Effect of Sitosterol on the Rate-limiting Enzymes in Cholesterol Synthesis and Degradation

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Attempts were made to develop an animal model for phytosterolemia. Infusion of Intralipid containing 0.2% sitosterol in rats gave circulating levels of sitosterol of about 2.5 mmol/l, which is similar to or higher than those present in patients with untreated phytosterolemia. In addition, the infusions gave serum levels of cholesterol nearly twice those obtained in rats infused with Intralipid alone or Intralipid containing 0.2% cholesterol. The hepatic HMG-CoA reductase activity was unaffected or slightly increased by the sitosterol infusions (not statistically significant). The cholesterol 7α-hydroxylase activity was slightly depressed (ca. 30%). In the case of 7α-hydroxylation of endogenous cholesterol, the depression reached statistical significance ($p < 0.05$). The microsomal content of sitosterol in the sitosterol-infused rats was about 30% of that of microsomal cholesterol. The effect of sitosterol on 7α-hydroxylation of cholesterol was investigated by incubations of acetone powder of rat liver microsomes with mixtures of cholesterol and sitosterol. Sitosterol mixed with cholesterol to a composition similar to that found in the above microsomal fraction had a depressing effect on 7α-hydroxylation of cholesterol. This degree of depression was of the same magnitude as that found in the sitosterol infusion experiments. The possibility is discussed that the hypercholesterolemia obtained in the β-sitosterol-infused rats is due to the inhibitory effect of sitosterol on the cholesterol 7α-hydroxylase.
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Phytosterolemia (sitosterolemia) is an inborn metabolic disease characterized by increased absorption of phytosterols and premature atherosclerosis (1). Some patients with sitosterolemia have moderately elevated plasma cholesterol levels (1). The reason for and significance of this finding is unknown.

The primary defect is most probably located in the intestine, and thus the hypercholesterolemia and atherosclerosis may be secondary to the high levels of circulating phytosterols. Studies of the effect of sitosterol and other plant steroids on cholesterol metabolism and turnover have hitherto been hampered by the lack of a suitable animal model. Because plant sterols are absorbed only to a very small degree in the normal intestine, feeding with phytosterol-enriched diet does not give levels of circulating phytosterols sufficiently high to simulate the condition in human phytosterolemia. In addition, sitosterol inhibits cholesterol absorption, thus interrupting the enterohepatic circulation of this sterol and thereby lowering the serum cholesterol. Sitosterol has, in fact, been used as an antihypercholesterolemic agent in patients with mild hypercholesterolemia, giving 10–20% reduction of plasma cholesterol (1). From the above information, one would expect a depressing effect on circulating cholesterol by dietary sitosterol and an increasing effect by administrations bypassing the restricted intestinal absorption. Results hitherto obtained from animal studies are, however, confusing and difficult to interpret. Administration of sitosterol intraperitoneally to rats and subcutaneously to chickens has been reported to lead to depressed levels of plasma cholesterol (2,3). Bhattacharyya and Lopez (4), however, found that sitosterol given orally to rabbits led to a 60% increase in plasma cholesterol concentration. There was no accumulation of cholesterol in the tissues of these animals, and the mechanism behind the increased plasma cholesterol levels could not be explained. Attempts in our laboratory to confirm the results by Bhattacharyya and Lopez have not been successful.

In the present study, we have infused rats intravenously with sitosterol and obtained circulating concentrations of this sterol similar to or higher than those seen in patients with phytosterolemia. Thus, we have an animal model suitable for studies on possible interactions of sitosterol on cholesterol metabolism.

MATERIALS AND METHODS

Unlabeled compounds. Sitosterol (24-ethyl-5-cholesten-3β-ol) (98% pure) was obtained from Alltech Assoc. Inc. (Deerfield, IL). The purity of the material was ascertained by HPLC prior to use. Cholesterol (CH-USP), epicoprostanol(5β-cholestan-3α-ol), mevalonic acid as well as all cofactors were purchased from Sigma Chemical Co. (St. Louis, MO). 3-Hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) was obtained from Pharmacia (Uppsala, Sweden). Other chemicals were commercial products of high purity.

Labeled compounds. [4-^{14}C]Cholesterol and [3-^{14}C]hydroxymethylglutaryl-CoA with specific activities of 55–60 mCi/mmol were obtained from New England Nuclear Corp. (Boston, MA). [$^{2}H_3$]-Labeled 7α-hydroxycholesterol was synthesized as described previously (5).

Animals. Male Sprague-Dawley rats, weighing ca. 250 g, were used. A polyethylene tube (Intramedic PE-50) was introduced into a jugular vein during ether anesthesia. The animals were given an infusion of sitosterol or cholesterol dissolved in soybean oil by sonication and mixed with Intralipid 10% (KabiVitrum AB, Stockholm, Sweden). The mixture was treated with Ultraturax to produce a stable emulsion. The added soybean oil constituted 10% (v/v) of the mixture, and the final sterol concentration was 2 mg/ml. The infusion was continuous for 38 hr at a flow rate of 0.8 ml/hr. A control group of rats received the same amounts of soybean oil and Intralipid without added sterol. The animals were kept in restraining cages with free access to water and ordinary pelleted food.

The animal operations were approved by the local ethical committee for animal experiments.

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Abbreviations: DTT, dithiothreitol; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; TMS, trimethylsilyl; TLC, thin layer chromatography.

Preparation of liver microsomes. A 10% (w/v) homogenate of the rat livers was prepared in 50 mM Tris-HCl buffer (pH 7.4) containing 0.3 M sucrose, 50 mM NaCl, 10 mM EDTA and 10 mM DTT (dithiothreitol). The microsomal fraction was prepared by centrifugation of the homogenate at 20,000 *g* for 15 min and recentrifugation of the supernatant at 100,000 *g* for 60 min. Half of the microsomal fraction was resuspended in the homogenizing medium and recentrifuged at 100,000 *g* for 60 min. The resulting fraction was suspended in 20 mM imidazol/HCl buffer (pH 7.4) containing 10 mM DTT and used for assay of HMG-CoA reductase. Half of the original microsomal fraction was suspended in a homogenizing medium lacking DTT and recentrifuged at 100,000 *g* for 60 min. This fraction was resuspended in 0.1 M potassium phosphate buffer and used for assay of cholesterol 7α-hydroxylase.

Protein was determined by a method of Lowry et al. (6).

Preparation of acetone powder of liver microsomes. Liver microsomes were prepared as above from rats fed a diet supplemented with 5% cholestyramine (Questran, Bristol-Myers, New York, NY) for six days. The microsomal fraction was washed in a 20 mM phosphate buffer containing 10 mM EDTA and resuspended in this buffer corresponding to 2 g original liver tissue per ml. Of the resulting suspension, 2 ml was added dropwise to 80 ml acetone at −10°C (7). After 30 min, this mixture was passed through an OOA filter and washed with 40 ml acetone and 40 ml ether. The acetone powder was dried in a desiccator and then dissolved in the phosphate buffer to a concentration of 14 mg per ml. For the assay of cholesterol 7α-hydroxylase activity, 1 ml of the acetone powder solution was fortified with 3 mg NADPH in 1.7 ml phosphate buffer and sterol dissolved in 0.3 ml Tween. The cholesterol concentration was varied from 26 to 130 μM. In a parallel series of incubations, sitosterol was added to the incubation in increasing amounts, constituting from 10 to 80% of the total sterol content. [$^2H_3$]-Labeled 7α-hydroxycholesterol was used as internal standard as described below.

Assay of HMG-CoA reductase activity. This assay was performed essentially as described by Brown et al. (8). Forty μl of the microsomal fraction was preincubated for 15 min at 37°C in a total volume of 200 μl, containing 0.1 M phosphate buffer (pH 7.4), 10 mM imidazole buffer (pH 7.4), 5 mM DTT, 10 mM EDTA, 1.5 mM NADP, 6 mM glucose-6-phosphate and 1 unit of glucose-6-phosphate dehydrogenase. The reaction was initiated by adding 95 nmol (0.5 μCi) of [3-^{14}C]HMG-CoA, dissolved in 25 μl of distilled water. The total substrate concentration was 475 μM. The incubation was run for 15 min and terminated by addition of 25 μl 6 M HCl. [^{3}H]Mevalonic acid (0.01 μCi) together with 3 mg of unlabeled mevalonic acid lactone was added as internal standard. The incubation mixture was further lactonized, subjected to thin layer chromatography (TLC) and analyzed for radioactivity.

Assay of cholesterol 7α-hydroxylase activity. Incubation of the microsomal fraction with [4-^{14}C]cholesterol was carried out as described previously (5). In this procedure, the conversion of [4-^{14}C]-labeled cholesterol into 7α-hydroxycholesterol is measured by radioscanning after separation of the product from the substrate by TLC. The conversion of the endogenous microsomal cholesterol into 7α-hydroxycholesterol is measured by isotope dilution-mass spectrometry after isolation of the product by TLC. It should be pointed out that in the latter procedure [4-^{14}C]-labeled 7α-hydroxycholesterol is not detected. For assay of unlabeled 7α-hydroxycholesterol by isotope dilution-mass spectrometry, [$^2H_3$]-labeled 7α-hydroxycholesterol is used as an internal standard, which is added to the incubation mixture prior to the extraction step. After isolation of 7α-hydroxycholesterol by preparative TLC, the material is converted into trimethylsilyl (TMS) derivative prior to determination of the ratio between labeled and unlabeled molecules by combined gas chromatography-mass spectrometry (5). In the latter analysis, the ions at *m/z* 456 and *m/z* 459 are used (corresponding to the M-90 ion in the mass spectrum of derivative of unlabeled and [$^2H_3$]-labeled 7α-hydroxycholesterol, respectively).

Conversion of unlabeled sitosterol into 7α-hydroxysitosterol was assayed as for conversion of cholesterol into 7α-hydroxycholesterol with the exception that the ion at *m/z* 484 was used in the mass fragmentography.

Determination of sterols in blood serum and liver microsomes. Epicoprostanol was added as internal standard before alkaline hydrolysis and lipid extraction twice with *n*-hexane, as described previously (9). The lipid extracts were taken to dryness under nitrogen. The sterols were converted to their TMS ether derivatives before quantitation by gas chromatography, using a Hewlett-Packard 5880A gas chromatograph with a 25 m capillary column (0.32 mm i.d.) CP-WAX 52 CB.

Student's t-test was used for statistical analysis.

RESULTS

Infusion of sitosterol led to an increase in serum levels of this sterol from 0.2 to 2.5 mmol/l, as shown in Table 1. Serum cholesterol concentration in the sitosterol-treated group was significantly elevated to almost twice the level of the control group ($p < 0.001$). Even compared with the rats infused with corresponding amounts of cholesterol, sitosterol infusion resulted in a significantly higher cholesterol concentration ($p < 0.025$). The total plasma sterol concentration in the sitosterol-treated rats was 10.3 mmol/l, of which 24% was attributed to the plant sterol.

Infusion of sitosterol resulted in an increase of microsomal sitosterol concentration from 1.2 to 5.8 μg/mg protein ($p < 0.001$). The microsomal cholesterol content decreased by approximately the same amount, resulting in an unchanged total microsomal content of sterol compared to the control group. Sitosterol constituted 22% of the microsomal sterols. Cholesterol infusion led to a significant increase in cholesterol concentration.

Table 2 shows the activity of hepatic HMG-CoA reductase and cholesterol 7α-hydroxylase. HMG-CoA reductase activity was slightly increased in the sitosterol-treated rats. This increase was, however, not statistically significant ($p > 0.05$). As expected, cholesterol treatment led to a significantly depressed activity of HMG-CoA reductase.

7α-Hydroxylation of endogenous microsomal cholesterol was significantly reduced in the sitosterol-treated rats ($p < 0.05$). The total 7α-hydroxylation of both endogenous- and exogenous-labeled substrate was also

TABLE 1

Effect of Infusion of Sitosterol and Cholesterol on Concentration of Cholesterol and Sitosterol in Serum and Liver Microsomes

	Serum		Microsomes	
	Cholesterol (mmol/l)	Sitosterol (mmol/l)	Cholesterol (μg/mg)	Sitosterol (μg/mg)
Rats treated with sitosterol (n = 5)	7.8 ± 0.7[a]	2.5 ± 0.3	20.2 ± 1.9	5.8 ± 0.4
Control rats (n = 6)	4.1 ± 0.3	0.2 ± 0.0	26.8 ± 2.1	1.2 ± 0.2
Rats treated with cholesterol (n = 5)	5.2 ± 0.6	0.2 ± 0.0	37.3 ± 1.6	1.5 ± 0.4

[a]Mean ± SEM.

TABLE 2

Effect of Infusion of Sitosterol and Cholesterol on Hepatic Cholesterol 7α-Hydroxylase and HMG-CoA Reductase

		Hepatic cholesterol 7α-hydroxylase activity	
	Hepatic HMG-CoA reductase activity (pmol/min/mg)	Endogenous substrate (pmol/min/mg)	Endogenous + exogenous substrate (pmol/min/mg)
Rats treated with sitosterol (n = 5)	660 ± 126[a]	21.2 ± 2.6	28 ± 4
Control rats (n = 6)	465 ± 92	29.2 ± 1.6	40 ± 5
Rats treated with cholesterol (n = 5)	73 ± 31	27.7 ± 7.1[b]	31 ± 8[b]

[a]Mean ± SEM.

[b]Result of an experiment with six rats different than those in which HMG-CoA reductase activity, serum cholesterol and microsomal content of sterol were assayed.

apparently reduced, but this reduction was not significant ($p > 0.05$).

Experiments with delipidated rat liver microsomes. The depressing effect of the sitosterol treatment on the 7α-hydroxylation of cholesterol may be due to the replacement of part of the microsomal cholesterol with sitosterol. Under normal conditions, the microsomal cholesterol 7α-hydroxylase is saturated or almost saturated with substrate (10). If sitosterol has no effect per se on 7α-hydroxylation of cholesterol, a replacement of 20% of the microsomal cholesterol would be expected to lead to about 20% reduction in formation of 7α-hydroxylated product. To study this, delipidated rat liver microsomes (acetone powder) were incubated with increasing amounts of substrate cholesterol (Table 3). In agreement with previous studies (10), saturation was achieved at about 120 μM, and the K_m was about 50 μM. With a cholesterol concentration corresponding to about 80% saturation, the cholesterol 7α-hydroxylase activity was about 70% of V_{max} (Table 3). Addition of sitosterol to yield a mixture of cholesterol and sitosterol in the ratio 4:1, similar to that found in liver microsomes from sitosterol-treated animals (Table 1), further reduced the cholesterol 7α-hydroxylase activity to about 60% of V_{max}. Thus, this degree of depression of the cholesterol 7α-hydroxylase is similar to that obtained in the experiments with infusion of sitosterol (Table 2). As shown in Table 3, addition of increasing amounts of sitosterol, together with decreasing amounts of cholesterol, depressed the 7α-hydroxylase activity to a level 10–60% lower than that expected if sitosterol has no effect per se on 7α-hydroxylation of cholesterol. In separate experiments, it was shown that the rate of 7α-hydroxylation of sitosterol by rat liver microsomes or acetone powder of such microsomes was about 2% of that of cholesterol. This finding is in agreement with a previous report (11).

TABLE 3

Rate of 7α-Hydroxylation of Cholesterol in the Presence of Increasing Concentrations of Cholesterol and Cholesterol + Sitosterol by Delipidated Rat Liver Microsomes

Cholesterol concentration (μM)	Sitosterol concentration (μM)	Rate of 7α-hydroxylation of cholesterol[a] (% of V_{max})
130	0	100
117	0	100
117	13	85
104	0	72
104	26	60
78	0	57
78	52	47
52	0	56
52	78	42
26	0	50
26	104	22

[a]The absolute rate of conversion was 26 pmol/min/mg microsomal protein.

DISCUSSION

The circulating levels of sitosterol obtained in the present animal model, about 2.5 mmol/l, are of the same magnitude as those reported for patients with phytosterolemia (up to 1.6 mmol/l) (12). The hypercholesterolemic effect

of the sitosterol infusion is of interest in relation to the fact that patients with phytosterolemia often have elevated serum cholesterol. It is tempting to suggest that the hypercholesterolemic effect of sitosterol is due to the demonstrated inhibitory effect on the rate-limiting enzyme in cholesterol degradation, the cholesterol 7α-hydroxylase. According to the present investigation, the inhibition is, in part, due to decreased availability of substrate cholesterol because of its replacement with sitosterol and, in part, due to an inhibitory effect of sitosterol on enzyme activity. Shefer and Salen (13) have recently reported, in preliminary form, that sitosterol is a competitive inhibitor of the cholesterol 7α-hydroxylase. It is well documented that sitosterol is a poor substrate for the cholesterol 7α-hydroxylase (11) and, under the present conditions, the rate of 7α-hydroxylation of sitosterol was only about 2% of that obtained with cholesterol as substrate.

The depressing effect of the sitosterol infusion on the cholesterol 7α-hydroxylase was relatively small—about 30%. Evidence has been presented previously, however, that even such a moderately depressing effect on the cholesterol 7α-hydroxylase may significantly increase circulating levels of cholesterol. Andersson and Boström (14) showed that disulfiram is a potent inhibitor of cholesterol 7α-hydroxylase and that treatment of rats with this drug gives a dose-related depression of the cholesterol 7α-hydroxylase. This depression of the choiesterol 7α-hydroxylase was paralleled by increasing serum concentrations of cholesterol. A depression of the cholesterol 7α-hydroxylase by 29% (statistically significant) was associated with an increase in serum cholesterol level by 86%. These effects are almost identical to those obtained here in the infusion experiments with sitosterol. It may be mentioned that Shefer and Salen (13) have assayed cholesterol 7α-hydroxylase activity in a liver biopsy from a patient with phytosterolemia and found it to be about 50% of normal. In view of the great interindividual variations (15), however, no firm conclusions can be drawn from this finding. A reduced cholesterol 7α-hydroxylase activity in patients with phytosterolemia would be expected to lead to reduced bile acid biosynthesis. Miettinen (16), however, found that quantitatively, bile acid synthesis was normal or even higher in a phytosterolemic subject than in controls.

