **8** was characterized by IR and ^1H NMR (proton nuclear magnetic resonance). The *bis*-TMS derivative of **8** gave a mass spectrum consistent with the structure of this derivative of a hydroxyacid (12). Bromination (14) of **8** with CBr_4 and triphenylphosphine in dioxane provided **9**. Commercially avail-

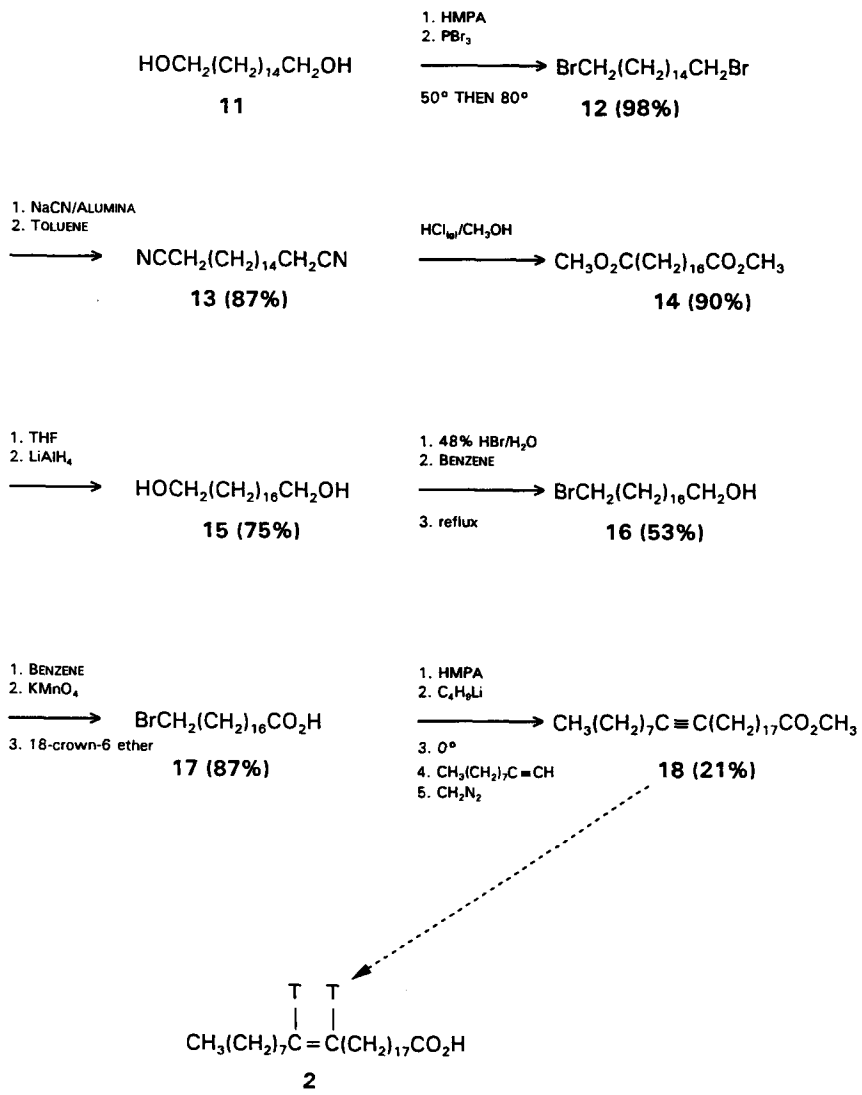


SCHEME 1

able 1-decyne was converted to the lithium salt and reacted with **9** in hexamethyl phosphoramide (HMPA) to give a relatively low 17% yield of **10** after distillation (the general procedure for synthesis of acetylenic carboxylic acids has been described) (15). Compound **10** is now available for tritiation to the proposed labeled precursor of the housefly pheromone **1**.

To insure that partial tritiation of the triple bond to the double bond with poisoned catalyst (Lindlar catalyst) is indeed practical with minimal scrambling of the hydrogen isotope, we partially reduced **7** with both hydrogen and dueterium in two separate experiments. The mass spectra of both products gave molecular ions having a mass difference of 2, indicating partial reduction of the triple bond by either isotope. The ^{13}C NMR spectrum of the hydrogenated **10** showed the expected single resonance for the ethylenic carbons at 129.9 ppm, whereas the spectrum of the deuterated compound **10** shows no peak at that frequency due to suppression by dueterium indicating partial reduction of the triple to the double bond. ^{13}C NMR showed a resonance at 27.2 ppm for the allylic carbons rather than at 32 ppm indicating *cis*-rather than *trans*-geometry. This corroborated the reduction to the ethylenic carbons and attachment of the isotope to the ethylenic carbons.

The commercially available diol **11** was converted to the dicyano-compound **13** *via* the dibromide **12** using a triphasic catalysis reaction consisting of NaCN adsorbed on alumina in toluene (16). Hydrolysis of **13** with methanolic HCl (17) gave the dimethyl ester **14**. Reduction of the diester **14** with $LiAlH_4$ provided the diol **15**. The monobromo compound **16** was obtained by reaction of **15** with 48% aqueous HBr in benzene solution. The progress of the reaction was followed by capillary gas chromatography (CGC) by observing the disappearance of the diol **15** and appearance of the monobromo **16** and dibromo products over a period of three days under reflux. Oxidation of the bromoalcohol **16** was accomplished with $KMnO_4$ in anhy-



SCHEME 2

drous benzene using 18-crown-6 ether as the phase transfer agent to afford **17**. The bromoacid **17** was coupled at 0°C with the lithio salt of 1-decyne in HMPA to give, in modest yield, ω9-octacosynoic acid, which was converted to the methyl ester (**18**) with diazomethane.

EXPERIMENTAL PROCEDURES

Melting points are uncorrected. Yields and purity of synthetic compounds were checked by CGC. We used a 12.5 m × 0.2 mm i.d. crossed linked methylsilicone fused silica column (0.33 μ film) mounted in a Varian model 3700 flame ionization instrument (Varian Associates, Palo Alto, CA). Samples were injected as solutions *via* a multipurpose type injector in the split mode fitted with a glass frit insert. The carrier gas was He at a flow rate of 0.77 mL/min (20 cm/seconds) and a head pressure of 12.5 psi. Mass spectra were obtained on a Hewlett-Packard model 5970 MSD interfaced to a 5890 gas chromatograph and a 59970 Chem Station. Infrared spectra were obtained with a Nicolet model 5MX FT-IR spectrometer. NMR spectra were recorded with a JEOL FX90Q Fourier transform spectrometer at 89.55 MHz for protons and at 22.5 MHz for carbon. Alternatively, a Bruker AM400 spectrometer with proton observation at 400 MHz and carbon observation at 100 MHz was used. Zero ppm was referenced to tetramethylsilane.

14-Hydroxy-2-tetradecynoic acid (**7**). A solution of 50 g (199 mmol) 11-bromoundecanol (**3**), 5.0 g (19.9 mmol) PPTS, 81.8 mL (897 mmol) DHP and 200 mL dry CH_2Cl_2 ($CaCl_2$) was stirred magnetically for 4 hr at room temperature (10). The reaction mixture was transferred to a separatory funnel with 100 mL diethyl ether and partitioned once against saturated brine (diluted by one half) to remove PPTS. The organic layer was separated and dried over anhydrous $MgSO_4$. The solvents were removed *in vacuo* and the excess dihy-

dropyran removed at 40°C at 0.75 mm Hg. The tetrahydropyranyl ether 4 was obtained in crude yield of 100% as a liquid. Attempts to distill the derivative at reduced pressure led to decomposition. Therefore, the product from replicate experiments was taken to the next step without further purification. IR (film) 2940 (s), 2845 (s), 1475 (m), 720 (w) (CH_2); 1098 (m), 813 (w) cm^{-1} (cyclic ether).

The crude material from above, 78.9 g (actual quantity of 4 was 69.7 g, 208 mmol, determined to be 88.3% of crude by CGC) was added dropwise to a cooled (8°C) and well stirred slurry of lithium acetylide-ethylene diamine complex (20.2 g, 213 mmol) in 107 mL dimethylsulfoxide (DMSO) under a nitrogen atmosphere (11). Addition of 4 was at such a rate so as to keep the temperature near 8°C. After addition, the solution was allowed to warm to room temperature and was stirred for an additional hour. Water (*ca.* 50 mL) was added slowly to the reaction mixture while keeping the temperature below 35°C. Then the mixture was poured into an additional 100 mL H_2O and extracted three times with hexane. The organic layer was separated and dried over $CaCl_2$. The filtered solvent was removed *in vacuo* to yield 57.4 g of a product that was 82.1% 5 by CGC (yield of product from 4 was 81%). IR (KBr) 2940 (s), 2845 (s), 1475 (m), 720 (w) (CH_2); 3300 (m), 2150 (w), 1260 (w), 630 (m) (alkyne); 1100 (m), 810 (m-s) (cyclic ether).

A solution of 47.2 g (160 mmol) 5 in sodium-dried diethyl ether was cooled to 3°C and 20 mL (2 M solution, 200 mmol) butyl lithium in hexane was added dropwise under N_2 and stirred for an additional hour. The contents of the reaction flask were poured over crushed, solid CO_2 contained in a crystallizing dish and allowed to warm to room temperature overnight. The viscous residue (6) was dissolved in diethyl ether and partitioned twice against 6N HCl, followed by H_2O. The organic layer was separated and dried over $MgSO_4$ and evaporated. The tetrahydropyranyl protecting group was removed by reacting the compound with PPTS catalyst in ethanol solution (10). The residue from the hydrolysis was mixed with 0.1 N KOH and partitioned against diethyl ether. The aqueous layer was acidified with 0.1 N HCl and the precipitated acid (7) collected by filtration and recrystallized from CH_2Cl_2 to yield 17.2 g (43% from 5) crystals, mp 84-86°C; IR (KBr) 2245 cm^{-1} (w, disubstituted alkyne conjugated with carbonyl) (ref. 18); MS for trimethylsily 14-trimethylsilyloxy-2-tetradecynoate (di-TMS derivative of 7), *m/z* (rel int) 384 (1.0, M^+), 369 (83, M − CH_3), 353 (10, M − CH_3O), 279 (16), 155 (17), 147 {90, $[(CH_3)_3Si{=}OSi(CH_3)_2]^+$}, (ref. 12), 133 (24), 103 {30, $[(CH_3)_3SiO{=}CH_2]^+$}, 95 (25), 93 (16), 81 (30), 75 {94, $[(CH_3)_2SiOH]^+$}, 73 [100, $(CH_3)_3Si^+$]; MS for methyl 14-trimethylsilyloxy-2-tetradecynoate (TMS ether of methyl ester of 7), *m/z* (rel int) 311 (35, M − CH_3), 295 (5, M − CH_3O), 279 (86, M − 47) (ref. 13) 75 {90, $[(CH_3)_2SiOH]^+$}, 73 [100, $(CH_3)_3Si^+$].

14-Hydroxy-tetradecanoic acid. (8). Compound 7, 11.0 g (45.8 mmol) was dissolved in 180 mL dioxane with warming on a steam bath and reduced in a H_2 atmosphere over 5% Pt/C catalyst at about 40°C. The isolated product was crystallized from $CHCl_3$ to give 8.6 g (78%) product, mp 89.5-90.5°C; IR (KBr) 3250 (br-m), 1312 (s), 1117 (m), 1051 (m) (primary alcohol), 3250 (br-m), 2561 (br-m), (OH of the acid), 1685 (s) (carbonyl stretch of the acid), 2920 (s), 2850 (s), 1475 (m), 719 (m) cm^{-1} (CH_2). ^1H NMR (400 MHz, THF-d_8) δ 1.32 (*s*, 22 H, -CH_2-), 2.20 (*t*, J = 8 Hz, 2H, -CH_2-COOH), 3.48 (*m*, 2H, -CH_2-OH), 5.5 (broad, -O*H*); MS *m/z* (rel intensity) for di-TMS derivative 388 (0.79, M^+), 373 (22, M − 15), 357 (4.6), 283 (9.2), 243 (1.4), 217 (6.5), 204 (8.9), 179 (2.5), 147 {23, $[(CH_3)_3SiOSi(CH_3)_2]^+$}, 132 [9.7, $(CH_3)_3SiOC(OH)CH_2^+$],117 [19.5, $(CH_3)_3SiOCO^+$], 103 {15.6, $[(CH_3)_3SiOCH_2]^+$}, 75 {66.5, $[(CH_3)_2SiOH]^+$}, 73 [100, $(CH_3)_3Si^+$].

14-Bromotetradecanoic acid (9). The procedure was a modification of that described for bromination of an ω-hydroxy acid (14). Into the flask containing a well stirred solution of 8 (5.4 g, 22 mmol) in 180 mL CH_2Cl_2, 48 mL dioxane and 9.3 g CBr_4 (28 mmol) was slowly added 8.7 g triphenylphosphine (33 mmol). After addition was completed, the solution was stirred at room temperature for an additional 4 hr. The solvents were removed *in vacuo* to leave a viscous residue which was mixed with 360 mL hexane/ethyl acetate (50:50, v/v) and filtered from the insoluble material. The filtrate was taken to dryness under vacuum and the residue applied to an activated silica gel column. Elution of the product from the column was with hexane/ethyl acetate (8:2, v/v). The compound was crystallized from hexane to yield 4.3 g (63%) of 9, mp 57-58°C; IR (KBr pellet) 3030 (br-m), 2650 (br-w), 1430 (m), 1410 (m), 945 (m-b) (OH); 2910 (s), 2845 (s), 1475 (m-s), 722 (m) (CH_2); 1690 (s), 1300 (m), 1225 (m), (C=O, C-C-O), 648 (m) cm^{-1} (CBr); ^1H NMR (400 MHz, $CDCl_3$); δ 1.26 (*s*, 16 H), 1.42 (*p*, J = 7.2 Hz, 2 H), 1.61 (*p*, J = 7.2 Hz, 2 H), 1.84 (*p*, J = 7.2 Hz, 2 H), 2.35 (*t*, J = 7.2, 2 H), 3.40 (*t*, J = 6.8, 2 H); MS *m/z* (rel intensity) for methyl ester derivative of 9 (only lowest mass of bromine isotopic cluster is reported) 320 (2.8, M^+), 289 (1.9 M − 15), 277 (3.2, M − 43), 241 (7.9, M − Br), 143 {13, $[(CH_2)_6COOCH_3]^+$}, 87 {54, $[(CH_2)_2COOCH_3]^+$}, 74 {100, $[CH_3C(=OH)OCH_2]^+$}.

15-Tetracosynoic acid (10, *ω9-tetracosynoic acid*). The general procedure for coupling 1-alkynes to alkylbromides is that described by Gilman and Holland (15). A solution of 2.4 mL 1-decyne (1.8 g, 13 mmol) in 15 mL HMPA was cooled to 0°C and 8.2 mL butyl lithium (1.6 M in hexane, 13 mmol) was added dropwise. Then compound 9 (2.0 g, 6.5 mmol) was added in one lot at 0°C and stirred at this temperature for 1 hr and then at room temperature for an additional 3 hr. The reaction mixture was poured into ice water and the aqueous mixture was acidified with dilute H_2SO_4. The mixture was extracted four times with diethyl ether. The combined ether extracts were washed with brine and dried over $MgSO_4$. The solvent was removed and the residue distilled, the yield was 400 mg (17%) of 10, b.p. 130°C/0.5 mm Hg; IR (KBr) 2975 (s), 1464 (m), 1380 (m) (CH_3), 2940 (s), 2880 (m), 1455 (m), 723 (m) (CH_2), 2212 (w) (C≡C), ≈3000 (w-b), 950 (m) (OH), 1740 (s) (C=O), series of peaks diagnostic of long-chain carboxylic acids 1345-1180 (m-w) cm^{-1} (ref. 19); MS of methyl ester of 10, *m/z* (rel int) M^+ 378 (1), 347 (M − 31, 5.3), 74 [21, $CH_2C(OCH_3){=}OH^+$], acetylene fragment series (refs. 20—22): 123 (18), 109 (37), 95 (82), 81 (100), 67 (92), and 166 (12), 152 (23), 138 (10), 124 (18), 110 (27),

96 (64), 82 (89), 68 (70); ^{13}C NMR (100 MHz, $CDCl_3$) δ 180 (-COOH), 80.1 (-C≡C-) (ref. 23).

Reduction of **10**. Compound **10** methyl ester, 20 mg, was reduced either in a hydrogen or a deuterium atmosphere as follows: A six-inch disposable glass pipette was dry packed with a 10% mixture of Lindlar catalyst on celite to a column height of 3 cm. Then a solution of **10** in 250 μL ethyl acetate containing 1% quinoline was applied to the column. The reducing gas was allowed to slowly flow through the column for 15 min and the partially reduced compound was eluted with 2-3 mL of ethyl acetate. The eluent was removed and the residue chromatographed on silica thin layers with hexane/diethyl ether (80:20, v/v) as solvent. *Methyl 15-tetracosenoate* was obtained in ≈100% (CGC) yield; MS *m/z* (rel int) M^+ 380 (7, $C_{25}H_{48}O_2$); ^{13}C NMR (100 MHz, $CDCl_3$) δ 129.9 (*s*, ethylenic carbon), 27.2 (allylic carbon with *cis*-geometry). *Methyl [15,16-d_2]tetracosenoate* was obtained in ≈100% (CGC) yield; MS *m/z* (rel int) M^+ 382 (7, $C_{25}H_{46}D_2O_2$);^{13}C NMR (400 MHz, $CDCl_3$) no ethylenic carbon resonance due to supression by deuterium.

1,16-Dibromohexadecane (**12**). The 1,16-hexanediol (**11**, 10.0 g, 39 mmol) was dissolved in 100 mL HMPA at 50°C with stirring. The PBr_3 (8.4 g, 90 mmol) was added dropwise *via* a corrosive-liquid funnel, and after addition was completed, the mixture was heated with stirring at 80°C for 4 hr, then at room temperature overnight. The reaction mixture was poured into ice water, and the precipitated material collected by filtration. The precipitate was dissolved in $CHCl_3$, treated with charcoal and filtered. The solution was concentrated *in vacuo* and the collected solid recrystallized from methanol to yield 14.6 g (98%) of **12**, mp 56-57°C, IR (KBr) 2940 (s), 2840 (s), 1475, (s), 717 (m) (CH_2), 642 cm^{-1} (CBr); MS *m/z* (rel int) 305 (2.7, M − Br), cycloalkylbromonium ion series: 247 (2.9), 233, (4.2), 219 (4.9), 205 (5.6), 191 (5.8), 177 (6.0), 163 (5.5), 149 (7.7), 135 (28).

1,18-Octadecanedinitrile (**13**). Alumina (Alcoa F-20) was impregnated with NaCN as described by Regen *et al.* (16). A 500-mL resin pot fitted with a mechanical stirrer, a condenser with a $CaCl_2$ drying tube and a N_2 inlet was charged with 14.6 g (38 mmol) 1.16-dibromohexadecane, 80 g alumina impregnated with 40 g NaCN (82 mmol) and 250 mL toluene. The mixture was heated with slow stirring at 100°C. The progress of the reaction was monitored by CGC. After five days, the reaction mixture was cooled and the alumina filtered from the organic solvent. Removal of the solvent gave **13** in a yield of 87%, mp 58-59°C; IR (KBr) 2918 (s), 2849 (s), 1472 (m-s), 715 (m) (CH_2), 2245 (m-w) cm^{-1} (C≡N); MS *m/z* (rel int) 275 (1.8, M − 1), 247 (2.9), 233 (5.9), 97 (100, cyclohexylimonium, $C_6H_{11}N^+$), C_nH_{2n-1} series (ref. 24): 208 (0.7), 194 (2.2), 180 (3.3), 166 (4.8), 152 (7.5), 138 (9.3), 124 (17.9), 110 (40.5).

bis-Methyl 1,18-octadecanedioate (**14**). A solution of 6.0 g (22 mmol) of **13** in 150 mL of methanolic hydrogen chloride (30% by wt) was stirred at 45°C for four days. The reaction was quenched by pouring into ice water and the ester extracted into diethyl ether. The ether was dried ($MgSO_4$) and evaporated. The product weighed 6.7 g (90% yield), mp 60-60.5°C; IR (KBr) 2970 (s), 1475 (m) (CH_3), 2930 (s), 2840 (s), 1460 (m), 725 (m-w) (CH_2), 1750 (s) (C=O), 1175 (m) (C-C-O), series of peaks diagnostic of carboxylic acids and esters: 1180-1345 (m) cm^{-1} (ref. 19); MS identical to that reported by Ryhage and Stenhagen (25).

1,18-Octadecanediol (**15**). The $LiAlH_4$ (1.5 g, 39 mmol) was dissolved in 50 mL dry THF under argon and cooled to 0°C and a solution of 6.7 g (19 mmol) of **14** in 100 mL dry THF was added dropwise over one hour. The reaction mixture was allowed to stir at room temperature overnight. Acetone was added slowly to consume the excess $LiAlH_4$ followed by dilute aqueous solution of HCl. The decomposed mixture was extracted three times with diethyl ether and the ether extract was dried over $MgSO_4$ and evaporated. The residue was crystallized from methanol (charcoal) to yield 4.2 g (75%), mp 97.5-98.5°C; IR (KBr) 3420 and 3380 (m), 1070 (m) (OH), 2945 (s), 2860 (s), 1465 (m), 731 (m-s) cm^{-1} (CH_2); MS *m/z* (rel int) *bis*-TMS ether derivative: 315 (2, M − 15), 399 (7), 325 (15, M − 105), 165 {8, $[(CH_3)_3SiOHSi(CH_3)_2OH]^+$}, 149 {100, $[(CH_3)_2SiOSi(CH_3)_2(OH)]^+$}, 147 {46, $[(CH_3)_2SiOSi(CH_3)_3]^+$}, 73 [97, $(CH_3)_3Si^+$].

18-Bromo-1-octadecanol (**16**). Monobromination of diol **15** was accomplished by the procedure described by Kang *et al.* (26). A mixture of 3.4 g (11.9 mmol) **15**, 1.5 mL 48% aqueous HBr and 100 mL benzene were heated under reflux for three days in a flask fitted with a water separation apparatus. Additional 1.5 mL aliquots of HBr were added as needed by monitoring product formation with CGC. The solvents were removed *in vacuo* and the residue percolated through a silica gel column. The product was eluted with benzene gave diethyl ether (65:35, v/v). Crystallization from methanol gave 2.2 g (53% yield) of product having a mp 59-61°C; IR (KBr) 2915 (s), 2850 (s), 1465 (m-s), 725 (m) (CH_2), 3250 (m), 1065 (m) (OH and -CO), 650 (m) cm^{-1} (CBr); MS of TMS derivative *m/z* rel int) 405 (1.1, M − CH_3), 73 [65, $(CH_3)_3Si^+$].

18-Bromo-octadecanoic acid (**17**). The procedure for oxidation was modified from that described by Sam and Simmons (27). A mixture of **16** (2.2 g, 6.3 mmol), $KMnO_4$ (2.9 g, 18.1 mmol), 18-crown-6 ether (160 mg 0.6 mmol) and 100 mL benzene was stirred at room temperature for 18 hr, the purple solution eventually turned brown. The mixture was poured over crushed ice and acidified with 1N HCl. The organic/aqueous mixture was then filtered through a bed of celite, the organic layer removed and the aqueous layer extracted three times with diethyl ether. The combined organic extracts were dried over $MgSo_4$ and evaporated. The residue was crystallized from acetone to yield 2.0 g (87%) of **17** having a mp of 74-75.5°C; IR (KBr) ≈3000 (br m-w), 2700 (br m-w), 1435 (m), 930 (m) (OH), 2915 (s), 2850 (s), 1475 (m), 718 (m) (CH_2), 1745 (s) (C=0), 1350 (m) (C-C-O), 645 (m) (CBr); MS *m/z* (rel int) 434 (M^+ for $C_{21}H_{43}BrO_2Si$, 6.8), 6.8), 419 (5.9, M − 15), 391 (2.9, M − 43), 355 (19, M − Br), 339 (18, M − BrO), 265 (7.8), 201 [16, $(C_{10}H_{21}O_2Si)^+$], 145 [35, $(C_6H_{13}O_2Si)^+$], 132 [65, $(CH_3)_3SiOC(=OH)^+CH_2\bullet$], 129, [47, $(CH_3)_2=Si^+OC(=0)CH\text{-}CH_2$], 117 [100, $(CH_3)_2Si^+OC(=O)CH_3$], 73 [82, $(CH_3)_3Si^+$].

Methyl 19-octacosynoate (methyl ω9-octacosynoate, **18**). A solution of 1.6 g (11.6 mmol) 1-decyne in 15 mL HMPA was cooled with stirring to 0°C under argon,

n-butyllithium (2.32 mL of a 2.5 M solution in hexane, 5.8 mmol) was added dropwise and the resulting solution stirred at that temperature for an additional hour. solid 18-bromooctadecanoic acid (2.0 g, 5.8 mmol) was added in one portion to the lithium acetylide and the resulting solution stirred for 18 hr. The reaction mixture poured onto crushed ice water. When the ice had melted the solution was filtered. The air-dried residue was dissolved in hexane/diethyl ether/formic acid (50:50:1, by vol) and percolated through a silica gel column with the same solvent system. After elution from the column the acid was esterified with diazomethane and further purified by TLC (hexane/diethyl kether, 95:5, v/v) to yield 0.6 g (18, 21%); mp, 42-43°C, IR (KBr) 2970 (m-s), 2860 (m), 1480 (m), 1380 (w-m) (CH_3), 2935 (s), 2860 (s), 1435 (m), 723 (m) (CH_2, 1732 (s) (C=O), 1227 (m) (C-C-O); MS *m/z* (rel int) 434 (1.9, M^+), 403 (16, M − 31), 350 (9.2), 336 (10), 318 (6.8), 304 (7), 276 (2.8), 193 (5.4), 95 (78), 81 (100), acetylenic series (ref. 21, 23): 166 (22), 152 (32), 138 (23), 124 (34), 110 (38), 96 (80), 82 (76), 68 (55), 54 (30); hydrogenated derivative of 18 (methyl octacosanoate, M_r = 438) MS *m/z* (rel int) 438 (59, M^+), 409 (3.6, M − 29), 407 (4.0, $CH_3(CH_2)_{26}CO^+$), 395 (23, M − 43), 339 (13, M − 99), 199, {18, $[(CH_2)_{10}C(=O)OCH_3]^+$}, 143, {45, $[(CH_2)_6C(=O)OCH_3]^+$}, 87 {76, $[(CH_2)_2C(=O)OCH_3]^+$}, 74 [100, $CH_3OC(=OH^+)CH_2$].