The activity of HMG-CoA reductase, the rate-limiting enzyme in cholesterol synthesis, was slightly increased in the sitosterol-infused rats. This increase was, however, not statistically significant. Most probably the increase is related to the effect of sitosterol on intestinal absorption of cholesterol. Part of the infused sitosterol must be secreted by the liver into the bile and may then reduce cholesterol reabsorption and thereby the feed-back inhibition of the hepatic HMG-CoA reductase by chylomicron cholesterol.

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Phosphatidylinositol 4,5-Bisphosphate Phospholipase C Activity in Particulate Preparations From Rat Brain

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We describe the hydrolysis of phosphatidylinositol 4,5-bisphosphate by a particulate rat brain preparation in the presence of the cationic detergent, cetrimide. The optimum cetrimide concentration was in the range 0.2–0.4 mg/ml; at higher or lower concentrations, the reaction rate diminished abruptly, suggesting that the electrical charge density of the micelle is critical for enzymatic attack. In other respects, such as its partial requirement for Ca^{++} and its pH optimum of about 7.0, the particulate enzyme seems similar to soluble preparations which have been reported previously. Interestingly, the particulate preparation could be stimulated about fourfold by a soluble brain extract in the presence of 1 mM guanosine triphosphate, confirming that the enzyme is the catalytic subunit in a membrane-bound signal-transduction system.
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The receptor-mediated breakdown of PIP_2 to diacylglycerol and inositol trisphosphate is now recognized to be of major importance in the transduction of signals across the plasma membrane of eukaryotic cells (1–4). The enzyme which catalyzes this reaction (EC 3.1.4.10) may be named either as a phospholipase C or as a phosphodiesterase; here, the former term is used. It is now clear that the membrane-bound system requires a guanosine triphosphate (GTP)-binding protein in addition to the receptor and the catalytic subunit (5).

The majority of early workers investigating this pathway used intact cells. Following prelabeling (e.g., with [^{3}H]-inositol) and stimulation with an appropriate "first messenger," the cells were disrupted and changes were demonstrated in the ratio of inositol phosphates (water-soluble) to the inositol-containing phospholipids (lipid-soluble). More recently, several workers have shown receptor-mediated release of inositol phosphates from prelabeled membrane fragments (6–8). In addition, a number of reports have appeared describing soluble preparations that catalyze the hydrolysis of exogenous PI, PIP or PIP_2. These include extracts from brain (9,10), heart muscle (11), seminal vesicles (12) and platelets (13).

We have now applied this last approach to particulate preparations from rat brain. Using the commonly used anionic detergents (such as deoxycholate), all attempts to demonstrate phospholipase C activity were uniformly negative; surprisingly, however, very high activity could be obtained using micelles prepared with a cationic detergent. Here, we describe the technique and report some properties of the particulate enzyme.

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Abbreviations: GTP, guanosine triphosphate; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate.

MATERIALS AND METHODS

Materials. Radioactive [inositol-2-^{3}H(N)]-PIP_2 was obtained from New England Nuclear, Doorn, The Netherlands. Unlabeled PIP_2, GTP and alkyltrimethylammonium bromide ("cetrimide," cat. no. M 7635) were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of analytical reagent grade and were purchased from Merck (Darmstadt, Federal Republic of Germany).

Preparation of samples and phospholipase C assay. All samples were weighed and homogenized in assay medium (20 mM tris, 1 mM $CaCl_2$ and 100 mM KCl, pH 7.4) using an ice-cooled all-glass Potter-type grinder. The homogenate was centrifuged (30 min, 40,000 *g*), the pellet washed twice by centrifugation in assay medium and the washed particles resuspended in the medium at an appropriate concentration ("crude membrane preparation").

PIP_2 stocks (hot and cold) were stored in chloroform/methanol (1:1) and cetrimide, as a solution, in ethanol. Immediately prior to assay, appropriate volumes of the stock solutions were mixed to yield a "cocktail" containing 50 nmol/ml (total) PIP_2, 0.5 μCi/ml [^{3}H]-PIP_2 and 0.4 mg/ml cetrimide. The solvent was removed in a stream of nitrogen, an equal volume of assay medium added and the preparation sonicated for 30 sec at 0°C.

The reaction was initiated by the addition of 10 μl of this reagent to 10 μl of the crude membrane preparation, usually at a concentration equivalent to 5 mg/ml fresh weight brain. After incubation for 10 min at 37°C, the reaction was stopped by the addition of 500 μl 1 N HCl and 500 μl chloroform/methanol (1:1). The mixture was agitated vigorously, the phases separated by a brief centrifugation and 500 μl of the upper (aqueous) phase removed for scintillation counting. All samples were measured in duplicate, and appropriate reaction blanks were included with each batch of assays. After correction for the blank, activity was calculated as pmol PIP_2 hydrolyzed/min/mg fresh weight of brain.

Preliminary experiments showed that siliconization of glassware was essential to avoid serious loss of PIP_2 by surface absorption. Using the technique described above, solubilization of [^{3}H]-PIP_2 (i.e., percentage of the radioactivity in the "cocktail" which was present in the aqueous micelle preparation) was usually better than 90%; at other concentrations of substrate or detergent, much lower figures were observed.

Alternative procedures for substrate solubilization. Other methods tested for solubilization of the PIP_2 in the reaction mixture included (a) anionic detergents (deoxycholate and taurodeoxycholate) with final concentrations in the range 0.02–5 mg/ml; (b) a neutral detergent (Triton X-100) with a final concentration in the range 0.02–2% v/v; (c) absorption onto bovine serum albumin; and (d) absorption onto a suspension of glass particles (diameter less than 1 μm). In certain experiments using cetrimide, the final concentration of the detergent varied from 0.04 to 0.6 mg/ml.

Kinetic studies. Experiments were carried out to investigate the effects of various parameters on the rate of hydrolysis of PIP_2. These included the incubation time (range 1–30 min), tissue concentration (up to 20 mg/ml), Ca^{++} concentration (0–2 mM) and pH in the range 6.5–9.0. In all experiments, the reaction conditions, other than the variable being studied, were as described in the standard protocol above.

Stimulation experiments. Because the crude membrane preparation may be expected to contain receptors for a range of different neurotransmitters, the supernatant from the original brain homogenate (at 100 mg/ml) was used as a source of mixed "first messengers." This was heated to 100°C for 5 min prior to use to destroy any soluble phospholipase C activity present, and appropriate dilutions prepared in assay medium.

A series of assays was set up containing crude membrane preparation (5 mg/ml), GTP (final 1 mM) and boiled supernatant at various concentrations. Reagent was added, the mixture incubated for 5 min and the measurements completed in the usual way. Blanks containing boiled supernatant but omitting the membrane preparation were included in addition to the normal reagent blank.

RESULTS

General. Attempts to demonstrate particulate phospholipase C using systems in which the substrate was "solubilized" with different concentrations of anionic or neutral detergents, by absorption onto protein or by absorption onto the surface of dispersed glass particles, were uniformly negative. By contrast, the use of micelles stabilized with a cationic detergent (cetrimide) resulted in high levels of enzymatic activity. The effects of different cetrimide concentrations are illustrated in Figure 1A.

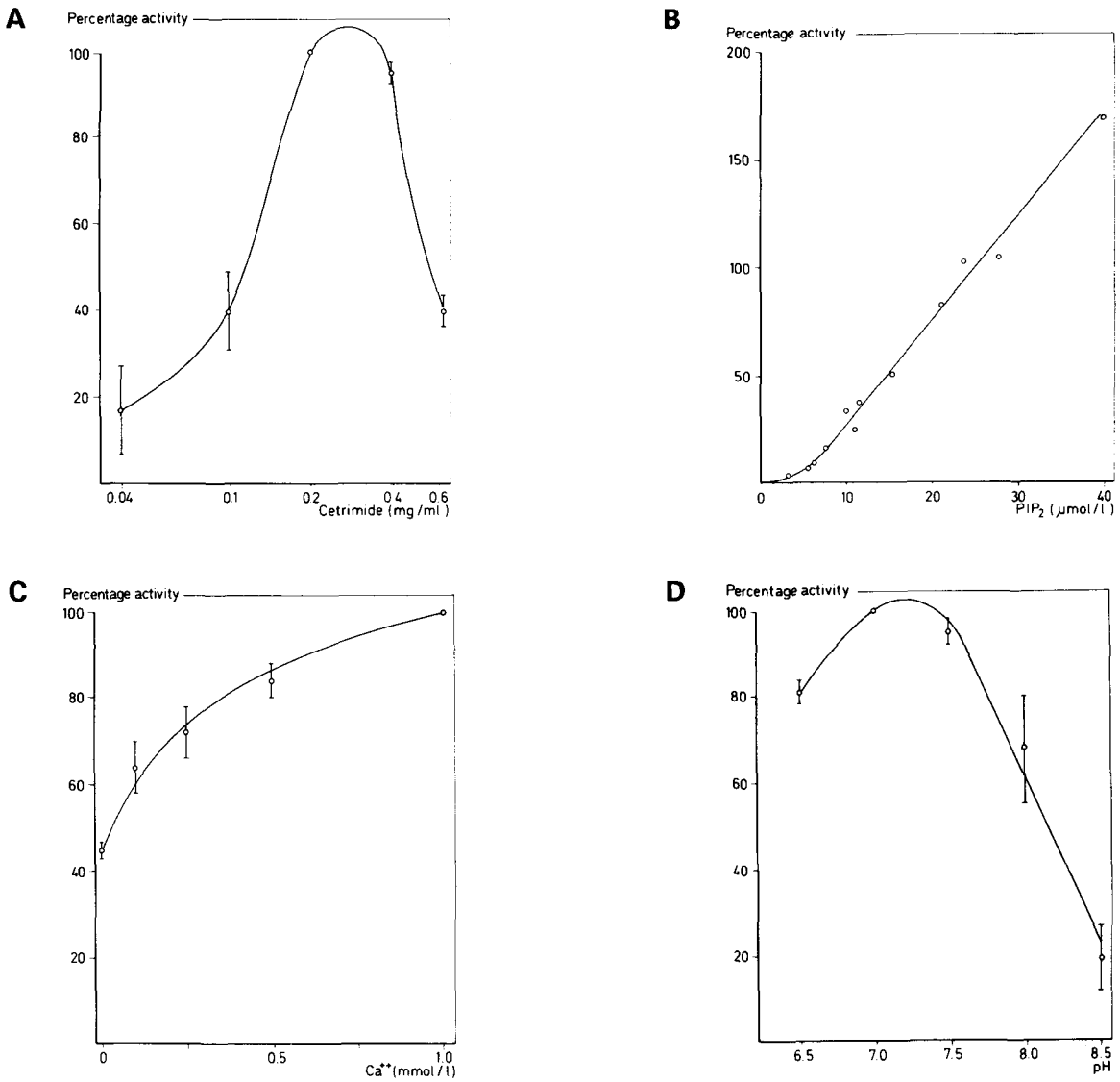

FIG. 1. Kinetics of the hydrolysis of PIP_2 by particulate preparations of rat brain in the presence of cetrimide. In all cases, the conditions, other than the variable being studied, were as described in the standard assay procedure. The absolute value obtained using the standard protocol (mean 165 $pmol.min^{-1}.mg^{-1}$) corresponds to 100%. In general, points represent the mean ± SE of 4–6 experiments; in (B), each point represents a single assay. (A) Cetrimide concentration; (B) PIP_2 concentration; (C) Ca^{++} concentration; (D) pH.

It is clear that maximum activity occurs around 0.2 mg/ml (final concentration in the reaction mixture).

The observed phospholipase C activity of rat brain, using the standard protocol, was 165 ± 39 pmol substrate hydrolyzed/min/mg fresh weight (mean ± SD, 23 different preparations).

Reaction kinetics. Regardless of the concentration of the tissue preparation, the reaction was reasonably linear until about 20% of the substrate had been cleaved, after which a sharp reduction in the rate of reaction occurred. Thus, using a rat brain preparation corresponding to 5 mg/ml fresh weight tissue, the time-curve was linear for 10–15 min; at lower concentrations, linearity was maintained for longer periods.

The variation of reaction rate with PIP_2 concentration is illustrated in Figure 1B. The reaction does not obey Michaelis-Menton kinetics, there being a distinct lag phase at low substrate concentrations. The curve appears to saturate above about 50 μM PIP_2 (not shown), but the reproducibility here became very poor, presumably because of the difficulty in maintaining a uniform micelle preparation. Ca^{++} dependence is shown in Figure 1C; there is clear stimulation, the activity at 1 mM being about double that observed in the absence of Ca^{++} (no added Ca^{++}, 1 mM EDTA). The incremental activity followed Michaelis-Menton kinetics, a Lineweaver-Burke plot showing an apparent K_m of 0.2 mM. Variation of activity with pH is shown in Figure 1D; optimum activity occurs in the range 7.0–7.5.

Stimulation experiments. The addition of 1 mM GTP to the particulate brain preparation resulted in a slight but definite reduction in activity (mean activity remaining 79%, 6 experiments). The further inclusion of "boiled supernatant" gave a clear concentration-dependent stimulation, illustrated in Figure 2. It is seen that addition of supernatant at a final concentration equivalent to 50 mg/ml brain increased the basal activity of the particulate preparation about fourfold. In the absence of particles, the "boiled supernatant" had no detectable phospholipase C activity.

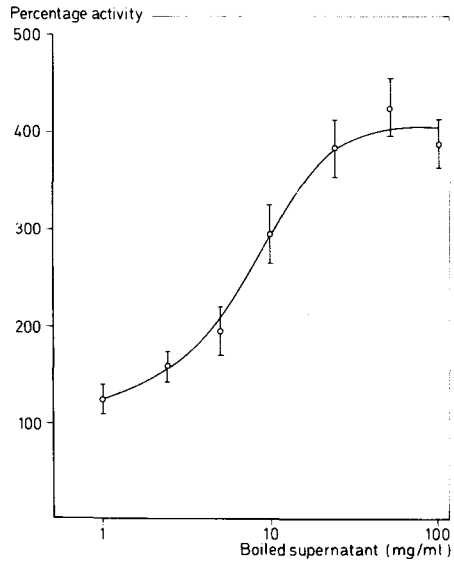

FIG. 2. Stimulation of particulate rat brain phospholipase C activity by the addition of a soluble brain extract ("boiled supernatant"). Points represent the means ± SE of 6 experiments; activity in the absence of the soluble preparation is taken as 100%. All assays contained GTP at a final concentration of 1 mM.

DISCUSSION

We show here that the use of a cationic detergent at an appropriate concentration permits direct measurement of the phospholipase C activity of particulate preparations using an exogenous substrate. Presumably, the critical factor which determines the rate of hydrolysis of the substrate under our conditions is the electrical charge density at the surface of the micelle. The PIP_2 molecule will, of course, carry a strongly negative charge at neutral pH, and it seems that a partial neutralization of this charge by the alkyltrimethylammonium ion is necessary for attack at a significant rate. It is not possible to calculate the net charge density from the data available, because we do not know the distribution of the cetrimide between the micelle and the aqueous phase.

The relationship between the particulate enzyme(s) and the various soluble preparations which have been described remains unclear. Brain has been shown to contain at least two immunologically distinct forms of soluble PI-specific phospholipases C, both of which may exist in different oligomeric forms (10). Further, the degree of phosphorylation of the substrate (PI, PIP or PIP_2) and the precise manner in which it is presented (for example, admixture of other phospholipids) seem to have a profound effect on the observed properties of the enzyme, such as its behavior with respect to pH and its requirement for metals (9). These latter workers reported that a soluble rat-brain preparation showed a major peak of activity against PIP_2 at pH 5.5 together with a minor peak around pH 7.5. Using pure PIP_2 as substrate and a low ionic strength buffer, they also demonstrated a threefold stimulation of this preparation by submicromolar concentrations of Ca^{++}; however, this shifts to the millimolar region in the presence of Mg^{++} and at higher ionic strength (9,14). Thus, the possibility of identity between the particulate enzyme and previously described soluble preparations cannot be excluded.

Nonetheless, the marked stimulation of activity obtained by the addition of a soluble extract to the particulate preparation in the presence of GTP strongly suggests that the enzyme reported here is, in fact, the rate-limiting step in this signal-transduction pathway. Our approach may well be of value in situations where speed, simplicity and sensitivity are of paramount importance, such as measurement of phospholipase C in human biopsy material. We are currently applying this technique to our investigations into the skin disease psoriasis, where an abnormality in the phosphoinositol cycle has been speculated (15).

ACKNOWLEDGMENTS

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Dietary N-6 and N-3 Fatty Acids and Salt-induced Hypertension in the Borderline Hypertensive Rat

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This study examined the effects of salt-loading on blood pressure (BP) development in the borderline hypertensive rat (BHR) and its modification by dietary n-3 and n-6 fatty acids. In experiment 1, 4 groups (n = 10/group) of male BHR receiving 1% NaCl as a replacement for tap water were placed on chow enriched with either olive oil (OL), sunflower oil (SUN), evening primrose oil (EPO) or fish oil for 6 weeks. BP, heart rate, body weight, water, Na^+ and K^+ intake and urinary output were measured weekly. SUN and fish oil reduced the pressor response to salt seen vs the OL group by 50%, and EPO abolished the pressor response, reducing BP below control levels. The BP response was unrelated to either food intake or water and electrolyte intake and excretion. In experiment 2, male BHR received water +/− 18:3n-6 (0.04 mg/hr in OL via ip pump) or 1% NaCl +/− 18:3n-6 (n = 12/group) for 12 weeks, followed by 2 weeks recovery on tap water. Salt increased BP, and 18:3n-6 decreased this response, but had no effect on animals receiving tap water. In experiment 3, effects of 3 doses of 18:3n-6 (0.04, 0.08, and 0.12 mg/hr) on the pressor response to 1% NaCl were examined. All doses reduced the BP response to salt vs controls with no dose-response. These data suggest that the BHR is genetically salt-sensitive, and that dietary n-3 and n-6 fatty acids can attenuate the cardiovascular response to salt in this model.
Lipids 24, 17-24 (1989).

The borderline hypertensive rat (BHR) is produced by backcrossing the spontaneously hypertensive rat (a genetically hypertensive animal) with its normotensive parent strain, the Wistar Kyoto rat. The offspring of this mating typically demonstrate a gradual increase in systolic blood pressure (BP), which stabilizes at maturity at 140-150 mm Hg (1,2). Certain types of chronic stressors have been demonstrated to act as neurogenic triggers in this animal, producing an irreversible hypertension similar to that of its genetically hypertensive parent, with BP in excess of 180 mm Hg (1-3). We have recently demonstrated that administration of 18:3n-6 is capable of diminishing the pressor and tachycardic response to chronic isolation stress in this animal model (4).

The BHR has recently been demonstrated to be salt-sensitive, developing a more severe hypertension when placed on chow containing 8% NaCl (2). The goals of the present study were to verify the salt-sensitivity of the BHR and to assess the effects of dietary supplementation with oils rich in various n-3 and n-6 fatty acids on the pressor response to salt-loading in this animal. Oils used were rich in 18:1n-9 (olive [OL]), 18:2 (sunflower [SUN]), 18:3n-6 (evening primrose oil [EPO]) and 20:5n-3/22:6n-3 (fish).

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Abbreviations: EPO, evening primrose oil; OL, olive oil; PL, phospholipid; SUN, sunflower oil; TG, triglyceride; BHR, borderline hypertensive rat; BP, blood pressure; ip, intraperitoneally.

METHODS

Experiment 1: Effects of dietary oils and salt-loading on BP and electrolyte excretion.

Diet and salt-load. Individually housed adult male BHR (n = 40), 9 wk of age, were used in the study. All animals received standard chow containing 5% vegetable oil (Master Feeds, London, Ontario), until the start of the test period. One wk following a pretest measure, all animals were placed on 1% NaCl, as a replacement for drinking water, for a 6-wk period. Concurrently, animals were randomly assigned to one of four diet groups receiving supplementary OL, SUN, EPO (Efamol) or fish oil (Maxepa), which was mixed into their food at a level of 10% total calories (n = 10/group). All animals remained on the supplemented diets and saline for the 6-wk period.

Measures. Weekly measures of BP and heart rate were made on conscious animals using the tail cuff technique, previously described (5). Body weight was also measured. In addition, animals were placed in metabolic cages at weekly intervals for 24-hr assessment of saline and food intake and urine output. From this information, weekly assessments of Na^+, K^+ and water intake and urinary excretion were made. Electrolyte analysis was performed by flame photometry (Corning 405). During measurements the experimenters were blind to the treatment grouping of the animals. Following the week-6 measures animals were killed with CO_2, and livers were removed and flash frozen in liquid nitrogen for fatty acid analysis of the phospholipid (PL) and triglyceride (TG) fractions.

Experiment 2: Effects of salt-loading and 18:3n-6 on BP and electrolyte excretion.

Treatment and salt-load. Forty-eight male BHR, bred at the University of Waterloo, were individually housed for a 9-wk post-weaning acclimation period. All animals received chow (Master Feeds) and tap water ad libitum during this period. Animals were then implanted ip with either 8-wk, constant flow (1.3 μl/hr) osmotic pumps (Alza Corp., Palo Alto, CA), releasing 0.04 mg 18:3n-6 (Sigma Chemical, St. Louis, MO)/hr in OL vehicle, or dummy pumps (n = 24/group) under halothane anesthesia, as previously described (4). Dummy pumps were used because OL administered by pump has been demonstrated to have no effect on BP in the Wistar Kyoto rat (6), and supplementation of the chow diet of 4 BHR rats with OL (10% cal) over 4 wk produced changes in BP of less than 4 mm Hg (unpublished), which approximates the reproducibility of the method in this laboratory. One week following surgery 12 rats/group were placed on 1%-NaCl solution as a replacement for tap water, whereas 12/group remained on tap water for a 12-wk experimental period. Weekly measures of BP, heart rate and body weight were taken, as described above.

Experiment 3: Dose-response of 18:3n-6 on BP and water and electrolyte excretion during salt-loading in BHR.

Treatment. Following a 5-wk acclimation period, 40 individually housed male BHR were rank-ordered by body

weight, distributed to 1 of 4 treatment groups, then implanted ip with either dummy pumps or 8-wk osmotic pumps releasing 0.04, 0.08 or 0.12 mg 18:3n-6/hr in OL (10/group). Following a 2-wk control period on tap water, all animals were placed on 1% NaCl, as previously described, for 6 wk. Weekly measures of BP; heart rate; body weight; food, water and NaCl intake; and urinary water and electrolyte excretion were made, as previously described. At the end of the salt-loading period, all animals were killed and their livers, kidneys and adrenals were removed and frozen for fatty acid analysis.

Lipid analysis. Lipids were extracted from ca. 1 g of tissue (except for the adrenals, in which lipids were extracted from the paired glands), which had been homogenized in chloroform/methanol (2:1, v/v) with 0.02% BHT at a ratio of 20:1 (v/w). One ml 0.9% saline was added to the homogenate, and it was allowed to stand overnight. The lower phase was then extracted and evaporated under nitrogen at 40°C, the residue was suspended in 750 μl chloroform and 300 μl was spotted on silica gel GF plates (Analtech Inc., Newark, DE) for separation of the fractions. Plates were then placed in a solution of hexane/diethyl ether/glacial acetic acid (90:10:1). Following separation, 2 ml BF_3 was added to the PL and TG fractions, which were then tightly capped and heated at 90°C for 30 min. Next, 5 ml hexane and 2 ml saline were added, the tubes vortexed and then centrifuged at 1,500 rpm for 2 min. The top layer was then removed and evaporated under nitrogen at 40°C, and the residue dissolved in 500 μl hexane. This was then transferred to a gas chromatograph autosampler vial, capped under nitrogen and analyzed on a 15-m Supelcowax 10 column.

Statistics. Blood pressure, heart rate, body weight and fluid and electrolyte data were analyzed using a 2-way analysis of variance with repeated measures. Where a significance of $p < 0.05$ was demonstrated, specific points of difference were compared using the Newman-Keuls test. Fatty acid data were analyzed using a 1-way ANOVA. Again, where significance of $p < 0.05$ was demonstrated, further comparisons were made using the Newman-Keuls test.

RESULTS

The fatty acid composition of the lab diet and dietary oil supplements are shown in Table 1.

TABLE 1

Selected Fatty Acid Composition (%) of Chow Diet, Olive Oil, Sunflower Oil, Evening Primrose Oil (Efamol) and Fish Oil (Maxepa)

	Chow	OL	SUN	EPO	Fish oil
14:0	—	—	—	—	0.2
16:0	15.2	9.9	7.3	7.8	19.9
18:0	2.9	2.6	4.1	2.0	3.1
18:1n-9	21.1	77.5	14.2	8.7	11.6
18:2	47.6	8.8	72.1	72.3	1.4
18:3n-6	—	—	—	8.1	—
18:3n-3	4.6	0.5	0.3	—	—
20:4	—	—	—	0.5	—
20:5	—	—	—	—	18.4
22:6	—	—	—	0.3	11.5

Experiment 1—blood pressure, heart rate and body weight. Systolic BP responses to variations in dietary fat during salt-loading are illustrated in Figure 1. Salt-loading produced a significant increase in BP in animals receiving OL ($p < 0.001$), SUN ($p < 0.01$) and fish ($p < 0.01$) oil vs presalt levels. However, the BP response to salt-loading was reduced by SUN and fish oil in comparison to OL ($p < 0.001$). In contrast to the above, supplementation with EPO abolished the pressor response to salt-loading and lowered BP vs presalt levels ($p < 0.001$). There were no differences in heart rate or body weight gain among treatment groups in the study (data not shown).

Urinary sodium and potassium excretion. Weekly measures of 24-hr water, Na and K intake and urinary excretion are illustrated in Figures 2 and 3. Replacement of drinking water with 1% NaCl resulted in a significant increase in both Na and water intake/unit body weight in all of the dietary groups vs control period values. Furthermore, Na and water intake in the EPO group alone was significantly greater than in the OL group for the duration of salt-loading. In contrast, K intake/unit body weight fell significantly in all groups during salt-loading, with no consistent differences among the dietary groups. The drop in K intake was a direct reflection of the decrease in food consumption during the experimental period (data not shown).

Urinary Na excretion increased ca. 5-fold during salt-loading vs the presalt condition. There were no significant differences in Na excretion among the various dietary groups, with the exception of the EPO group, which had a greater Na excretion than the OL group at week 1, and SUN, which had a lower Na excretion than the OL group at week 2. Urinary K excretion fell significantly during salt-loading in all groups except the SUN group, in which excretion was significantly elevated, vs

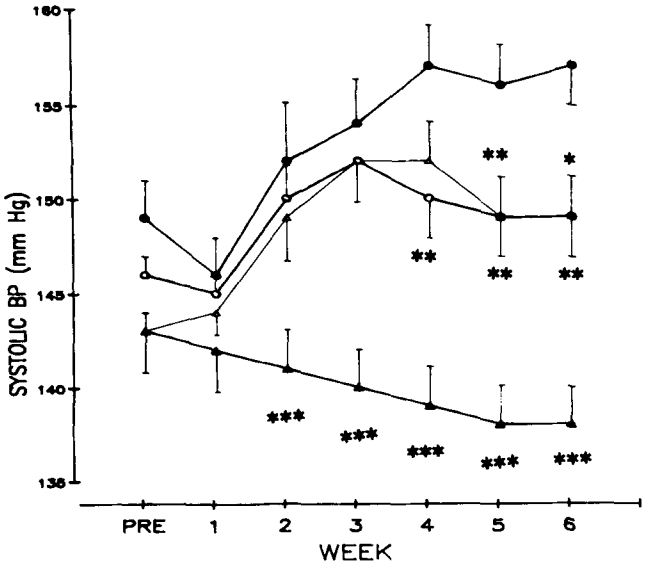

FIG. 1. Systolic BP ($\bar{X} \pm$ SEM) prior to (PRE) and during salt-loading with 1% NaCl as a replacement for drinking water in male BHR (n = 10/group) receiving chow plus 10% (cal) olive oil (OL), sunflower oil (SUN), fish oil (Maxepa) or evening primrose oil (EPO, Efamol). *, $p < 0.05$ vs olive oil. **, $p < 0.01$ vs olive oil. ***, $p < 0.001$ vs olive oil. △, Maxepa; ●, olive oil; ▲, evening primrose oil; ○, sunflower oil.

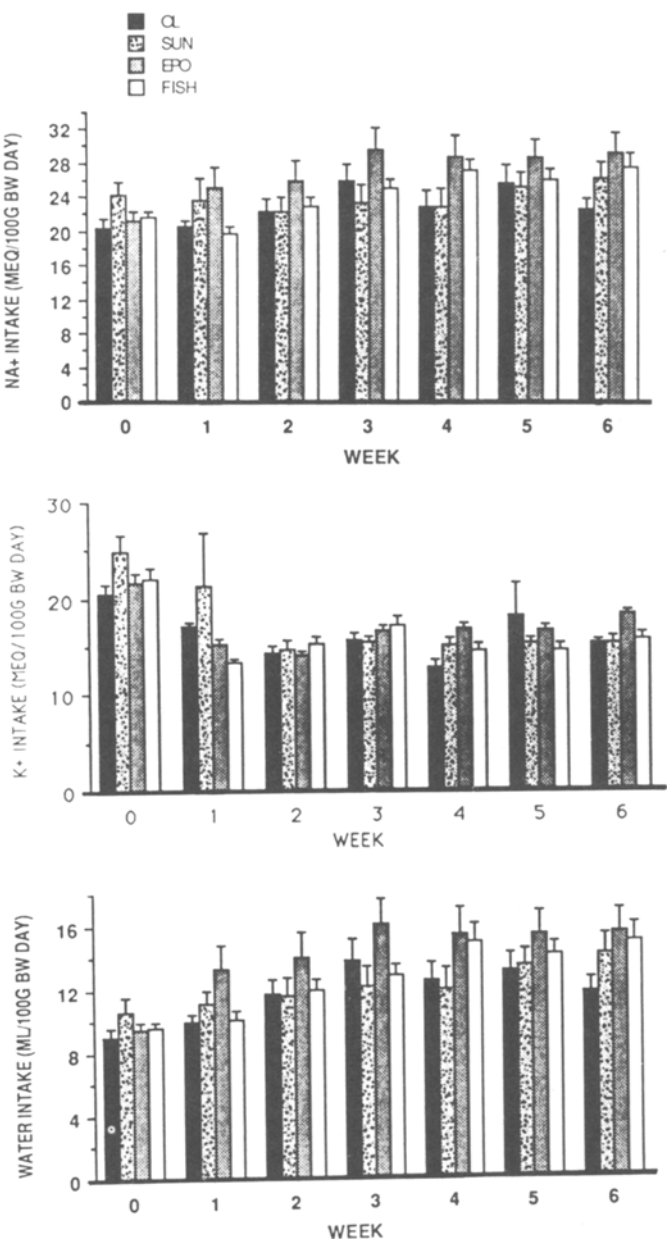

FIG. 2. Twenty-four-hr water, Na^+ and K^+ ($\bar{X} \pm$ SEM) intake prior to (week 0) and during salt-loading in BHR receiving chow plus OL, SUN, EPO or fish oil. Details as in Figure 1.

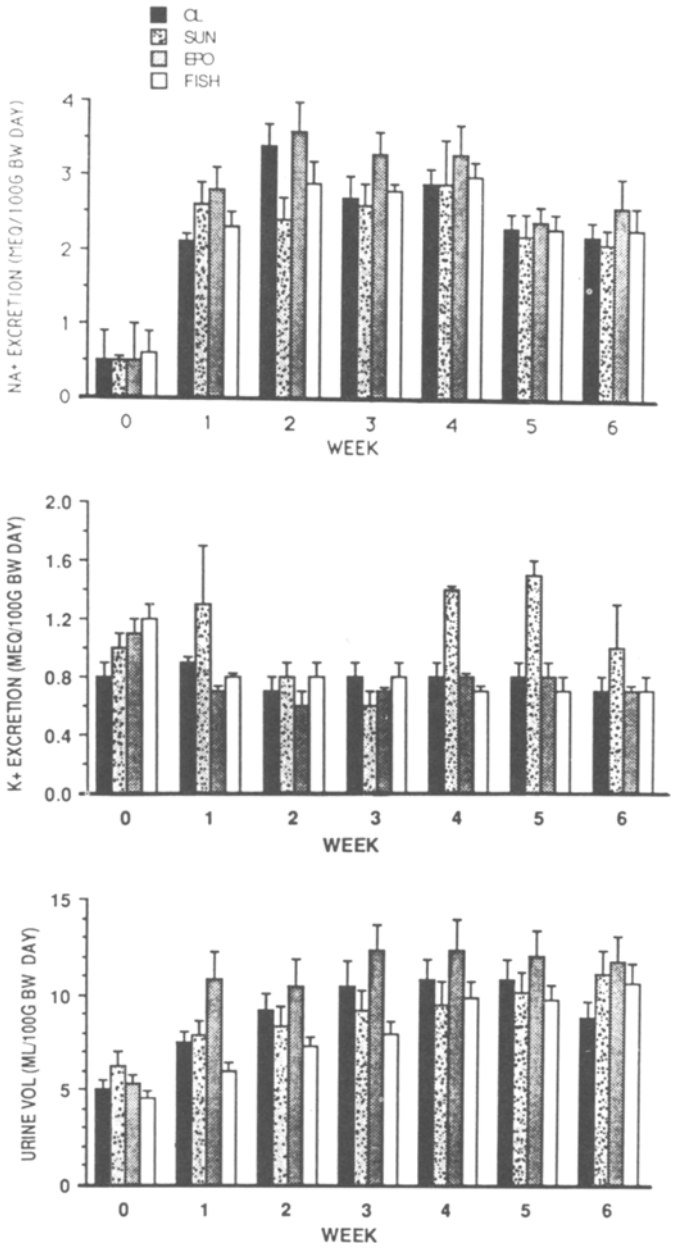

FIG. 3. Twenty-four-hr urinary Na^+, K^+ and water excretion ($\bar{X} \pm$ SEM) prior to (week 0) and during salt-loading in BHR receiving chow plus OL, SUN, EPO or fish oil. Details as in Figure 1.

that of the OL group at weeks 1, 4 and 6, as well as vs the presalt value in the SUN group.

The increase in Na excretion seen during salt-loading was accompanied by an increase in urine volume/unit body weight in all diet groups. In addition, the urine volume of the EPO group was significantly higher than that of the OL group at weeks 1 and 6, and that of the SUN group was elevated vs the OL group at week 6. In contrast, the group receiving fish oil had a reduced urine volume vs OL at weeks 1, 2 and 3.

Liver fatty acid composition. Fatty acid composition of liver PL and TG fractions are shown in Table 2. In liver PL 16:0 was increased by EPO and fish oil and decreased by SUN, whereas 18:0 was increased by SUN and EPO. Olive oil supplementation led to greater 18:1 levels than in all other groups. SUN, EPO and fish increased 18:2 vs OL, and SUN and EPO increased 18:3n-6 and 22:4, whereas fish oil decreased 22:4 vs OL. Liver PL 20:3 was increased by fish oil and was decreased by SUN vs OL, and both EPO and fish oil lowered 20:4 vs OL, although the fish oil effect was more pronounced. Fish oil alone reduced 22:5n-6 vs OL. SUN increased the proportion of 18:3n-3 and 20:5 in liver PL, and fish oil increased 22:5n-3 and 22:6n-3 vs OL. EPO, in contrast, reduced 22:6n-3 vs OL.