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Effects of Dietary n-3 and n-6 Polyunsaturated Fatty Acids on Macrophage Phospholipid Classes and Subclasses

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This study examined the effects of n-3 and n-6 polyunsaturated fatty acid alimentation on murine peritoneal macrophage phospholipids. Mice were fed complete diets supplemented with either corn oil predominantly containing 18:2n-6, borage oil containing 18:2n-6 and 18:3n-6, fish/corn oil mixture containing 18:2n-6, 20:5n-3 and 22:6n-3, or fish/borage oil mixture containing 18:2n-6, 18:3n-6, 20:5n-3 and 22:6n-3. After two weeks, the fatty acid levels of glycerophosphoserines (GPS), glycerophosphoinositols (GPI), sphingomyelin (SPH), and of the glycerophosphocholine (GPC) and glycerophosphoethanolamine (GPE) phospholipid subclasses were determined. We found that mouse peritoneal macrophage GPC contain primarily 1-*O*-alkyl-2-acyl (range for the dietary groups, 24.6–30.5 mol %) and 1,2-diacyl (63.2–67.2 mol %), and that GPE contains 1-*O*-alk-1′-enyl-2-acyl (40.9–47.4 mol %) and 1,2-diacyl (44.2–51.2 mol %) subclasses. In general, fish oil feeding increased macrophage 20:5n-3, 22:5n-3 and 22:6n-3 levels while simultaneously reducing 20:4n-6 in GPS, GPI, GPE and GPC subclasses except for 1-*O*-alk-1′-enyl-2-acyl GPC. Administration of 18:3n-6 rich diets (borage and fish/borage mixture) resulted in the accumulation of 20:3n-6 (2-carbon elongation product of 18:3n-6) in most phospholipids. In general, the novel combination of dietary 18:3n-6 and n-3 PUFA produced the highest 20:3n-6/20:4n-6 phospholipid fatty acid ratios. This study demonstrates that marked differences in the responses of macrophage phospholipid classes and subclasses exist following dietary manipulation. The reduction of 20:4n-6, while simultaneously increasing 20:3n-6 and n-3 PUFA levels, may be important in relation to the putative beneficial effects of 20:3n-6 and fish oil on macrophage eicosanoid and platelet activating factor (PAF) biosynthesis.
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The macrophage plays a central role in the immune system and is capable of executing or modifying a number of important biological functions (1). In addition to its ability to sequester, ingest and destroy microorganisms, this cell type can take part in both the amplification and suppression of immune responses (1,2). Macrophages are also known to secrete large quantities of arachidonic acid (20:4n-6) derived cyclooxygenase and lipoxygenase oxidative products (1,3). This is significant because the 20:4n-6 derived eicosanoids are capable of influencing many of the regulatory activities of macrophages (4,5). In addition, excess or imbalanced production of 20:4n-6 derived eicosanoids may exacerbate pathophysiological conditions such as asthma, arthritis and psoriasis (6,7). Therefore, an understanding of the mechanisms regulating macrophage eicosanoid production is vital in controlling inflammation and ameliorating pathophysiological states.

The cascade of 20:4n-6 release and metabolism is regulated in part by its specific phospholipid class distribution (8,9). Previous studies have proposed different classes of phospholipids, *i.e.*, glycerophosphocholines (GPC), glycerophosphoethanolamines (GPE), glycerophosphoinositols (GPI) and glycerophosphoserines (GPS) as sources of eicosanoid fatty acid precursors (10,11). It has been shown recently that considerable amounts of the phospholipid subclasses of GPC and GPE are present in alveolar macrophages (12). These subclasses of phospholipid are made up of 1-*O*-alkyl-2-acyl, 1-*O*-alk-1′-enyl-2-acyl and 1,2-diacyl species, each differing in the covalent linkage of the aliphatic chain at the *sn*-1 position of the glycerol backbone. The 1-*O*-alkyl and 1-*O*-alk-1′-enyl phospholipid subclasses are unique because they contain significantly higher levels of 20:4n-6 than diacyl species in certain cell types (12–14) and could be important pools of polyunsaturated fatty acids (PUFA) in macrophages. This is noteworthy since eicosanoid production can be regulated merely by altering the distribution of 20:4n-6 within the cell so that it is not released from phospholipids for eicosanoid synthesis (14). Thus, although a phospholipid may have a certain prototypic structure, it is in reality a family of related molecules with distinct metabolic and physical properties (15).

It is well established that alteration in the dietary content of fatty acids can modulate membrane bound receptors, enzyme activities, and eicosanoid production (16–18). One of the most interesting approaches to the dietary modification of the eicosanoid system has been the possible prophylactic role of dietary γ-linolenic acid (18:3n-6) and fish oil on thrombo-embolic (19,20) and chronic inflammatory disorders (21,22). Since borage oil, derived from the borage plant (*Borago officinalis*), contains large amounts of 18:3n-6 and fish oil contains large amounts of eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3), interest has focused on the possibility of competition between n-6 and n-3 fatty acids at the sites of membrane phospholipid storage and eicosanoid production (7,23). However, to date, detailed studies documenting the ability of dietary 18:3n-6 and fish oil to modify macrophage phospholipid classes and GPE and GPC subclasses are lacking.

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Abbreviations: 8-Anilino-1-naphthalenesulfonic acid ammonium salt; 20:4n-6, arachidonic acid; 20:3n-6, dihomogammalinolenic acid; 22:6n-3, docosahexaenoic acid; 22:5n-3, docosapentaenoic acid; 20:5n-3, eiocosapentaenoic acid; FAME, fatty acid methyl ester; 18:3n-6, γlinolenic acid; GPC, glycerophosphocholine; GPE, glycerophosphoethanolamine; GPI, glycerophosphoinositol; GPS, glycerophosphoserine; HPLC, high performance liquid chromatography; 18:2n-6, linoleic acid; PAF, platelet activating factor; PUFA, polyunsaturated fatty acids; SPH, sphingomyelin; TLC, thin-layer chromatography.

We have recently demonstrated (24) that fish oil feeding can suppress the activation of macrophage tumoricidal capability *in vitro*. These results suggest that fish oil feeding may play a regulatory role in host protection against neoplastic and infectious diseases. Prompted by these observations, we are now investigating how fish oil supplementation may act to influence host responsiveness. In order to address this complex issue, it is first necessary to determine the ability of n-3 fatty acids to alter macrophage membrane phospholipid profiles. Therefore, we report i) the levels of mouse peritoneal macrophage phospholipid subclasses; and ii) the effect of n-6/n-3 dietary fatty acid mixing on macrophage phospholipid class and subclass composition.

MATERIALS AND METHODS

Materials. Fatty acid methyl ester standards and monopentadecanoin were from NuChek Prep (Elysian, MN). Silica gel 60 plates and silica gel G were from E. Merck (Darmstadt, Federal Republic of Germany). Phospholipase C (*Bacillus cereus*, Type V) was from Sigma Chemical Co. (St. Louis, MO). 8-Anilino-1-naphthalenesulfonic acid ammonium salt was from Eastman Kodak (Rochester, NY). Benzoic anhydride and 4-dimethylaminopyridine were from Aldrich Chemical Co. (Milwaukee, WI). Corn and borage oil were donated by Traco Labs (Champaign, IL) and menhaden fish oil was donated by Zapata-Haynie (Reedville, VA). All chemicals were of high performance lipid chromatography (HPLC) grade.

Dietary treatments and macrophage isolation. Specific pathogen-free male C57BL/6 mice (Harlan, Indianapolis, IN) weighing 15–18 g were used. Mice were fed, *ad libitum*, one of four purified diets (Table 1) which were adequate in all nutrients (25). The diets were changed daily and varied only in the type of oil fed, *i.e.*, either corn, borage, fish/corn or fish/borage mixtures in a 1:1 ratio at 10% of the diet by weight, and were stored at −20°C. Under these conditions, fatty acid oxidative breakdown products are not detected (24,26). The fatty acid composition of the different diets is shown in Table 2. After two weeks of feeding the experimental diets, primary cultures of peritoneal macrophages were established from responsive cells as previously described (27). Selected cell monolayers were solubilized in 0.1 M sodium hydroxide for protein determination using the method of Lowry *et al.* (28).

Separation of phospholipid classes and subclasses. Macrophages were extracted by the method of Folch *et al.* (29). The individual phospholipid classes were separated by thin-layer chromatography (TLC) on silica gel 60 plates using chloroform/methanol/acetic acid/water (50:37.5:3.5:2, by vol.) as previously described (25). Bands were detected under ultraviolet light after spraying with 0.1% 8-anilino-1-naphthalenesulfonic acid ammonium salt (ANS). A known amount of monopentadecanoin as an internal standard was added to isolated GPS, GPI and SPH phospholipid classes prior to transesterification (25,30). The resultant fatty acid methyl esters (FAME) were further purified on silica gel G plates run in a toluene solvent system, detected using ANS, and extracted using methanol/hexane/water (1:1:0.5, by vol.) prior to gas chromatographic analysis. The isolated choline (GPC) and ethanolamine (GPE) glycerophospholipid bands were extracted using chloroform/methanol/water (5:5:1, by vol.) followed by the addition of 2.25 mL chloroform and 1 mL 50 mM Tris buffer, pH 9.0 (31). The upper aqueous layer was discarded and the solvent from the lower chloroform phase was removed under N_2. The isolated GPC and GPE fractions were converted to benzoate derivatives following phospholipase C hydrolysis (32,33). Briefly, approximately 0.4 mg of phospholipid was suspended in 2 mL peroxide-free diethyl ether containing 0.005%

TABLE 1

Composition of Experimental Diets

Ingredient[a]	Amount (g/100 g diet)
Oil[b]	10.00
Casein (vitamin free)	20.00
DL-methionine	0.30
Sucrose	44.00
Corn starch	14.98
Cellulose	6.00
Mineral mix[c]	3.50
Vitamin mix[d]	1.00
Choline chloride	0.20
t-Butylhydroquinone[e]	0.02

[a]All dietary components were purchased from U.S. Biochemicals (Cleveland, OH), except where noted.
[b]All diets provide approximately 22% energy from lipid.
[c]Provided at the following amount in grams/kilogram of salt mix as per AIN 76 mixture: $CaHPO_4$, 500.0; NaCl, 74.0; K-citrate, 220.0; K_2SO_4, 52.0; MgO, 24.0; manganous CO_3, 3.5; ferric citrate, 6.0; $ZnCO_3$, 1.6; $CuCO_3$, 0.3; KIO_3, 0.01; Na_2SeO_3, 0.01; and $CrK(SO_4)_2$, 0.55.
[d]Provided at the following amount in grams/kilogram of vitamin mix (except as noted) as per AIN 76 mixture; thiamin HCl, 0.6; riboflavin, 0.6; pyridoxine HCl, 0.7; nicotinic acid, 3.0; Ca pantothenate, 1.6; folic acid, 0.2; biotin, 0.02; cyanocobalamin, 1 mg/kg; retinyl palmitate, 1.6 (250,000 I.U./g); dl-α-tocopheryl acetate, 20 (250 I.U./g); cholecalciferol, 2.5 mg (400,000 I.U./g); and menaquinone, 5.0 mg.
[e]Eastman Kodak Chemicals (Rochester, NY).

TABLE 2

Fatty Acid Composition of Diets

Fatty Acid[a]	Corn	Borage	Fish[b]/Corn	Fish[b]/Borage
14:0	tr	0.3	4.5	4.5
16:0	12.1	10.3	14.1	13.4
16:1n-7	0.2	0.1	6.1	5.9
18:0	1.7	3.2	2.6	3.5
18:1n-9	25.7	14.7	16.4	10.8
18:2n-6	59.0	39.2	30.6	20.4
18:3n-6	tr	25.6	0.3	13.0
20:4n-6	tr	tr	0.4	0.4
20:5n-3	tr	tr	7.6	6.8
22:5n-3	tr	tr	1.0	1.0
22-6n-3	tr	tr	3.9	4.1

[a]Values are expressed as mg/100 mg total fatty acids present. Only the major fatty acids are presented (tr=trace amounts, less than 0.1%).
[b]Menhaden fish oil.

butylated hydroxy toluene and 2 mL of 50 mM Tris-HCl buffer, pH 7.4, containing 5 mM calcium chloride. The mixture was agitated for 3 hr at room temperature. The diradylglycerols were extracted two times with 2 mL diethyl ether. The combined extracts were passed through Pasteur pipets containing anhydrous sodium sulfate and dried under N_2. Samples were dissolved in 0.2 mL of benzene containing 10 mg benzoic anhydride and 0.1 mL of benzene containing 4 mg of 4-dimethylaminopyridine and incubated for 1 hr at room temperature (32). The reactions were stopped by placing the samples on ice and slowly adding 1 mL of concentrated ammonium hydroxide and 2 mL hexane (34). The diradylglycerobenzoates were extracted three times with hexane and washed once with 1 mL water. The solvent was removed under N_2 and the diradylglycerobenzoates were separated on silica gel G plates using benzene/hexane/diethyl ether (50:45:4, by vol.) as solvents (32). The 1-*O*-alk-1′-enyl-2-acyl-, 1-*O*-alkyl-2-acyl-, and 1,2-diacyl- glycerobenzoates were visualized using ANS spray. The fractions were scraped from the plates and transesterified in the presence of known amounts of monopentadecanoin for mass determinations (25). The FAME were purified by TLC using toluene, as described above. The concentration and fatty acid composition of the GPI, GPS, SPH and the three subclasses of GPC and GPE were analyzed on a Hewlett Packard 5890A gas chromatograph (Hewlett Packard, Norwalk, CT) equipped with a DB-225 30-meter fused silica capillary column (I.D. 0.25 mm, J&W Scientific, Folsom, CA). Hydrogen served as carrier gas at a flow rate of 1.78 mL/min. The split ratio was 1:66, and the oven temperature was kept at 200°C. Fatty acids were identified by comparing their retention times with those of known standards. Fatty acid concentrations were corrected for recovery of phospholipid classes and subclasses and for background noise as measured by parallel analysis of blanks as previously described (25). Since the acyl group composition of the 1-*O*-alk-1′-enyl-2-acyl and 1-*O*-alkyl-2-acyl subclasses is derived exclusively from the *sn*-2 position, whereas the acyl groups from the diacyl subclasses represent the *sn*-1 plus *sn*-2 positions, the molar amounts of the total FAME from the diacyl subclasses were divided by two for relative subclass composition analysis.

Statistical analysis. The data expressed as nmol fatty acid/mg protein or mol % were analyzed by one-way analysis of variance using multiple comparisons, with the upper level of significance chosen at $P<0.05$ (35).

RESULTS

Table 3 shows the fatty acid composition of serine-containing glycerophospholipids from murine peritoneal macrophages. Borage and borage/fish feeding significantly increased ($P<0.05$) dihomo-γ-linolenic acid (20:3n-6) levels relative to corn and fish/corn feeding. As expected, diets containing n-3 fatty acids (fish/corn and fish/borage) significantly increased ($P<0.05$) 20:5n-3, 22:5n-3 and 22:6n-3 levels relative to diets containing corn and borage oils. It is noteworthy that the absolute 20:3n-6 levels in GPS exceeded 20:4n-6 levels following fish/borage mixture feeding. Data describing GPI fatty acid concentrations are shown in Table 4. Animals fed diets containing 18:3n-6 (borage and fish/borage) had significantly increased ($P<0.05$) 20:3n-6 levels. Similar to GPS, GPI 20:3n-6 levels were highest in fish/borage mice relative to borage, corn and fish/corn. The composition of the fatty acyl moieties in sphingomyelin are provided in Table 5. Dietary manipulation had a minimal effect on SPH-PUFA composition. PUFA n-3 feeding produced a small but significant ($P<0.05$) increase in 22:6n-3 in fish/corn mice. Alterations in the predominant acyl chain components of SPH, of which 16:0 and 24:1n-9 comprise approximately 80%, were noted following dietary manipulation.

The compositions of the fatty acyl moieties in the

TABLE 3

Fatty Acid Composition of Macrophage Glycerophosphoserines (GPS)*

Fatty acid	Corn	Borage	Fish/Corn	Fish/Borage
16:0	3.35 ± 0.33	4.36 ± 1.52	2.77 ± 0.48	2.80 ± 0.12
16:1n-7	0.13 ± 0.01	0.10 ± 0.05	0.12 ± 0.01	0.12 ± 0.01
18:0	9.44 ± 1.10	8.02 ± 0.37	9.02 ± 0.31	9.28 ± 0.53
18:1n-9	2.40 ± 0.24^a	1.27 ± 0.12^b	1.47 ± 0.26^b	1.74 ± 0.08^b
18:1n-7	0.61 ± 0.07	0.42 ± 0.11	0.50 ± 0.05	0.44 ± 0.03
18:2n-6	3.32 ± 0.43^a	0.99 ± 0.18^b	1.82 ± 0.45^b	1.42 ± 0.02^b
20:2n-6	0.29 ± 0.03^a	0.10 ± 0.02^b	tr^b	tr^c
20:3n-6	0.68 ± 0.07^b	1.00 ± 0.14^b	0.30 ± 0.09^c	1.57 ± 0.05^a
20:4n-6	2.35 ± 0.30^a	1.34 ± 0.24^b	0.65 ± 0.12^c	1.04 ± 0.05^b
20:5n-3	tr^c	tr^c	0.32 ± 0.03^a	0.16 ± 0.01^b
22:4n-6	2.06 ± 0.35^a	1.10 ± 0.21^b	tr^d	0.36 ± 0.04^c
22:5n-6	1.30 ± 0.33^a	0.16 ± 0.04^b	tr^c	tr^c
22:5n-3	0.04 ± 0.03^b	tr^b	1.67 ± 0.37^a	1.48 ± 0.12^a
22:6n-3	0.17 ± 0.02^b	0.14 ± 0.01^b	0.64 ± 0.11^a	0.68 ± 0.03^a

*Results are expressed as nmol/mg protein. Each Figure represents the mean ± SEM (n=4). Values with the same or no superscripts are not significantly different ($P>0.05$). tr, Trace amounts, less than 0.1 nmol/mg protein.

TABLE 4

Fatty Acid Composition of Macrophage Glycerophosphoinositols (GPI)*

Fatty acid	Corn	Borage	Fish/Corn	Fish/Borage
16:0	1.97 ± 0.31^a	0.98 ± 0.04^b	2.09 ± 0.48^a	1.48 ± 0.13^b
16:1n-7	tr	0.11 ± 0.06	0.10 ± 0.05	tr
18:0	7.42 ± 0.85^a	5.30 ± 1.16^b	3.40 ± 0.88^b	6.36 ± 0.54^b
18:1n-9	0.79 ± 0.10	0.55 ± 0.10	0.48 ± 0.09	0.57 ± 0.04
18:1n-7	0.27 ± 0.05	0.28 ± 0.04	0.28 ± 0.06	0.18 ± 0.01
18:2n-6	0.74 ± 0.09^a	0.44 ± 0.11^b	0.24 ± 0.11^b	0.36 ± 0.02^b
20:3n-6	0.26 ± 0.05^c	0.52 ± 0.17^b	0.19 ± 0.04^c	0.84 ± 0.03^a
20:4n-6	4.80 ± 0.17^a	3.66 ± 0.65^a	2.17 ± 0.65^b	4.34 ± 0.22^a
20:5n-3	tr^c	tr^c	0.34 ± 0.04^a	0.25 ± 0.02^b
22:4n-6	0.71 ± 0.14^a	0.56 ± 0.25^{ab}	tr^c	0.20 ± 0.01^b
22:5n-3	tr^b	tr^b	0.46 ± 0.14^a	0.69 ± 0.05^a
22:6n-3	tr^b	tr^b	0.16 ± 0.02^a	0.25 ± 0.04^a

*Refer to Table 3 for legend details. Results are expressed as nmol/mg protein.

TABLE 5

Fatty Acid Composition of Macrophage Sphingomyelin (SPH)*

Fatty acid	Corn	Borage	Fish/Corn	Fish/Borage
16:0	13.61 ± 1.84^a	10.05 ± 1.07^b	14.47 ± 0.48^a	14.58 ± 1.21^a
16:1n-7	tr^b	0.18 ± 0.11^{a,b}	0.25 ± 0.07^a	tr^b
18:0	1.08 ± 0.13	1.88 ± 0.54	1.39 ± 0.22	0.68 ± 0.10
18:1n-9	0.24 ± 0.03^{a,b}	0.59 ± 0.30^{a,b}	0.84 ± 0.35^a	0.13 ± 0.04^b
18:1n-7	tr^b	0.30 ± 0.21^{a,b}	0.53 ± 0.02^a	tr^b
18:2n-6	0.14 ± 0.03^b	0.25 ± 0.12^{a,b}	0.96 ± 0.48^a	0.08 ± 0.01^b
20:4n-6	0.16 ± 0.01^c	0.78 ± 0.02^a	0.34 ± 0.06^b	0.12 ± 0.02^c
22:1n-9	1.14 ± 0.54^b	1.56 ± 0.88^b	3.11 ± 0.32^a	0.18 ± 0.01^c
22:6n-3	tr^b	tr^b	0.62 ± 0.29^a	tr^b
24:0	0.93 ± 0.09^a	0.48 ± 0.07^b	0.94 ± 0.12^a	0.89 ± 0.02^a
24:1n-9	5.04 ± 0.69^a	2.96 ± 0.69^b	2.60 ± 0.33^b	3.86 ± 0.23^{a,b}

*Refer to Table 3 for legend details. Results are expressed as nmol/mg protein.

three subclasses of GPE are shown in Table 6. The major PUFA in all GPE subclasses was 20:4n-6 regardless of the diet fed. Consistent with previous published reports on rabbit alveolar macrophages (12) and guinea pig peritoneal macrophages (36), the content of 20:4n-6 was highest in the 1-*O*-alk-1′-enyl-2-acyl (plasmalogen) subclass relative to the 1,2-diacyl and 1-*O*-alkyl-2-acyl-GPE fractions. Fish oil supplementation consistently reduced n-6 PUFA in all GPE subclasses while concomitantly increasing n-3 PUFA levels, especially 22:5n-3. Animals consuming the borage diet exhibited significantly elevated ($P<0.05$) levels of 20:3n-6 relative to other diets in all three subclasses of GPE. Relative to the corn, borage and fish/corn dietary groups, fish/borage feeding reduced diradyl GPE 20:4n-6 levels 2–3-fold, while only slightly decreasing or even increasing 20:3n-6 levels. Table 7 shows the effect of dietary manipulation on GPC subclass fatty acyl composition. The predominant fatty chain was 16:0 in each GPC subclass. This contrasts with macrophage GPE subclasses which contained 18:0 as the predominant fatty acid chain. Similar to GPE subclasses, markedly higher levels of n-3 PUFA as 20:5n-3, 22:6n-3 and especially 22:5n-3 were found in all GPC subclasses following fish oil supplementation. The magnitude of the n-3 PUFA increase was greatest in the 1,2-diacyl GPC subclass. Borage/fish feeding resulted in higher 20:3n-6 levels relative to corn, fish or fish/corn groups in all GPC subclasses.

For comparative purposes, the relative class compositions as mol % of GPC and GPE are shown in Figures 1 and 2. High levels of 1,2-diacyl and 1-*O*-alk-1′-enyl-2-acyl were associated with macrophage GPE. In contrast, macrophage GPC contained primarily 1,2-diacyl and 1-*O*-alkyl-2-acyl linkages. Minimal amounts of 1-*O*-alkyl-2-acyl and 1-*O*-alk-1′-enyl-2-acyl were detected in GPE and GPC, respectively. This is consistent with Sugiura *et al.* (12,36) who reported similar subclass composition data for rabbit and guinea pig macrophages. The relative distribution of GPE 1,2-diacyl and 1-*O*-alkyl-2-acyl subclasses was significantly ($P<0.05$) different between fish oil supplemented dietary groups (fish/corn and fish/borage) and the corn and borage diet groups. Dietary treatment had no measurable effect on subclass contributions to total GPC (Fig. 2).

DISCUSSION

In order to determine the molecular mechanisms of how dietary fish oils influence macrophage immune responsiveness, we have initially chosen to examine

TABLE 6

Fatty Acid Composition of Macrophage Glycerophosphoethanolamines (GPE)*

Fatty acid	Corn	Borage	Fish/Corn	Fish/Borage
		Diacyl		
16:0	5.79 ± 1.09	4.32 ± 0.49	4.24 ± 0.09	4.64 ± 0.39
16:1n-7	0.21 ± 0.06^a	tr^b	0.17 ± 0.05^a	0.19 ± 0.01^a
18:0	11.99 ± 1.31^b	9.16 ± 0.89^a	9.61 ± 1.71^a	9.34 ± 0.63^a
18:1n-9	3.94 ± 0.12^b	2.57 ± 0.30^a	2.78 ± 0.44^a	2.10 ± 0.03^a
18:1n-7	1.17 ± 0.15^b	0.68 ± 0.08^a	0.94 ± 0.14^{a,b}	0.71 ± 0.03^a
18:2n-6	4.54 ± 0.18^c	1.72 ± 0.36^a	2.49 ± 0.44^a	1.31 ± 0.14^b
20:2n-6	0.66 ± 0.02^b	0.42 ± 0.12^a	tr^c	tr^c
20:3n-6	0.73 ± 0.02^b	1.50 ± 0.33^a	0.29 ± 0.15^b	1.23 ± 0.14^a
20:4n-6	7.32 ± 1.24^a	5.38 ± 1.01^{a,b,c}	2.72 ± 0.38^b	2.92 ± 0.16^{b,c}
20:5n-3	tr^c	tr^c	1.47 ± 0.31^a	0.39 ± 0.03^b
22:4n-6	1.80 ± 1.09^{a,b}	2.33 ± 0.40^a	0.22 ± 0.11^b	0.36 ± 0.01^b
22:5n-3	tr^c	tr^c	2.51 ± 0.49^a	1.24 ± 0.07^b
22:6n-3	0.52 ± 0.26^b	tr^b	1.46 ± 0.27^a	0.79 ± 0.06^a
		Alkylacyl		
16:0	0.67 ± 0.29	0.60 ± 0.15	0.68 ± 0.16	0.77 ± 0.23
16:1n-7	tr	tr	0.10 ± 0.08	0.15 ± 0.07
18:0	0.97 ± 0.20	0.96 ± 0.36	0.77 ± 0.19	0.53 ± 0.19
18:1n-9	0.87 ± 0.27	1.42 ± 0.72	0.28 ± 0.09	0.39 ± 0.12
18:1n-7	tr	tr	tr	tr
18:2n-6	0.56 ± 0.14	0.47 ± 0.10	0.20 ± 0.08	0.29 ± 0.13
20:2n-6	tr	tr	tr	tr
20:3n-6	0.11 ± 0.11^a	0.35 ± 0.13^a	tr^a	tr^a
20:4n-6	0.79 ± 0.17^b	1.19 ± 0.58^a	0.12 ± 0.06^b	0.21 ± 0.01^b
20:5n-3	tr	tr	tr	tr
22:4n-6	0.33 ± 0.04^b	tr^a	tr^a	tr^a
22:5n-3	tr^a	tr^a	0.16 ± 0.03^b	0.14 ± 0.01^b
22:6n-3	tr	tr	tr	tr
		Alkenylacyl		
16:0	0.46 ± 0.16	0.53 ± 0.14	0.49 ± 0.21	0.35 ± 0.01
16:1n-7	tr	tr	tr	tr
18:0	0.18 ± 0.07	0.39 ± 0.20	0.28 ± 0.15	0.33 ± 0.14
18:1n-9	0.73 ± 0.11^a	0.58 ± 0.22^{a,b}	0.46 ± 0.01^{a,b}	0.25 ± 0.03^c
18:1n-7	0.15 ± 0.02^a	tr^b	tr^b	tr^b
18:2n-6	0.66 ± 0.18	0.47 ± 0.03	0.45 ± 0.01	0.43 ± 0.03
20:2n-6	tr	tr	tr	tr
20:3n-6	0.62 ± 0.08^b	1.13 ± 0.15^a	tr^c	0.78 ± 0.01^b
20:4n-6	10.56 ± 1.35^a	7.92 ± 0.66^b	3.18 ± 0.20^c	4.62 ± 0.38^c
20:5n-3	tr^c	tr^c	1.93 ± 0.28^a	0.77 ± 0.05^b
22:4n-6	6.54 ± 0.59^a	3.96 ± 0.39^b	tr^d	0.59 ± 0.06^c
22:5n-3	0.18 ± 0.18^b	tr^b	2.60 ± 0.33^a	2.48 ± 0.25^a
22:6n-3	tr^c	tr^c	1.52 ± 0.29^a	0.68 ± 0.27^b

*Refer to Table 3 for legend details. Results are expressed as nmol/mg protein.

the ability of n-6 and n-3 fatty acids to alter macrophage membrane phospholipid class and subclass composition. In addition, prompted by the fact that no attempt has been made to quantitate ether lipid contribution to murine peritoneal macrophage phospholipids, the relative abundance of 1-*O*-alkyl-2-acyl, 1-*O*-alk-1′-enyl-2-acyl and 1,2-diacyl subclasses of peritoneal macrophage GPC and GPE was determined. Similar to rabbit alveolar (12) and guinea pig peritoneal (36) macrophages, mouse peritoneal macrophage GPC contains primarily 1-*O*-alkyl-2-acyl and 1,2-diacyl, and GPE contains 1-*O*-alk-1′-enyl-2-acyl and 1,2-diacylsubclasses. Examination of the data in Tables 3–7 demonstrates that dietary fatty acids are nonuniformly distributed among macrophage cellular phospholipids. The subclasses of GPC and GPE are characterized by different levels of PUFA, especially 20:4n-6. We report that mouse peritoneal macrophage 1,2-diacyl and 1-*O*-alk-1′-enyl-2-acyl GPE and 1-*O*-alkyl-2-acyl GPC subclasses are highly enriched in 20:4n-6. Similarly, GPI is highly enriched with respect to 20:4n-6. The specific distribution of 20:4n-6 into certain phosphoglyceride pools is maintained by a variety of phospholipid remodeling mechanisms (8,10).