Liver TG were also altered by diet. Fish oil elevated both 16:0 and 18:0 vs OL, and EPO decreased 16:0. As in the PL, 18:1 was higher in the OL group than in the

TABLE 2

Fatty Acid Composition of Liver PL and TG in Salt-loaded BHR Receiving Lab Chow Plus 10% OL, SUN, EPO or Fish Oil

	OL	SUN	EPO	Fish
Liver PL				
16:0	21.8 ± 0.3	18.6 ± 0.2***	23.7 ± 0.4***	23.7 ± 0.4***
18:0	18.0 ± 0.3	21.1 ± 0.3***	19.4 ± 0.3**	17.3 ± 0.3
18:1n-9	10.2 ± 0.3	5.2 ± 0.2***	7.4 ± 0.2***	6.8 ± 0.2***
18:2n-6	8.5 ± 0.3	10.8 ± 0.3***	11.0 ± 0.4***	12.6 ± 0.4***
18:3	0.3 ± 0.0	0.4 ± 0.0***	0.4 ± 0.0***	0.2 ± 0.0
20:3	0.5 ± 0.0	0.2 ± 0.0***	0.4 ± 0.0	1.0 ± 0.0***
20:4	29.1 ± 0.2	28.9 ± 0.5	26.2 ± 0.2***	17.9 ± 0.7***
22:4	0.4 ± 0.0	0.6 ± 0.0***	0.7 ± 0.0***	0.1 ± 0.0***
22:5	0.3 ± 0.0	0.4 ± 0.1	0.3 ± 0.0	0.2 ± 0.2**
18:3n-3	tr	0.2 ± 0.0***	tr	0.1 ± 0.0
20:5	tr	0.1 ± 0.0**	tr	tr
22:5	1.1 ± 0.1	1.1 ± 0.0	0.9 ± 0.1	1.9 ± 0.1***
22:6	5.9 ± 0.2	6.3 ± 0.2	4.6 ± 0.1***	10.4 ± 0.3***
Liver TG				
16:0	26.8 ± 0.7	22.3 ± 0.6***	23.2 ± 0.3**	33.3 ± 1.3***
18:0	1.8 ± 0.1	0.5 ± 0.3**	1.6 ± 0.0	2.7 ± 0.4*
18:1n-9	40.0 ± 1.9	22.2 ± 1.0***	16.8 ± 0.4***	30.1 ± 1.0***
18:2n-6	17.9 ± 0.8	35.7 ± 0.7***	37.2 ± 1.1***	18.3 ± 1.1
18:3	0.3 ± 0.1	0.7 ± 0.1***	1.3 ± 0.1***	0.1 ± 0.0*
20:3	0.2 ± 0.1	0.4 ± 0.1**	0.9 ± 0.0***	0.2 ± 0.0
20:4	2.4 ± 0.2	5.8 ± 0.5***	7.8 ± 0.3***	1.1 ± 0.1**
22:4	1.1 ± 0.2	2.0 ± 0.2***	3.0 ± 0.2***	0.1 ± 0.0***
22:5	0.4 ± 0.1	0.5 ± 0.1	0.6 ± 0.0*	0.2 ± 0.1
18:3n-3	0.4 ± 0.1	0.6 ± 0.1	0.5 ± 0.0	0.4 ± 0.1
20:5	tr	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
22:5	0.7 ± 0.2	0.6 ± 0.1	1.0 ± 0.1	1.1 ± 0.2
22:6	0.9 ± 0.3	0.8 ± 0.1	1.1 ± 0.1	1.8 ± 0.3**

Details are as in Figure 1. All values represent $\bar{X}$ ± SEM.

other diet groups. Groups receiving SUN and EPO demonstrated higher levels of 18:2, 18:3n-6, 20:3, 20:4 and 22:4 vs OL, whereas the fish oil group demonstrated less 18:3n-6, 20:4 and 22:4. Levels of 22:5n-6 were also increased by EPO. The only change seen in liver TG was an increase in 22:6n-3 by the fish oil group.

Experiment 2—blood pressure, heart rate and body weight. The systolic BP response to salt loading with and without 18:3n-6 is shown in Figure 4. Administration of 18:3n-6 had no effect on BP in the absence of salt-loading, with the exception of week 2, when it increased BP vs animals with dummy pumps. In BHR with dummy pumps, BP increased significantly within 2 weeks of salt-loading vs BHR on tap water and remained significantly higher than animals on tap water at weeks 5, 6, 7 and 10, with BP not significantly different than that of animals on tap water during recovery. Furthermore, BP in the salt-loaded BHR receiving 18:3n-6 was significantly lower than in salt-loaded BHR without 18:3n-6 at weeks 3, 7, 8, 9, 10, 11 and at week 1 of recovery. Neither salt-loading nor 18:3n-6 administration significantly affected heart rate or body weight gain in this study.

Experiment 3—blood pressure, heart rate and body weight. The effects of various doses of 18:3n-6 on the systolic BP response to salt-loading are shown in Figure 5. Control animals receiving OL responded to salt-loading with a significant increase in BP within 1 week. Administration of 18:3n-6 during salt-loading, although not completely eliminating the pressor response, significantly diminished the BP increase vs control animals throughout the entire period of salt-loading. Generally, all doses of 18:3n-6 had similar effects on BP, with the exception that the BP response to salt was reduced to a greater extent by administration of 18:3n-6 at 0.12 mg/hr than 0.04 mg/hr at weeks 4 and 5 of salt-loading.

Changes in body weight gain from the end of the control (presalt) period are shown in Figure 6. Body weight rose linearly throughout the control and experimental period in the control group. In contrast, all doses of 18:3n-6 led to a decline in body weight during the control period, which differed significantly from the response of the control animals. No dose response was seen in this parameter. The body weight gain during salt-loading was reduced vs controls in groups receiving 0.04 and 0.08 mg 18:3n-6/hr, but not in those receiving 0.12 mg 18:3n-6/hr. Heart rate declined throughout the salt-loading period in all groups, with no significant treatment effects (data not shown).

Food, water and electrolyte regulation. During salt-loading, 24-hr urine volume and Na excretion significantly increased, and 24-hr K excretion decreased vs the presalt-loading values (data not shown). However, no differences

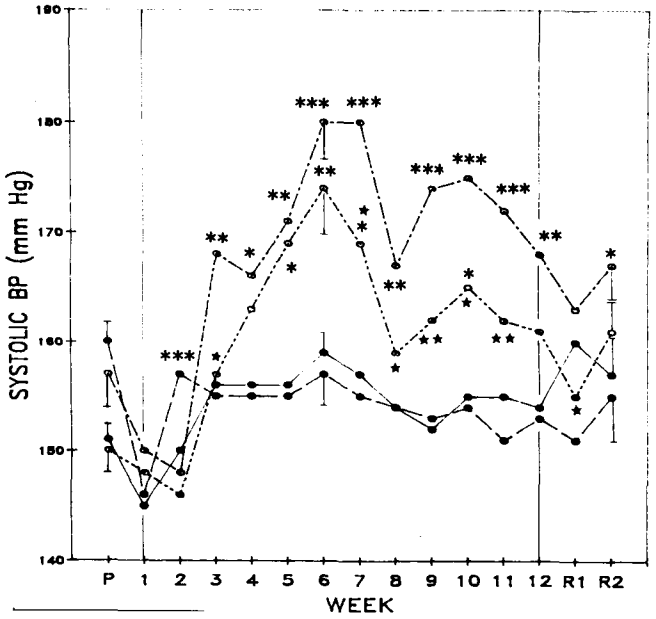

FIG. 4. Systolic BP ($\bar{X} \pm$ SEM) prior to (P), during (1-12), and following (R1-R2) salt-loading with 1%-NaCl replacement for tap water (or tap water in controls) in male BHR implanted ip with either osmotic pumps releasing 0.04 mg 18:3n-6/hr in OL or sham pumps (n = 12/group). *, $p < 0.05$ vs no salt. **, $p < 0.01$ vs no salt. ***, $p < 0.001$ vs no salt. ★, $p < 0.05$ vs salt + 18:3n-6. ★★, $p < 0.01$ vs salt + 18:3n-6. —●, No salt-sham; – – –●, no salt-GLA; —○, salt-sham; – – –○, salt-GLA.

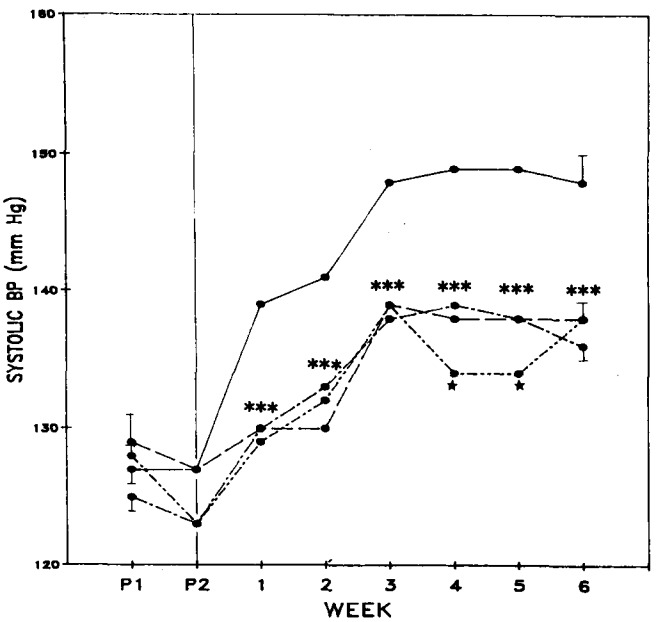

FIG. 5. Systolic BP ($\bar{X} \pm$ SEM) in male BHR implanted ip with either sham pumps or osmotic pumps releasing 0.04, 0.08 or 0.12 mg 18:3n-6(GLA)/hr in OL prior to (P1-P2) and during salt-loading with 1%-NaCl replacement for tap water (n = 10/group). ***, $p < 0.001$ vs sham. ★, $p < 0.05$ vs 0.04 and 0.08 mg 18:3n-6/hr. —●, Salt-0.0 GLA; – –●, salt-0.04 GLA; —·—●, salt-0.08 GLA; – – –●, salt-0.12 GLA.

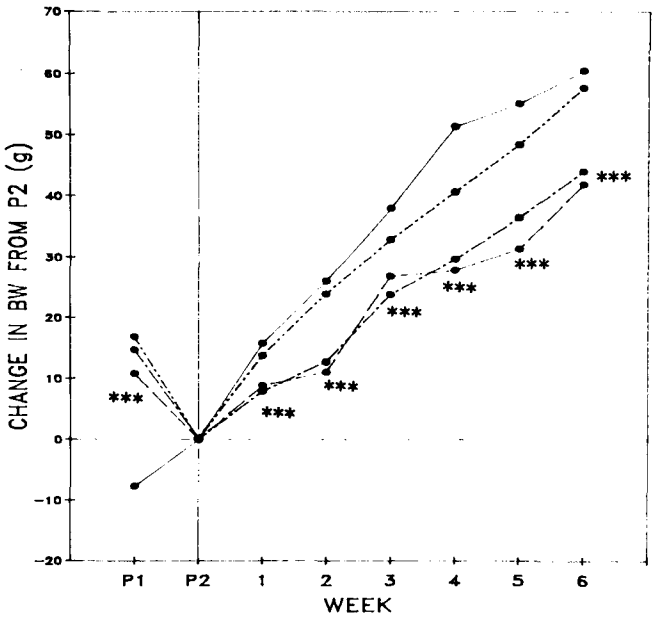

FIG. 6. Change in body weight from day 1 of salt-loading ($\bar{X}$) in BHR implanted with either sham pumps or pumps releasing 0.04, 0.08 or 0.12 mg 18:3n-6/hr. Details as in Fig. 5. ***, $p < 0.001$ vs sham. —●, Salt-0.0 GLA; – –●, salt-0.04 GLA; —·—●, salt-0.08 GLA; – – –●, salt-0.12 GLA.

among treatment groups were seen in either total or weight-adjusted daily food, saline or electrolyte intake, urine production or Na and K excretion (data not shown).

Tissue fatty acid composition. The composition of selected liver, adrenal and kidney PL fatty acids is shown in Table 3. Liver composition was unaffected by fatty acid administration, with the exception that 18:3n-6 and 20:3n-6 were significantly increased in the groups receiving 0.08 and 0.12 mg 18:3n-6/hr, respectively. In the kidney PL, all doses of 18:3n-6 increased 18:1n-9 and decreased 22:5n-6 vs controls. In addition, 0.08 mg/hr increased 20:3n-6 and 22:4n-6, and both 0.08 and 0.12 mg/hr increased 18:0 and decreased 20:4n-6 vs controls. In the adrenal PL, all doses of 18:3n-6 increased 18:2n-6 and decreased 18:3n-6, 20:3n-6 and 22:5n-6. In this tissue, 0.04 mg/hr alone increased 20:4n-6 vs controls.

DISCUSSION

In a preliminary report, Lawler and Cox observed that salt-loading with chow containing 8% NaCl significantly increased BP in the BHR by 19 mm Hg over a 6-wk period (2). The results of the present study, in which salt-loading was achieved via the replacement of drinking water with 1%-saline solution, confirm the salt-sensitivity of the cardiovascular system in this animal model and suggest that salt-induced hypertension in the BHR may be achieved by lower levels of salt than previously believed. In addition, the failure of BP to return to control levels during the recovery period, following 12 wk of 1% saline, suggests that the hypertension produced by chronic salt-loading may be irreversible, due to a neurogenic triggering effect which has been described previously (1-3). However, additional work is needed to verify this hypothesis, as the hypertension observed in the

TABLE 3

Fatty Acid Composition (%) of Liver, Kidney and Adrenal PL in Salt-loaded BHR Implanted ip With Sham Pumps or Pumps Releasing 0.04, 0.08 or 0.12 mg 18:3n-6/hr in OL

	0.00	0.04	0.08	0.12
Liver				
16:0	22.4 ± 0.6	21.7 ± 0.6	23.2 ± 1.0	20.6 ± 0.8
18:0	15.3 ± 0.9	16.5 ± 0.5	16.8 ± 1.3	18.5 ± 0.3
18:1n-9	4.5 ± 1.1	4.5 ± 0.5	6.6 ± 0.7	5.8 ± 0.3
18:2n-6	13.5 ± 1.6	14.3 ± 0.5	14.8 ± 0.4	14.5 ± 0.2
18:3	0.2 ± 0.1	0.3 ± 0.1	0.5 ± 0.1*	0.3 ± 0.1
20:3	0.4 ± 0.1	0.4 ± 0.0	0.3 ± 0.0	0.7 ± 0.1**
20:4	24.8 ± 1.0	26.4 ± 0.6	23.3 ± 0.7	24.4 ± 0.6
22:4	0.5 ± 0.1	0.5 ± 0.0	0.3 ± 0.0	0.5 ± 0.1
22:5	0.4 ± 0.1	0.1 ± 0.0	0.3 ± 0.1	0.2 ± 0.1
18:3n-3	0.2 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
20:5	0.2 ± 0.1	tr	tr	tr
22:5	2.1 ± 0.6	1.3 ± 0.1	1.3 ± 0.1	1.4 ± 0.1
22:6	6.0 ± 0.8	7.2 ± 0.5	5.4 ± 0.5	5.7 ± 0.3
Kidney				
16:0	19.6 ± 0.2	20.2 ± 0.9	19.6 ± 0.2	19.6 ± 0.2
18:0	13.0 ± 0.4	14.0 ± 0.5	15.6 ± 0.5**	16.3 ± 0.2**
18:1n-9	6.6 ± 0.1	7.1 ± 0.2*	7.2 ± 0.2**	7.0 ± 0.1*
18:2n-6	13.9 ± 0.2	13.7 ± 0.4	13.7 ± 0.6	13.4 ± 0.3
18:3	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
20:3	0.8 ± 0.0	0.9 ± 0.0	0.9 ± 0.0*	0.8 ± 0.0
20:4	34.8 ± 0.6	33.6 ± 0.9	31.9 ± 0.5**	32.3 ± 0.3**
22:4	0.4 ± 0.0	0.4 ± 0.1	0.6 ± 0.1*	0.5 ± 0.0
22:5	0.2 ± 0.0	0.1 ± 0.0*	0.1 ± 0.0*	— **
18:3n-3	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
20:5	tr	tr	0.1 ± 0.0	tr
22:5	0.3 ± 0.0	0.2 ± 0.0	0.4 ± 0.0	0.3 ± 0.0
22:6	2.4 ± 0.0	0.3 ± 0.1	2.2 ± 0.0	2.3 ± 0.0
Adrenal				
16:0	13.1 ± 0.9	12.1 ± 0.7	15.3 ± 1.1	14.6 ± 0.8
18:0	18.8 ± 1.2	20.9 ± 1.6	21.0 ± 1.2	25.5 ± 1.4
18:1n-9	9.5 ± 0.6	8.0 ± 0.4	10.7 ± 1.0	11.7 ± 1.2
18:2n-6	5.7 ± 0.4	8.0 ± 0.6**	7.7 ± 0.5**	7.4 ± 0.4**
18:3	1.4 ± 0.5	0.1 ± 0.1*	tr ***	tr ***
20:3	1.3 ± 0.4	0.1 ± 0.1*	tr ***	tr ***
20:4	34.7 ± 3.7	45.7 ± 1.2**	37.7 ± 2.5	33.3 ± 2.0
22:4	0.8 ± 0.2	0.5 ± 0.2	0.4 ± 0.3	0.8 ± 0.5
22:5	0.5 ± 0.3	tr *	tr *	tr *
18:3n-3	tr	tr	tr	tr
20:5	tr	tr	tr	tr
22:5	tr	0.1 ± 0.1	tr	tr
22:6	0.2 ± 0.1	0.2 ± 0.2	0.2 ± 0.1	0.7 ± 0.6

Details are as in Figure 5. All values represent $\bar{X}$ ± SEM.

*, $p < 0.05$ vs sham. **, $p < 0.01$ vs sham. ***, $p < 0.001$ vs sham.

recovery period of the present study was monitored for only two wk and was of a modest degree. More severe salt-loads for longer exposures and longer follow-up periods must be examined in order to confirm the ability of this stimulus to act as a neurogenic trigger.

The results of the present study also suggest that the pressor response to salt is modifiable by dietary fatty acid manipulation. Specifically, the present data suggest that EPO, at a level of 10% of total calories, is capable of completely abolishing the pressor response to salt-loading with 1% saline in the BHR. Furthermore, SUN and fish oil, at a level of 10% of total calories, are ca. 50% as effective as EPO in reducing the pressor response to salt. The greater effectiveness of EPO vs SUN may be due to the additive effects of 18:2 (ca. 72% in both SUN and EPO) and 18:3n-6 (ca. 8% in EPO). Although SUN and fish oil appeared to be equally effective in this model, one may not conclude from these results that 18:2 and 20:5/22:6 are of equal potency in this model, because the dose of 18:2 in SUN was ca. 3-fold that of 20:5/22:6 in the fish oil used. Further studies are required in which the individual fatty acids contained in these oils are directly compared on an equimolar basis.

The present data also suggest that administration of

18:3n-6 per se attenuates, but does not eliminate, the pressor response to salt-loading in the BHR, with no significant dose-response relationship demonstrable over the range studied. Possible explanations for the absence of a dose-response are: (1) the threshold dose of nonesterified 18:3n-6 administered ip is ≤0.04 mg/hr, (2) the fatty acid may exert a permissive action in the system which does not demonstrate a dose response or (3) the dose range examined was not broad enough to detect a dose-response relationship. The reduced effect of ip 18:3n-6 on the BP response to salt in comparison with that of EPO may result from: (1) the additive effect of 18:2 and 18:3n-6 in EPO, (2) the greater dosage of 18:3n-6 associated with dietary EPO (ca. 50-fold that of the pump study) or (3) the differences in bioactivity of the TG form of 18:3n-6 found in EPO vs the nonesterified form administered by pump.

The absence of an effect of 18:3n-6 on baseline BP in the BHR is in agreement with an earlier report (4) and supports the concept that 18:3n-6 acts via modifying BP regulation in the perturbed system. In contrast, the current observation that EPO leads to a modest, but significant reduction in baseline BP in the BHR suggests that, at a higher dose and/or in combination with 18:2, 18:3n-6 may alter basal BP regulation.

The present observation that administration of 18:3n-6 by osmotic pump does not alter resting HR is in contrast to an earlier report (5). This difference in effect may be due to the use of the normotensive Wistar-Kyoto strain in the earlier study vs the BHR in the present or the use of a fat-free diet during the experimental period in the earlier study vs chow in the present.

The reduction of BP seen following fatty acid manipulation in the present study was not associated with any consistent alterations in food, water, Na or K intake or urinary water, Na or K excretion. The only suggestion of an alteration in either food or water and electrolyte metabolism was the altered body weight gain seen in animals receiving 18:3n-6 in OL. However, similar body weight changes were not observed in animals receiving EPO. This inconsistency between experiments 1 and 2, and experiment 3 can be explained, in part, by the fact that the animals in experiment 3 were rank ordered by weight prior to assignment to treatment groups, whereas animals in experiments 1 and 2 were randomly assigned to groups. This variation in procedure resulted in a reduction of the body weight variance in experiment 3 vs 1 and 2, and allowed for a more sensitive discrimination of body weight changes resulting from treatment in the final experiment. However, the relationship between body weight regulation and pressor response to salt is unclear, as the weight gain and BP response do not closely coincide. These indicate that the cardiovascular actions of SUN, EPO and fish-oil supplements in this model act via mechanisms other than the alteration of salt and water metabolism.

Dietary 18:2 has been previously shown to diminish the pressor response to salt-loading that occurs in genetically normotensive rats raised on an essential fatty acid-deficient diet (7,8). Several mechanisms of action have been proposed for 18:2 in this model, including suppression of circulating epinephrine and angiotensin, altered vascular reactivity to pressor agents and increased excretion of Na, K and water (7–9). Although the salt-loaded BHR of the present study were maintained on an essential fatty acid-repleted diet, 18:2, 18:3n-6 and 20:5/22:6 supplementation may utilize mechanisms similar to those of 18:2 in the essential fatty acid-deficient normotensive rat to reduce BP during salt-loading in the BHR. In support of this are the observations that 18:3n-6 and 20:5 reduce vascular reactivity to circulating angiotensin in both animals (4–6) and humans (10) and that EPO, SUN and linseed oil decrease sympathetic activity in the spontaneously hypertensive rat (11).

In the present study, dietary supplementation with various vegetable and marine oils rich in n-3 and n-6 fatty acids produced significant changes in liver PL and TG fatty acids, reflecting the major constituents of the oils and the effects of these constituents on fatty acid elongation and desaturation. Specifically, OL increased 18:1, and SUN, EPO and fish oil increased 18:2 (vs OL). Fish oil also increased long-chain n-3 fatty acids vs the other groups. In addition, both EPO and fish oil reduced 20:4, and fish oil reduced long-chain n-6 fatty acids. The levels of 18:3n-6 and 20:3 were unchanged by EPO in liver PL, but 22:4 and 22:5 increased, suggesting that 18:3n-6 was elongated in this group. In liver TG, OL increased 18:1 vs other groups, and SUN and EPO elevated 18:2. In addition, EPO increased 18:3 and 20:3 levels. Again, fish oil elevated long-chain n-3 fatty acids and reduced long-chain n-6 fatty acids. In animals receiving 18:3n-6 ip, there were no consistent changes in tissue PL fatty acids, with the exception of an increase in 18:2 and a reduction in 18:3n-6 and 20:3n-6 in the adrenal PL of all groups receiving 18:3n-6, possibly reflecting an inhibition of desaturation of 18:2.

The proposal that dietary n-3 and n-6 fatty acid-induced changes in BP in both salt-loaded and control animals are related to endogenous prostanoid synthesis, particularly those derivatives of 20:4 (prostaglandins E_2 and I_2), 20:3 (prostaglandin E_1) and 20:5 (prostaglandin I_3), is controversial (12–15). Support for this relationship is often found in the proportion of these fatty acid precursors in tissue PL or actual prostanoid production/excretion (12,13). A commonly proposed mechanism for the physiological effects of dietary 18:3n-6 is that it is incorporated into tissue PL and subsequently metabolized to prostaglandin E_1 (16,17), which can subsequently reduce BP via peripheral vasodilation (18) or diuresis (19,20). However, there appears to be no relationship between tissue fatty acid composition and the pressor responses to salt, as might be predicted, based on the prostaglandin E_1 theory. This suggests that in this model, (1) the levels of prostanoid synthesis are not reflected in the levels of PL or TG fatty acid precursors, (2) some tissue other than the liver, kidney or adrenals is the site of relevant increases in 20:3 or (3) the effects of dietary n-3 and n-6 fatty acids on BP are mediated through processes that do not involve altered prostanoid synthesis. This last proposal is consonant with several other reports in the literature (14,15). It appears from the data that changes in PL fatty acid composition are not prerequisite for a physiologic effect. As levels of dietary supplementation are often decided because of their effect on tissue PL composition, these data suggest that physiologically significant changes may be achieved at lower levels of fatty acid supplementation than commonly believed.

The BHR is a useful model in the investigation of the

relationship between genetic and environmental factors in the regulation of BP. The results of this study suggest that the BHR is salt-sensitive and that dietary n-3 and n-3 fatty acid modification, particularly with 18:3n-6, may attenuate the triggering of deleterious cardiovascular effects of salt in this genetically predisposed model.

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Serum Lipoprotein and Apoprotein Concentrations in 4-(4-Chlorophenyl)-2-Hydroxytetronic Acid and Clofibrate-treated Cholesterol and Cholic Acid-fed Rats

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Influence of clofibrate and an aci-reductone, 4-(4-chlorophenyl)-2-hydroxytetronic acid (CHTA) on lipoproteins and apoproteins was studied in cholesterol- plus cholic acid-fed rats. CHTA (0.4 mmol/kg body wt, twice daily) significantly lowered serum total cholesterol and triglyceride concentrations at both 10 and 16 days, whereas clofibrate at the same dose did not alter serum cholesterol levels, but elevated serum triglyceride concentrations at 16 days. The abnormal cholesterol-rich very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL) and low density lipoproteins (LDL) produced by cholesterol plus cholic acid were significantly reduced in their cholesterol content by treatment with CHTA, a compound having an oxidation reduction potential. Conversely, clofibrate administration increased VLDL-cholesterol with concomitant decreases in IDL- and LDL-cholesterol concentrations. Administration of CHTA to cholesterol- plus cholic acid-fed rats significantly increased concentrations of VLDL and IDL, but had no effect on HDL protein. Both CHTA and clofibrate administration to cholesterol- plus cholic acid-fed rats significantly lowered IDL protein concentrations.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) studies of apoproteins revealed that clofibrate treatment significantly reduced apoC-III and C-II in VLDL, C-II in IDL, and apoA-IV and A-I in HDL. Rats treated with CHTA significantly raised apoC-II and C-III in HDL.

Isoelectric focusing (IEF) of VLDL apoproteins showed a significant decrease in apoC-II, C-III-0 and apoC-III-3 in clofibrate-treated animals. Thus, the mechanism for antilipidemic action of the oxidation reduction compound, CHTA, which differs markedly from the prototype drug, clofibrate, is independent from major apoprotein modification.

Lipids 24, 25–32 (1989).

Clofibrate and related analogues are among the most widely used hypolipidemic drugs for treating "Broad Beta" hyperlipidemic patients (1). We have been involved for many years with investigating the synthesis (2), effects on lipid and lipoprotein parameters (3), modes of action (2,3) and metabolism (4) of these substances. In this article, we discuss the antilipidemic activity of 4-(4-chlorophenyl)-2-hydroxytetronic acid (CHTA), an aci-reductone [-C(OH)=C(OH)-C=O] having a proposed (5) biologically relevant oxidation reduction potential that may modify lipogenesis oxidation reduction enzyme systems. We have reported both the hypocholesterolemic and platelet anti-aggregatory properties of this compound and found that CHTA was superior to clofibrate as a hypocholesterolemic drug and modifier of the heparin-$MnCl_2$ precipitated lipoprotein cholesterol to alpha-lipoprotein cholesterol ratio (5).

Rats (6), swine (7), rabbits (8) and dogs (9) fed cholesterol have been shown to induce β-migrating very low density lipoprotein (β-VLDL), intermediate density lipoprotein (IDL) and high density lipoprotein (HDL)-cholesterol (C) with a marked increase in arginine-rich peptide (apoE). We employed the cholesterol- plus cholic acid-fed rat model to determine the antilipidemic activity of CHTA, a compound related to clofibrate by its chlorophenyl group, but unrelated because of its aci-reductone characteristics. Clofibrate administration to rats fed a chow diet has been reported to cause a decrease in plasma triglycerides, cholesterol, HDL-C and apoAI, B and C-III (10). Recently, Krause and Newton (11) observed decreased plasma cholesterol, cholesterol in apoB-containing lipoprotein and apoB without alteration of HCL-C, apoA-I and apoE concentrations in cholesterol-fed rats treated with clofibrate.

Because apoproteins are considered to play a major role in lipid and lipoprotein metabolism, we compared clofibrate and CHTA for effects on lipoprotein and apolipoprotein distribution in rats fed cholesterol plus cholic acid. We specifically evaluated CHTA to determine if the oxidation-reduction portion of the molecule would confer greater hypolipoproteinemic effects, because this compound might integrate into cellular membranes and modify receptors which may, in turn, alter enzymatic activity necessary for lipid metabolism.

In view of the altered apoprotein distribution in cholesterol-induced hypercholesterolemia and in an attempt to further our knowledge of the role of clofibrate and CHTA with apoproteins, we have analyzed and now report the apoprotein distribution in this model.

MATERIALS AND METHODS

Chemicals. Acrylamide, N-N'-methylene-bis-acrylamide, riboflavin-5-phosphate, urea, ampholines, Coomassie Brilliant Blue R-250 and sodium dodecylsulfate were obtained from Bio-Rad Laboratories (Rockville Center, NY). A-Gent Cholesterol and A-Gent Triglyceride reagents

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Abbreviations: β-VLDL, beta-very low density lipoprotein; C, cholesterol; CHTA, 4-(4-chlorophenyl)-2-hydroxytetronic acid; HDL, high density lipoprotein; IDL, intermediate density lipoprotein; (V)LDL, (very) low density lipoprotein; ANOVA, analysis of variance; dl, deciliters; hsd, honestly significantly different; IEF, isoelectric focusing; PI, pH at isoelectric point; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis.

were obtained from Abbott Laboratories, Diagnostic Division (Chicago, IL). Rat semisynthetic diet and high-cholesterol fortified diets were obtained from U.S. Biochemicals (Cleveland, OH). Clofibrate [2-(*p*-chlorophenoxy)-2-methylpropionic acid ethyl ester] was purchased from Sigma (St. Louis, MO). CHTA was synthesized in our laboratory, as described earlier (5).

Animals and experimental protocol. Male Sprague-Dawley rats (250–260 g) were maintained on a semisynthetic diet (dextrin, 48%; casein, 18%; sucrose, 11%; coconut oil, 10%; USP X VII salt mixture, 4%; celufil, 2%; liver concentrate NF, 0.4%; choline chloride, 0.2%; D-methionine, 0.2%; vitamin supplements, 4%) or a high-cholesterol diet (semisynthetic diet ingredients plus cholesterol USP, 1% and cholic acid, 0.5%) for a period of 1 wk prior to vehicle or drug-plus-vehicle treatment. Rats were housed in a vivarium at 25–26°C with an alternate 12-hr light and dark cycle with food and water provided ad libitum. After 1 wk of feeding with semisynthetic diet (8 rats, group I) and high-cholesterol diet (24 rats), blood was withdrawn from the orbital plexus of all ether-anesthetized animals. Serum cholesterol was determined in these blood samples and, on the basis of cholesterol levels, rats fed high-cholesterol diet (24 rats) were distributed into three groups (II, III and IV; 8 rats in each group) by a stratified random sampling to minimize variations in the initial cholesterol values (mean serum cholesterol concentrations in these three groups were 238,237 and 236 mg/dl, respectively). Groups III and IV received either 0.4 mmol/kg body wt twice daily of CHTA or clofibrate through intragastric administration with 0.25% methylcellulose as a vehicle, for 16 days. Groups I (control) and II (CH-control) were administered the same amounts of vehicle only and, during drug treatment, rats were maintained on the respective diets.

All rats were fasted 16–18 hr before blood collection. Blood was drawn from the orbital plexus under light ether anesthesia on the day before (Day −1) and 10 days after drug treatment (Day +10). After 16 days of drug treatment, blood was collected by exsanguination from the abdominal aorta. All blood samples were kept for 2–3 hr at 4°C and, after coagulation, serum was separated by centrifugation at 2000 *g* for 10 min.

Lipoprotein isolation and characterization. Serum samples were combined to obtain 3 pools from each group of animals (two rat sera in each pool), and these samples were used for lipoprotein isolation. Serum was fractionated into VLDL (d < 1.006 g/ml), IDL (d 1.006–1.019 g/ml), low density lipoprotein (LDL) (d 1.019–1.063g/ml) and HDL (d 1.063–1.21 g/ml) classes by differential ultracentrifugation according to the procedure of Havel et al. (12). Lipoprotein fractions were collected with a Spinco tube slicer and were dialyzed against 0.15 M NaCl–0.005 M EDTA, pH 7.5 at 4°C. Cholesterol recovery based on total serum cholesterol concentrations ranged from 65–70%. All reported values have been normalized to 100% recovery. The purity of the lipoprotein fractions was checked by agarose gel electrophoresis. In cholesterol-fed rats, contamination of HDL-C in LDL fractions occurs because both float at similar densities (6). Thus, the LDL fraction was not used for sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) or isoelectric focusing (IEF) studies. The protein content of purified VLDL, IDL and HDL was measured by the method of Lowry et al. (13), as modified by Sata et al. (14). Apoprotein B concentrations of both VLDL and IDL were calculated to be the difference in protein concentration found before and after 2-propanol precipitation (15). Appropriate amounts of VLDL, IDL and HDL (400 μg for IEF and 150 μg for SDS-PAGE) were lyophilized and delipidated according to the procedure of Brown et al. (16) and Scanu and Edelstein (17).

Apoprotein electrophoresis. Apoproteins of VLDL, IDL and HDL were separated by SDS-PAGE, as described by Shore et al. (18). In all SDS-PAGE of apoproteins, a distinct band was observed having a molecular mass similar to bovine serum albumin. We observed similar IEF patterns in VLDL, IDL and HDL fractions at the albumin isoelectric points. The gels were stained with 0.2% Coomassie Brilliant Blue R-250 (18) and scanned at 570 nm in a Gilford model 240 spectrophotometer equipped with a Gilford Model 2410-S linear transport system connected to an Apple II computer. The percentage of the total area under each peak was computed by the ADALAB Chromatochart program. Apolipoproteins were identified on the basis of molecular mass (18).

IEF of apoVLDL, IDL and HDL was carried out at 4°C in 7.5% polyacrylamide gel containing 8 M urea and 2% ampholines, pH 3.5–10 (19). Amounts of protein used for IEF with respect to the linearity of the dye binding and chromogenicity of apoCs have been established in our laboratory (20). For the present study, appropriate amounts of apoprotein and corrections were used. For each set of gels, one IEF gel was run without applying any sample to monitor pH gradient. Following electrophoresis, gels were stained with Coomassie Brilliant Blue R-250 and destained as described by Vesterberg and Hansen (21). The gels were scanned at 570 nm, and areas under each peak and apoprotein proportions were computed as described above. The gel, without the sample applied to monitor pH gradient, was sliced into 2 mm segments. Each of these segments was transferred to an Eppendorff tube containing 1.2 ml of distilled water. The tubes were left in the cold room overnight. The pH was measured at room temperature on a Beckman Model 3550 digital pH meter (22). Apoproteins were characterized on the basis of pI values (20,22,23).

Analyses. Serum cholesterol and lipoprotein-cholesterol levels were determined by the method of Allain et al. (24) with A-Gent cholesterol reagent on the Abbott Biochromatic analyzer (ABA-100). Serum triglycerides were measured on the ABA-100 with A-Gent triglyceride reagent according to the procedure of Sampson et al. (25).

Statistical analyses. Cholesterol and triglyceride concentration values were analyzed by a two-way analysis of variance (ANOVA). Significance of cholesterol distributions among lipoproteins and apoprotein concentrations were analyzed by a one-way analysis of variance. On those ANOVAs that demonstrated significance due to groups, post-hoc Tukey (hsd) tests for significance among groups were performed.