Dietary consumption of fish oil containing n-3 PUFA significantly altered fatty acyl moieties of macrophage phospholipids as compared to animals receiving corn oil (rich in 18:2n-6). In general, increases in 20:5n-3, 22:5n-3 and 22:6n-3 were associated with a concomitant reduction of 20:4n-6 in GPS, GPI, GPE

TABLE 7

Fatty Acid Composition of Macrophage Glycerophosphocholines (GPC)*

Fatty acid	Corn	Borage	Fish/Corn	Fish/Borage
		Diacyl		
16:0	32.19 ± 1.90	28.91 ± 2.64	29.57 ± 3.43	32.95 ± 2.13
16:1n-7	0.89 ± 0.09	0.73 ± 0.18	0.94 ± 0.59	1.19 ± 0.06
18:0	9.17 ± 1.03	7.45 ± 0.38	6.27 ± 0.93	8.00 ± 2.76
18:1n-9	9.88 ± 0.84	7.41 ± 0.26	6.60 ± 1.27	7.85 ± 1.41
18:1n-7	2.74 ± 0.22	1.70 ± 0.07	2.30 ± 0.59	2.46 ± 0.69
18:2n-6	14.07 ± 0.66^a	6.40 ± 0.60^b	9.43 ± 2.24^a	7.21 ± 1.81^a
20:2n-6	1.62 ± 0.15^a	0.48 ± 0.03^b	0.25 ± 0.13^b	0.38 ± 0.10^b
20:3n-6	1.19 ± 0.11^b	3.35 ± 0.26^a	0.43 ± 0.22^c	3.96 ± 0.67^a
20:4n-6	7.38 ± 0.26^a	6.65 ± 0.62^a	2.98 ± 0.30^b	6.37 ± 1.60^a
20:5n-3	tr^b	tr^b	2.87 ± 1.00^a	0.35 ± 0.17^b
22:4n-6	1.19 ± 0.43^a	1.25 ± 0.37^a	tr^b	0.24 ± 0.12^b
22:5n-3	tr^c	tr^c	5.06 ± 0.82^a	1.69 ± 0.58^b
22:6n-3	0.16 ± 0.16^b	tr^b	1.07 ± 0.53^a	1.64 ± 0.42^a
		Alkylacyl		
16:0	6.03 ± 1.14	4.57 ± 0.94	2.48 ± 0.30	4.85 ± 1.14
16:1n-7	0.54 ± 0.22	0.35 ± 0.24	0.35 ± 0.11	0.35 ± 0.10
18:0	0.58 ± 0.03	0.60 ± 0.13^a	0.24 ± 0.06^b	0.96 ± 0.27^a
18:1n-9	2.25 ± 0.76	1.60 ± 0.08	1.82 ± 0.18	1.70 ± 0.13
18:1n-7	0.19 ± 0.08	0.28 ± 0.10	0.20 ± 0.10	0.33 ± 0.15
18:2n-6	4.09 ± 1.14^a	1.80 ± 0.38^b	3.86 ± 0.53^a	2.27 ± 0.02^b
20:2n-6	tr	tr	tr	tr
20:3n-6	0.43 ± 0.10^b	0.90 ± 0.18^a	tr^b	1.07 ± 0.20^a
20:4n-6	5.91 ± 1.47^a	4.46 ± 0.86^a	1.25 ± 0.08^b	2.92 ± 0.71^b
20:5n-3	tr^b	tr^b	0.68 ± 0.10^a	0.15 ± 0.07^b
22:4n-6	1.21 ± 0.42^a	0.92 ± 0.21^a	tr^b	0.28 ± 0.14^a
22:5n-3	tr^b	tr^b	0.83 ± 0.14^a	0.80 ± 0.25^a
22:6n-3	tr^b	tr^b	0.27 ± 0.14^a	0.48 ± 0.12^a
		Alkenylacyl		
16:0	1.17 ± 0.42	1.30 ± 0.59	0.48 ± 0.04	0.79 ± 0.47
16:1n-7	tr	tr	tr	tr
18:0	0.57 ± 0.19	1.59 ± 0.84	0.21 ± 0.19	0.53 ± 0.41
18:1n-9	0.48 ± 0.24	0.29 ± 0.13	0.98 ± 0.48	0.44 ± 0.17
18:1n-7	tr^b	tr^b	tr^b	0.10 ± 0.04^a
18:2n-6	0.19 ± 0.07^a	tr^b	0.20 ± 0.03^a	0.11 ± 0.03^{a,b}
20:2n-6	tr	tr	tr	tr
20:3n-6	tr	tr	tr	tr
20:4n-6	0.64 ± 0.14	0.36 ± 0.12	0.48 ± 0.15	0.30 ± 0.15
20:5n-3	tr	tr	tr	tr
22:4n-6	tr	tr	tr	tr
22:5n-3	tr^b	tr^b	0.34 ± 0.12^a	0.10 ± 0.05^a
22:6n-3	tr	tr	tr	tr

*Refer to Table 3 for legend details. Results are expressed as nmol/mg protein.

and GPC subclasses except for 1-*O*-alk-1′-enyl-2-acyl GPC. This is significant because PUFA of the n-3 series can modulate macrophage function (24) and eicosanoid synthesis and may ameliorate chronic inflammatory pathophysiological states (6,7,37). The reduction of 1-*O*-alkyl-2-arachidonoyl-GPC by n-3 PUFA administration is of interest because it is a precursor to platelet activating factor (PAF, 1-*O*-alkyl-2-acetyl-GPC). PAF is a potent bioactive phospholipid with broad pro-inflammatory properties and is produced by stimulated macrophages (38,39). The regulation of PAF production is important in the control of inflammation, since unregulated production could result in pathological effects (38,40). Several recent studies (41,42) have demonstrated a possible modulatory effect of dietary fish oil on PAF synthesis.

Administration of 18:3n-6 rich diets (borage and fish/borage mixture) to mice resulted in the accumulation of 20:3n-6 (2-carbon elongation product of 18:3n-6) in GPI, in 1-*O*-alk-1′-enyl-2-acyl and 1,2-diacyl GPE and in 1-*O*-alkyl-2-acyl and 1,2-diacyl GPC. Comparison of macrophage fatty acid composition showed that ether-containing GPE and GPC were less enriched with 20:3n-6 than the 1,2-diacyl species. The accumulation of 20:3n-6 is noteworthy, because this fatty acid can serve as a precursor for the synthesis of eicosanoids which may possess anti-inflammatory properties (43,44). The absence of 18:3n-6 and elevation of 20:3n-6 support our earlier observations in which the presence of an active long chain elongase and modest Δ5 desaturase activities in the peritoneal macrophage were documented (25,27).

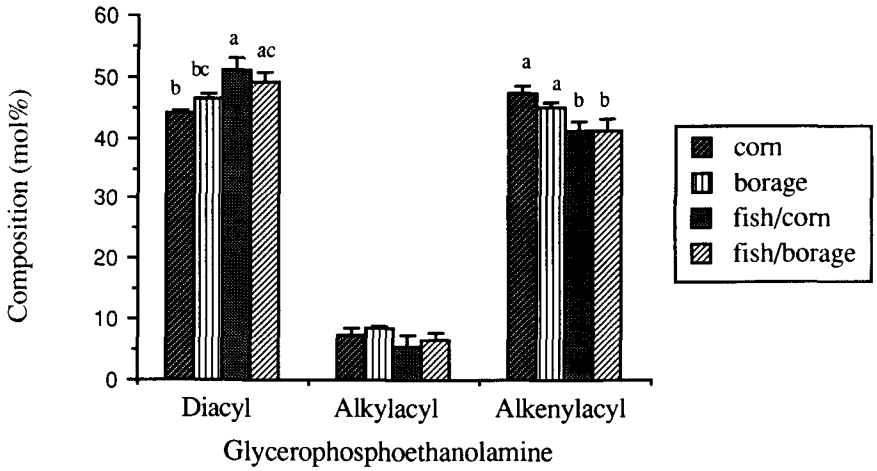

FIG. 1. Subclass composition of glycerophosphoethanolamines. Results represent mean ± SEM (n=4). Values with the same or no superscripts are not significantly different (P>0.05).

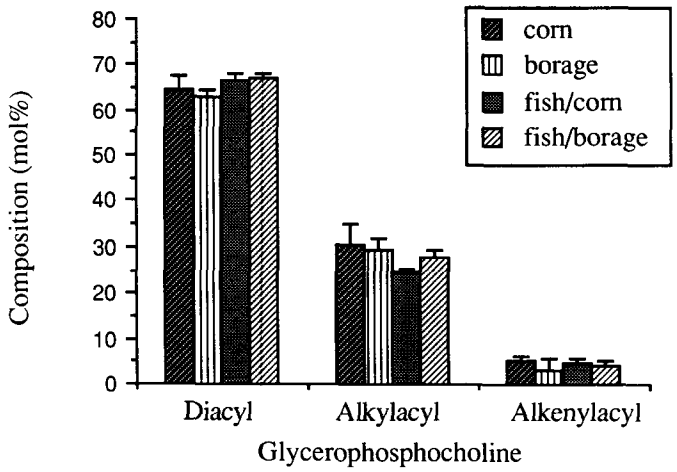

FIG. 2. Subclass composition of glycerophosphocholines. Refer to Figure 1 for legend details.

The combination of dietary 18:3n-6 and n-3 PUFA (fish/borage) produced the highest 20:3n-6/20:4n-6 fatty acyl ratio. GPS was the most malleable phospholipid with 20:3n-6 levels exceeding 20:4n-6 by approximately 1.5 times. In contrast, SPH and 1-*O*-alk-1′-enyl-2-acyl GPC contained only trace amounts of 20:3n-6. These results demonstrate that the phospholipid 20:3n-6/20:4n-6 levels can be more effectively manipulated by combined 18:3n-6 and n-3 PUFA administration than with 18:3n-6 administration alone. The increase in 20:3n-6 relative to 20:4n-6 is consistent with the ability of n-3 PUFA to inhibit Δ5 desaturation of 20:3n-6 to 20:4n-6 (25). The significance of the simultaneous reduction of 20:4n-6, a potentially pro-inflammatory eicosanoid antecedent, while increasing 20:3n-6, 20:5n-3, 22:5n-3 and 22:6n-3 levels (eicosanoid antecedents with anti-inflammatory potential) remains to be determined.

The mechanisms that control the initial incorporation and subsequent remodeling of PUFA among macrophage phospholipids are undoubtedly complex. Data from this study demonstrate that marked differences exist in the responses of phospholipid classes and subclasses following dietary lipid manipulation. Further studies are required to determine if specific macrophage phospholipid subclasses provide PUFA precursors for select eicosanoid products.

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Identification of Naturally Occurring *trans, trans* Δ5,9 Fatty Acids from the Sponge *Plakortis halichondroides*

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The first *trans* fatty acids from a sponge have been isolated from *Plakortis halichondroides*. The sponge was shown to contain the *iso* acids (5E,9E)-19-methyl-5,9-eicosadienoic, (5E,9E)-20-methyl-5,9-heneicosadienoic and (5E,9E)-21-methyl-5,9-docosadienoic acid, as well as the *anteiso* acids (5E,9E)-19-methyl-5,9-heneicosadienoic and (5E,9E)-20-methyl-5,9-docosadienoic acid together with the straight chain (5E,9E)-5,9-docosadienoic acid. The acids were shown by gas chromatography, Fourier transform infrared and ^{13}C nuclear magnetic resonance to contain the *trans* configuration. An eicosadienoic acid, namely (6E,14E)-6,14-eicosadienoic acid, and 12-methyl-5-octadecenoic acid were also identified in a sponge for the first time. The fatty acids were shown to be the principal constituents of phosphatidylethanolamine and phosphatidylcholine. No sterols were found in the sponge. The results presented in this work should be helpful in taxonomy of Homoscleromorpha.
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It is now well established that the phospholipids of marine sponges are a rich source of structurally unusual and biosynthetically interesting fatty acids. Common phospholipid fatty acids from marine sponges include 5,9-pentacosadienoic acid (25:2) and 5,9-hexacosadienoic acid (26:2), which can be found in many sponges as described originally by Litchfield and co-workers (1). Interestingly enough, branched fatty acids more than 26 carbons long also have been encountered recently in several sponges. For example, work by Ayanoglu *et al.* (2) and Carballeira *et al.* (3) with the sponges *Petrosia ficiformis* and *Jaspis stellifera* has revealed the presence of the unusual phospholipid fatty acids, 25-methyl-5,9-hexacosadienoic acid and 24-methyl-5,9-hexacosadienoic acid, which are interesting examples of *iso/anteiso* terminal methyl branching in these so called "demospongic" acids. Recently, we reported that the sponge *Ectyoplasia ferox* also contained members of the novel 28:2 *iso/anteiso* series, *i.e.,* the very long chain phospholipid fatty acids 25-methyl-5,9-heptacosadienoic acid and 26-methyl-5,9-heptacosadienoic acid, these being the longest (5Z,9Z) *iso/anteiso* phospholipid fatty acids described from any marine sponge known to date (4). Fatty acids of the *iso/anteiso* Δ5,9 series with chain lengths in the range between 21 and 25 carbons have not been described previously. Sponges analyzed to date have only been reported to contain (5Z,9Z)-branched fatty acids with chain lengths between 26 and 28 carbons. There are also no reports on naturally occurring (5E,9E)-dienoic fatty acids in sponges.

In our search for novel acids of unusual biochemical origin in Caribbean sponges around Puerto Rico we have found that the sponge *Plakortis halichondroides* (subclass Homoscleromorpha) contains the hitherto unreported 19-methyl-5,9-eicosadienoic acid (*iso*-21:2), as well as the 19- and 20-methyl-5,9-heneicosadienoic acids (the *iso/anteiso*-22:2 series), the straight chain 5,9-docosadienoic acid (22:2), and the novel 20- and 21-methyl-5,9-docosadienoic acids (the *iso/anteiso*-23:2 series). Furthermore, we have found that these fatty acids possess the unusual (5E,9E) configuration. In addition, we have encountered in *P. halichondroides* a new eicosadienoic acid, namely (6E,14E)-6,14-eicosadienoic acid (20:2) possessing the *trans,trans* arrangement, and the hitherto unknown fatty acid 12-methyl-5-octadecenoic acid.

EXPERIMENTAL PROCEDURES

Plakortis halichondroides was collected in July, 1989, near the shelf edge of La Parguera, Puerto Rico at a depth of 24 m. The sponge (500 g) was washed in sea water, carefully cleaned of all nonsponge debris and cut into small pieces. Immediate extraction with 700 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.* (5). The phospholipid classes were fractionated by preparative thin-layer chromatography (TLC) on silica gel G using chloroform/methanol/water (25:10:1, v/v/v) as solvent. ^{31}P nuclear magnetic resonance (NMR) and ^{13}C NMR spectra were measured at 22°C on a GN 300 Fourier transform (FT)-NMR spectrometer. In a typical ^{31}P NMR run, phospholipids (20–30 mg) were dissolved in 3 mL of deuterated chloroform/methanol (2:1, v/v) containing triphenylphosphine as internal reference. $CDCl_3$ was used as solvent for ^{13}C NMR measurements. The fatty acyl components of the phospholipids were converted to methyl esters by reaction with methanol hydrogen chloride (6), 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 (GC-MS) using either a Hewlett Packard 5995 A gas chromatograph/mass spectrometer or a Hewlett Packard 59970 MS ChemStation (Hewlett-Packard, Palto Alto, CA) equipped with a 30 m × 0.25 mm nonpolar fused silica column coated with DB-1. GC/FT-IR (infrared) spectra were recorded on a Nicolet 740 FT-IR spectrometer. For the location of double bonds, *N*-acylpyrrolidide derivatives were prepared by direct treatment of the methyl esters with pyrrolidine/acetic acid (10:1, v/v) in a capped vial (3 hr at 100°C), followed by ethereal extraction from the acidified solution and purification

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Abbreviations: ECL, equivalent chain length; FT-IR, Fourier transform infrared spectroscopy; GC-MS, gas chromatography/mass spectrometry; HPLC, high performance liquid chromatography; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; PE, phosphatidylethanolamine; TLC, thin-layer chromatography.

by preparative TLC. Hydrogenations were carried out in 10 mL of absolute methanol and catalytic amounts of platinum oxide (PtO_2).

Mass spectral data for the key fatty acids described are presented below: 12-Methyl-5-octadecen-pyrrolidide. MS(70 eV) *m/z* (relative intensity), 349(M^+,3), 335(0.3), 334(0.2), 320(0.2), 306(0.3), 294(0.1), 292(0.3), 288(0.1), 279(0.2), 278(0.7), 265(0.2), 264(0.6), 237(0.4), 236(1.8), 223(0.2), 222(0.5), 209(0.1), 208(0.6), 196(0.2), 195(0.4), 194(1.4), 182(0.3), 181(0.5), 180(2.5), 168(0.4), 166(1.4), 152(0.4), 140(0.7), 138(0.2), 126(6.8), 113(100), 98(9), 85(8), 70(6), 67(2), 57(4), 55(8).

6,14-Eicosadien-pyrrolidide. MS (70 eV), *m/z* (relative intensity) 361(m^+,4.7), 346(0.1), 332(0.2), 318(0.4), 305(0.2), 304(0.8), 291(0.3), 290(0.8), 276(0.4), 264(0.2), 250(0.3), 237(0.6), 236(4), 224(0.1), 222(0.5), 209(0.2), 208(0.7), 195(0.4), 194(1.6), 182(0.7), 181(0.7), 180(2.8), 166(1.5), 154(0.4), 140(0.7), 126(9), 114(7), 113(100), 98(9), 95(1.6), 85(8.5), 71(3), 70(7.5), 55(11).

19-Methyl-6,9-eicosadien-pyrrolidide. MS (70 eV), *m/z* (relative intensity) 375(M^+,2.4), 361(0.3), 360(0.5), 333(0.1), 332(0.3), 305(0.1), 304(0.3), 291(0.1), 290(0.4), 276(0.2), 262(0.2), 249(0.1), 248(0.3), 237(0.2), 236(1.3), 235(0.2), 234(0.6), 221(0.4), 220(0.5), 207(0.2), 206(0.3), 196(0.2), 195(0.2), 194(0.7), 182(0.7), 181(3), 180(19), 168(0.6), 167(0.5), 166(1.2), 153(0.4), 152(0.5), 140(1.1), 138(0.5), 126(12.5), 113(100), 98(14), 81(2.6), 79(2), 70(8), 55(11).

20-Methyl-5,9-heneicosadien-pyrrolidide. MS (70 eV), *m/z* (relative intensity) 389(M^+,2), 374(0.6), 234(1.4), 220(0.8), 181(4), 180(25), 166(0.8), 126(14), 113(100), 98(15), 85(10), 55(12).

19-Methyl-5,9-heneicosadien-pyrrolidide. MS (70 eV), *m/z* (relative intensity) 389 (M^+, 2.3), 180(23), 166(0.8), 152(0.5), 140(1.2), 126(14), 113(100), 98(15), 85(7.7), 79(3), 72(7), 70(9), 55(12).

5,9-Docosadien-pyrrolidide. MS (70 eV) *m/z* (relative intensity) 389(M^+, 2), 234(0.8), 220(0.9), 181(4.6), 180(23), 166(1.2), 140(1.4), 126(13), 113(100), 98(15), 85(6), 79(3), 55(13).

21-Methyl-5,9-docosadien-pyrrolidide. MS (70 eV), *m/z* (relative intensity) 403(M^+,2), 388(0.6), 360(0.2), 346(0.2), 332(0.1), 318(0.1), 304(0.2), 290(0.2), 276(0.1), 262(0.2), 248(0.5), 234(1), 220(0.8), 206(0.3), 196(0.1), 194(0.5), 181(5), 180(30), 166(0.7), 152(0.4), 140(1), 138(0.5), 126(15), 113(100), 98(15), 85(7), 72(7), 55(10).

20-Methyl-5,9-docosadien-pyrrolidide. MS (70 eV), *m/z* (relative intensity) 403(M^+,1.7), 388(0.3), 374(0.4), 341(0.2), 290(0.2), 276(0.2), 248(0.6), 234(1), 220(0.7), 206(0.4), 194(0.4), 181(5), 180(29), 166(1), 152(0.4), 140(1), 138(0.5), 126(15), 113(100), 98(14), 85(8), 72(6), 70(8), 55(11).

RESULTS

The phospholipid fatty acids from *P. halichondroides* are shown in Table 1. The fatty acid mixture showed a variety of branched fatty acids (60%) of which 25% were long chain fatty acids ranging from 21 to 23 carbons in length. Most of the shorter chain fatty acids occurred as mixtures of saturated *iso/anteiso* acids, which were characterized by their typical equivalent chain length (ECL) values and by the mass spectra of their pyrrolidides (7). Of the mixture of saturated branched short-chain fatty acids, 2-methyloctadecanoic acid is of special interest because it is the first 2-methyl substituted fatty acid found in a phospholipid of a sponge. The mass spectrum of the methyl ester displayed a molecular ion peak at *m/z* 312 and fragmentation peaks at *m/z* 101, 157, and 222. The base peak at *m/z* 88 was the key for characterization. It arises from a McLafferty rearrangement and matches what is reported for the 2-methyloctadecanoic acid methyl ester.

Another interesting fatty acid methyl ester from *P. halichondroides* showed a molecular ion peak at *m/z* 310, which is indicative of a nonadecenoic acid. A key fragment ion in the mass spectrum was a peak at *m/z* 255 (1.3%), which is not commonly produced by fatty acid methyl esters and which was considered indicative of methyl branching at C-12. In fact, upon catalytic hydrogenation, this acid was converted to a saturated-branched 19:0 fatty acid (M^+,312). The pyrrolidide of the original acid was the key for the definitive characterization, since it produced a diminished C_{12} fragment (*m/z*250) with concurrently enhanced C_{11} (*m/z*236) and C_{13} (*m/z* 264) fragments, consistent with methyl substitution at C-12 (Fig. 1). The double bond position was determined from the observed difference of 12 amu between C_5 (*m/z* 152) and C_4 (*m/z* 140), which is indicative of Δ5 unsaturation (7). Therefore, the compound in question was identified as 12-methyl-5-octadecenoic acid, which had not been encountered in nature before. Due to the small amounts of this acid present, we were not able to unequivocally determine the double bond configuration.

The second new fatty acid methyl ester in the mixture (11% abundance) displayed a molecular ion at *m/z* 322 which pointed towards an eicosadienoic acid methyl ester. The key fragments observed in this case at *m/z* 55, 67, 74, 81, 95, and 96 suggested the presence of a new eicosadienoic acid structure. Upon catalytic hydrogenation, the new acid was readily converted to the eicosanoic acid methyl ester which co-chromatographed in GC with an authentic standard. The spectrum of the corresponding pyrrolidide derivative was critical for determining the double bond location (Fig. 1). Differences of 12 amu were encountered between *m/z* 264 (C_{13}) and *m/z* 276 (C_{14}), which is indicative of Δ14 unsaturation, while the second double bond was found to be at carbon 6 based on the 12 amu difference between *m/z* 154 (C_4) and *m/z* 166 (C_5). A most interesting feature of this acid turned out to be its GC/FT-IR spectrum. The most striking finding was a reasonably strong band at 968 cm^{-1} and no absorption at *ca* 722 cm^{-1}, excluding the presence of any *cis* double bond. The 968 cm^{-1} band is known to be due to CH out of plane deformation of *trans* double bonds (8). A second band at 1304 cm^{-1}, due to the *trans* double bond CH in plane deformation, confirmed the results. We therefore concluded that the acid in question is the novel (6E,14E)-6,14-eicosadienoic acid.

A series of very interesting fatty acid methyl esters in *P. halichondroides* were six new branched *iso/anteiso* Δ5,9 acids (21 to 23 carbons in length). The new structures included the *iso* acids 19-methyl-5,9-

TABLE 1

Fatty Acids from the Phospholipids of *Plakortis halichondroides*

Fatty acid	ECL	Abundance (%)
12-Methyltridecanoic (*i*-14:0)	13.62	0.3
Tetradecanoic (14:0)	14.00	1.5
13-Methyltetradecanoic (*i*-15:0)	14.61	6.6
12-Methyltetradecanoic (*ai*-15:0)	17.73	2.7
3-Methylpentadecanoic (16:0)	14.92	1.0
Pentadecanoic (15:0)	15.00	2.2
14-Methylpentadecanoic (*i*16:0)	15.60	0.5
13-Methylpentadecanoic (*ai*-16:0)	15.73	1.8
9-Hexadecenoic (16:1)	15.80	1.0
11-Hexadecenoic (16:1)	15.83	0.3
Hexadecanoic (16:0)	16.00	6.0
15-Methylhexadecanoic (*i*-17:0)	16.60	6.6
15-Methyl-9-hexadecenoic (*i*-17:1)	16.42	2.0
2-Methyloctadecanoic (19:0)	16.76	4.3
14-Methylhexadecanoic (*ai*-17:0)	16.69	2.4
Heptadecanoic (17:0)	17.00	1.1
Methylheptadecanoic (18:0)	17.21	2.1
Octadecanoic (18:0)	18.00	1.3
12-Methyl-5-octadecenoic (19:1)[a]	18.28	6.0
17-Methyloctadecanoic (*i*-19:0)	18.59	5.0
16-Methyloctadecanoic (*ai*-19:0)	18.80	1.0
Nonadecanoic (19:0)	19.00	3.2
(6E,14E)-6,14-Eicosadienoic (20:2)[a]	19.80	11.0
(5E,9E)-19-Methyl-5,9-eicosadienoic (*i*-21:2)[a]	20.46	8.9
(5E,9E)-20-Methyl-5,9-heneicosadienoic (*i*-22:2)[a]	21.36	2.7
(5E,9E)-19-Methyl-5,9-heneicosadienoic (*ai*-22:2)[a]	21.50	1.0
(5E,9E)-5,9-Docosadienoic (22:2)[a]	21.75	3.8
(5E,9E)-21-Methyl-5,9-docosadienoic (*i*-23:2)[a]	22.47	11.2
(5E,9E)-20-Methyl-5,9-docosadienoic (*ai*-23:2)[a]	22.58	1.4
5,9-Tetracosadienoic	23.39	0.9

[a]These compounds have not previously been described to occur in nature.

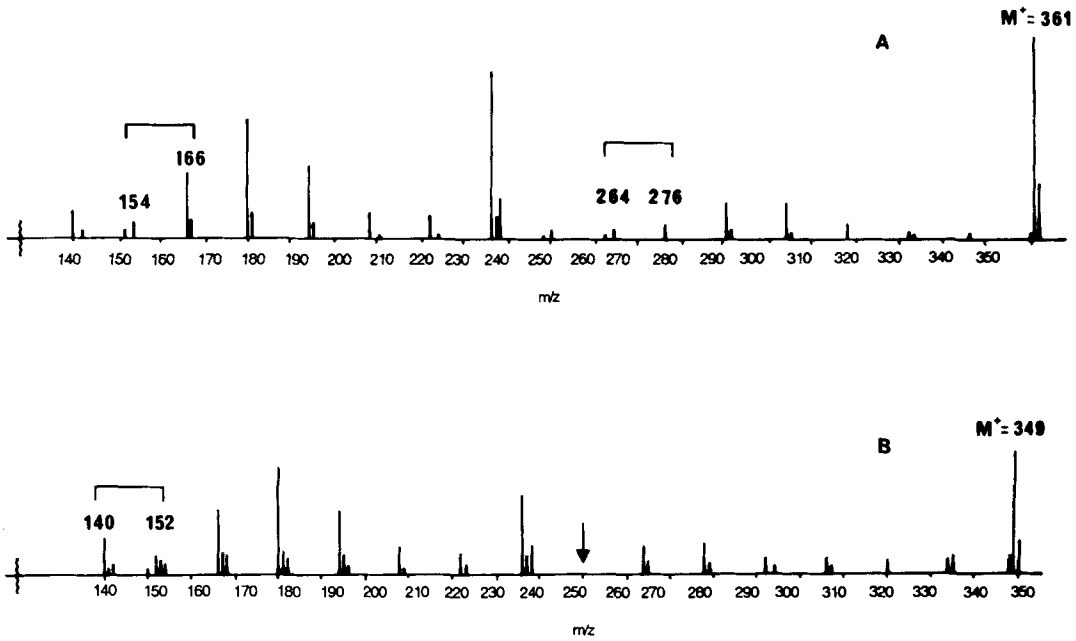

FIG. 1. (A) Partial mass spectrum of *N*-(6,14-eicosadienoyl)pyrrolidine. (B) Partial mass spectrum of *N*-(12-methyl-5-octadecenoyl)pyrrolidine.

eicosadienoic (*i*-21:2), 20-methyl-5,9-heneicosadienoic (*i*-22:2) and 21-methyl-5,9-docosadienoic (*i*-23:2) acid. The pyrrolidides of each of these acids were again the key to their characterization. All of the latter compounds displayed the characteristic difference of 12 amu between fragments *m/z* 152 (C_5) and *m/z* 140 (C_4) indicating unsaturation at carbon-5, while a second characteristic difference of 12 amu between fragments *m/z* 206 (C_9) and *m/z* 194 (C_8) established the second double bond at carbon-9. These derivatives also exhibited a very intense peak at *m/z* 180 due to doubly activated allylic cleavage between C_7 and C_8, resulting from the Δ5,9 unsaturation, as well as other peaks due to allylic cleavage between C_3 and C_4 (*m/z* 126) and between C_{11} and C_{12} (*m/z* 234). The Δ5,9 unsaturation was also consistent with occurrence of the base peak at *m/z* 81 of the corresponding fatty acid methyl esters since all Δ5,9 fatty acid methyl esters isolated to date display this peak, which is not found in other double bond combinations (2). Critical to the identification of *iso* branching was the observation that these hydrogenated fatty acid methyl esters displayed ECL values of 20.62, 21.63 and 22.69, which are all typical of *iso* acids (Fig. 2). On the other hand, from the pyrrolidide derivatives we were also able to clearly identify the methyl substitution at the ω−2 carbon. For example, the spectrum of *N*-(21-methyl-5,9-docosadienoyl)pyrrolidine (M^+, 403) displayed a peak of diminished intensity at *m/z* 374 with enhanced flanking peaks at *m/z* 360 and *m/z* 388 (Fig. 3). Similarly, the spectrum of *N*-(19-methyl-5,9-eicosadienoyl) pyrrolidine (M^+,375) displayed a less intense peak at *m/z* 346 with enhanced flanking peaks at *m/z* 332 and *m/z* 360. Since the *iso* acids 19-methyl-5,9-eicosadienoic (*i*-21:2) and 21-methyl-5,9-docosadienoic (*i*-23:2) acid were present in reasonable quantities (9 and 11% abundance, respectively), we performed GC/FT-IR analyses of their corresponding fatty acid methyl esters. In both cases we found a strong band at 968 cm^{-1} (*trans* double bond) in the absence of a band at *ca.* 722 cm^{-1} (*cis* double bond), and observed other informative bands at 1759, 1459, 1439, 1305, and 1169 cm^{-1}. The *trans* double bond stereochemistry in these Δ5,9 acids was also confirmed by ^{13}C NMR spectroscopy. The two methylenic carbons adjacent to *cis* double bonds occur at higher field (*ca.* to 26.4-27.4 ppm) than those bonded to *trans* double bonds, which resonate at 31.5-33.7 ppm (9,10). In order to obtain a reasonable ^{13}C NMR spectrum from the small amount of material, we isolated the total C_{21}-C_{23} *iso/anteiso* Δ5,9 acids from *P. halichondroides* by reverse phase high performance liquid chromatography (HPLC) for ^{13}C NMR analysis. For comparison, we also isolated *bona fide* (5Z,9Z)-fatty acid methyl esters from the sponge *Cribrochalina vasculum,* which contain the complete *iso/anteiso* C_{26}-C_{28} series (11). By having the ^{13}C NMR spectrum of a pure (5Z,9Z) mixture, we were able to compare the region between 30-35 ppm with the one obtained for *P. halichondroides,* and could identify the signals responsible for the methylenic carbons directly bonded to the *trans* double bonds, while we could eliminate common absorptions in the 30-35 ppm region arising from the ω−3 or C(2) carbons, the *iso/anteiso* terminal methyl branching, and others. In the Δ5,9 acids from *P. halichondroides,* we were able to identify the C-4, C-7, C-8, and C-11 carbons at 30.80, 32.27, 32.51, and 31.24 ppm, characteristic of methylenes adjacent to *trans* double bonds (Table 2). A second interesting fact that confirms the 5E,9E stereochemistry came from the ^{13}C NMR signal of the ester carbonyls. Mena *et al.* (9) had reported chemical shifts of 174.02 and 174.01 ppm, respectively, for the carbonyls of the 5Z,9Z and 5Z,9E isomers of 5,9-hexacosadi-

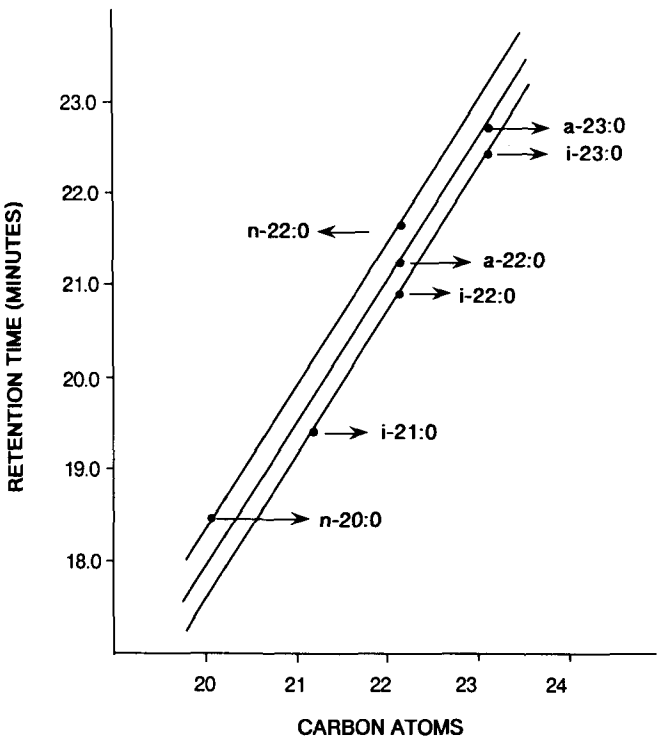

FIG. 2. Plot of retention time (minutes) *vs* number of carbon atoms for the hydrogenated long chain fatty acids (C_{20}-C_{23}) from *P. halichondroides.*

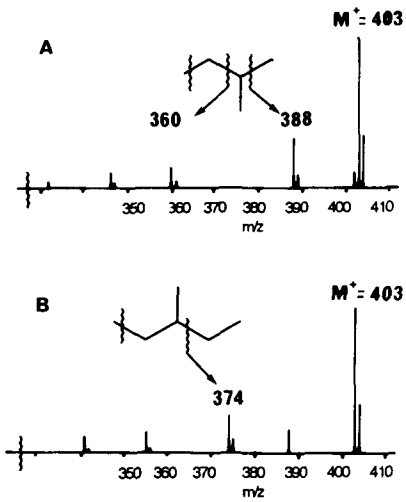

FIG. 3. (A) Partial mass spectrum of *N*-(21)-methyl-5,9-docosadienoyl)pyrrolidine. (B) Partial mass spectrum of *N*-(20-methyl-5,9-docosadienoyl)pyrrolidine.

enoic acid methyl ester, while the 5E,9Z isomer had a shift of 173.22 ppm and the 5E,9E isomer a shift of 173.45 ppm. In our purified C_{21}-C_{23} mixture, we only observed a signal at 173.55 ppm, quite consistent with the 5E,9E configuration. On the other hand, the purified *cis* -C_{26}-C_{28} mixture from *C. vasculum* only showed a carbonyl absorption at 174.35 ppm. The GC/FT-IR and NMR results clearly confirm the (5E,9E) fatty acid structure which has been found for the first time in nature.

The *anteiso* acids 19-methyl-5,9-heneicosadienoic (*ai*-22:2) and 20-methyl-5,9-docosadienoic (*ai*-23:2) acid were also identified in the mixture even though they were present in smaller amounts—namely at 1 and 1.4% abundance, respectively. The Δ5,9 unsaturation was established in a similar fashion as described above. The *anteiso* branching was established based on the fact that the hydrogenated fatty acid methyl esters gave ECL values of 21.79 and 22.79, which are typical for *anteiso* compounds (Fig. 2). The pyrrolidides also confirmed the structures since, for example, the spectrum of *N*-(20-methyl-5,9-docosadienoyl) pyrrolidide (M^+, 403) displayed a small peak at *m/z* 360 and an intense peak at *m/z* 374 (Fig. 3). GC/FT-IR analysis of these acids again showed the 968 cm^{-1} band indicative of trans unsaturation. The only remaining new fatty acid in the mixture, namely 5,9-docosadienoic (22:2) acid was easily characterized since it displayed the typical spectral characteristics for Δ5,9 unsaturation and was transformed into docosanoic acid upon catalytic hydrogenation and co-chromatographed in GC together with an authentic standard.

The phospholipid mixture from *P. halichondroides* was analyzed with the help of ^{31}P NMR and found to consist mainly of phosphatidylethanolamine (42%; PE) and phosphatidylcholine (35%; PC). Traces of phosphatidylinositol, phosphatidylserine and phosphatidylglycerol were also observed. The new acids described in this work were mostly concentrated in PE and PC. Interestingly in this context we were not able to identify any sterols in this sponge. This is surprising, since all eucaryotes are thought to contain complex sterols (12).

TABLE 2

Partial ^{13}C NMR Chemical Shifts of *iso/anteiso* Δ5,9 Fatty Acid Methyl Esters[a]

5Z,9Z	5E,9E
—	30.80
30.95	30.96
—	31.24
31.92[b]	31.93[b]
—	32.27
—	32.51
32.74	32.77
33.45	33.48
34.11[c]	34.12[c]

[a]The Z,Z isomers (C_{26}-C_{28}) were isolated from *C. vasculum* while the E,E isomers (C_{21}-C_{23}) are from *P. halichondroides.* Chemical shifts are given in ppm downfield from tetramethylsilane.
[b]These correspond to the ω−3 carbons.
[c]These correspond to the C-2 carbons.

DISCUSSION

Our finding of *iso/anteiso* (5E,9E) fatty acids in *P. halichondroides,* ranging from 21 to 23 carbons in length, presents evidence for new biosynthetic possibilities available to sponges. Some of the Porifera analyzed to date, such as *Ectyoplasia ferox* and *Cribrochalina vasculum* (4), have revealed that the typical *iso/anteiso* very long chain fatty acids with the Δ5,9 unsaturation found in Porifera are those ranging in length between 26 and 28 carbons. However, our present findings indicate that in *P. halichondroides,* fatty acids only reach chain lengths between 21 and 23 carbons and, more importantly, introduce *trans* Δ5,9, double bonds. *trans* Double bonds are rare in nature and are not produced in mammalian biosynthesis, but are formed by microorganisms in the gastrointestinal tract of ruminants. *trans* Double bonds are also produced in substantial amounts during industrial hydrogenation of *cis*-unsaturated lipids (13-14). Some plants have been shown to possess fatty acids with *trans* double bonds at either the Δ5 or Δ9 positions. For example, a 18:1 (5*t*) acid has been isolated from a *Thalictum sp.* and a 18:2 (9*t*, 12*t*) acid was found in *Chilopsis linearis* (14). It is also of interest in this context that, as we can see from Table 1, larger amounts of the *iso* acids are present in this sponge. In fact, only the *iso* 21:2 (Δ5,9) acid was detected and large amounts of the *iso*23:2 (Δ5,9) were observed (11%) *vs* 1.4% for the *anteiso* 23:2 (Δ5,9). As to the biosynthetic origin of the acids isolated in this work, we could postulate that they may arise from shorter chain saturated *iso/anteiso* acids that can be elongated and then desaturated by an unusual Δ5,9, dehydrogenase system that is only capable of introducing *trans* double bonds (15). A more likely alternative could be bond position shifts involving a *cis*-to-*trans* isomerization mechanism.

An interesting fatty acid from *P. halichondroides* is certainly 6,14-eicosadienoic acid (20:2), which has not been reported previously. Only few eicosadienoic acids are known, the most ubiquitous being (11Z,14Z)-11,14-eicosadienoic acid. However, there have been some reports on *trans* fatty acids in the marine environment, specifically those of the *trans*-6-hexadecenoic acid, which was first isolated from lipids of the ocean sunfish *Mola mola* (16) and later from the fats of the leatherback turtle *Dermochelys coriacea coriacea* (17). The origin of the 6,14-eicosadienoic acid identified in this work is quite uncertain at this point.

The final new compound we identified, namely 12-methyl-5-octadecenoic acid, could be biosynthesized from a 10-methylhexadecanoic acid which could be further elongated to 12-methyloctadecanoic acid and desaturated at carbon 5. While several isomers of both the C_{16} and C_{18} midchain, monomethyl-branched fatty acids (principally, 10-methyl 16:0, and 12-methyl 18:0) have been reported in bacteria (18), the Δ5 acid isolated in this work has not yet been reported. We therefore believe that this acid is probably of cyanobacterial origin.

In summary, our findings establish the presence of trans Δ5,9 double bonds in fatty acids of sponges. The information could be useful in the taxonomy of the species *P. halichondroides,* which belongs to the small

subclass Homoscleromopha and the order Homosclerophorida. A key question remains as to whether other members of this subclass also contain *trans* phospholipid fatty acids or whether the *trans* unsaturation is unique for the Plakinide. Work is in progress to answer this question of taxonomic relevance.

ACKNOWLEDGMENT

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Purification and Specificity of Lipases from *Geotrichum candidum*[1]

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A crude, commercial *Geotrichum candidum* lipase (EC 3.1.1.3) preparation (Amano GC-20) was purified by hydrophobic interaction chromatography on Octyl Sepharose. The purified enzyme is a microheterogeneous glycoprotein containing isozymes varying in molecular weight, pI and specificity. It consists of 64, 62 and 59 kDa species as determined by denaturing polyacrylamide gel electrophoresis. Five isozymes (pI 4.40, 4.47, 4.58, 4.67 and 4.72) are detected by isoelectric focusing using both silver and activity stains. Chromatofocusing was used to separate the isozymes according to pI. Although all the isozymes are specific for oleate *vs* stearate esters, one isozyme (pI 4.72) is also specific for oleate *vs* palmitate. The number of isozymes is reduced to two (pI 4.67 and 4.72) after carbohydrate removal using endoglycosidase F/N-glycosidase. These isozymes may be products of two lipase genes.
***Lipids 25,* 841–848 (1990).**

Lipases (EC 3.1.1.3) catalyze the hydrolysis of ester bonds at a lipid-water interface. Lipases may exhibit specificity for the position or type of fatty acid in a triglyceride, their natural substrate (1). Lipases also exhibit stereochemical specificity when reacting with a wide variety of substrates in organic solvents (2).

Lipases from *Geotrichum candidum* are known to show specificity for the hydrolysis of unsaturated fatty acids with a *cis*-double bond at the 9-position (oleic, linoleic, linolenic acids) *vs* the corresponding saturated fatty acid (stearic) (3). Some differences in specificity among different strains have been reported (3–8). The molecular bases for this specificity are unknown, although partial purifications of various lipases from *G. candidum* (9–12) have been reported. A low resolution X-ray structure (13) and a DNA sequence (14) have been reported for strain ATCC 34614 (9). The existence of two genes for *G. candidum* lipase from this strain has been reported very recently (15). A second lipase was found upon further purification of a preparation (14) previously believed to be homogeneous. The two lipases had the same substrate specificities, and similar amino acid compositions, but showed slight differences in molecular mass (15).

Unfortunately, the strain of *G. candidum* originally characterized (3) is no longer available (7). We have previously studied crude lipase from several strains of *G. candidum* (7) and found that while all the lipases were specific for the hydrolysis of oleate (O) *vs* stearate (S) esters, the fatty acid specificity for oleate *vs* palmitate (P) varied from 1.0 to 50 (that is, from no selectivity to high selectivity). In order to investigate the bases for these differences, we chose to study a commercially available strain, GC-20 (Amano), in detail. The purification, characterization and specificity of isozymes of GC-20 lipase are reported here.

MATERIALS AND METHODS

Materials. G. candidum lipase GC-20 lot 80106TS20, was a generous gift from Amano International Enzyme (Troy, VI). *G. candidum* lipase was also obtained from Germe (Marseille, France). Endoglycosidase F/N-glycosidase (endo F) was obtained from Boehringer Mannheim (Indianapolis, IN). Commercial olive oil was purified according to the procedure of Linfield *et al.* (16), or purified olive oil was obtained from Sigma (St. Louis, MO). Chromatography media were obtained from Pharmacia LKB Biotechnology (Piscataway, NJ) or Bio-Rad Laboratories (Richmond, CA). 4-Methylumbelliferyl (4-MUMB) esters were obtained from Sigma. All other materials were reagent grade or better.

Assays. Lipase activity was quantitated titrimetrically by an initial rate assay using an olive oil-gum arabic emulsion containing 11 mM $CaCl_2$ at pH 8.2, as described previously (17). A unit of lipase activity is defined as the release of one micromole of free fatty acid (FFA)/min at room temperature.

Lipase activity was qualitatively measured by detection of fluorescence on a Rhodamine-olive oil-agar plate (18). This method is more sensitive than the titrimetric method, and was used to monitor column fractions and native electrophoretic gels. An unstained native gel was laid on the surface of a Rhodamine-olive oil plate. Bands due to active lipase were visible under fluorescent light after about 30 min.

The method of protein assay was chosen to avoid interfering substances in the sample buffer. The bicinchoninic acid (BCA) method (19), available as a kit from Pierce (Rockford, IL), was used when detergent was present, and the Bradford method (20) (kit from Bio-Rad) was used when chromatofocusing buffer was present. Bovine serum albumin (Sigma) was used as standard.

The specificity of lipase for oleate *vs* stearate or palmitate was calculated from the initial rate of hydrolysis of the respective esters of 4-methylumbelliferone. Slight modifications were made to the procedure of Dooijewaard-Kloosterziel and Wouters (21). A stock solution of 1 mM substrate in *N,N*-dimethylacetamide (Alfa, Danvers, MA) was diluted to a final concentration of 50 μM in a buffer (pH 8.0) of 0.1 M

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Abbreviations: BCA, bicinchoninic acid; con A, concanavalin A; endo F, endoglycosidase F/N-glycosidase; FFA, free fatty acids; IEF, isoelectric focusing; kDa, kilodalton; 4-MUMB, 4-methylumbelliferyl; O, free or esterified oleic acid; P, free or esterified palmitic acid; PAS, periodic acid-Schiff reagent; S, free or esterified stearic acid; SDS PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TFMS, trifluoromethanesulfonic acid; Tris, tris(hydroxmethyl)aminomethane hydrochloride.

tris(hydroxymethyl)aminomethane hydrochloride (Tris), 5 mM $CaCl_2$. Fluorescence of the product was measured on a Sequoia-Turner Model 450 Fluorometer (360 nm excitation filter, 415 nm emission filter), standardized with 4-methylumbelliferone. The specificity for one substrate (A) *vs* another substrate (B) at one min reaction time was calculated using the Eq. [1] (22):

$$\text{Specificity}_{A/B} = \frac{V_A\,[B]}{V_B\,[A]} \qquad [1]$$

where the rate, V, was multiplied by the substrate concentrations at one minute. Note that low values of specificity (around 1) can be measured much more accurately than large values (around 100).

Electrophoresis. Polyacrylamide gel electrophoresis was performed using the pHast system (Pharmacia) with 7.5% or 12.5% acrylamide gels or isoelectric focusing (IEF) gels (pH 4–6.5) according to instructions provided by the manufacturer. Two-dimensional electrophoresis was run on the pHast system using native IEF gels (pH 4–6.5) and 12.5 acrylamide denaturing gel. Mini-slab (8 × 10 cm) vertical electrophoresis was also performed. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) was run according to Laemmli (23). Molecular weight standards were obtained from Diversified Biotech (Newton Centre, MA). pI Standards were obtained from Sigma (pH 3.6–6.6). Molecular weight measurements are averages of three to six denaturing gels of both 7.5 and 12.5% acrylamide; all measurements had a standard deviation of 1 kDa. Proteins were silver stained according to the method of Morrissey (24). Lipase activity was detected in native gels by an esterase stain (17) which was more sensitive than silver stain. It was confirmed that the esterase stain identifies lipase activity by comparing the position of esterase-positive bands with the position of active, fluorescent lipase bands from a duplicate gel placed on a Rhodamine-olive oil-agar plate. Periodic acid-Schiff reagent (PAS) (25) was used for staining carbohydrate.

Carbohydrate was also detected by concanavalin A (con A) labeled with peroxidase (Sigma). After SDS PAGE, samples were transferred to nitrocellulose by diffusion blotting. The nitrocellulose was blocked with deglycosylated bovine serum albumin (26), incubated with con A-peroxidase, and washed. Peroxidase activity was detected with 4-chloro-1-naphthol/H_2O_2.

Carbohydrate analysis. The amount of sugar in the purified lipase was measured by the phenol-sulfuric acid procedure (27), using mannose as the standard. Carbohydrate was removed by hydrolysis with trifluoromethanesulfonic acid (TFMS) for 2 hr (26) or by treatment with endo F (26) for 24 hr.

Purification. All procedures were performed at room temperature (21–25°C). GC-20 (3.6 g) was dissolved in a solution (pH 6.8) of 1 M ammonium sulfate, 10 mM imidazole-Cl, and 0.1 mM ethylenediaminetetraacetic acid, to a final concentration of 50 mg/mL. The sample was applied to an Octyl Sepharose column (1.5 × 48 cm, flow rate of 25 mL/hr) previously equilibrated in the same buffer, and the unbound proteins were washed off. When the absorbance at 280 nm returned to baseline, the buffer was switched to 10 mM imidazole-Cl (pH 6.8), and additional contaminants were eluted. Lipase was eluted by a linear gradient of 365 mL each of 10 mM imidazole and 10 mM imidazole with 0.5% polyoxyethylene 10-tridecyl ether (Emulphogene BC-720, Sigma). This is a non-ionic detergent very similar to Triton X-100, but non-absorbing at 280 nm (28).

Chromatofocusing was used for isozyme isolation. Active fractions were dialyzed against 0.025 M piperazine-Cl (pH 5.4), and applied to a column (0.7 × 50 cm, flow rate of 13 mL/hr) of Polybuffer exchanger 94 equilibrated in the same buffer. The amount of protein applied to the column was kept minimal in order to maintain high resolution. The column was later scaled-up (1 × 111 cm, flow rate of 28 mL/hr) to accommodate larger samples. Lipase was eluted with Polybuffer 74, pH 3.5 (1 column volume Polybuffer 74 diluted with 12 column volumes of water). Polybuffer was removed by desalting on Bio-Gel P-30 in water or 10 mM ammonium bicarbonate. The active fractions were lyophilized.

In a separate procedure, Con A-Sepharose was also used to partially purify the crude lipase. A Con A-Sepharose column was equilibrated in a buffer (pH 6.0) of 0.1 M sodium acetate, 0.2M NaCl, 0.01% thimersol and 1 mM each of $MnSO_4$, $MgCl_2$, and $CaCl_2$. Lipase was dissolved in the equilibrating buffer, applied, and subsequently eluted with 0.2 M α-methyl D-mannoside. However, due to tailing and poor recovery of activity, this method of purification was not pursued. Lipase that had been partially purified on the Con A-Sepharose column ("con A lipase") was used as a standard marking the position of the lipase isozymes in isoelectric focusing; it was very similar to the material referred to as lipase A below.

Initial attempts to purify the lipase by gel filtration on Sephacryl S-200 or Bio-Sil TSK 250 were abandoned because of a tendency of the lipase to be retained and to elute in broad bands on both columns.