RESULTS

Serum total cholesterol levels in control, CH-control and drug-treated rats are recorded in Table 1. Feeding a cholesterol-rich diet for 1 wk (Day −1) significantly increased serum cholesterol levels which remained stable

TABLE 1

Effect of CHTA and Clofibrate on Serum Cholesterol Concentrations in Cholesterol-fed Rats[a]

	Serum cholesterol (mg/dl)[b]		
	Day −1	Day +10	Day +16
I. Control	119 ± 6.5[c]	103 ± 8.4	98.5 ± 10.2[d]
II. Cholesterol-rich (CH)	238 ± 72.1	195 ± 36.6[e]	221 ± 67.4[e]
III. CH + CHTA	237 ± 69.8	137 ± 19.2[f]	155 ± 36.5[f]
IV. CH + Clofibrate	236 ± 65.2	186 ± 33.1[g]	218 ± 58.5[g]

[a]Groups III and IV received either 0.4 mmol/kg body wt twice daily of CHTA or clofibrate through intragastric administration with 0.25% methylcellulose as a vehicle for 16 days. Day +10 and Day +16 represent 10 and 16 days of drug treatment. Day −1 represents initial values before drug treatment.

[b]Mean ± SD, N = 8 rats per group.

[c]$p < 0.005$, Group I vs II, III and IV.

[d]N = 5.

[e]$p < 0.005$, Group II vs I, Group II vs III.

[f]$p < 0.005$, Group III vs I.

[g]$p < 0.005$, Group IV vs I, Group IV vs III.

TABLE 2

Effect of CHTA and Clofibrate on Rat Serum Triglyceride Concentrations[a]

	Serum triglycerides (mg/dl)[b]		
	Day −1	Day +10	Day +16
I. Control	119 ± 30.4	112 ± 22.5	119 ± 26.1[c]
II. Cholesterol-rich (CH)	126 ± 30.0	128 ± 13.0[d]	130 ± 17.3[d]
III. CH + CHTA	130 ± 33.7	100 ± 7.13[e]	97 ± 16.0[e]
IV. CH + clofibrate	131 ± 26.3	136 ± 37.0[f]	162 ± 39.1[f]

[a]Drug administration is the same as described in Table 1.

[b]Mean ± S.D., n = 8 rats per group.

[c]N = 5.

[d]$p < 0.005$, Group II vs I, III.

[e]$p < 0.005$, Group III vs II, IV.

[f]$p < 0.005$, Group IV vs III, I.

at Day +10 and Day +16. Intragastric administration of CHTA (0.4 mmol/kg body wt, twice daily) to hypercholesterolemic rats significantly lowered serum cholesterol levels at 10 and 16 days compared with CH-controls, whereas clofibrate treatment did not alter serum cholesterol levels at either Day +10 or Day +16.

Serum triglyceride concentrations in control, cholesterol-fed and drug-treated animals are listed in Table 2. Cholesterol feeding did not significantly alter serum triglyceride levels. Administration of CHTA to CH-controls, however, significantly decreased serum triglyceride concentrations when compared with initial values before drug treatment or with those of the CH-control group. Clofibrate treatment for 10 days did not alter serum triglycerides, but, following 16 days of drug treatment, serum triglyceride concentrations were elevated when compared with either initial values or with CH-control rat serum triglyceride concentrations.

Cholesterol feeding to rats markedly increased VLDL-, IDL- and LDL-C and decreased HDL-C levels when compared with those of the semisynthetic diet-fed group (Table 3). CHTA treatment significantly lowered VLDL-, IDL- and LDL-C concentrations. No change was observed in HDL-C levels. Similarly, clofibrate treatment also lowered IDL- and LDL-C levels, but there was a marked increase in VLDL- and a modest rise in HDL-C concentrations when compared with CH-controls. VLDL-apoB concentrations were increased with CHTA treatment when compared with CH-controls (Table 4). Inclusion of cholesterol in the semisynthetic diet increased VLDL and IDL protein concentrations (Table 5).

The SDS-PAGE pattern of VLDL apoproteins exhibited apoE, apoC-III and C-II as major and apoA-IV and A-I as minor bands. A faint unknown band with a molecular mass of about 21,000 was also observed. Cholesterol feeding significantly increased apoE concentrations compared with CH controls (Table 6). None of the apoprotein proportions changed in CHTA-treated animals, whereas clofibrate treatment produced smaller proportions of both ApoC-II and apoC-III. With SDS-PAGE of IDL apoproteins (Table 7), apoC-II was decreased with clofibrate treatment, but no other apoprotein proportions were altered with drug treatment. A distinct unknown band, similar to one found in total lipoproteins (26) and having a molecular mass of about 21,000 (similar to the unknown VLDL apoprotein band), was detected in IDL apoproteins.

Separation of apoA-IV, E, A-I, C-III and C-II from HDL was obtained. Whereas cholesterol feeding significantly increased the proportion of ApoA-IV, A-I and E in HDL with proportional decreases in apoC-II, clofibrate reduced both A-I and A-IV toward control values, but had no effect on ApoE (Table 8). CHTA treatment increased apoC-II, but had no effect on other apoproteins.

With IEF, VLDL ApoC peptides were focused in the pI range 4.42–4.73, and had four bands, which are characterized as apoC-II (pI 4.73), apoC-III-0 (pI 4.66), apoC-III-2(-1) (pI 4.49) and apoC-III-3 (pI 4.42). ApoE peptides showed 4–5 isoforms characterized as E-4 (pI 5.52), E-3 (pI 5.47), E-2 (pI 5.40), E-1 (pI 5.33) and at pI 5.26. In CH-controls, a distinct band appeared at pI 5.07 and a faint band at pI 4.92, which together accounted for 4–6% of the total apoprotein. Clofibrate treatment did not alter the ApoE isoform concentration compared with that of CH-controls (Table 9), whereas CHTA administration caused a small but significant reduction in $apoE_2$. ApoC-III-2(1) concentrations were significantly decreased with clofibrate treatment compared with CH controls and CHTA (Table 10). ApoC-II in clofibrate-treated animals was only significantly decreased when compared with CHTA.

IEF of IDL apoproteins showed faint bands in the apoA-I region characterized as apoA-I-1 (pI 5.91), A-I-2 (pI 5.89), A-I-3 (pI 5.64) and A-I-4 (pI 5.57). Similar to apoE isoforms in VLDL, apoE isoforms in IDL were focused in the pI range of 5.52–5.26. In CH-controls, IEF produced two unidentified peaks at pI 5.07 and 4.92, a result similar to that observed for apolipoproteins in VLDL. Apo A-I-1 was significantly increased with clofibrate administration (Table 11). Decreased proportions of apoC-III-2 were observed in clofibrate-treated rats (Table 12) when compared with CH controls and CHTA-

TABLE 3

Effect of CHTA and Clofibrate on Serum Lipoprotein Cholesterol Concentrations in Cholesterol-fed Rats[a]

	Lipoprotein cholesterol (mg/dl serum)[b]			
	VLDL	IDL	LDL[c]	HDL
I. Control[d]	8.7 ± 0.7	—	25.9 ± 4.1	73.9 ± 0.2
II. Cholesterol-rich (CH)	106 ± 18.6[e]	68.3 ± 11.9	49.9 ± 5.8[e]	26.4 ± 4.3[f]
III. CH + CHTA	71.2 ± 8.3[g]	40.7 ± 6.3[h]	27.1 ± 4.7[h]	26.2 ± 1.9[i]
IV. CH + Clofibrate	135 ± 15.1[j]	32.9 ± 4.4[k]	15.4 ± 7.0[j]	32.8 ± 5.7[j,k]

[a]Drug administration is the same as described in Table 1.

[b]Mean ± S.D., N = 3.

[c]LDL cholesterol may be overestimated because HDL_c is included in this fraction from cholesterol-cholic acid-fed rats.

[d]N = 2.

[e]$p < 0.005$, Group II vs I, III, IV.

[f]$p < 0.0001$, Group II vs I, IV.

[g]$p < 0.005$, Group III vs I, IV.

[h]$p < 0.005$, Group III vs II, IV.

[i]$p < 0.0001$, Group III vs I, IV.

[j]$p < 0.005$, Group IV vs I.

[k]$p < 0.005$, Group IV vs II.

TABLE 4

ApoB Concentrations in VLDL and IDL From Cholesterol-fed Rats Treated With Either CHTA or Clofibrate[a]

	ApoB concentrations (mg/dl)[b]		
	VLDL	IDL	LDL
II. Cholesterol-rich (CH)	6.82 ± 0.256	7.00 ± 1.46	11.1 ± 3.84
III. CH + CHTA	4.49 ± 0.581[c]	5.17 ± 0.832	7.78 ± 0.958
IV. CH + Clofibrate	7.40 ± 0.434	4.97 ± 0.407	7.22 ± 0.958

[a]Drug administration is the same as described in Table 1.

[b]Values are means ± SD of three pooled samples.

[c]Statistically significant at $p < 0.05$, Group IV vs II, III.

TABLE 5

Protein Concentrations of Serum Lipoproteins From Cholesterol-fed Rats Treated With CHTA or Clofibrate[a]

	Protein concentration (mg/dl)[b]		
	VLDL	IDL	HDL
I. Control	12.7 ± 5.93[c]	5.29 ± 3.37[c,d]	57.2 ± 2.20[d]
II. Cholesterol-rich (CH)	21.7 ± 2.60	19.5 ± 2.05	49.9 ± 8.67
III. CH + CHTA	16.6 ± 1.24	15.2 ± 0.685	39.3 ± 0.895[e]
IV. CH + Clofibrate	25.9 ± 3.42	13.8 ± 2.61	46.2 ± 2.31

[a]Drug administration is the same as described in Table 1.

[b]Mean ± SD of 3 pooled samples.

[c]Statistically significant, $p < 0.05$, Group I vs II, III, IV.

[d]N = 2.

[e]Statistically significant, $p < 0.05$, Group III vs I, II, IV.

TABLE 6

Relative Concentrations of VLDL Apolipoproteins Measured by SDS-PAGE on Serum Lipoproteins From Cholesterol-fed Rats With and Without CHTA or Clofibrate Treatment[a]

	Apolipoproteins (% of total)[b]			
	ApoA-IV	ApoE	ApoC-III	ApoC-II
II. Cholesterol-rich (CH)	3.24 ± 0.35	43.7 ± 1.72	26.8 ± 1.15	20.9 ± 0.84
III. CH + CHTA	1.52 ± 1.95	46.5 ± 4.53	28.7 ± 2.89	21.1 ± 0.96
IV. CH + Clofibrate	3.80 ± 0.53	56.0 ± 7.04	12.7 ± 7.38	12.1 ± 3.28[c]

[a]Drug administration is the same as described in Table 1.

[b]Mean ± SD of 3 pooled samples.

[c]$p < 0.005$, Group IV vs II, III.

TABLE 7

Relative Concentrations of IDL Apolipoproteins Measured by SDS-PAGE From Serum of Cholesterol-fed Rats Treated With CHTA or Clofibrate[a]

	Apolipoproteins (% of total)[b]			
	ApoE	ApoC-III	ApoC-II	Unidentified
II. Cholesterol-rich (CH)	31.4 ± 3.44	39.9 ± 7.31	13.9 ± 1.65	11.1 ± 3.29
III. CH + CHTA	30.0 ± 1.46	44.1 ± 2.25	11.1 ± 1.16	14.7 ± 1.73
IV. CH + clofibrate	34.6 ± 6.20	28.2 ± 8.94	7.21 ± 4.05	12.4 ± 2.40

[a]Drug administration is the same as described in Table 1.

[b]Mean ± SD of 3 pooled samples.

TABLE 8

Relative Concentrations of HDL Apolipoproteins Measured by SDS-PAGE From Serum of Cholesterol-fed Rats Treated With CHTA or Clofibrate[a]

	Apolipoproteins (% of total)[b]				
	ApoA-IV	ApoE	ApoA-I	ApoC-III	ApoC-II
I. Control	4.46 ± 2.18	36.0 ± 4.98[c]	32.9 ± 2.26	13.0 ± 0.39	13.5 ± 0.90
II. Cholesterol-rich (CH)	11.8 ± 1.75[d]	5.64 ± 0.49	62.8 ± 2.73[d]	11.9 ± 4.16	7.80 ± 1.40[e]
III. CH + CHTA	11.0 ± 2.02[d]	5.49 ± 4.32	61.7 ± 8.72[d]	13.7 ± 2.91	9.62 ± 1.50[f]
IV. CH + clofibrate	4.80 ± 1.36	5.11 ± 3.15	43.5 ± 9.30	35.3 ± 8.03[g]	10.6 ± 1.90

[a]Drug administration is the same as described in Table 1.

[b]Mean ± SD of 3 pooled samples.

[c]$p \leq 0.05$, Groups II, III, IV vs I.

[d]$p \leq 0.05$, Groups II, III vs I, IV.

[e]$p \leq 0.05$, Group II vs I, IV.

[f]$p \leq$ Group II vs III.

[g]$p \leq 0.05$, Group IV vs I, II, III.

TABLE 9

IEF Distributions of VLDL Apolipoprotein E in Rats Treated With CHTA and Clofibrate[a]

	Apolipoprotein Es (% of total)[b]			
	E4	E3	E2	E1
II. Cholesterol-rich (CH)	8.8 ± 1.4	44.9 ± 1.9	30.6 ± 1.6	15.7 ± 1.2
III. CH + CHTA	5.4 ± 2.9	43.5 ± 3.8	26.0 ± 1.7[c]	11.9 ± 2.6
IV. CH + Clofibrate	9.0 ± 3.6	40.9 ± 7.3	32.1 ± 12.0	18.0 ± 14.3

[a]Drug administration is the same as described in Table 1.

[b]Mean ± SD, n = 3 per group.

[c]$p \leq 0.01$, Group III vs II.

TABLE 10

IEF Distributions of VLDL Apolipoprotein C-III in Rats Treated With CHTA and Clofibrate[a]

	Apolipoprotein C-IIIs (% of total)[b]		
	III-0	III-2(1)	III-3
II. Cholesterol-rich (CH)	60.0 ± 6.0	12.8 ± 3.7	27.2 ± 6.8
III. CH + CHTA	51.0 ± 15.5	16.1 ± 3.6	32.9 ± 12.3
IV. CH + Clofibrate	31.5 ± 29.3	0 ± 0[c]	35.2 ± 32.2

[a]Drug administration is the same as described in Table 1.

[b]Mean ± SD, n = 3 per group.

[c]$p \leq 0.05$, Group IV vs II, III; III below detectable limits.

TABLE 11

IEF Distributions of IDL Apolipoprotein AI in Rats Treated With CHTA and Clofibrate[a]

	Apolipoprotein A-Is (% of total)[b]			
	1	2	3	4
II. Cholesterol-rich (CH)	30.3 ± 1.2	35.3 ± 3.5	10.7 ± 1.6	34.4 ± 4.3
III. CH + CHTA	30.4 ± 5.9	42.5 ± 2.3	6.7 ± 1.2	20.4 ± 6.8
IV. CH + Clofibrate	39.8 ± 6.0[c]	31.4 ± 5.2	9.5 ± 6.8	19.3 ± 9.3

[a]Drug administration is the same as described in Table 1.

[b]Mean ± SD, n = 3 per group.

[c]$p \leq 0.05$, Group IV vs II, III.

TABLE 12

IEF Distributions of IDL Apolipoprotein C-III in Rats Treated With CHTA and Clofibrate[a]

	Apolipoprotein C-IIIs (% of total)[b]		
	III-0	III-2(1)	III-3
II. Cholesterol-rich (CH)	69.7 ± 9.3	10.2 ± 3.5	20.1 ± 6.0
III. CH + CHTA	66.5 ± 6.6	11.0 ± 2.4	22.5 ± 4.7
IV. CH + Clofibrate	48.2 ± 1.4[c]	26.1 ± 1.0[c]	25.7 ± 2.4

[a]Drug administration is the same as described in Table 1.

[b]Mean ± SD, n = 3 per group.

[c]$p \leq 0.01$, Group IV vs II, III.

TABLE 13

IEF Distributions of HDL Apolipoprotein A-I in Rats Treated With CHTA[a]

	Apolipoprotein A-Is (% of total)[b]			
	1	2	3	4
I. Control	35.9 ± 9.47[c]	28.6 ± 0.554	18.2 ± 2.33[c]	31.4 ± 0.753
II. Cholesterol-rich (CH)	24.9 ± 5.0	27.1 ± 8.6	21.7 ± 3.1	26.4 ± 10.8
III. CH + CHTA	28.7 ± 3.7	31.0 ± 3.6	23.1 ± 1.3	17.1 ± 5.6

[a]Drug administration is the same as described in Table 1.

[b]Mean ± SD, n = 3 per group.

[c]$p \leq 0.05$, I vs II, III.

TABLE 14

IEF Distributions of HDL Apolipoprotein C-III in Rats Treated With CHTA[a]

	Apolipoprotein C-IIIs (% of total)[b]		
	III-0	III-2(1)	III-3
I. Control	50.0 ± 1.43	16.7 ± 2.36	33.2 ± 3.46[c]
II. Cholesterol-rich (CH)	55.0 ± 10.9	22.5 ± 12.3	22.6 ± 3.0
III. CH + CHTA	52.6 ± 10.8	19.4 ± 3.9	28.0 ± 7.6

[a]Drug administration is the same as described in Table 1.

[b]Mean ± SD, n = 3 per group.

[c]$p \leq 0.05$, Group I vs II, III.

treated rats, whereas apoC-III-0 concentrations were only significantly decreased when compared with CH controls.

IEF-analyzed HDL apoprotein revealed separated isoforms of apoA-I, apoC-III and apoE + A-IV. CHTA treatment did not alter any of these apoprotein concentrations (Table 13). The IEF pattern of HDL apoprotein of clofibrate-treated animals did not indicate any measurable bands in pI regions of apoA-I, apoE and apoCs due to insufficient sample sizes. However, cholesterol feeding did increase apoAI-1 and AI-3 (Table 14).