Amino acid analysis. Lipase was hydrolyzed in 6 N HCl at 110°C for 24, 48 and 72 hr. The hydrolyzed amino acids were analyzed on a Beckman 119Cl amino acid analyzer (Beckman, Fullerton, CA). Serine and threonine values were determined by extrapolation to 0 hr. Cysteine and cystine were determined as cysteic acid, and methionine as methionine sulfone, after performic acid oxidation. Tryptophan was determined by pyrolysis gas chromatography (29).

pH Stability. Purified lipase was dissolved (0.5 mg/mL) in deionized water or 0.2 M buffer containing 5 mM $CaCl_2$. Citrate-phosphate buffer was used at pH 4, 5, 6 and 7, and Tris buffer at pH 7, 8 and 9. The samples were incubated at 30°C, and aliquots were assayed for activity titrimetrically at pH 8.2 as a function of time. The control was incubated in water at 4°C.

RESULTS

Purification. Conventional methods of enzyme purification showed the lipase separation to be rather complex. We have developed a rapid and relatively simple two-step procedure for purification of lipases involving Octyl Sepharose and chromatofocusing.

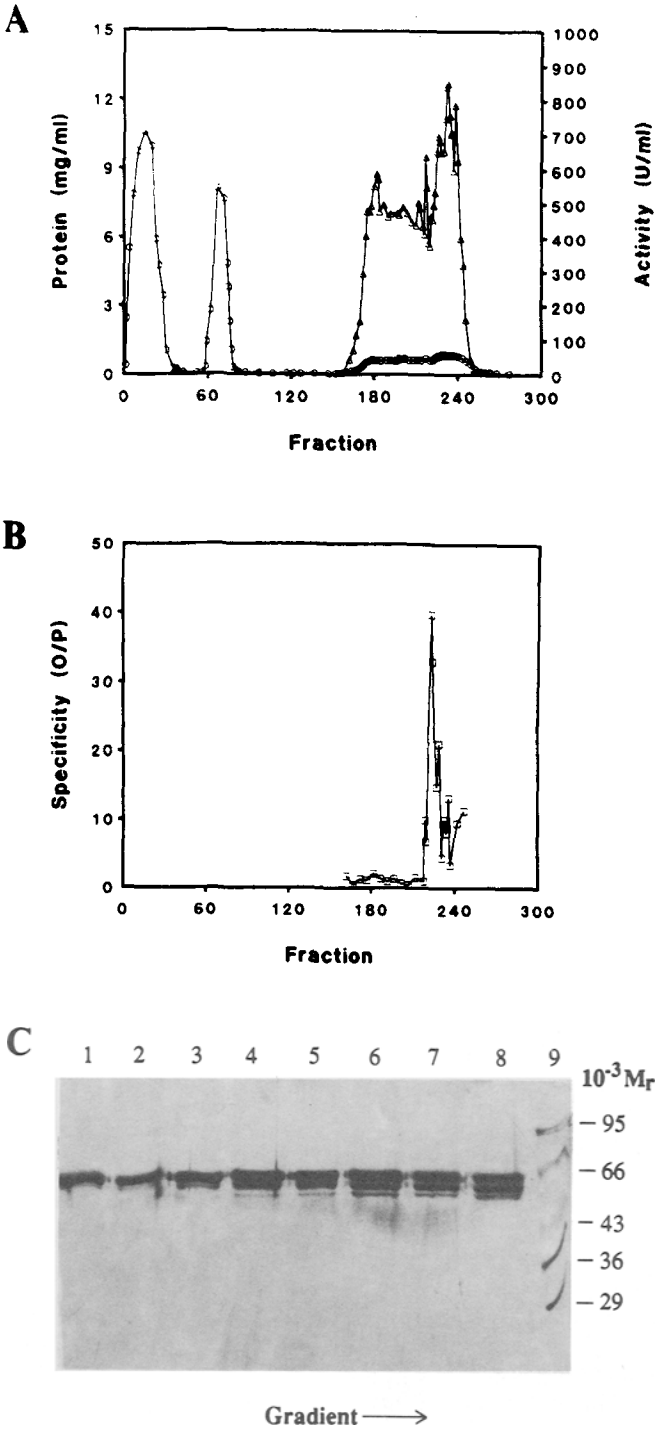

FIG. 1. Chromatography on Octyl Sepharose. Crude lipase was applied as described in Materials and Methods. Low ionic strength buffer was started at fraction 50, and the detergent gradient was started at fraction 120. Lipase B started eluting at fraction 211. Shown are (A), lipase activity (Δ), protein (○); and (B), specificity O/P (□). (C) SDS PAGE of detergent gradient fractions showing the transition from lipase A (lanes 1-3) to lipase B (lanes 4-8). Lane 9, molecular weight standards.

Chromatography of lipase on Octyl Sepharose is shown in Figure 1. Colored, nonactive material which reacts strongly in the BCA protein assay is not bound to Octyl Sepharose. Additional colored material is eluted with the low ionic strength buffer. Total lipase activity is eluted by the detergent gradient as two relatively broad peaks (Fig. 1A). However, lipase specific for oleate *vs* palmitate is found primarily in a narrow region of the second peak (Fig. 1B). All the fractions contain a number of components on SDS PAGE. The first peak (fractions 165 to 211) contains one main species of 64 kDa and a minor one of 62 kDa—it is referred to as lipase A. The second peak (fractions 212 to 246) contains the components in lipase A plus an additional species at 59 kDa—it is referred to as lipase B. SDS PAGE of fractions from a representative Octyl Sepharose column at the transition between lipase A (lanes 1-3) and lipase B (lanes 4-8) is shown in Figure 1C. The lipases contain no other contaminating bands by SDS electrophoresis at loadings of 0.5 to 3 μg, with silver staining. The purification achieved is shown in Table 1.

In order to investigate the identity of the multiple molecular weight species in lipases A and B, the samples were studied by IEF. Lipase A contains four main components by both silver stain and activity stains. Lipase B contains the same four components plus an additional component at more basic pI.

Chromatofocusing was then used to separate the components of lipase A and lipase B. In theory, this method separates proteins according to pI; however, proteins do not necessarily elute at their pI. The separation of representative aliquots of lipases A and B is shown in Figures 2 and 3, respectively. Lipase A contains 5 isozymes eluting at pH 4.25, 4.11, 3.99, 3.93 and 3.83 (minor). The aliquot of lipase B shown in Figure 3 contains 4 isozymes eluting at pH 4.24 (shoulder), 4.20, 4.09 and 3.93 (minor). The reproducibility of peak position *vs* pH among different chromatofocusing runs is good.

The purification factors for the chromatofocusing columns are shown in Table 1. The recovery is based on the sum of all peaks. A small increase in the specific activity was achieved. In order to obtain pure isozyme, the fractions were pooled according to isozyme content. Yield was sacrificed for purity.

Analytical IEF of the five isozymes of *G. candidum* lipase is shown in Figure 4. The isozymes have pIs of 4.72, 4.67, 4.58, 4.47 and 4.40, and are identified by the numbers 0, 1, 2, 3 and 4, respectively. Lipase A contains isozymes 1 to 4. Lipase B contains isozyme 0 in addition to isozymes 1 to 4. Con A lipase contains mainly isozymes 1-4; isozyme 0 is also found after extended incubation or with higher concentration samples.

The isozymes elute from the chromatofocusing column approximately, but not exactly, in order of decreasing pI; although band 0 is the most basic isozyme (pI 4.72), it elutes at pH 4.20, after isozyme 1 (pI 4.67, pH 4.25). This suggests that isozyme 0 differs in surface charge distribution and/or solubility as compared to the other isozymes.

Specificity. The specificity of GC-20 lipase at various stages of purification is summarized in Table 2. *G. candidum* lipase favors reaction with oleate over stearate at all stages; for the crude lipase, the specificity for 4-MUMB esters (specificity O/S) is 17. For lipase A the

TABLE 1

Purification of *G. candidum* Lipase

Step	Protein (mg)	Activity (U)	Specific activity (U/mg)	Recovered activity (%)	Purification factor
Octyl Sepharose					
Applied	1,030	81,828	79	100	1
Recovered	127	91,584	721	112	9
Chromatofocusing					
Lipase A					
Applied	11.1	8,748	790	10.78[a]	10
Recovered	7.7	7,869	1,023	9.6	13
Lipase B					
Applied	10.9	8,335	763	10.2[a]	10
Recovered	2.5	3,602	1,464	4.4	18

[a]Chromatofocusing was run on aliquots to avoid overloading the column.

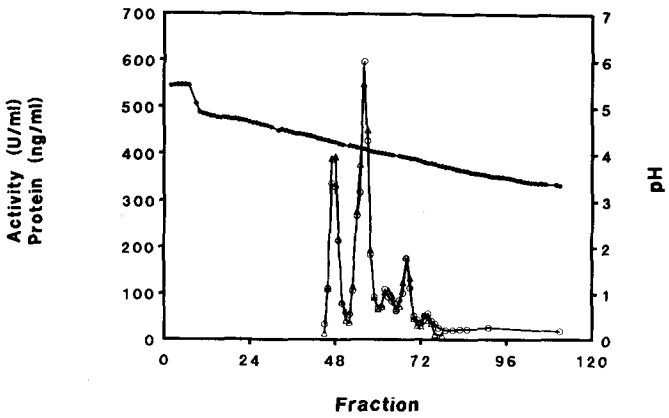

FIG. 2. Chromatofocusing of lipase A. Lipase A from the Octyl Sepharose column (21 mL of pooled fractions 165 to 197) was applied to a 0.7 × 50 cm polybuffer exchanger 94 column. Elution conditions are given in Materials and Methods. Shown are pH, (●); lipase activity (Δ); and protein, (○). The specificity O/P is 1 for all peaks.

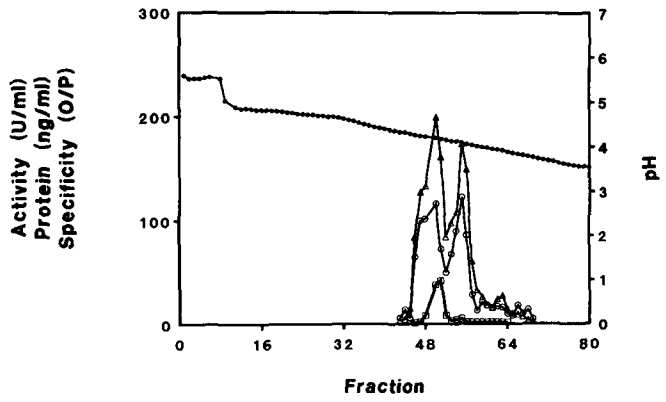

FIG. 3. Chromatofocusing of lipase B. Fractions 220 to 229 from the Octyl Sepharose column were applied to the column. Conditions as in Figure 2. Also shown is specificity O/P, (□).

specificity O/S is 15 ± 11 [averaged for 12 pure or mixed samples of isozyme(s) 1 to 4 after chromatofocusing]; for lipase B, it is 128 ± 105 (11 samples).

The specificity for 4-MUMB oleate *vs* 4-MUMB palmitate (specificity O/P) of the crude lipase (1.5 ± 0.4) is the same as that of isozymes 1 to 4 (1.4 ± 0.4); there is very little selectivity between oleate and palmitate. However, in the lipase B samples, the specificity O/P increases as the isozyme O content increases. The specificity O/P is 29 ± 8 in samples of isozyme 0 pure or mixed with a small amount of isozyme 1 (5 samples). Thus, the specificity for oleate *vs* palmitate characteristic of lipase B appears to be found only in isozyme 0.

Comparison of lipase A and lipase B. Lipases A and B were initially separated because of the presence of an additional, lower molecular weight component in SDS PAGE of lipase B (Fig. 1C). Lipase B also shows an additional, higher molecular weight component compared to lipase A on native PAGE with silver or activity stains (not shown). Lipase B has an additional, basic isozyme in IEF, and has higher specificity for 4-MUMB oleate compared to lipase A. However, amino acid analysis (Table 3) shows no significant difference between lipases A and B. Originally, it appeared that Con A-Sepharose separated lipases A and B, but small amounts of isozyme 0 (characteristic of lipase B) are indeed found in con A lipase.

The contribution of carbohydrate side chains to lipases A and B was investigated. Carbohydrate was removed from lipases A and B enzymatically by treatment with endo F. The endo F-treated lipases no longer stain with con A-peroxidase, indicating successful removal of the carbohydrate sites responsible for con A binding. Figure 5 shows native and endo F-treated lipases A and B after native IEF and incubation on a Rhodamine-olive oil plate. Activity is maintained after endo F treatment. Isozymes 2 to 4 have collapsed into isozyme 1 in both lipase A and B. Thus isozymes 2 to 4 appear to differ from isozyme 1 only by extent of glycosylation. Isozyme 0 appears to be unaffected by endo F according to IEF.

These conclusions were extended by two-dimen-

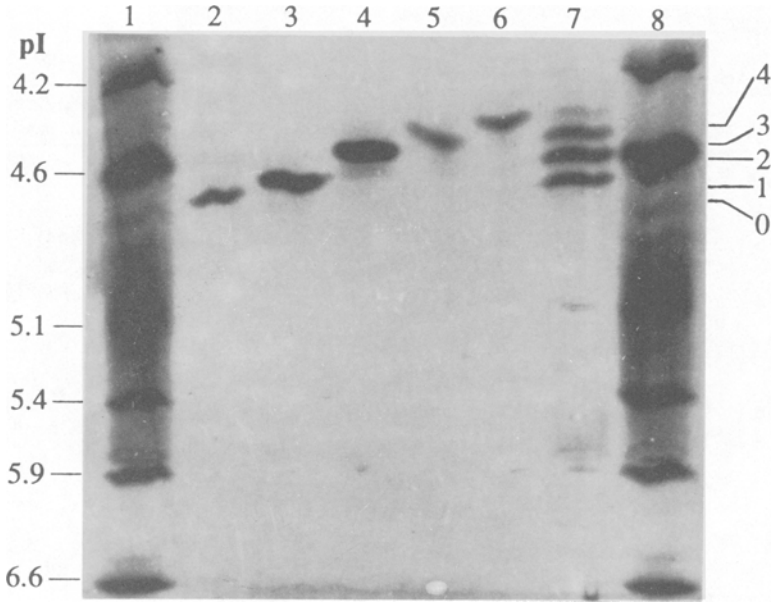

FIG. 4. IEF of lipase isozymes. The gel was run at 15°C and silver stained. Lanes 1 and 8, pI standards; lanes 2 to 6, isozymes 0 to 4, respectively; lane 7, con A lipase.

TABLE 2

Specificity of GC-20 Lipase at Various Stages of Purification

Sample	Specificity O/P	Specificity O/S
Crude lipase	1.50	17.0
Con A lipase	1.33	15.8
Lipase A	2.04	47.0
Lipase B	14.5	302
	8.68	68.8
Isozyme 0	36.7	246
Isozymes 0, 1	31.6	124
	24.7	51.6
	32.9	303
	17.4	95.8
Isozymes 0, 1, (2)[a]	14.9	47.1
Isozymes 0, 1, 2	20.2	92.6
Isozymes (0), 1	2.07	34.6
Isozymes (0), 1, 2	1.82	36.8
	3.28	64.4
	4.26	154
Isozymes (0), 1, 2, 3	3.27	25.0
Isozymes 1	1.00	7.4
	2.15	6.8
Isozymes 1, 2	1.68	12.3
Isozymes 1, 2, 3	1.04	9.0
Isozymes 2, 3	0.96	11.1
	1.55	19.2
Isozyme 3	1.41	7.6
	1.28	19.0
Isozymes 3, 4	0.82	9.1
	1.46	10.4

[a]Isozymes in parentheses are minor components. Multiple entries refer to different preparations, which may differ slightly in the ratio of isozymes present.

sional electrophoresis (Fig. 6). After endo F treatment, the four chain spots of lipase A are reduced to one. The five chain spots of lipase B are reduced to two, one equivalent to lipase A and an additional, more basic component. Endo F-treated lipase A has a main component of 60 kDa, while endo F-treated lipase B has species of 60 and 58 kDa (Fig. 7).

Characterization of lipase A. Lipase A was further studied because it appears to be a single protein varying only in degree of glycosylation, and is readily available. Lipase A contains 11.4% sugar by the phenol-sulfuric acid method. The carbohydrate was also hydrolyzed with TFMS; after this treatment, lipase A no longer stained with PAS stain or con A-peroxidase, indicating successful removal of the carbohydrate side chains. The hydrolysis results in a decrease in size to 60 kDa, similar to endo F treatment. Lipase A is compared to TFMS- and endo F-treated A in Figure 7.

Lipase A was found to be N-terminal-blocked when sequencing was attempted. Shimada *et al.* (14) also reported N-terminal blocking, and determined the N-terminal amino acid to be pyroglutamic acid. Lipase A and *G. candidum* lipase from Germe are very similar in molecular weight. The Germe lipase contains 2 isozymes apparently corresponding to isozymes 2 and 3 in lipase A (not shown).

Lipase A proved to be quite stable. After 24 hr at 30°C, 99 ± 4% of the initial activity remains in the control and in samples incubated in buffers at pH 5, 6, 7, 8 and in H_2O. After 90 hr, 86 ± 5% activity remains. At pH 4 and 9, activity is 90 ± 2% of initial at 24 hr, and after 90 hr, 58% at pH 4 and 73% at pH 9.

DISCUSSION

We have previously reported (7) significant variations among different strains of *G. candidum* lipase in the specificity for oleic *vs* palmitic acid esters of methanol or butanol. We can only speculate on the molecular bases for these differences and on their utility for the organism. At present, the mechanism of lipase catalysis and substrate binding is unknown. Information on the

TABLE 3

Amino Acid Composition (mole %) of *G. candidum* Lipase

Amino acid	Lipase A	Lipase B	Tsujisaka *et al.* (9)	Shimada[a] *et al.* (14)	Germe[b]	Vandamme *et al.* (11)
Lys	4.0	4.3	3.8	4.0	4.0	6.6
His	2.5	2.2	2.4	2.0	2.6	1.9
Arg	3.3	3.0	3.7	3.7	3.9	3.8
Asx	12.8	12.3	13.1	12.1	14.8	12.3
Thr	5.1	6.0	5.2	4.8	5.5	6.1
Ser	8.1	8.8	6.8	8.1	7.7	7.1
Glx	7.7	7.9	7.4	7.0	7.7	4.7
Pro	6.4	6.1	6.5	6.1	3.0	3.3
Gly	9.1	9.2	9.8	10.1	9.5	11.3
Ala	7.8	9.0	8.2	8.1	8.2	7.5
Cys	0.8[c]	0.9[c]	0.0	0.9	0.0	0.0
Val	5.4	5.7	6.0	5.7	5.2	9.4
Met	2.4[c]	2.2[c]	0.0	2.2	2.1	2.8
Ile	4.4	4.7	4.3	4.6	3.9	5.7
Leu	9.1	8.7	9.9	9.0	9.6	9.4
Tyr	4.8	3.9	4.4	4.8	4.8	4.2
Phe	5.4	4.9	6.6	5.5	5.7	2.8
Trp	0.8	ND[d]	1.8	1.3	ND	0.9

[a]Deduced from the nucleotide sequence.
[b]As reported in Vandamme *et al.* (11).
[c]Determined after performic acid oxidation.
[d]Not determined.

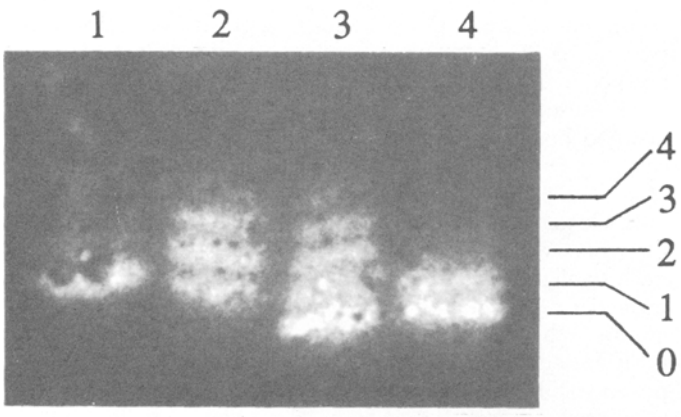

FIG. 5. IEF of native and endo F-treated lipases A and B with lipase activity stain. Lane 1, endo F-treated lipase A; lane 2, lipase A; lane 3, lipase B; and lane 4, endo F-treated lipase B.

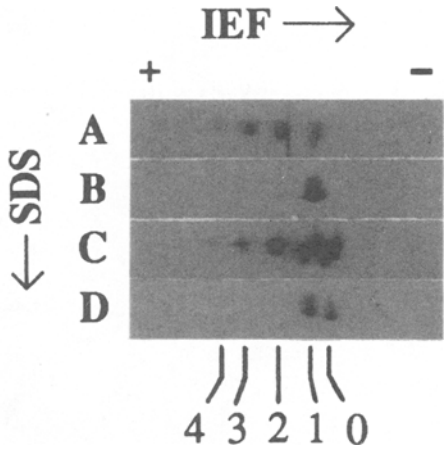

FIG. 6. Two-dimensional PAGE of (A) lipase A; (B) endo F-treated lipase A; (C) lipase B; (D) endo F-treated lipase B. The position of isozymes 0 to 4 is indicated.

actual differences among these strains and the isozymes present would contribute to the understanding of lipase and enzyme structure/function in general. As a means to investigate the biochemical basis for specificity differences, we undertook the purification of Amano GC-20 lipase.

Upon discovering the presence of isozymes in the crude lipase, we looked for, and found, specificity differences among the isozymes. Although the crude Amano *G. candidum* lipase does not select for oleate over palmitate, isozyme 0 (pI 4.72) does, while also retaining specificity against stearates. We will continue to study the basis for this specificity in this, and other, strains and with additional substrates.

The amino acid compositions of lipases A and B from Amano and those previously reported (9,11,14) are quite similar. The composition of the patented lipase (11) varies in Lys, Glx, Gly, Val and Phe content. Cysteic acid was found in lipases A and B after performic acid oxidation; no cysteines had been reported previously (9,11). The presence of cysteine is confirmed by the DNA sequence (14). Tsujisaka *et al.* (9) found no methionine, while greater than 2 mole percent is reported for each of the other lipases.

The lipase isozymes isolated here and the lipase from Germe are quite similar in molecular weight to those recently reported (12,14,15). Tsujisaka *et al.* (9) and Kroll *et al.* (10) report lipases of significantly lower molecular weights, as determined by gel filtration. A tendency for the lipase to be retained by gel filtration was observed; this would result in lower molecular weight determinations by this method. The molecular weight reported for the patented *G. candidum* lipase (11) is significantly lower, and suggests that either an

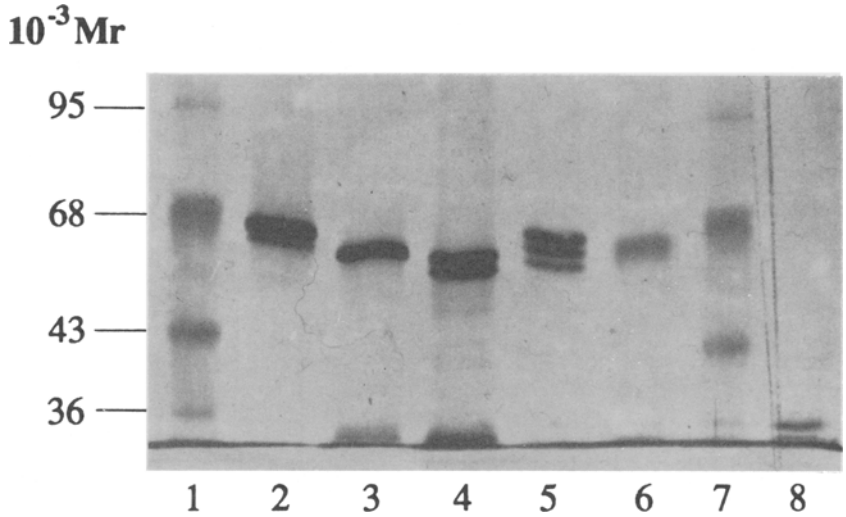

FIG. 7. SDS PAGE (7.5% polyacrylamide) of lipases A and B, endo F-treated lipases A and B, and TFMS-treated lipase A. Lanes 1 and 7, molecular weight standards; lane 2, lipase A; lane 3, endo F-treated lipase A; lane 4, endo F-treated lipase B; lane 5, lipase B; lane 6, TFMS-treated lipase A; lane 8, endo F.

active fragment or a distinct lipase is involved. The differences in amino acid composition also suggest this.

The heterogeneity of the isozymes of GC-20 lipase is partly due to microheterogeneity in the carbohydrate side chains, since the isozymes of lipase A reduce to one isoform (isozyme 1) after carbohydrate removal by endo F. However, both isozyme 0 and isozyme 1 remain after enzymatic carbohydrate removal from lipase B. Moreover, isozyme 0 of lipase B shows other significant differences beyond specificity differences. Isozyme 0 seems to be slightly more hydrophobic, and to have a slightly different charge distribution than the other isozymes, as shown by its longer retention time on Octyl Sepharose and chromatofocusing columns, respectively. This heterogeneity may be due to proteolytic or degradative breakdown. Heterogeneity in *Candida cylindracea* is postulated to be due to a conformational change induced during purification (30). However, it seems likely that isozymes 0 and 1 are distinct proteins from different genes, analogous to strain ATCC 34614 (15). The differences reported among the various strains of *G. candidum* may be due to the expression of multiple lipase genes.

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Formation of 8,15-Dihydroxy Eicosatetraenoic Acid *via* 15- and 12-Lipoxygenases in Fish Gill

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Metabolites of arachidonic acid are important regulators of biological function in a variety of mammalian tissues. We have demonstrated similar lipoxygenase enzyme activities in fish gills and mammalian lungs suggesting that their metabolites may have matching functions. Fish gills were investigated for their ability to generate dioxygenated metabolites of polyunsaturated fatty acids. Fatty acids, including arachidonic acid, were incubated with crude tissue homogenates and polar metabolites were extracted, derivatized and analyzed by high performance liquid chromatography, gas chromatography and mass spectrometry. The major dihydroxy metabolite of arachidonic acid was characterized as 8(L_R), 15(L_S)-dihydroxyeicosatetraenoic acid. This product was formed by the sequential action of the 15- and 12-lipoxygenases in the tissue. The formation of the dihydroxyeicosatetraenoic acid by crude tissue homogenates was significantly enhanced by the addition of 1 mM reduced glutathione. The metabolism of other polyunsaturated fatty acids, including eicosapentaenoic acid and docosahexaenoic acid, to dihydroxy acids was consistent with their relative ability to serve as substrates for the initial 15-lipoxygenase reaction.
***Lipids* 25, 849–853 (1990).**

The biosynthesis of oxygenated metabolites of polyunsaturated fatty acids is catalyzed by a diverse group of enzymes including cyclooxygenases, lipoxygenases and cytochromes (1–3). The high bioactivities of these metabolites has led to considerable research on the mechanisms by which they are formed (4,5). Recent evidence indicates that in certain pathways, one enzyme can catalyze the formation of several different classes of compounds, while in others, several enzymes act sequentially to form similar products. Thus, the 5-lipoxygenase catalyzes the initial addition of oxygen to arachidonic acid at position 5 and the hydroperoxide formed is either released by the enzyme leading to 5-HPETE (hydroxyeicosatetriaenoic acid) or further metabolized by the same lipoxygenase to a specific epoxide, leukotriene A_4 (LTA_4) (6). Recent evidence suggested that the 11,12- and 5,12-diHETE (dihydroxyeicosatetraenoic acid) formed from arachidonic acid are generated in platelets by the 12-lipoxygenase alone *via* an unstable epoxide intermediate (7). Similarly, the soybean lipoxygenase I generates two diHETE, the 8(S),15(S) and 5,15 products (8). Alternatively, in leukocytes the production of 8,15- and 14,15-diHETEs was shown to be catalyzed sequentially by a 15-lipoxygenase and a 12-lipoxygenase (6,9). Since these metabolites and their analogs exhibit different bioactivities (10–12), the regulation of this initial reaction and the divergence of products is of physiological importance. We have been investigating the 12- and 15-lipoxygenases isolated from fish gills as a model to study the reactions catalyzed by this enzyme class and the multitude of products which can be traced to this activity.

In this paper we report the synthesis of an 8,15-diHETE by fish gill tissue homogenates, and we show that the initial oxygenation reaction is catalyzed by the 15-lipoxygenase and that the subsequent formation of the 8,15-diHETE is catalyzed by the 12-lipoxygenase. The net balance of products formed was dependent upon the fatty acid substrate oxygenated and the concentration of reduced glutathione.

MATERIALS AND METHODS

Enzyme preparation. Fresh water rainbow trout and marine rockfish *sebastes* were used as sources for the enzyme. Both freshwater and marine species showed similar levels of enzyme activity, and the product profiles from arachidonic acid were also similar. From freshly killed animals, 3–20 g of gill tissue were removed and homogenized in 4 volumes of 0.05 M sodium phosphate buffer, pH 7.8. The crude homogenate was then partially purified by i) low-speed centrifugation at 15,000 × *g* for 15 min; ii) high-speed centrifugation at 70,000 × *g* for 90 min; iii) ammonium sulfate precipitation (30–45% saturation); and iv) hydroxy appatite chromatography (Bio-rad Laboratories, Richmond, VA). Enzyme preparations were used immediately or frozen in liquid nitrogen as 50 μL beads. Soybean lipoxygenase type 1 was purchased from Sigma Chemical Co. (St. Louis, MO) and 100 units/mL were dissolved in the reaction buffer immediately prior to incubation.

Enzyme assay. Enzyme preparations were added to 0.5 M sodium phosphate buffer pH 7.8 and incubated for 15 min with or without cofactors; glutathione, esculetin (Sigma), gold thioglucose (kindly provided by Dr. A. Tappel, UC Davis), epoxide hydrolase or phenyl chalcone (gifts of Dr. B. Hammock), and fatty acids (NuChek Prep, Elysian, MN) in ethanol were added with or without radiolabeled tracer ([^{14}C] arachidonic acid, NEN Research Products, Boston, MA) at the appropriate final concentrations. In radiotracer experiments, lipid products were extracted with 2 volumes of cold ethyl acetate from buffer acidified to pH 3.5 with dilute formic acid. In experiments without tracers, the lipid products were separated using solid phase extraction columns (Analytichem, Harbor City,

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Abbreviations: diHETE, dihydroxyeicosatetraenoic acid; HPETE, hydroperoxyeicosatetraenoic acid; LTA_4, leukotriene A_4, LTB_4, leukotriene B_4; GC/MS, gas chromatography/mass spectrometry; HPLC, high performance liquid chromatography; HPTLC, high-performance thin-layer chromatography; PUFA, polyunsaturated fatty acids; RP, performance reverse phase.

CA) following methods which we employed previously (13).

Product analysis. Radiolabeled products were separated by thin-layer chromatography high performance thin-layer chromatography (HPTLC) plates (Merck, Darmstadt, West Germany) as solvent system (14). Radiolabeled compounds were visualized by autoradiography. High performance liquid chromatographic (HPLC) analyses of chromophore-containing products were performed on a Hewlett Packard 1090 HPLC (Hewlett Packard, Norwalk, CT) equipped with a RP C-18 column (Supelco, Inc., Bellefonte, PA). The compounds were eluted using a linear gradient solvent system consisting of methanol/water (70:30–85:15, v/v) buffered with 5 mM ammonium acetate to an apparent pH of 5.7 with 0.5 mM EDTA added. UV spectra of eluting compounds were recorded automatically using the diode array detector. Retention times, extinction coefficients and spectra were compared and standardized with authentic compounds (leukotriene B_4, LTB_4; 12-HETE) which were kindly provided by Merck-Frosst (Montreal, Canada) or purchased from Cayman (Detroit, MI). For GC/MS analyses, a Hewlett-Packard Model 5890 GC interfaced to a VG Masslab system with integrated NBS-library was used. Chromatographic conditions were 180°C to 230°C at 10°C/min using a 25-m DB 23 column (J&W Scientific, Folsom, CA). Ionization energy was 70 eV, scan time was 1 second, and transfer line temperature was 240°C. Gas chromatography/mass spectrometry (GC/MS) analyses were performed on products after methylation with ethereal diazomethane and after forming trimethylsilyl ethers of free hydroxyl groups with BSTFA (Supelco).

RESULTS AND DISCUSSION

Most of the oxygenated products in fish gill tissue derived from exogenous arachidonic acid were ascribable to the 12-lipoxygenase reaction. The dominance and positional specificity of this enzyme is apparent from HPLC analyses of the monohydroxy derivatives shown in Figure 1. The enzyme generates a hydroperoxide which, after reductive metabolism, becomes the 12-hydroxy derivative of arachidonic acid. This reaction recently has been shown to be highly stereospecific and to favor the 12(S) hydroxyl product (15,16).

Further metabolism of the arachidonic acid was apparent from TLC analyses of the radiolabeled products. We had previously shown that in the absence of glutathione (16) or in the presence of glutathione peroxidase inhibitors, such as gold thioglucose (17), a variety of non-enzymatic breakdown products of the 12-hydroperoxide are found, including trihydroxy derivatives. These products indicate a non-specific breakdown of the labile 12(S) hydroperoxide formed from arachidonic acid by the lipoxygenase. With the addition of glutathione, these non-specific peroxidation products were found to decrease and the enzymatic reduction product, hydroxy fatty acid 12(S) HETE increased (17). Closer inspection revealed that in addition to the monohydroxy derivative, a more polar metabolite of arachidonic acid increased in response to glutathione addition. We therefore sought to investigate the nature of this compound and its mechanism of formation.

The polar arachidonic acid product exhibited HPLC chromatographic behavior and an UV absorption spectrum very similar to the diHETE compounds gener-

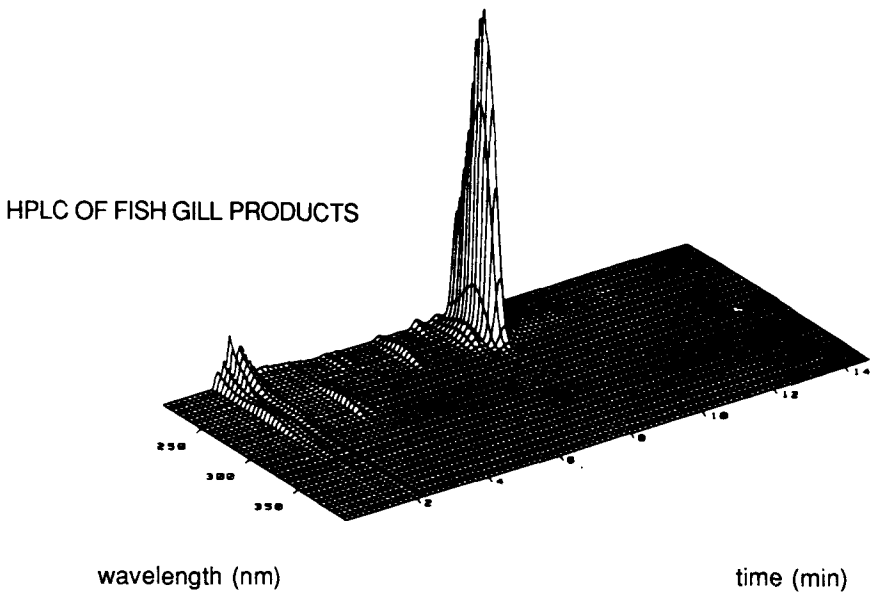

FIG. 1. Reverse phase HPLC separation and full spectral UV detection of the products of exogenous arachidonic acid metabolism by crude fish gill homogenate. Arachidonic acid was added to homogenate (final concentration 20 μM) and incubated for 10 min. Products were extracted with ethyl acetate and chromatographed as described. Major peak eluting with maximum absorbance at 233 nm corresponds to 12-HETE.

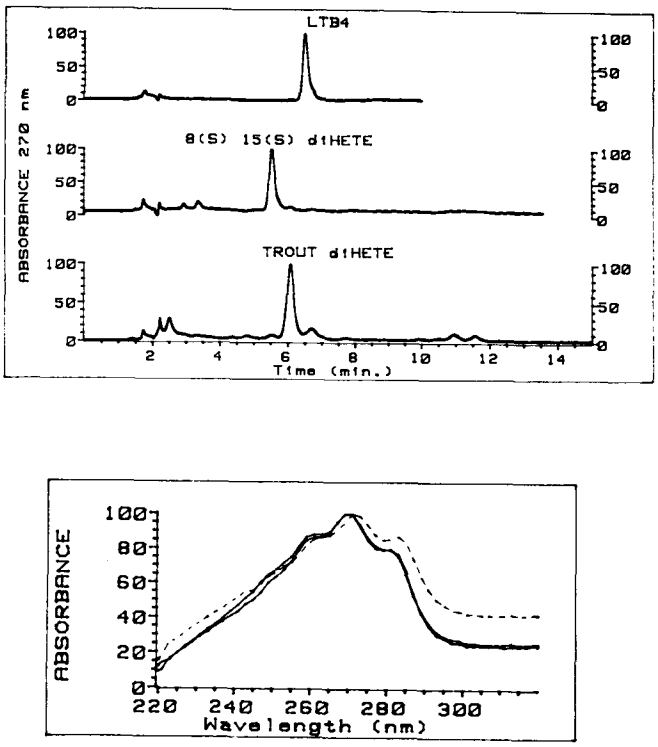

FIG. 2. Reverse phase HPLC separation (A) of diHETE material produced from arachidonic acid by trout gill preparation, soybean lipoxygenase 1 [8(S),15(S)-diHETE] and authentic LTB_4 run under identical conditions and UV spectra; and (B) of eluting trout diHETE (——), 8(S),15(S)-diHETE (——) and LTB_4 (---). Absorption spectra of the trout diHETE and 8(S),15(S)-diHETE were indistinguishable.

ated by mammalian leukocytes, *i.e.*, LTB_4 (Fig. 2a). High resolution HPLC and matching of absorption spectra, however, revealed that the retention time was shorter and the absorption spectrum was blue shifted by 2 nm (Fig. 2b). Therefore, the data indicated that the diHETE was not LTB_4 but suggested another arachidonic acid metabolite similar in structure. The compound was analyzed by GC/MS and a spectrum consistent with that of the 8,15-dihydroxy derivative of arachidonic acid was obtained (Fig. 3). Thus fish gill preparations generated an 8,15-diHETE from exogenous arachidonic acid. It was formed quantitatively at approximately 3% of 12-HETE in both marine and fresh water teleost fishes (data not shown).

We next sought to determine how this diHETE was produced. Support for a lipoxygenase reaction was provided by the observation that inhibition with the specific lipoxygenase inhibitor esculetin eliminated both the 12(S) monoHETE and the diHETE products. This failed to explain, however, how the 12-lipoxygenase managed to generate an 8,15-diHETE.

This seeming paradox was resolved by partial purification of the 12-lipoxygenase activity over hydroxyapatite. Elution of the enzyme material using a salt gradient resolved not only the 12-lipoxygenase, but a previously undetected 15(S)-lipoxygenase activity (German and Creveling, unpublished data). The 15-lipoxygenase was apparently inhibited in the presence of an as yet undetermined component from the crude homogenate, and separation over hydroxyapatite relieved the inhibition and gave rise to significantly enhanced 15-lipoxygenase activity. A series of remixing experiments and the elution of the lipoxygenases over hydoxylapatite (Fig. 4) demonstrated that the 8,15-diHETE arose as a consequence of initial oxygenation by the 15(S)-

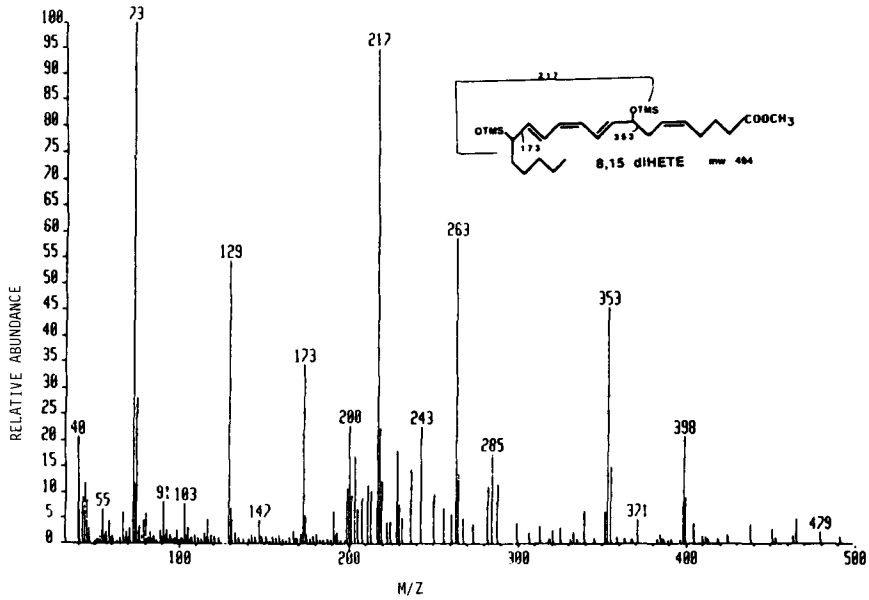

FIG. 3. Mass spectrum of diHETE material separated by GC as the methyl ester/trimethylsilyl ether derivative. Relative abundances are magnified ten-fold above *m/z* 200.

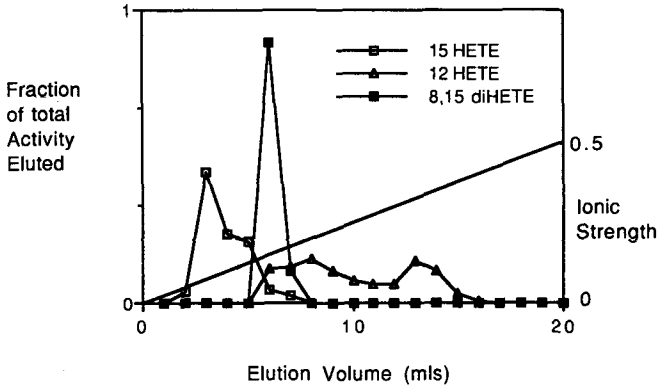

FIG. 4. Elution of fish lipoxygenase activities from an hydroxylapatite column. Fractions eluting from the hydroxylapatite were assayed using exogenous arachidonic acid (20 μM). Products were extracted and analyzed by reverse phase HPLC as described. 8,15-diHETE was the major diHETE observed and it was detectable only in fractions exhibiting both 15- and 12-lipoxygenase activities.

lipoxygenase, followed by a second reaction of the 15-lipoxygenase product with the 12-lipoxygenase. The product was not generated in detectable quantities by either enzyme alone. Evidence against an epoxide as an intermediate in the formation of the diHETE was provided by incubation studies with exogenous epoxide hydrolase or the epoxide hydrolase inhibitor phenyl chalcone. Addition of neither to the enzyme preparation altered the relative production of diHETE relative to 12-HETE.

The stereochemistry of the compound was determined as 8(L_R),15(L_S) diHETE by two pieces of evidence. Both the 15- and 12-lipoxygenases show very strong chiral selectivity and both produce the specific (L_S) monoHETE isomer. Therefore, the initial product of the 15-lipoxygenase was the 15(L_S). The second abstraction at carbon 10 and oxygen addition at carbon 8 (−2) would either retain the specificity of the 12(L_S) lipoxygenase for the L hydrogen and yield the 8(L_R) product or reverse the specificity, to yield the 8(D_S) product, analagous to the soybean reaction. To resolve the chirality of the second oxygenation, the diHETE from trout was analyzed on reverse phase (RP) HPLC relative to the 8(D_S),15(L_S) compound formed by soybean lipoxygenase 1 (8). The retention of the trout product was significantly greater than that of the soybean 8 (D_S),15(L_S)-diHETE product, but less than that of LTB_4 (Fig. 2), indicating an 8(L_R),15(L_S) structure. These results suggest that the chiral specificity of the 12-lipoxygenase for abstraction is retained but the oxygen addition is reversed to −2 to form the second 8 oxygen addition product.

The physiological function of the 8,15-diHETE is not known, but has been the subject of prior investigations. Kirsch *et al.* (18) have recently demonstrated that 8(S),15(S)-diHETE in canine trachea exhibited chemotactic properties comparable to LTB_4. Our discovery of production of 8(L_R),15(L_S)-diHETE as a single isomer in an evolutionary precursor to the lung, namely the fish gill, suggests that there may be a physiological function attributable to this pathway specific to these tissues.

Alternatively, the ability of lipoxygenases to catalyze the peroxidation of tissue components in mammals, including circulating low density lipoprotein has been well recognized (19). The results suggest that the high concentration of lipoxygenases in lung tissue may be a potentially damaging source of acyl hydroperoxides, especially if the activity of the lipoxygenase is uncoupled from natural controls, such as by a decrease in endogenous peroxidases.

Several different substrate PUFA were incubated with the tissue preparation to determine substrate preferences for the production of the respective diHETE generated by this pathway. Arachidonic acid (20:4n-6), dihomogammalinoleic acid (20:3n-6), eicosapentaenoic acid (20:5n3) and docosahexaenoic acid (22:6n-3) were all, in essence, equally active substrates for the 12-lipoxygenase. The production of the diHETE products however, differed significantly. Arachidonic acid and eicosapentaenoic acids were similarly metabolized by each enzyme and were metabolized to diHETE products to essentially equivalent extents. Docosahexaenoic acid, although metabolized readily by the 12-lipoxygenase to the 14 hydroxy product, is a poor substrate in terms of 15-lipoxygenase activity (German and Creveling, unpublished data). Although the dihydroxy product was formed, it was produced in significantly lower quantities than from the 20 carbon substrates. These results, if extended to mammalian metabolism *in vivo*, imply that the replacement of arachidonic acid with eicosapentaenoic acid in tissue would not cause quantitative changes in this pathway. Replacement of arachidonic acid by dihomogammalinoleic acid, or docosahexaenoic acids, however, would be predicted to have a significant suppressive effect on the amount of total metabolites produced.

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The Metabolism of 20- and 22-Carbon Unsaturated Acids in Rat Heart and Myocytes as Mediated by Feeding Fish Oil

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When rats were fed 5% corn oil, the heart phospholipids contained large amounts of 22-carbon (n-6) acids. When half of the corn oil was replaced with fish oil, the reduced level of arachidonate and 22-carbon (n-6) acids in phospholipids was accompanied by increases in the levels of 22-carbon (n-3) acids while only small amounts of 20:5(n-3) were acylated. Heart myocytes readily took up and acylated [1-^{14}C]-labeled 20:4(n-6), 20:5(n-3) and 22:6(n-3) into phospholipids. The uptake and acylation of 20:4(n-6) was greater than for 20:5(n-3) but the intracellular labeling profiles were similar. Uptake and acylation of 22:6(n-3) was somewhat lower. In addition the intracellular labeling profile differed in that more 22:6(n-3) was incorporated into the ethanolamine-containing phospholipids than when 20:4(n-6) or 20:5(n-3) were the substrates. Neither 20:4(n-6) nor 20:5(n-3) was chain elongated. When [3-^{14}C]-labeled 22:4(n-6) and 22:5(n-3) were the substrates, it was not possible to detect radioactive 22:5(n-6) or 22:6(n-3). Both [3-^{14}]-labeled substrates were acylated into phospholipids and retroconverted with the subsequent esterification of radioactive 20:4(n-6) and 20:5(n-3) into triglycerides and phospholipids. These studies show that cardiomyocytes lack the ability to make 22-carbon acids from 20-carbon precursors but they retroconvert 22-carbon acids to 20-carbon acids. The high levels of 22-carbon polyunsaturated acids in total heart lipids thus cannot be attributed to the synthetic capacities of cardiomyocytes.
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Heart phospholipids contain linoleate, arachidonate and 22:6(n-3) as the principal polyunsaturated fatty acids (1–3). The factors regulating the unsaturated fatty acid composition of heart phospholipids are poorly understood. Several studies suggest that heart has a very limited ability to desaturate and elongate fatty acids. Brenner (4) reported that heart microsomes converted small amounts of linoleate to 18:3(n-6). Heart contains malonyl-CoA (5,6) as well as an acetyl-CoA carboxylase which differs from the liver enzyme (7). However, we were unable to show that heart myocytes had the capacity to either desaturate or elongate fatty acids (8). Lefkowith *et al.* (9) reported that when [1-^{14}C]-arachidonate was given to rats raised on a fat-free diet, it was initially incorporated into liver lipids. Over time, there was movement of the labeled arachidonate to heart and kidney lipids. These results suggest that liver plays a major role in the uptake and modification of dietary fatty acids prior to their release into plasma for subsequent uptake and acylation into extraheptatic tissue such as heart.

In recent years there has been considerable interest in supplementing diets with (n-3) fatty acids to modify physiological processes. Dietary fat change obviously has the potential of modifying the fatty acid composition of all membrane lipids. Indeed, several studies have described how fish oil supplements alter heart phospholipid fatty acid composition (3,10–12). In the study reported here, we fed male weanling rats a diet containing either 5% corn oil or a diet in which half of the corn oil was replaced by fish oil. The fatty acid composition of individual heart phospholipids was analyzed and experiments were carried out with myocytes to define how they metabolized 20- and 22-carbon (n-3) and (n-6) fatty acids.

MATERIALS AND METHODS

Materials. Phospholipase C, essentially fatty acid-free bovine serum albumin, L-glutamine, pyruvic acid and *N*-2-hydroxyethyl piperazine-*N'*-2-ethane sulfonic acid (Hepes) were obtained from Sigma Chemical Co. (St. Louis, MO). Benzoic anhydride and 4-dimethylaminopyridine were from Aldrich Chemical Co. (Milwaukee, WI). Basal medium Eagle's amino acid solution (BME)- and minimum essential medium (MEM) non-essential amino acids were from Gibco Laboratories (Grand Island, NY). Collagenase was from Worthington Biochemical Co. (Freehold, NJ). LK5 and LK6 thin layer plates were from Whatman, Inc. (Clifton, NJ). All [1-^{14}C]- and [3-^{14}C]-labeled fatty acids (55 Ci/mol) were made by total synthesis using established methodology (13). Phospholipid standards were from Avanti Biochemical Inc. (Birmingham, AL) while methyl esters were from Nu-Chek Prep. (Elysian, MN) with the exception of 7,10,13,16,19-docosapentaenoic acid which was from Ocean Organics (Peace Dale, RI). All solvents were either reagent or high-performance liquid chromatography (HPLC) grade.

Animals and diets. Male weanling 21-day-old Sprague-Dawley rats were divided into two groups. One group was fed the AIN-76 diet which contained 5% corn oil (ICN-Biomedicals, Inc., Cleveland, OH). The second group received a modified diet in which 50% of the corn oil was replaced by menhaden oil. The composition of this batch of oil was previously described (14). The rats were maintained on these diets for six weeks.

Lipid isolation and separation. The animals were sacrificed using ether anesthesia. The hearts were minced in isotonic saline to remove blood, and lipids were extracted by the method of Bligh and Dyer (15). Neutral lipids were separated from phospholipids by

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Abbreviations: BME, basal medium Eagle's amino acid solution; CPG, choline-containing phospholipids; EPG, ethanolamine-containing phospholipids; GPE, *sn*-glycero-3-phosphoethanolamine; Hepes, *N*-2-hydroxyethyl piperazine-*N'*-2-ethane sulfonic acid; HPLC, high-performance liquid chromatography; MEM, minimum essential medium; PI, phosphatidylinositol; PS, phosphatidylserine.

silicic acid column chromatography by sequential elution with $CHCl_3$ and MeOH. The phospholipids were fractionated on LK5 plates which were developed with $CHCl_3$/MeOH/40% methylamine (60:20:5, v/v/v) (16). The lipids were visualized by spraying the plates with 0.1% (w/v) 2′,7′-dichlorofluorescein in ethanol. Individual phospholipid classes were recovered by extracting the silica gel twice with 5 mL of $CHCl_3$/MeOH/H_2O (5:5:1, v/v/v). To 10 mL of extracting medium was added 4.5 mL of $CHCl_3$ and 2 mL of H_2O. The upper layer was discarded and the solvent from the lower phase was removed under a stream of N_2. Ethanolamine-containing phospholipids (EPG) were reacted with phospholipase C (17). The resulting diradylglycerols were converted to benzoates according to Blank *et al.* (18). The 1,2-diacylglycerobenzoates were separated from the 1-*O*-alk-1′-enyl-2-acyl-glycerobenzoates by thin-layer chromatography on LK6 plates using benzene/hexane/diethyl ether (50:45:5, v/v/v) as solvent (19).

Individual phospholipids, as well as the two lipids derived from EPG, were reacted with 5% anhydrous HCl in methanol at 80°C for 1 hr. Methyl esters and dimethylacetals were recovered by the addition of water followed by extraction with hexane. Aliquots of hexane were injected into a Varian Vista 6000 gas chromatograph which contained a 10 ft by 2 mm i.d. glass column packed with 10% SP-2330 on 100/200 mesh Supelcoport (Supelco, Bellefonte, PA). Helium was the carrier gas (30 mL/min); the temperatures of the injector and detector were 240°C and 250°C, respectively. The oven temperature was held at 180°C for 17 min and then increased at 2°C/min to 190°C where it was maintained until the methyl ester of 22:6(n-3) had eluted. Methyl esters and dimethylacetals were identified by comparing retention times with authentic standards. Dimethylacetals were synthesized as described previously (20).