DISCUSSION

Although the rat, which is resistant to hypercholesterolemia and atherosclerosis, has limited use as an experimental model, cholesterol feeding (6) provides a lipoprotein pattern resembling that in miniature swine and rabbit models (7,8). Features include: (a) presence of β-VLDL, (b) increase in IDL, a remnant composite resulting from chylomicron and VLDL metabolism (27-30), (c) increase in LDL and (d) measurable concentrations of HDL-C (6-8). Additionally, in these models, higher concentrations of apoE were observed in VLDL, IDL and HDL (6-8). We also observed an increase in VLDL apoE proportions in cholesterol- plus cholic acid-fed rats when compared with controls (Controls, 32.7 ± 3.81%; CH-control, 43.7 ± 1.72%). In our studies with CH-controls compared with animals on a basal diet, VLDL cholesterol concentrations increased 12.2 times, whereas protein increased 1.7-fold. Additionally, with CHTA treatment, the ratio for cholesterol in VLDL became 8.18, whereas the protein ratio became 1.31. Our observations are compatible with the rat cholesterol feeding studies of Mahley and Holcombe (6), DeLamatre and Roheim (26) and Lasser et al. (31).

Nonetheless, clofibrate has no effect on serum total cholesterol or triglyceride in this cholesterol-fed rat model at +10 and +16 days of treatment. Concurrent studies wherein cholesterol concentrations were measured at 4, 7 and 14 days showed that clofibrate administration caused a transient lowering of total cholesterol levels on days 4 and 7 (Day +4, 164 ± 51.5; Day +7, 204 ± 77.4; Day +14, 171 ± 72 mg/dl). This transient effect (not observed with CHTA) may be due to offsetting changes in VLDL, LDL and HDL. At Day +16 the following changes were observed: VLDL and HDL cholesterol concentrations were significantly higher, and IDL- and LDL-cholesterol levels were significantly lower than those of CH-controls. The elevation in VLDL-cholesterol levels may be attributed to a decreased catabolism of the particles leading to lower IDL- and LDL-cholesterol concentrations. Decreased catabolism of VLDL in clofibrate-treated cholesterol-fed rats may be due to the decreased apoC-II concentrations because this apoprotein enhances lipoprotein lipase activity (32). Alternatively, there may be inhibition of lipoprotein lipase activity with no reduction in the usual transfer rates of apoC-III to HDL and apoE to VLDL. Our findings of no effect on total serum cholesterol concentration at +10 and +16 days are similar to those of Seri et al. (33) who observed no reduction in serum cholesterol levels in rats fed a chow diet supplemented with 2% cholesterol, 1% sodium cholate and 5% coconut oil for 4 days. However, in some studies with clofibrate treatment of rats fed cholesterol-containing diet, there was a decreased serum cholesterol concentration with no alteration in HDL-cholesterol levels, but this change did not result in an increased proportion or an absolute increase in apoA levels. In fact, we observed a significant decrease in the proportions of apoA-I with a concomitant increase in apoC-III in contrast to the usual finding of a direct relationship of ApoA-I and HDL-C, as was observed with other drugs in both rats (11,34) and human subjects (35). However, Kitazacki et al. also observed a significant decrease in apoA-I levels (36) in cholesterol-fed EXHC rats. In contrast, Krause and Newton (11) observed no alteration in apoA-I concentration.

CHTA is more effective than clofibrate in reducing total serum cholesterol levels in this model. The action of this aci-reductone appears to differ markedly from that of clofibrate because CHTA lowers the concentration of VLDL-, IDL- and LDL-cholesterol without altering C apoprotein proportions when compared with CH-controls. Clearly, in contrast to the action of clofibrate in this model, CHTA does not inhibit, but appears to enhance, VLDL catabolism. Interestingly, absence of alteration in the apoC-II to apoE ratios by CHTA treatment suggests that this oxidation reduction compound acts by a mechanism independent of apoprotein regulation of triglyceride hydrolysis.

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Cardiac Membrane Vitamin E and Malondialdehyde Levels in Heart Muscle of Normotensive and Spontaneously-hypertensive Rats

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The vitamin E (α-tocopherol) and free and bound malondialdehyde (MDA) in ventricular heart muscle and myocardial membrane from Wistar-Kyoto (W/K) normotensive and spontaneously hypertensive (SH) rats have been measured directly by high performance liquid chromatography (HPLC). Thiobarbituric acid-reactive substance (TBA-RS) in the myocardium and heart-muscle membrane of the two strains was also quantified by a colorimetric TBA test. It was found that SH-rat myocardium and myocardial membrane contained more than 3-fold less α-tocopherol than did heart muscle and cardiac membrane of the normotensive rat. Coincident with this relative vitamin E deficiency were several-fold greater amounts of MDA and TBA-RS in SH-rat myocardium and myocardial membrane. Most (87%) of the MDA in SH-rat heart muscle, but only 40% in W/K-rat heart muscle, was free (i.e., unbound). These results offer direct evidence that SH-rat myocardium is vitamin E-deficient and highly peroxidative, relative to cardiac muscle of the normotensive W/K parent strain. The lower vitamin E content of SH-rat myocardium is particularly striking, because SH-rat myocardial membrane was found to contain ~35% more phospholipid than myocardial membrane in the W/K rat. Although the amounts of myocardial TBA-RS are greater in the SH strain, they do not reflect the actual MDA profiles of the heart muscles or the heart membranes and cannot be used as a quantitative index of cardiac oxidative-injury status due to non-MDA TBA-RS in both strains.
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Peroxidation of polyunsaturated fatty acid (PUFA) moieties in membrane phospholipids is a causal contributor to the pathophysiology of many diseases (1). The heart muscle damage associated with cardiac ischemic and reperfusion injury may result, at least in part, from radical-mediated myocardial membrane lipid peroxidation, perhaps through superoxide-dependent, iron-promoted oxygen chemistry (2). Vitamin E (α-tocopherol), an essential antioxidant nutrient (3), is present in cardiac muscle (4) and may help prevent myocardial oxidative injury from free radicals produced in both the normal and diseased heart (3,5). Clinical data suggest that increased cardiac-membrane tocopherol tone effectively protects the heart from irreversible necrosis: Dietary supplementation with vitamin E is associated with a reduced risk of human mortality from cardiovascular disease (6), and vitamin E has been used therapeutically in ischemic heart-disease patients (7).

Despite such evidence, quantitative information on the relationship between myocardial membrane vitamin E content and cardiac membrane oxidative stress is lacking. The male spontaneously hypertensive (SH) rat appears to be a particularly suitable study-object in this regard. The SH rat, derived from the Wistar-Kyoto (W/K) normotensive strain, is an accepted model of human cardiovascular disease (8), because it displays a progressive, postweaning increase in systolic blood pressure (9). Although the plasma vitamin E concentration in both strains (when fed the same diet) is identical (10), whole-heart homogenate from the SH rat contains ~25% less vitamin E than does normotensive W/K-rat heart homogenate. Furthermore, recent methodological advances for direct quantification of MDA (11-13), a secondary end product of lipid hydroperoxide decomposition (1), have obviated reliance on the thiobarbituric acid (TBA) test (14) to estimate tissue and membrane oxidative-injury status.

We report here investigation of the relationships among cardiac membrane vitamin E level, TBA-reactivity and MDA content in the myocardial membrane of SH and W/K normotensive rats. The membrane data are placed within the context of the vitamin E, TBA-reactive substance (TBA-RS), and bound and free MDA levels in the myocardium of each strain.

EXPERIMENTAL PROCEDURES

Materials. N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), tris(hydroxymethyl)aminomethane (Tris), hydroxylamine hydrochloride, TBA, and tetramethoxypropane were from Sigma Chemical Co. (St. Louis, MO). Catalase (hydrogen-peroxide:hydrogen-peroxide oxidoreductase, EC 1.11.1.6; thymol-free analytical preparation from beef liver; 260,000 units/mg) and superoxide dismutase (SOD; superoxide:superoxide oxidoreductase, EC 1.15.1.1; analytical preparation from bovine erythrocytes; 5000 units/mg) were from Boehringer-Mannheim (Indianapolis, IN). Desferal (desferrioxamine B methanesulphonate) was a gift from Ciba A.G. (Basle, Switzerland). Chelex 100 was from Bio-Rad (Richmond, CA). Vitamin E (*d*-α-tocopherol) and [3,4-^{3}H]vitamin E (297 μCi/mg, sp. act.) were synthesized and purified by Hoffmann-La Roche. All organic solvents were of analytical grade (Burdick and Jackson, Muskegon, MI).

Animals. SH and W/K rats (10-wk-old) were purchased from Charles River Breeding Labs (Wilmington, MA) and maintained on Purina Rodent Chow (Ralston Purina, St. Louis, MO) and tap water ad libitum on a 12-hr light/dark cycle. When 12 wk of age, the rats were decapitated. Just prior to sacrifice, blood pressures and heart rates of conscious animals were taken by the indirect tail-cuff method (15), and body weights were recorded. The systolic blood pressures of the rats used in the studies described were: SH, 214 ± 2.9 mm Hg; W/K, 120 ± 3.9 mm Hg. Their

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Abbreviations: HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; MDA, malondialdehyde; PUFA, polyunsaturated fatty acid; SOD, superoxide dismutase; TBA-RS, thiobarbituric acid-reactive substance; TCA, trichloroacetic acid; Tris, tris(hydroxymethyl)aminomethane; HPLC, high performance liquid chromatography; SH, spontaneously hypertensive; W/K, Wistar-Kyoto.

heart rates were: SH, 420 ± 16 beats/min; W/K, 397 ± 9 beats/min. Their body weights were: SH, 273 ± 12 g; W/K, 282 ± 14 g (means ± S.D.; n ⩾ 30).

Isolation of myocardial membrane. The procedure was carried out in a dehumidified cold room (3°C) under amber lighting. Hearts of SH and W/K rats were rapidly excised, perfused ex vivo via the aorta with 50 ml ice-cold 10 mM HEPES buffer, pH 7.4, and trimmed. The ventricular myocardium from each strain was homogenized (100 mg tissue/ml HEPES) for 3 × 5 sec with a Tissuemizer (Tekmar, Cincinnati, OH) at "maximal" setting. The respective homogenates were filtered through 4-ply cheesecloth, and a portion of each filtrate was set aside for immediate lipid, vitamin E, TBA-RS, MDA and protein analyses (below). The remainder of each filtrate was subjected to salt extraction and differential centrifugation for myocardial membrane isolation, as detailed (11). The washed, purified membranes were resuspended in 10 mM HEPES buffer, pH 7.4, and used immedidately.

Extraction and quantification of lipid and vitamin E. During lipid and α-tocopherol extractions, care was taken to avoid sample exposure to air; only solvents purged with highly purified argon were used. Lipids were extracted from myocardial homogenate and membranes by a modified (16) Bligh-Dyer procedure (17). Lipid recovery was quantitative by the chemical criteria detailed (18): >98% of the radioactivity associated with the organic fraction obtained by direct saponification (18) of membrane metabolically labeled in vivo from [^{14}C]acetate, sodium salt (54.0 mCi/mmol, sp. act. [New England Nuclear, Boston, MA]), was recovered in the saponified fraction from the lipid extracted out of an identical amount of labeled lmembrane. Lipid ester was quantified by the hydroxamate reaction with L-α-phosphatidylcholine dipalmitate (Avanti Polar Lipids, Birmingham, AL) as standard (19).

Vitamin E was extracted into acetone according to procedures developed for rat liver (20). Tissue samples and extracts for vitamin E analysis were exposed to amber lighting only. In preliminary experiments, extraction efficiency was estimated by adding a known amount of [^{3}H]vitamin E to the myocardial suspension prior to homogenization and extraction and likewise supplementing the myocardial membrane prior to extraction. Because >96% of the ^{3}H-labeled vitamin E was recovered in the final tissue and membrane acetone extracts, the procedure was considered quantitative for vitamin E extraction from myocardium. Vitamin E determination was carried out fluorometrically by reverse-phase high performance liquid chromatography (HPLC) on a μBondapak C_{18} 3.9 mm × 30 cm column preceded by a radially-packed C_{18} precolumn (Waters/Millipore, Bedford, MA). A ternary-gradient HPLC system (Beckman Instruments, Palo Alto, CA) with flourescence detector (Perkin-Elmer, Norwalk, CT) was used. The mobile phase was methanol/water (97:3, v/v) at a flow rate of 2.0 ml/min (10). Calculation of vitamin E content in the experimental samples was by computer-assisted regression analysis from a standard peak-area response curve of varying amounts of pure *d*-α-tocopherol.

TBA test. TBA-reactivity as MDA-equivalents was determined colorimetrically (11). In brief, myocardial homogenate or membrane was acidified with 0.15 ml ice-cold 76% trichloroacetic acid (TCA)–2.3 N HCl/1.0 ml tissue suspension. Next was added 0.35 ml of a mixture of H_2O/7.14 M butylated hydroxytoluene in ethanol/1.51% TBA in 0.2 M Tris, pH 7.0 (1:1:5, v/v/v). After thorough mixing, the samples (pH = 2.4) were incubated in an 80°C shaking water bath for 30 min. The tubes were then chilled, and 0.5 ml ice-cold 91% TCA, followed by 2.0 ml $CHCl_3$, was added. After centrifugation for 30 min at 2000 × *g*, 4°C, the absorbance of the upper phase was read at 532 nm. MDA standard, prepared by hydrolysis of 1,1,3,3-tetramethoxypropane with 76% TCA-2.3 N HCl, was subjected to the identical TBA-test procedure to generate a standard curve of TBA-reactivity as MDA-equivalents. The molar amounts of TBA-RS (as MDA-equivalents) in the biological samples were calculated by computer-assisted regression analysis of the standard curve.

Isolation and quantification of MDA. MDA was isolated as its enolate anion by ion-pair HPLC with spectrophotometric detection at 267 nm (11). The myocardial homogenate or membrane suspension was prepared for HPLC analysis by rapid ultrafiltration through a YM2 membrane (Amicon, Danvers, MA) to exclude high mol-wt (>1000) molecules and particulates. The clear filtrate was then subjected to HPLC for "free" (unbound) MDA quantification.

In order to liberate any bound tissue MDA prior to HPLC analysis, samples were hydrolyzed in base (NaOH) at pH 12.0, 60°C, for 30 min (11,21). The hydrolysis reaction mixtures were then subjected to ultrafiltration (as above), and the clarified filtrates were neutralized (pH ~7.4) with HCl prior to HPLC analysis for "total" MDA. The difference in sample MDA prior to and after hydrolysis (i.e., between 'free" and "total" MDA) was considered "bound" MDA. Recovery of standard MDA in these procedures was >96%.

MDA for HPLC calibration chromatograms was prepared by hydrolysis of malonaldehyde-bisdiethylacetal (Merck, Darmstadt, West Germany) with H_2SO_4 and purification of MDA product as described (22). Absolute MDA concentration in the solution was checked by UV spectrophotometry at 267 nm with the extinction coefficient 34,000 M^{-1} cm^{-1} (22) in a Beckman DU-7 spectrophotometer.

Protein determination. Protein was quantified by a dye-binding microassay (23).

Statistical analysis. Comparisons of two group means were made by Student's *t*-test (24). Unless otherwise noted, differences between SH-rat and W/K-rat group means were significant ($p \leqslant 0.01$).

RESULTS

Biochemical characteristics of SH and W/K rat myocardial membrane. The properties of the membrane isolated from SH and W/K rat ventricular myocardia are summarized in Table 1. For both strains, ~70% of the total cardiac-muscle homogenate lipid was recovered in the isolated myocardial membrane. Because the phospholipid is localized almost exclusively in heart-muscle membrane (25), these data demonstrate an equivalent, high myocardial membrane recovery from both strains. The dramatically lower protein content of the membrane with respect to starting homogenate illustrates the efficiency of soluble myocardial (particularly contractile) protein extraction

TABLE 1

Biochemistry of SH and W/K Rat Myocardial Membrane[a]

Fraction	Lipid				Protein		Lipid ester: protein
	Ester		Phosphate				
	(%)	(mEquivalents/heart)[b]	(%)	(μg/heart)[b]	(%)	(mg/heart)[b]	
Heart muscle homogenate							
SH	100	9.9 ± 0.3	100	536.3 ± 31.4	100	53.2 ± 2.7[d]	0.18 ± 0.01
W/K	100	6.8 ± 0.6	100	367.7 ± 28.7	100	49.6 ± 2.4[d]	0.14 ± 0.01
Myocardial membrane							
SH	74.8 ± 3.2[d]	7.4 ± 0.4[c]	76.5 ± 4.4[d]	410.3 ± 23.4	23.1 ± 1.3[d]	12.3 ± 0.8[d]	0.60 ± 0.04
W/K	69.1 ± 4.5[d]	4.7 ± 0.2[c]	68.0 ± 4.9[d]	250.1 ± 16.3	24.2 ± 1.4[d]	12.0 ± 0.7[d]	0.40 ± 0.02

[a]Myocardial membrane was isolated from cardiac venticular muscle homogenates of SH and W/K rats by salt extraction and differential centrifugation, as described (11). Results are means ± S.D. ($n \geq 7$).

[b]One heart-equivalent represents ~1.0 g tissue wet-weight.

[c]89.9 ± 3.2% of the lipid ester was found in membrane phospholipid; the remainder was associated with neutral lipid.

[d]Difference between respective SH and W/K group means not statistically significant ($p > 0.01$).

during membrane isolation. The high proportion of lipid recovered in the myocardial membrane and the ~3-fold greater lipid ester:protein ratio of the membrane over the respective source homogenate indicate that the isolated membrane is representative of the myocardial membrane in situ. Further support for this conclusion rests with the finding that, after density-gradient centrifugation of either the isolated SH-rat or W/K-rat myocardial membrane, the resultant distribution of membrane types (~90% mitochondria, ~7% sarcoplasmic reticulum, ~3% sarcolemma) approximates the relative membrane areas of the three organelles in the intact heart-muscle cell (26). The present state of development of myocardial subfractionation procedures does not allow isolation of rat-heart sarcoplasmic reticulum and sarcolemma to purity and with high yield (27). Consequently, the washed particulate total-membrane fraction from SH and W/K myocardia was used for the studies herein.

Myocardial membrane of the SH rat contained ~35% more lipid ester and phospholipid, but not more protein, than did the membrane of W/K rat-heart muscle (Table 1). From the high tissue (phospho)lipid recovery in the isolated membrane and the similar (phospho)lipid enrichment in SH-rat myocardial homogenate relative to W/K-rat myocardial homogenate, the greater heart-muscle membrane (phospho)lipid content of the SH rat would appear to reflect the situation in situ.