Metabolic studies with heart myocytes. Cardiac myocytes were isolated from 250–300 g male chow-fed Sprague-Dawley rats as previously described (21). The cells (2.5 mg protein) were incubated in Hepes buffer containing in mM: 25 Hepes, 118 NaCl, 4.8 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 1 $CaCl_2$, 5 glucose, 5 pyruvate, a complete mixture of amino acids (MEM and BME) and 2% (w/v) essentially fatty acid-free bovine serum albumin. The incubation volume was 1 mL and the fatty acid concentration was 0.1 mM with a specific activity of 10 mCi/mmol. Incubations were initiated by addition of the fatty acid complexed to bovine serum albumin. The incubations were carried out at 37°C in 95% O_2; 5% CO_2 in a shaking water bath. The incubations were terminated by addition of $CHCl_3$/MeOH (2:1, v/v). Lipids were extracted (15) and separated into neutral lipids and phospholipids by column chromatography. Neutral lipids were separated on LK6 plates using hexane/diethyl ether/acetic acid (80:20:2, v/v/v) while phospholipids were separated as described above. Individual lipids were scraped into scintillation vials and counted in ScintiVerse E (Fisher Scientific, Fairlawn, NJ). Phosphorus analysis was carried out as described by Rouser *et al* (22). Aliquots of triglycerides and total phospholipids were saponified by stirring overnight with 1M KOH in 95% ethanol. Following acidification with 4N HCl, the free fatty acids were recovered by extraction with hexane. The metabolism of fatty acid was quantified by HPLC using a 0.46 × 250 mm Zorbax ODS column. Acetonitrile/water (70:30, v/v) was used as solvent. The pH of the water was adjusted to 2.4 with H_3PO_4. The flow rate was 1 mL/min. Radioactivity was measured with a flow-through detector (Radiomatic Instruments, Tampa, FL). The flow rate of ScintiVerse LC (Fisher Scientific) was 3 mL/min.

RESULTS

The fatty acid composition of the choline-containing phospholipids (CPG) and the diacyl and alkenylacyl components from the EPG is shown in Tables 1 and 2. When fish oil was included in the diet there was a reduction in the level of arachidonate which ranged from 45 to 47% in the three lipid fractions. Surprisingly, the addition of fish oil to the diet resulted in 1.8-, 1.9- and 1.2-fold increases in linoleate, respectively, in CPG, diacyl-*sn*-glycero-3-phosphoethanolamine and 1-*O*-alkenyl-2-acyl-GPE. Only relatively small amounts of 20:5(n-3) were incorporated into any of the three lipid fractions in the fish oil-supplemented animals. Corn oil is a poor source of (n-3) fatty acids. There was thus the expected reduction in the level of 22-carbon (n-3) acids which was accompanied by an increase in the analogous 22-carbon (n-6) acids. However, this type of dietary fat change did not markedly alter the total content of 22-carbon acids. In the corn oil-fed animals the CPG, diacyl-GPE and the 1-*O*-alkenyl-2-acyl-GPE contained 6.1, 23.5 and 16.6 percent 22-carbon unsaturated acids, respectively. For the fish oil-fed animals, the corresponding values were 9.2, 26.9 and 23.7. Although the amounts of 22:5(n-3) and 22:6(n-3) were depressed in the corn oil-fed animals, the ratio of 22:6(n-3) to 22:5(n-3) was relatively independent of the type of dietary fat. The 22:6(n-3)/22:5(n-3) ratio in the corn oil-fed rats for GPG, diacyl-GPE and 1-*O*-alkenyl-2-acyl-GPE was respectively 1.5, 7.3 and 3.5. When fish oil was added to the diet, these ratios were 1.9, 6.3 and 2.6.

The fatty acid composition of phosphatidylserine (PS) and phosphatidylinositol (PI) is shown in Table 3. PS contained only 9.5% arachidonate. When fish oil was added to the diet, there was a 34% reduction in the arachidonate content. PI from corn oil-fed animals contained 32.8% arachidonate and its level was reduced by only 9% by feeding fish oil. PS, unlike PI, is characterized by a high level of 22-carbon unsaturated fatty acids. PS from the corn oil-fed animals contained 27.7% 22-carbon acids. When fish oil was added to the diet, this value was 32.7%. Again, both these lipids contained only small amounts of 20:5(n-3).

The compositional studies show that in general heart lipids tend to accumulate both arachidonate, and 22-carbon (n-3) acids but not 20:5 (n-3). In an attempt to further define what regulates heart lipid fatty acid composition, we studied the time-dependent metabolism of [1-^{14}C]-labeled 20:4(n-6), 20:5(n-3) and 22:6(n-3) in isolated cardiac myocytes. As shown in Figure 1, all three fatty acids were acylated into myocyte phospholipids. At all four time points, the specific activity of arachidonate was greater than for 20:5(n-3). The specific activity of [1-^{14}C]22:6(n-3) was consistently less

TABLE 1

Fatty Acid Composition of Heart CPG from Rats Fed Corn Oil or an Equal Mix of Corn Oil and Fish Oil[a]

Fatty acid	Wt % of total fatty acids diet	
	Corn oil	Corn oil-fish oil
16:0	18:1 ± 0.9	21.3 ± 0.6
18:0	24.7 ± 2.5	18.4 ± 0.4
18:1	8.7 ± 0.4	11.3 ± 0.6
18:2(n-6)	9.5 ± 2.6	16.7 ± 1.4
20:3(n-6)	0.2 ± 0.0	0.7 ± 0.0
20:4(n-6)	28.8 ± 2.1	15.3 ± 0.9
20:5(n-3)	—	1.5 ± 0.1
22:4(n-6)	1.6 ± 0.2	0.3 ± 0.0
22:5(n-6)	1.7 ± 0.1	—
22:5(n-3)	1.1 ± 0.3	3.1 ± 0.1
22:6(n-3)	1.7 ± 0.3	5.8 ± 0.4

[a]Values are means ± SE for three animals/dietary group.

TABLE 2

Fatty Chain Composition of EPG Subclasses of Heart from Rats Fed Corn Oil or an Equal Mix of Corn Oil and Fish Oil[a]

Fatty chain	Wt % of total fatty acids diet			
	Diacyl		Alkenylacyl	
	Corn oil	Corn oil-fish oil	Corn oil	Corn oil-fish oil
16:0D[b]	—	—	20.3 ± 0.6	23.7 ± 1.2
16:0	9.2 ± 0.3	10.6 ± 0.7	2.8 ± 0.8	2.5 ± 0.6
18:0D[b]	—	—	6.3 ± 0.1	7.5 ± 0.3
18:0	32.5 ± 0.1	29.9 ± 0.4	4.7 ± 0.6	1.8 ± 0.2
18:1D[b]	—	—	6.9 ± 0.9	9.6 ± 1.0
18:1	5.2 ± 0.5	6.2 ± 0.2	1.9 ± 0.4	2.0 ± 0.1
18:2(n-6)	4.0 ± 0.4	7.7 ± 0.8	1.6 ± 0.2	2.0 ± 0.2
20:3(n-6)	—	0.4 ± 0.1	—	—
20:4(n-6)	22.9 ± 0.8	11.8 ± 0.3	30.8 ± 1.9	17.0 ± 0.5
20:5(n-3)	—	1.8 ± 0.1	—	3.1 ± 0.2
22:4(n-6)	2.6 ± 0.1	0.2 ± 0.00	4.8 ± 0.3	0.9 ± 0.4
22:5(n-6)	10.9 ± 0.2	0.4 ± 0.1	4.1 ± 0.2	0.3 ± 0.2
22:5(n-3)	1.2 ± 0.1	3.6 ± 0.1	1.7 ± 0.2	6.3 ± 0.2
22:6(n-3)	8.8 ± 0.4	22.7 ± 1.5	6.0 ± 1.0	16.2 ± 0.7

[a]Values are means ± SE for three animals/dietary group.
[b]Dimethylacetals.

TABLE 3

Fatty Chain Composition of Serine and Inositol-Containing Phospholipids of Hearts from Rats Fed Corn Oil or an Equal Mix of Corn Oil and Fish Oil[a]

Fatty acid	Wt % of total fatty acids diet			
	PS		PI	
	Corn oil	Corn oil-fish oil	Corn oil	Corn oil-fish oil
16:0	7.1 ± 2.4	5.1 ± 0.3	6.0 ± 1.2	3.5 ± 0.2
18:0	38.1 ± 1.0	38.7 ± 1.9	40.2 ± 3.4	37.2 ± 0.3
18:1	6.5 ± 1.2	6.8 ± 1.0	2.7 ± 0.7	6.1 ± 0.6
18:2(n-6)	3.1 ± 0.2	4.3 ± 0.2	3.4 ± 0.8	6.4 ± 0.1
20:3(n-6)	1.5 ± 0.3	2.2 ± 0.1	1.0 ± 0.2	2.0 ± 0.2
20:4(n-6)	9.5 ± 1.6	6.3 ± 0.6	32.8 ± 3.2	29.8 ± 0.1
20:5(n-3)	—	0.9 ± 0.2	—	1.4 ± 0.1
22:4(n-6)	7.1 ± 1.2	2.0 ± 0.8	1.2 ± 0.2	1.2 ± 0.4
22:5(n-6)	9.2 ± 1.5	0.8 ± 0.3	0.6 ± 0.1	0.3 ± 0.1
22:5(n-3)	4.2 ± 1.6	6.1 ± 0.4	1.9 ± 0.8	3.4 ± 0.2
22:6(n-3)	7.2 ± 1.5	23.8 ± 2.7	1.2 ± 0.7	3.9 ± 0.3

[a]Values are means ± SE for three animals/dietary group.

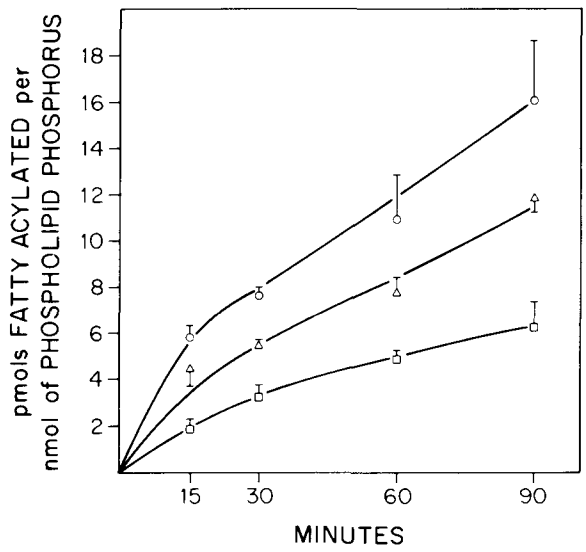

FIG. 1. Time-dependent incorporation of arachidonic acid (0), 5,8,11,14,17-eicosapentaenoic acid (Δ) and 4,7,10,13,16,19-docosahexaenoic acid (□) into total myocyte phospholipids. Results are the average ± range for two separate experiments.

than for either of the 20-carbon unsaturated fatty acids. The distribution of radioactivity in the individual phospholipids after a 60-min incubation is shown in Table 4. No major differences were observed in the percent distribution over time (data not shown). The percent distribution of arachidonate and 20:5(n-3) in myocyte phospholipids was similar. Both of these acids were preferentially incorporated in CPG and PI with smaller amounts in PS and EPG. When [1-^{14}C]-22:6(n-3) was the substrate, a greater percentage of the radioactivity was associated with EPG and smaller amounts were incorporated into PI than were found for the 20-carbon substrates. The results in Figure 2 show that there also was a time-dependent increase in the incorporation of all three unsaturated acids into myocyte triglycerides. Again arachidonate was more efficiently acylated than was either 22:6(n-3) or 20:5(n-3). At longer time intervals more 22:6(n-3) was incorporated into triglycerides than was 20:5(n-3). This effect may not reflect substrate specificity but may simply be due to the slower depletion of this substrate since arachidonate and 20:5(n-3) were both incorporated into phospholipids more efficiently than was 22:6(n-3).

In order to further examine the ability of heart myocytes to modify fatty acids, they were incubated with [1-^{14}C]arachidonate, [1-^{14}C]20:5(n-3), [3-^{14}C]22:4(n-6) or [3-^{14}C]22:5(n-3). When cells were incubated with either of the [1-^{14}C]-labeled 20-carbon acids, all of the radioactivity in the triglycerides and phospholipids was recovered as unmodified substrate. It was not possible to detect any chain elongated products, *i.e.*

TABLE 4

Percent Distribution of Radioactivity in Phospholipids After Incubating Cardiomyocytes for 60 Min with [1-^{14}C]-labeled 20:4(n-6), 20:5(n-3) or 22:6(n-3)[a]

	Substrate		
Lipid	[1-^{14}C]20:4(n-6)	[1-^{14}C]20:5(n-3)	[1-^{14}C]22:6(n-3)
EPG	8.2 ± 0.6	8.8 ± 2.3	28.5 ± 8.0
CPG	50.8 ± 4.1	41.9 ± 2.8	47.1 ± 8.5
PS	5.9 ± 0.2	7.1 ± 1.6	2.0 ± 0.3
PI	21.3 ± 1.4	27.5 ± 3.6	10.7 ± 0.6

[a]Values are means ± the range from two separate cardiomyocyte preparations.

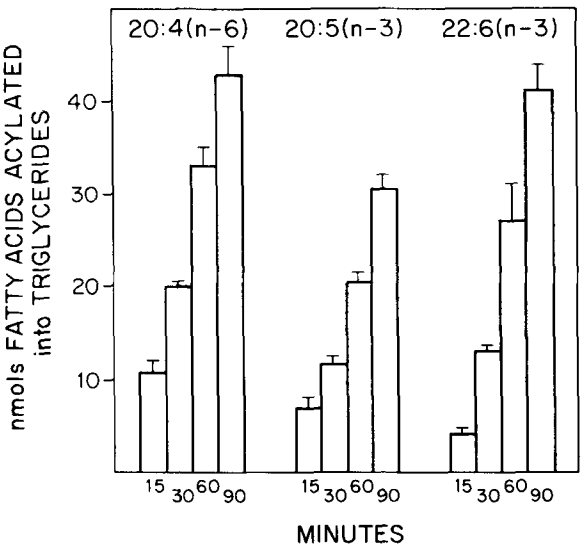

FIG. 2. Time-dependent incorporation of unsaturated acids into the triglycerides. Myocytes (2.5 mg of protein) were incubated with 0.1 mM substrate. Results are the average ± range for two separate experiments.

TABLE 5

Distribution of Radioactivity in Fatty Acids After Incubating Cardiomyocytes with Either [3-^{14}C]-labeled 22:4(n-6) or 22:5(n-3)[a]

Component	Phospholipids	Triglycerides
	[3-^{14}C]22:4(n-6)	
22:4(n-6)	2.8 ± 0.2 (52)[b]	88.6 ± 13.0 (93)
20:4(n-6)	2.6 ± 0.4 (48)	6.3 ± 1.7 (7)
	[3-^{14}C]22:5(n-3)	
22:5(n-3)	3.7 ± 0.1 (88)	96.3 ± 5.1 (96)
20:5(n-3)	0.5 ± 0.1 (12)	3.6 ± 0.9 (4)

[a]Values are means of analyses from three separate experiments in which results are expressed as nmols ± SE.
[b]Values in parentheses are percent distribution of radioactivity esterified in phospholipids and triglycerides.

radioactive 7,10,13,16-22:4 or 7,10,13,16,19-22:5. The results in Table 5 show how the two [3-^{14}C]-labeled 22-carbon fatty acids were metabolized. When [3-^{14}C]22:4-(n-6) was the substrate, 93% of the radioactivity in triglycerides was unmodified substrate and 7% was arachidonate. A very similar radioactive distribution pattern was found for [3-^{14}C]22:5(n-3). A markedly different labeling pattern was found in the total phospholipids. When [3-^{14}C]22:4(n-6) was the substrate, 48% of the radioactivity in phospholipids was 20:4(n-6) and the remaining 52% was unmodified substrate. When [3-^{14}C]22:5(n-3) was incubated with myocytes, only 12% of the radioactivity in phospholipids was 20:5(n-3) with the remainder being the substrate. With neither substrate was it possible to detect the product of a 4-desaturase, *i.e.* 4,7,10,13,16-22:5 or 4,7,10,13,16,19-22:6.

DISCUSSION

In the present study, rats were fed only 5% fat which is similar to the level found in chow. This study differs from several reports in the literature where higher levels of fish oil were fed to determine how heart lipid fatty acid composition was modified by dietary (n-3) acids (3,10-12). In addition, the fish oil-fed rats also received an equal amount of corn oil. The observed compositional changes thus cannot solely be attributed to the lack of linoleate for metabolism to longer chain (n-6) acids. In fact, the linoleate content in phosphoglycerides was somewhat higher in the fish oil-fed rats *versus* those that received only corn oil. When corn oil was fed as the sole source of fat, the heart phosphoglycerides contained relatively large amounts of 22-carbon (n-6) fatty acids. When fish oil was added to the diet, the decline in arachidonate content was accompanied by the almost total disappearance of 22-carbon (n-6) acids which in turn were replaced by 22-carbon (n-3) metabolites. This type of dietary fat change modified the types of 22-carbon acids in heart lipids but not the total combined level, *i.e.* (n-3) plus (n-6) 22-carbon acids. Only relatively small amounts of 20:5(n-3) were incorporated into phosphoglycerides. Clearly, mechanisms exist to selectively incorporate arachidonate *versus* 20:5(n-3) into heart phosphoglycerides. Conversely, there is a preference for esterifying 22-carbon (n-3) *versus* (n-6) acids.

The mechanism whereby heart obtains its unsaturated fatty acids for phospholipid synthesis is unclear. Stremmel (23) reported that fatty acid uptake by rat heart myocytes was mediated by a membrane protein. It is not known whether this protein exhibits specifici-

ties for various fatty acids. The studies reported here do show that arachidonate, 20:5(n-3) and 22:6(n-3) were all taken up and incorporated into both phosphoglycerides and triglycerides. The uptake and acylation of 20:5(n-3) into phospholipids was somewhat less than for arachidonate. However, neither arachidonate nor 20:5(n-3) was chain elongated. When cardiomyocytes were incubated with [3-^{14}C]-labeled 22:4(n-6) or 22:5(n-3), neither substrate was desaturated at position-4. These findings suggest that myocytes are not able to convert 20:4(n-6) or 20:5(n-3), respectively, to 22:5(n-6) and 22:6(n-3) *via* chain elongation and subsequent desaturation at position-4. When cultured myocytes were incubated in chemically defined media, the phosphoglycerides were enriched with the polyunsaturated fatty acids from the media (24,25). These studies also suggest that myocytes lack the ability to convert 18- (24) or 20-carbon (25) polyunsaturated fatty acids to 22-carbon (n-6) and (n-3) acids.

When [3-^{14}C]-labeled 22:4(n-6) and 22:5(n-3) were incubated with myocytes, 93% and 96% of the radioactivity in triglycerides were due to the respective substrates. A totally different type of labeling pattern was found in phospholipids. When [3-^{14}C]22:4(n-6) was the substrate, 48% of the phospholipid-associated radioactivity was arachidonate *versus* only 12% 20:5(n-3) when the cells were incubated with [3-^{14}C]22:5(n-3). The specificities for retroconversion and subsequent acylation are consistent with compositional findings for heart lipids. When 22:5(n-3) was presented to myocytes, it was preferentially acylated into lipids rather than converted to 20:5(n-3) followed by acylation. Conversely, much of the 22:4(n-6) was retroconverted to arachidonate followed by its acylation into membrane lipids.

These and our previous studies (8) suggest that cardiomyocytes do not have the capacity to synthesize unsaturated fatty acids for their own or heart membrane phosphoglyceride synthesis. Conversely, cardiomyocytes have the capacity to modify exogenous 22-carbon (n-3) and (n-6) acids by retroconversion to provide a supply of arachidonate and perhaps 20:5(n-3).

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METHODS

Separation of 20:4n-6 and 20:4n-7 by Capillary Gas-Liquid Chromatography

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The use of a capillary column coated with 100% cyanopropyl polysiloxane (CP™Sil 88) allows the separation of several fatty acids associated with fat deficiency. Starting with liver mitochondrial phospholipids of weanling rats fed a fat-free diet, an unusual fatty acid was isolated, along with 20:4n-6, by thin-layer chromatography on $AgNO_3$-impregnated silica gel plates. After partial hydrazine reduction of these acids, the resulting monoenes were isolated and subjected to ozonolysis in BF_3/methanol. The resulting monomethyl and dimethyl esters were identified by gas chromatography/mass spectrometry. Our data indicate that the unusual component corresponds to 20:4n-7. Based on published biochemical and analytical studies and on our own chromatographic retention data, some of the other unusual fatty acids were tentatively identified as 18:2n-7, 20:2n-7 and 20:3n-7. The CP™Sil 88 column appears to be a simple and useful tool for the separation of fatty acids of the palmitoleate series. ***Lipids 25*****, 859–862 (1990).**

Several investigators (1–11) have observed that fat-deficient diets can lead to the appearance of polyunsaturated fatty acids of the n-7 series in phospholipids. These acids include 18:2Δ8,11 (4,5,7,8,11), 18:3Δ5,8,11 (2,8,11), 20:2Δ10,13 (11), 20:3Δ7,10,13 (1,4–8,10,11) and 20:4Δ4,7,10,13 (3–9,11) acids. Unfortunately, these acids are usually not well resolved from similar fatty acids by gas-liquid chromatography (GLC) on packed columns (6,7,9,12,13). Complex procedures were thus needed to detect these components (1–10). On the other hand, n-7 acids are expected to be better separated on highly efficient capillary columns (14,15).

In our study of the fatty acid profile of liver mitochondrial phospholipids from rats fed a fat-free diet, several small peaks associated with fat-deficiency were noted on chromatograms obtained by GLC on different capillary columns. In this report, we demonstrate that one of these peaks corresponds to 20:4n-7 and that it can be separated easily by GLC on a CP™Sil 88 column. We also give some chromatographic data indicating that other members of the n-7 series are also well resolved on this column.

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Abbreviations: $AgNO_3$-TLC, thin-layer chromatography on silica gel impregnated with $AgNO_3$; DME, dimethyl esters; ECL, equivalent chain length; EFA, essential fatty acid; FAME, fatty acid methyl esters; FCL, fractional chain length; GC/MS, gas-liquid chromatography/mass spectrometry; GLC, gas-liquid chromatography; MME, monomethyl esters; TLC, thin-layer chromatography.

MATERIALS AND METHODS

Isolation of tetraenes. Lipids were extracted with a mixture of chloroform and methanol (16) from liver mitochondria of four weanling rats fed a fat-free diet for 66 days (17). Phospholipids were separated from neutral lipids by thin-layer chromatography (TLC) on silica gel H (Kieselgel H 60, Merck, Darmstadt, Germany) coated plates with diethyl ether/acetone (90:30, v/v) as developing solvent (18). The fatty acids in the resulting phospholipid fraction were methylated with 14% boron trifluoride in methanol (19). Fatty acid methyl esters (FAME) were then separated according to their number of double bonds by preparative TLC on plates coated with silica gel containing 5% $AgNO_3$ by weight. The solvent used was a mixture of hexane/diethyl ether/acetic acid (94:4:2, v/v/v) (20). The bands were detected under ultraviolet light, after spraying with a 0.2% solution of 2′,7′-dichlorofluorescein in ethanol, and scraped off into glass centrifuge tubes. The gel was suspended in a mixture of methanol/water/acetic acid (1:1:0.1, v/v/v) and FAME were extracted with hexane (18). Each fraction was then analyzed by GLC and the tetraene fractions were pooled for further analyses.

Structure determination. Tetraenoic FAME were submitted to hydrazine reduction according to Conway *et al.* (21), except that the reaction was allowed to proceed for only 2 hr. The reaction products were separated by $AgNO_3$-TLC on commercial plates (DC-Fertigplatten Kieselgel H, Merck) modified as described by Grandgirard *et al.* (22) and using a mixture of hexane/diethyl ether/acetic acid (94:4:2, v/v/v) as developing solvent (20). FAME were then extracted from the silica gel as described above. The resulting monoene fractions were submitted to oxidative ozonolysis in BF_3/methanol (23). The monomethyl and dimethyl esters (MME and DME) were analyzed first by GLC relative to authentic standards and further characterized by gas-liquid chromatography/mass spectrometry (GC/MS).

Gas-liquid chromatography (GLC). Most GLC analyses of FAME were done on a Carlo Erba 5160 chromatograph (Carlo Erba, Milano, Italy) equipped with a flame ionization detector. FAME were separated on a fused silica capillary column (50 m × 0.22 mm I.D.) coated with 100% cyanopropyl polysiloxane (0.22 μm film) (CP™Sil 88, Chrompack, Middelburg, Holland). Analyses were performed at 185°C using hydrogen as carrier gas (inlet pressure: 1.3 kg/cm^2). Injections were made through a split injector. A few analyses were also made using a CP™Wax 52 CB capillary column (50 m × 0.22 mm I.D.; Chrompack) operated at 195°C with a hydrogen pressure of 0.9 kg/cm^2. Equivalent chain lengths (ECL) were calculated (24) using the CP™Sil 88 column. Standards were purchased from Sigma (St. Louis, MO).

Gas-liquid chromatography/mass spectrometry (GC/

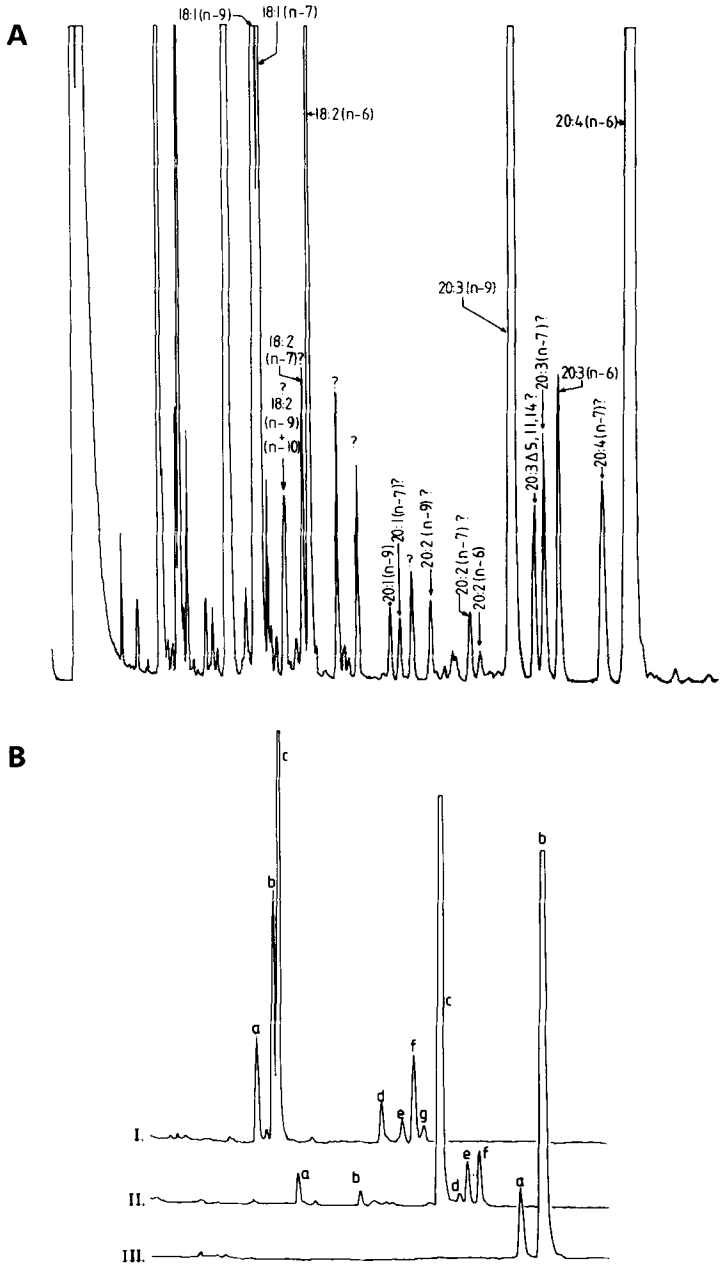

FIG. 1. A, part of a typical gas-liquid chromatogram of FAME prepared from liver mitochondria phospholipids of rats fed a fat-free diet. B, chromatograms of FAME present in the diene (I), triene (II) and tetraene (III) bands separated by $AgNO_3$-TLC from the preceeding FAME. All chromatograms were obtained using a CP™Sil 88 column.

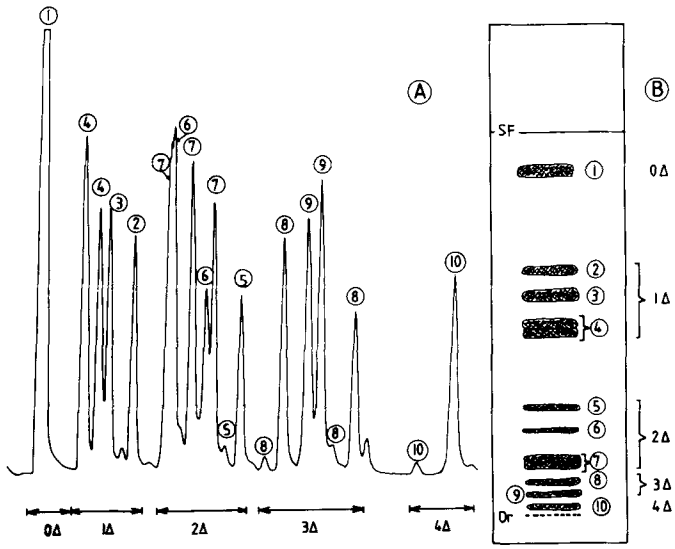

FIG. 2. A, chromatogram of FAME prepared by partial hydrazine reduction of the tetraenes isolated by $AgNO_3$-TLC from FAME prepared from liver mitochondria phospholipids of rats fed a fat-free diet (see Fig. 1b, chromatogram II). This chromatogram was obtained using a CP™Sil 88 column. B, $AgNO_3$-TLC separation of FAME obtained by partial hydrazine reduction of the tetraene fraction isolated as described preceedingly. Solvent mixture: hexane/diethyl ether/acetic acid (94:4:2, v/v/v) (20). A common peak and band numbering indicates that a peak in the chromatogram of Fig. 2a has been found in the corresponding band in Fig. 2b.

TABLE 1

Positions of Ethylenic Bonds in Monoenes Originating from the Tetraene Fraction[a]

$AgNO_3$-TLC		Ozonolysis products identified by GC/MS		Position of the ethylenic bonds
Band[b]	Rf	MME	DME	
2	0.64	$C_6 + C_7$	$C_{13} + C_{14}$	Δ13,Δ14
3	0.57	$C_9 + C_{10}$	$C_{10} + C_{11}$	Δ10,Δ11
4	0.49 0.47	$C_{12} + C_{13}$ $+ C_{15} + C_{16}$	$C_4 + C_5$ $+ C_7 + C_8$	Δ4,Δ5,Δ7,Δ8

[a]Tetraenes containing arachidonic acid methyl esters and an unknown component were isolated by $AgNO_3$-TLC from FAME prepared with fatty acids of phospholipids from liver mitochondria of rats fed a fat-free diet for 66 days. This fraction was then submitted to partial hydrazine reduction and monoenes (purified by $AgNO_3$-TLC) were submitted to ozonolysis in BF_3/methanol. The resulting fragments (MME and DME) were identified by GC/MS.

[b]The band numbers refer to Figure 2b.

MS). MME and DME were analyzed with a Hewlett-Packard 5890 gas chromatograph coupled with a 5970 Mass Selective Detector (Hewlett-Packard, Palo Alto, CA). A DB-WAX column (30 m × 0.25 mm I.D., 0.5 μm film; J & W Scientific Inc., Folsom, CA) was used. The temperature was programmed from 50 to 200°C at 20°C/min, held at 200°C for 25 min, then programmed from 200 to 220°C and held at 220°C until completion of the analyses (22).

RESULTS AND DISCUSSION

Identification of 20:4n-7. Figure 1a shows a typical resolution of FAME by gas chromatography on a CP™Sil 88 capillary column. These FAME were prepared from liver mitochondrial phospholipids of rats fed a fat-free diet for 66 days (17). Three major peaks of FAME with 18 carbon atoms [ECL = 19.21 (Ia), 19.50 (Ib) and 19.59 (Ic)] and at least four peaks with

TABLE 2

Retention Data[a] for the Tentative Identification of Some Minor Polyenes Associated with Fat-Deficiency

Fatty acid	Or.[b]	FCL[c]	ECL[d] Exp.	ECL[d] Calc.	δ[e]	Cor. ECL[f]
20:1Δ5	E	0.42	—	—	—	—
20:1Δ7	I	0.51	—	—	—	—
20:1Δ8	E	0.53	—	—	—	—
20:1Δ10	I	0.57	—	—	—	—
20:1Δ11	E,S	0.60	—	—	—	—
20:1Δ13	S	0.69	—	—	—	—
20:1Δ14	E	0.76	—	—	—	—
20:2Δ11,14	E,S	—	21.50	21.36	0.14	—
20:2Δ10,13	E	—	21.41	21.26	(+0.14)	21.40
20:2Δ8,11	E	—	21.27	21.13	(+0.14)	21.27
20:3Δ8,11,14	E,S	—	22.13	21.89	0.24	—
20:3Δ5,8,11	E	—	21.76	21.55	0.21	—
20:3Δ7,10,13	E	—	22.02	21.77	(+0.23)	22.00

[a]Results were obtained on a CP™Sil 88 fused silica capillary column (50 m × 0.22 mm I.D.) operated at 185°C; carrier gas (H_2): 1.3 kg/cm^2.
[b]Origin of the sample. E, experimental; I, interpolated value; S, authentic commercial standard.
[c]FCL = ECL − 20.00.
[d]Exp., experimental value; Calc., calculated value (20.00 + Σ FCL).
[e]Differences between experimental and calculated ECL values. Values in parentheses (ethylenic function adjustment) are used for the calculation of corrected ECL.
[f]Corrected ECL (20.00 + Σ FCL + δ).

20 carbon atoms [ECL = 21.05 (Id), 21.27 (Ie), 21.41 (If) and 21.49 (Ig)] were present in the diene fraction isolated by $AgNO_3$-TLC (Fig. 1b). Peaks Ic and Ig (Fig. 1b) had the same ECL as did authentic 18:2n-6 (19.59) and 20:2n-6 (21.49), respectively. The triene fraction contained two octadecatrienoic acids (peaks IIa and IIb) which did not correspond to either 18:3n-6 or 18:3n-3, and four peaks corresponding to 20:3 isomers. Peak IIc (ECL = 21.76) and peak IIf (ECL = 22.13) correspond to 20:3n-9 and 20:3n-6, respectively. Peak IId (ECL =21.89) is not associated with EFA deficiency and probably corresponds to 20:3Δ 5,11,14 (17). On the other hand, peak IIe (ECL = 22.02) is present only in fat-deprived rats (17). The tetraene fraction showed a minor peak (8% of the total fraction) with an ECL of 22.45 (peak IIIa) and a major peak (IIIb; ECL = 22.62) corresponding to 20:4n-6. Analysis of this fraction on the CP™Wax 52 CB gave only one peak.

A chromatogram of FAME produced by partial hydrazine reduction of the tetraene fraction is shown in Figure 2a. The fractionation of these FAME by $AgNO_3$-TLC gave 12 bands (Fig. 2b). Band 1 (Rf = 0.90) contained 20:0. Four monoene bands (Rf = 0.64, 0.57, 0.49 and 0.47) were observed, but two of these were poorly resolved and hence were combined (bands 2, 3, and 4 in Fig. 2b). No cross-contamination occurred with either dienes (first diene band: band 5; Rf = 0.28) or trienes (first triene band: band 8; Rf = 0.08) (Fig. 2b).

The structure of monoenes in bands 2, 3 and 4 was established by GC/MS of the fragments obtained after ozonolysis (Table 1). Besides major components originating from 20:4n-6 (Table 1), we have identified MME: C_7, C_{10}, C_{13}, C_{16}; and DME: C_4, C_7, C_{10}, C_{13}. Due to some contaminants in the final solutions of DME and MME, we could establish only a few mass ratios between major and minor components: DME C_{13}/DME C_{14} = 0.10; DME C_{10}/DME C_{11} = 0.10; and DME C_7/DME C_8 = 0.11. These values are similar to the initial mass ratio between the unknown tetraene and 20:4n-6. This component isolated by $AgNO_3$-TLC with arachidonic acid is 20:4Δ4,7,10,13.

Tentative identification of minor fatty acids associated with EFA deficiency. Data obtained in this study indicate that GLC analysis of FAME on CP™Sil 88 columns is a simple and convenient means of separating 20:4n-7. Other members of the n-7 family are probably also resolved from the n-6 isomers on this type of column. Peak Ib (Fig. 1b) is presumably 18:2n-7, the major octadecadienoic acid which increases in the carcass of rats during EFA deficiency (5). Peak Ia (Fig. 1b) appears too broad to contain a single component. It may thus correspond to the mixture of n-9 and n-10 isomers, which are also known to accumulate, but to a lesser extent than 18:2n-7 (5). Peaks Ia and Ib are also well resolved when a CP™Wax 52 CB is used. The small eicosatriene (peak IIe, Fig. 1b) associated with fat deficiency has an ECL which is compatible with the ECL of 20:3n-7 calculated according to Ackman *et al.* (25). This calculation is based on the summation of the fractional chain lengths (FCL) of the individual monoenoic elements and a correction term which takes into account the number and the relative positions of ethylenic bonds (25). The values for the FCL (FCL = ECL −20.00) of the Δ 7 and Δ 10 isomers have been interpolated from the FCL values of the Δ5, Δ8, Δ11 and Δ14 isomers originating from partial hydrazine reduction of 20:2n-6 and of authentic Δ13 isomer (Table 2). The appropriate adjustment for the interactions due to the 3 ethylenic bonds was established by calcu-

lating the difference δ between the experimental ECL (exp. ECL) and the calculated ECL of 20:3n-6 (δ = exp. ECL $-[20.00 + FCL(\Delta 8) + FCL(\Delta 11) + FCL(\Delta 14)]$) and of 20:3n-9. The ECL value of the unknown triene is quite close to the ECL value calculated for 20:3n-7 (22.00 *vs* 22.02; Table 2). Similar calculations indicate that peak If (ECL = 21.41) and peak Ie (ECL = 21.27) (Fig. 1b and Table 2) could correspond to 20:2Δ10,13 and 20:2Δ8,11, respectively. Peak Id (Fig. 1b) has the same ECL (21.05) as does 20:2Δ5,11 derived from 20:4n-6 after partial reduction with hydrazine (first peak of band 7, Fig. 2a and b). All these eicosadienoic acids were previously identified in the liver lipids of rats fed a fat-free diet (11).

Fatty acids of the n-7 series account for a low percentage of liver mitochondrial phospholipid fatty acids at the end of the feeding period (about 3.3%). However, it should be emphasized that this low level is nevertheless higher than the amount of n-3 acids which remains at this stage (2.7%). Consequently, n-7 acids cannot be considered as quantitatively unimportant compounds, and this should be taken into account when fatty acid compositions are tabulated.

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COMMUNICATIONS

The Effect of Bleomycin on the Uptake and Incorporation of [^{14}C]Choline into Phospholipids in Hamster Lung Tissue Slices

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Intratracheal administration of the anticancer drug bleomycin to hamsters produced an increase in the uptake and incorporation of [^{14}C]choline into phospholipids of lung slices *in vitro*. The stimulatory effect is opposite to the results obtained previously using [^{14}C]acetate and would appear to occur distal to cytidine diphosphocholine. Although alternate explanations are possible, the results are consistent with morphological evidence, published by others, indicating an increase in lung phospholipid following bleomycin treatment, and illustrate the significance of precursor selection when evaluating the effects of xenobiotics on phospholipid synthesis.
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Bleomycin is an anticancer drug whose major limitation in humans is the production of pulmonary fibrosis following chronic high doses (1). In addition to this fibrogenic response, bleomycin is also known to decrease lung beta receptor density and affinity for beta-agonists (2) as well as possibly affecting membrane phospholipid as evidenced by increased circulating levels of eicosanoids (3). We have also reported previously that the lungs of bleomycin-treated hamsters have increasingly and significantly higher levels of cyclic guanosine monophosphate (4) and calcium (4,5).

The pulmonary fibrogenic response to bleomycin is characterized by endothelial damage, epithelial type I injury and proliferation of type II pneumocytes containing giant lamellar bodies laden with phospholipid (6). This obvious accumulation of phospholipid is vexing in view of a previous report from these laboratories describing inhibition of phospholipid synthesis in lung from bleomycin-treated hamsters (7). However, in our previous study [^{14}C]acetate was used as the precursor. The results suggested that perhaps bleomycin might be producing an inhibitory effect on the conversion of acetate to fatty acids *via* inhibition of the fatty acid synthetase complex. The present study was carried out to investigate the effect of bleomycin on a distinctly separate pathway of phospholipid synthesis in the lung using [^{14}C]choline as the label.

EXPERIMENTAL PROCEDURES

Animals. Male Golden Syrian hamsters weighing 90–120 g were purchased from Simonsen, Inc. (Gilroy, CA). They were housed in groups of four in plastic cages and allowed one week to adapt to the new surroundings. They were maintained on laboratory chow and tap water *ad libitum* throughout the study.

Chemicals. Bleomycin Sulfate (Blenoxane®) was a gift from Bristol Myers Company (Syracuse, NY). All radiochemicals including [^{14}C]choline chloride (specific activity, 58.5 mCi/mmol), [^{14}C]phosphocholine (specific activity, 51.5 mCi/mmol) and [^{14}C]cytidine diphosphocholine (specific activity, 42.4 mCi/mmol) were purchased from New England Nuclear (Boston, MA). Phosphatidylcholine and sphingomyelin were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were purchased from standard laboratory sources.

The hamsters were weighed and anesthetized with sodium pentobarbital (80–90 mg/kg i.p.). A single dose of bleomycin sulfate freshly dissolved in sterile isotonic saline was introduced into the lung *via* the transoral route. The dose of bleomycin was 7.5 U/5 mL saline/kg body weight, and the control group animals received saline 5 mL/kg body weight.

Groups of control and treated animals were sacrificed by exsanguination under sodium pentobarbital anesthesia at 1, 2, 4, 7, and 14 days. The lungs were perfused *in situ* during exsanguination with approximately 60 mL of ice-cold isotonic saline from the right ventricle through the pulmonary vasculature and out the orifice created by left auriclectomy.

The lung lobes were dissected from the hilus and cleaned of extraneous tissue, rinsed three times in oxygenated saline and placed in ice-cold oxygenated Krebs-Ringer bicarbonate buffer supplemented with 10 mM glucose, 1 mM pyruvate and 21 amino acids prepared according to Kirkland and Bray (8). Lung slices of uniform thickness (0.5 mm) were prepared from all lobes using a Stadie-Riggs Microtome in a cold room at 4–6°C. The lung slices for each animal were pooled in 25-mL Erlenmeyer flasks containing 9.5 mL of modified Krebs-Ringer buffer. One μCi [^{14}C]choline chloride in a total volume of 0.5 mL of buffer was added to each flask and mixed by swirling. A 25-μL aliquot was taken from each flask and transferred to scintillation vials containing 10 mL Tritosol (9) for determination of preincubation radioactivity.

The flasks were incubated at 37°C in a Dubnoff metabolic shaker for 3 hr under an atmosphere of 95% O_2 + 5% CO_2 (v/v). After incubation, another 25-μL aliquot was taken to determine post-incubation radioactivity. The contents of the flask were then poured off onto a cheesecloth-covered beaker to recover lung slices. The slices were rinsed several times with ice-cold isotonic saline to remove unincorporated [^{14}C]choline from the surfaces.

The slices were transferred to a plastic extraction tube and homogenized in 3.5 mL isotonic saline using a polytron homogenizer. A 0.5-mL aliquot from each sample was frozen at −20°C for measurement of hydroxyproline as

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Abbreviations: BLM, bleomycin; C, choline; CDPcholine, cytidine diphosphocholine; PC, phosphatidylcholine; PPC, phosphocholine; SE, standard error; SM, sphingomyelin; UV, ultraviolet.

an index of collagen. To the remaining homogenate, 10 mL of chloroform/methanol (4:1, v/v) was added for lipid extraction. Three mL of water was added to the homogenate to create a two-phase system which was vortexed for 30 sec. The samples were then centrifuged in an International centrifuge (model EXD) at 2,500 rpm for 5 min to establish a clear two-phase system. The organic phase was transferred to 40-mL glass conical tubes and evaporated to dryness under nitrogen in an Organomation N evaporator. The aqueous phase was transferred to another set of 40-mL extraction tubes and the pellets discarded. The aqueous phase was dried in the same manner as the organic phase.

The organic phase was reconstituted in 520-μL of chloroform/methanol (4:1, v/v), and 25 μL aliquot was taken to determine the total radioactivity in the extract. Twenty five μL of the reconstituted sample was spotted on a 20 × 20 cm Whatman LK6 silica gel thin-layer chromatography plate and developed in a tank with a mobile phase of chloroform/methanol/acetic acid/H_2O (50:37.5:3.5:2, by vol) to a height of 15 cm. Sphingomyelin (SM) and phosphatidylcholine (PC) were visualized by spraying the plate with a saturated solution of fluorescein in methanol and exposing it to ammonia vapor in a developing tank. The plate was illuminated with an ultraviolet (UV) lamp to clearly differentiate the bands which were circled and scraped into scintillation vials to which 10 mL of Tritosol was added for radioactivity assay. Initial identification of PC and SM was achieved using individual and mixed standards of PC and SM in the same system. When developing samples, a mixed standard solution was spotted on the plate to assure correct identification.

The aqueous phase was reconstituted in 500 μL methanol and a 100-μL aliquot was taken to determine total radioactivity. Ten μL of sample was spotted on a 20 × 20 cm piece of Whatman #1 chromatography paper and developed in an Eastman chromagram plate chamber and through a system with a mobile phase of n-butanol/acetic acid/water (5:1.6:4, v/v/v) to a height of 16 cm. Cytidine diphosphocholine (CDPcholine), phosphocholine (PPC) and choline (C) were located using radioactive standards on the same sheet as the samples. After development, the paper was cut into 1-cm horizontal strips corresponding to each sample column. The strips were numbered according to distance from origin and placed in scintillation vials with 10 mL of Tritosol to quantitate radioactivity in the CDPcholine, PPC and C fractions. All liquid scintillation counting was done using a Beckman (LS-5801) scintillation counter.

Lung hydroxyproline. For hydroxyproline assay, a 0.5-mL aliquot of the homogenate after thawing was precipitated with 0.125 mL of 50% trichloroacetic acid, centrifuged, and the precipitate hydrolyzed in 2 mL of 6 N HCl overnight (16–20 hr) at 110°C. [^{3}H]Hydroxyproline (1 × 10^5 dpm) was added to each sample to determine recovery and the hydroxyproline content was measured by the technique described by Woessner (10). The recovery of [^{3}H]hydroxyproline ranged from 82 to 97% and was used to correct the final amount of hydroxyproline as a measure of collagen for each sample.

Statistical analysis. The data are reported as the mean ± standard error of mean (SE). Means of control and treated groups were compared using unpaired Student's t-test. The P values ≤0.05 were considered significant.

RESULTS AND DISCUSSION

The first parameter determined in the present study was the development of fibrosis in the hamster lung over the 2-week period of time following bleomycin administration. Table 1 indicates that, of the time points studied, collagen (hydroxyproline) first became significantly elevated 7 days following treatment. The accumulation continued to increase for an additional week. This profile is similar to that reported previously (4) and this time frame was used for subsequent experiments investigating phospholipid formation.

We next assessed the uptake of choline into lung slices from control and bleomycin-treated animals. This was determined by assaying the disappearance of radioactivity from the medium after the incubation of lung slices (11). In Table 2, the values presented include the [^{14}C]-choline-associated radioactivity in the lung slices. Bleomycin was found to produce an initial 24% and 13% decrease in choline uptake at days 1 and 2 post-treatment that was not statistically significant. However, at days 4, 7 and 14, [^{14}C]choline uptake was increased, being statistically significant at the latter two time points.

TABLE 1

Effects of Intratracheal Instillation of Bleomycin (BLM) (7.5 units/kg) on the Lung Hydroxyproline Content in Hamsters

Time after treatment[a] (days)	Hydroxyproline (μg/lung/100 g body weight)	P values between control and BLM
Control	649.7 ± 27 (20)[b]	
Bleomycin		
1	634.6 ± 43.4 (6)	NS
2	676.5 ± 39.4 (6)	NS
4	733.3 ± 31.9 (6)	NS
7	989.6 ± 93.4 (6)	<0.01
14	1133.3 ± 90.8 (9)	<0.001

[a]See methods for treatment details.

[b]Each value represents mean ± SE with number of animals shown in parentheses.

TABLE 2

Effects of Intratracheal Instillation of Bleomycin (BLM) (7.5 units/kg) on the Uptake of [^{14}C]Choline by Lung Slices[a]

Time after treatment (days)	% Change	Uptake of [^{14}C]choline (dpm/lung/100 g body weight)	P value between control and BLM
Control		553609 ± 59894 (19)[b]	
Bleomycin			
1	−24	419154 ± 50677 (5)	NS
2	−13	482066 ± 45950 (6)	NS
4	+11	616276 ± 52162 (6)	NS
7	+104	1129680 ± 123779 (5)	<0.001
14	+57	871045 ± 95910 (8)	<0.01

[a]See methods for technique and treatment details.

[b]Each value represents mean ± SE with number of animals shown in parentheses.

TABLE 3

Effects of Intratracheal Instillation of Bleomycin (BLM) (7.5 units/kg) on the Incorporation of [^{14}C]choline into Choline Containing Lipids[a]

Time after treatment (days)	[^{14}C]Choline incorporation (dpm/lung/100 g body weight)[b]			
	Sphingomyelin	P value between control and BLM	Phosphatidylcholine	P value between control and BLM
Control	9748 ± 513 (18)		165741 ± 7802 (18)	
Bleomycin				
1	14903 ± 1724 (6)	<0.01	223529 ± 32284 (6)	NS
2	10692 ± 2144 (6)	NS	171978 ± 13724 (6)	NS
4	13186 ± 2630 (6)	NS	198008 ± 10562 (6)	<0.05
7	12933 ± 1388 (5)	<0.05	258980 ± 20775 (6)	<0.001
14	16436 ± 1914 (9)	<0.01	264336 ± 32555 (9)	<0.01

[a]See methods for technique and treatment details.

[b]Each value represents mean ± SE with number of animals shown in parentheses.

TABLE 4

Effects of Intratracheal Instillation of Bleomycin (BLM) (7.5 units/kg) on the Incorporation of [^{14}C]Choline into Water-Soluble Intermediates of Phosphatidylcholine[a]

Time after treatment (days)	Label (dpm/lung/100 g body weight)[b]		
	Choline	Phosphocholine	CDPcholine
Control	7409 ± 1912 (14)	20684 ± 4726 (18)	156923 ± 12432 (19)
Bleomycin			
1	11273 ± 3559 (4)	11177 ± 2716 (5)	116088 ± 23917 (5)
2	10530 ± 3891 (6)	19012 ± 7390 (5)	118864 ± 19722 (6)
4	6282 ± 1804 (4)	13743 ± 5844 (4)	162222 ± 25384 (6)
7	8557 ± 5758 (5)	22934 ± 12860 (5)	177667 ± 34172 (6)
14	10364 ± 3477 (6)	21209 ± 5324 (6)	154298 ± 37113 (8)

[a]See methods for technique and treatment details.

[b]Each value represents mean ± SE with number of animals shown in parentheses.

Table 3 summarizes the effect of bleomycin on the incorporation of [^{14}C]choline into the choline containing lipids, phosphatidylcholine and sphingomyelin. Bleomycin produced a significant increase in the incorporation of [^{14}C]choline into phosphatidylcholine on days 4 (20%), 7 (56%) and 14 (60%) as well as sphingomyelin on days 1 (52%), 7 (33%) and 14 (69%) following treatment. The incorporation of [^{14}C]choline into phosphatidylcholine was 17-fold higher than that of sphingomyelin in lung slices from control hamsters, and this finding confirms the observation in goats that lung slices have a much greater capacity for the incorporation of [^{14}C]choline into phosphatidylcholine than they have for sphingomyelin (11).

Table 4 demonstrates the effect of bleomycin on the accumulation of radioactivity into water-soluble precursors of phosphatidylcholine and sphingomyelin. In the control slices, the greatest accumulation of ^{14}C radioactivity occurred in CDPcholine with an order of magnitude less in phosphocholine. These results differ from those reported for goat lung (11) and suggest that cholinephosphate cytidylyltransferase is not rate-limiting in hamster lungs. Treatment with bleomycin produced no significant change in the incorporation of [^{14}C]choline into any of the intermediates at any of the time points studied. In the present study, intratracheally administered bleomycin was found to produce a significant increase in the incorporation of [^{14}C]choline into the principal phospholipid components of hamster lung, phosphatidylcholine and sphingomyelin. In our previous studies, using [^{14}C]-acetate as the label (7), disparate inhibitory results were obtained.

The increased incorporation of [^{14}C]choline into phospholipid that we observed could be due to increased pool size. However, if the increased incorporation of label into the phospholipids was due to [^{14}C]choline concentration, one would expect a consistent effect throughout the pathway. This is not the case, however, since bleomycin treatment produced no change in the radioactivity in free choline, phosphocholine or CDPcholine.

Bleomycin treatment did produce a significant increase in the uptake of [^{14}C]choline by lung slices at 7 and 14 days. The data suggest that bleomycin may enhance choline uptake and, hence, increase phospholipid labeling. However, the level of free [^{14}C]choline within lung slices at these time points was not significantly altered. The increased phospholipid synthesis induced by bleomycin does not appear to be caused, therefore, by an increase in the substrate concentration within the lung slices. Furthermore, phosphatidylcholine synthesis was already

significantly elevated at least 3 days prior to increased uptake.

In conclusion, the present studies have shown that bleomycin treatment causes an increase in the uptake of choline from the medium as well as its incorporation into the phospholipids, namely phosphatidylcholine and sphingomyelin. In view of the fact that no alteration occurred in the ^{14}C radioactivity associated with choline, phosphocholine or CDPcholine of the lung, the stimulatory effect of bleomycin would appear to occur distal to CDPcholine, possibly involving cholinephosphotransferase, or alternatively, involving phosphatidic acid phosphatase and diacylglycerol formation. There are indications that these two enzymes could be rate-limiting in a species-dependent manner (12). However, it must be acknowledged that the results of the present study do not exclude alternate possibilities such as stimulation of the choline base exchange reaction or transphosphatidylation. In any event, the increased incorporation of choline into phospholipid observed in the present study is consistent with the morphological observations made by others (11) and suggests caution in using either acetate or choline alone when assessing the effect of xenobiotics on phospholipid synthesis.

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