Vitamin E contents of SH and W/K rat myocardia and myocardial membranes. SH and W/K rat-heart muscle and myocardial membrane were analyzed for their vitamin E content (Table 2). The isolated membrane from both strains represented a high proportion (~70%) of starting homogenate vitamin E, as was the case with tissue (phospho)lipid (Table 1). The ventricular cardiac muscle of the W/K normotensive rat contained some threefold more vitamin E than did the myocardium of the SH strain. A similar relative vitamin E deficit in SH-rat heart-muscle membrane was underscored by normalizing vitamin E mass to lipid mass: Because SH-rat myocardial membrane contains ~35% more lipid than W/K-rat myocardial membrane (Table 1), the vitamin E content of heart-muscle membrane from the SH rat is about fivefold less than that of the W/K-derived membrane, when normalized to lipid mass.

TBA-RS in SH and W/K rat myocardia and myocardial membranes. The content of TBA-RS in heart muscle and muscle membrane of SH and W/K rats is given in Table 3 as MDA-equivalents. Normalized to protein, the amount of TBA-RS in the ventricular myocardium in the SH rat was ~4-fold greater than in the normotensive W/K strain. Likewise, SH-rat myocardial membrane contained ~3-fold more TBA-RS (normalized to membrane protein) than did the membrane in the W/K rat. Between the two animal strains, the amounts of heart-muscle protein and myocardial membrane protein were equivalent, but SH-rat myocardium had the greater lipid content (Table 1). Consequently, when normalized to lipid mass, the differences in homogenate and membrane-associated TBA-RS between the two strains were diminished slightly, but not eliminated. The TBA-RS appears to be an endogenous tissue/membrane constituent, for it was present even when myocardial homogenization and membrane isolation were carried out in the presence of a battery of anti(per)oxidants and metal chelators, including SOD (10 nM, final conc.), catalase (20 μM, final conc.) and Desferal (100 μM, final conc.), demonstrated to inhibit TBA-RS formation from peroxidized heart lipids (11). Buffer ultrapurification with Chelex resin to remove trace metals likewise did not influence the TBA-RS content.

MDA contents of SH and W/K rat myocardia and myocardial membranes. The nonspecificity of the TBA test would appear to prohibit its use in the quantitative analysis of tissue or membrane MDA (11,13). Consequently, the MDA contents of ventricular muscle and myocardial membrane were determined by direct chromatographic MDA isolation (11). Myocardial homogenates and membrane samples were hydrolyzed to release any MDA bound to biomolecules such as proteins and nucleic acids (28), and the contents of MDA after hydrolysis ("total" MDA) were compared to those of parallel samples

TABLE 2

Vitamin E Contents of SH and W/K Rat-Heart Muscle and Myocardial Membrane

Fraction	Vitamin E[a] (%)	(μg/heart)	(μg/g protein)	(μg/mEquivalents lipid ester)	(μg/mg lipid phosphate)
Heart muscle homogenate					
SH	100	12.6 ± 0.9	236.8 ± 16.3	1.3 ± 0.1	23.5 ± 1.5
W/K	100	40.1 ± 2.3	808.5 ± 42.6	5.9 ± 0.4	109.1 ± 7.4
Myocardial membrane					
SH	76.9 ± 5.5[b]	9.7 ± 0.7	788.6 ± 41.7	1.3 ± 0.1	23.6 ± 1.5
W/K	70.1 ± 4.5[b]	28.1 ± 2.0	2341.7 ± 121.3	6.0 ± 0.3	112.4 ± 7.7

[a]Results are means ± S.D. ($n \geq 8$).

[b]Difference between respective SH and W/K group means not statistically significant ($p > 0.01$).

TABLE 3

TBA-Reactive Substances in SH and W/K Rat-Heart Muscle and Myocardial Membrane

Fraction	TBA-reactive substances[a] (μg MDA-equivalents/g protein)	(μg MDA-equivalents/mEquivalents lipid ester)
Heart muscle homogenate		
SH	196.43 ± 9.31	1.06 ± 0.07
W/K	47.98 ± 2.33	0.35 ± 0.02
Myocardial membrane		
SH	289.43 ± 12.01	0.48 ± 0.02
W/K	111.67 ± 5.37	0.29 ± 0.01

[a]Results are means ± S.D. ($n \geq 8$).

TABLE 4

MDA Contents of SH and W/K Rat-Heart Muscle and Myocardial Membrane

Fraction	MDA[a] (μg/g protein) Total	Bound	Free	(μg/mEquivalent lipid ester) Total	Bound	Free
Heart muscle homogenate						
SH	60.34 ± 3.61	8.08 ± 0.46	52.26 ± 2.61	0.32 ± 0.02	0.04 ± 0.01	0.28 ± 0.02
W/K	23.99 ± 1.24	14.31 ± 0.79	9.68 ± 0.75	0.18 ± 0.01	0.11 ± 0.01	0.07 ± 0.01
Myocardial membrane						
SH	185.37 ± 9.21	173.17 ± 9.94	nd[b]	0.31 ± 0.02	0.29 ± 0.01	nd[b]
W/K	35.01 ± 2.01	32.51 ± 1.92	nd[b]	0.09 ± 0.01	0.08 ± 0.01	nd[b]

[a]Results are means ± S.D. ($n \geq 8$).

[b]nd, not detected.

not hydrolyzed ("free" MDA), the difference considered 'bound" MDA.

As normalized to protein, the amount of myocardial MDA in the SH rat was ~2-fold greater than in the W/K normotensive animal, and the amount of myocardial membrane MDA was ~5-fold greater (Table 4). Most (87%) of the MDA in SH-rat heart muscle, but only ~40% of the total W/K-rat myocardial MDA, was free MDA. All membrane-associated MDA in both strains was bound and could be released only upon hydrolysis. When MDA contents were normalized to lipid mass, the inter-strain differences diminished slightly, because SH-rat myocardium is relatively enriched in (phospho)lipid (Table 1), but the ratio of bound MDA:free MDA in each strain's heart muscle or myocardial membrane did not change. As was the case with TBA-RS, the endogenous myocardial and membrane MDA amounts were not influenced by the presence of anti(per)oxidants during

tissue homogenization and membrane isolation or by buffer ultrapurification.

DISCUSSION

The present study offers direct evidence that heart muscle membranes in the SH rat are vitamin E-deficient relative to the myocardial membranes of the parent normotensive strain. Furthermore, the lower membrane vitamin E content is accompanied by greater amounts of endogenous membrane (and muscle tissue) MDA, an index of oxidative PUFA damage in biological systems and foods (29). This coincidence between low vitamin E status and high MDA content strongly indicates that SH-rat myocardium is highly peroxidative relative to W/K normotensive-rat myocardial tissue.

Bound MDA predominates in W/K-rat myocardium, whereas almost all of the MDA in SH-rat heart muscle is free MDA. The ~10-fold greater free MDA:bound MDA ratio in SH-rat myocardium indicates that the intrinsic capacity for MDA production in the heart muscle of the SH rat far exceeds the capacity for MDA condensation and cross-linking with tissue biomolecules (28,30).

The net amounts of myocardial and myocardial membrane MDA may be influenced to some extent by oxidative catabolism. Although mouse heart has been shown to convert ^{14}C-labeled MDA to $^{14}CO_2$ (and other products) (31), the relative abilities of SH- and W/K-rat myocardia to oxidize MDA are not known. The slightly (~1.8-fold) greater amount of bound MDA in SH-rat myocardium relative to the W/K-rat tissue, in spite of the ~5-fold greater free-MDA content in SH-rat heart muscle, demonstrates that SH-rat myocardium contains the larger MDA pool readily available for metabolic and/or excretory clearance (32). The relative sizes of the free MDA pools may reflect a greater efficiency of MDA clearance in the W/K rat relative to the SH strain. These data imply that, even though total myocardial MDA binding capacity is ~8-fold greater in the SH myocardium, net MDA production in the relatively vitamin E-deficient SH-rat myocardium is far greater than in the W/K-rat heart.

Lee and Csallany (21) have demonstrated that livers from Sprague-Dawley rats fed a vitamin E-deficient diet for 43 wk contained ~10-fold less α-tocopherol and ~3-fold more MDA than did livers from rats fed a normal diet. After 10 weeks on a vitamin E-deficient diet, rats are in a severe negative nitrogen and food balance (10,33). The SH and W/K rats used in this study were not in a wasting state. These physiological and metabolic differences notwithstanding, the association between depressed tissue α-tocopherol levels and enhanced tissue MDA content in a dietary animal model of vitamin E-deficiency (21) and in the SH rat (Tables 2 and 4) implies that a dynamic balance exists in situ between vitamin E level and tissue peroxidative status.

Alterations in this balance may have profound effects on membrane function, as our data at the membrane level, demonstrating an inverse correlation between myocardial membrane tocopherol tone and membrane-bound MDA, would suggest. It is known that reactivity of MDA with membrane constituents changes membrane properties (34), and the resistance of hepatomas to peroxidative damage is a reflection of their high α-tocopherol content relative to normal liver tissue (35). We have recently demonstrated, in fact, that the low membrane vitamin E content in the SH-rat heart enhances the susceptibility of cardiac membrane phospholipid to oxidative injury when exposed to oxy-radical chemistry (36).

The amounts of TBA-RS in SH-rat myocardium and myocardial membrane are significantly higher than in normotensive W/K-rat heart tissue. However, TBA-reactivity was not a true indicator of the peroxidative state of the myocardium. Neither the absolute amounts of TBA-RS nor the magnitudes of the relative inter-strain differences in TBA-RS quantitatively reflect the actual MDA profiles of the heart muscle or its membrane. These findings support our previous contention (11) that the TBA test cannot be used as anything other than an empirical indicator of membrane oxidative injury and extend our conclusion to the tissue level. Therefore, the TBA test may not be able to measure rat-heart MDA as a pathophysiological index of oxidative injury (cf. 37,38) or to quantify changes in liver and brain lipid peroxidation with dietary α-tocopherol (cf. 21,39).

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Effect of Dietary *trans* Fatty Acids on Some Membrane-associated Enzymes and Receptors in Rat Heart

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Three groups of male weanling Sprague-Dawley rats were fed diets containing 20% corn oil, 20% partially hydrogenated soybean oil (PHSBO) or 18% PHSBO + 2% corn oil. PHSBO contained about 48% of its total fatty acids as *trans*-octadecenoate. Rats were killed after 16–18 weeks of feeding the various diets, hearts were dissected and crude sarcolemma was prepared by differential centrifugation. The activities of ouabain-sensitive (Na^+ + K^+)ATPase were significantly lower in membranes of rats fed 20% PHSBO than the control rats fed 20% corn oil. The feeding of 2% corn oil with 18% PHSBO resulted in partial restoration of the enzyme activity. The maximum number of [^{3}H]ouabain-binding sites (B_{max}) was also lower in cardiac membranes of rats fed 20% PHSBO than those fed 20% corn oil. Similar to (Na^+ + K^+)-ATPase activity, some restoration of the number of [^{3}H]ouabain-binding sites was observed when 2% corn oil was fed with 18% PHSBO-containing diet. There was no difference in the binding affinity of the radioligand for the receptor among the 3 dietary groups. Adenylate cyclase activities (fluoride-, isoproterenol- and forskolin-stimulated) were lower in membranes of rats fed 20% PHSBO or 18% PHSBO + 2% corn oil than in the control group fed 20% corn oil. Density of the β-adrenergic receptor was the lowest in cardiac membranes of rats fed 20% PHSBO. The feeding of 2% corn oil with 18% PHSBO resulted in partial restoration of the maximum number of [^{3}H]dihydroalprenolol (DHA)-binding sites. The affinity of the binding sites was, however, not affected by the type of the dietary fat. The results of this study suggest that dietary *trans* fatty acids can affect the activities of (Na^+ + K^+)ATPase and adenylate cyclase and the density of digitalis and β-adrenergic receptors in rat heart.
Lipids 24, 39–44 (1989).

Because the type and the level of dietary fat is known to affect cardiovascular health and disease, there is a great deal of interest in understanding the mechanism of action of the dietary fats at the cellular level. Some of the cellular effects of dietary lipids on heart function may be exerted by their ability to induce changes in the structural lipids of cardiac sarcolemma. A number of studies have shown that the dietary lipids can influence the lipid composition of cardiac membranes (1–7). The diet-induced structural changes in membrane lipids were associated with modification of membrane-associated enzymes and receptors (1,4–8).

(Na^+ + K^+)ATPase and adenylate cyclase are membrane-associated enzymes, the activities of which have been shown to be dependent on the nature of the membrane lipids. Diet-induced changes in membrane lipids have been correlated with alterations in the activities of (Na^+ + K^+)ATPase (9–14) and adenylate cyclase in several tissues (1,4–8,15,16–18). However, there are few studies on the diet-induced changes in cardiac adenylate cyclase system. In one study, Wince and Rutledge (7) reported a decrease in adenylate cyclase activity in atrial homogenates of rats fed sunflower oil relative to the coconut oil. The K_D and B_{max} of [^{3}H]dihydroalprenolol (DHA)-binding sites were also decreased in atrial homogenates from rats fed sunflower oil. We have shown that the activity of adenylate cyclase was reduced and the characteristics of β-adrenergic receptors were altered in essential fatty acid (EFA) deficiency in rat heart (19). In another study (20) we found that the feeding of diets containing 10% menhaden oil, a rich source of ω3 fatty acids, resulted in an increase in adenylate cyclase activity and in the density of [^{3}H]forskolin-binding sites. In both of the above studies, there were changes in the fatty acid composition of cardiac membranes. An increase in catecholamine-stimulated adenylate cyclase activity with dietary cholesterol-supplementation and a reduction in the number of β-adrenergic receptors in rat heart also have been reported (4). The adenylate cyclase activity and β-receptor concentration was not affected by the dietary lipids (sunflowerseed oil vs sheep kidney fat) in this study. A similar increase in cardiac adenylate cyclase activity with a concomitant decrease in β-adrenergic receptor content has been reported recently in marmoset monkeys as a result of feeding diets supplemented with sheep kidney fat (6). These changes in adenylate cyclase system were attributed to alterations in the membrane cholesterol to phospholipid ratios.

trans Fatty acids, the geometric isomers of naturally occurring *cis* fatty acids, can also induce changes in membrane lipids. We have reported their incorporation into the exocrine gland membrane lipids (21–23) and changes in adenylate cyclase system (22,23). The purpose of the present study was to determine the effects of feeding diets containing *trans* fatty acids on cardiac adenylate cyclase and (Na^+ + K^+)ATPase activities and on β-adrenergic and digitalis receptors.

MATERIALS AND METHODS

All the dietary ingredients were purchased from TEKLAD (Madison, WI). Partially hydrogenated soybean oil (PHSBO) was supplied by Kraft, Inc. (Glenview, IL). All organic solvents were of nanograde and were obtained from Mallinckrodt (Paris, KY). All the biochemical reagents were purchased from Sigma Chemical Co. (St. Louis, MO). Cyclic [^{3}H]AMP (41 Ci/mmol), 1-[propyl-2,3-^{3}H]dihydroalprenolol (55 Ci/mmol) and [^{3}H]ouabain (42 Ci/mmol) were purchased from Amersham (Arlington Heights, IL). α[^{32}P]ATP (619–650 Ci/mmol) was obtained from ICN (Cleveland, OH).

Three groups of male weanling Sprague-Dawley rats (Holtzman Co., Madison, WI) were fed ad libitum semipurified diets containing 20% corn oil, 20% PHSBO or

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Abbreviations: DHA, dihydroalprenolol; EFA, essential fatty acid; FAME, fatty acid methyl ester; PHSBO, partially hydrogenated soybean oil; A-TLC, argentation-thin layer chromatography.

18% PHSBO + 2% corn oil. The diets were similar to the AIN-76A (24,25), except for high fat and sucrose content. Composition of the diets, including the fatty acid composition of the dietary lipids, has been reported (23). PHSBO contained about 48% of the total fatty acids as *trans* octadecenoic acid (t-18:1).

Rats were decapitated after 16–18 wk of feeding the various diets, hearts were dissected, washed several times with ice-cold physiological saline, chopped into small pieces with scissors and homogenized in a Potter-Elvejhem homogenizer with 9–10 volumes of a buffer containing 0.32 M sucrose, 0.05 M Tris-buffer, pH 7.4 and 1 mM $MgCl_2$. The homogenates were filtered through four layers of cheese cloth, and crude sarcolemmal membranes were prepared by differential centrifugation (26). The purity of the membrane preparations was determined by monitoring 5′-nucleotidase activity using the method of Aronson and Touster (27). The mitochondrial contamination was measured by succinic dehydrogenase activity (28) and microsomal contamination by glucose-6-phosphatase activity (27).

The crude cardiac sarcolemma prepared by the above method was used for all the studies (adenylate cyclase activity, β-receptor, fatty acid analyses and $Na^+ + K^+$-ATPase activities) except those for [^{3}H]ouabain binding studies. The latter were conduced in membranes prepared in an identical manner, except the tissues were homogenized in the presence of 0.1% Na-deoxycholate. The purpose of including 0.1% Na-deoxycholate in the homogenization medium was to expose all the potentially available binding sites in membranes which otherwise remain masked (29,30). In addition to measuring the [^{3}H]-ouabain-binding sites, ($Na^+ + K^+$)ATPase activities were also measured in membranes prepared in the presence of 0.1% deoxycholate.

Adenylate cyclase activity was measured in triplicate samples of the membranes (50 μg protein) by monitoring the conversion of α[^{32}P]ATP to cyclic-[^{32}P]AMP using the procedure of Sinensky et al. (31). The product, cyclic [^{32}P]AMP, was isolated by sequential column chromatography using Dowex and Alumina columns (32). Cyclic [^{3}H]AMP was added prior to column chromatography to monitor the recovery of cyclic [^{32}P]AMP. Details of the assay procedure have been described (18).

Beta-receptor-binding assays were performed by incubating 150–350 μg of the membrane protein (in duplicate) with 0.5 to 20 nM [^{3}H]DHA in a final volume of 0.5 ml of the incubation buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.4) for 15 min, followed by a rapid filtration on Whatman GF/A glass fiber filters, washing the filters three times, each with 4 ml of ice-cold buffer. Filters were dried and counted in a Liquid Scintillation Spectrometer (Beckman LS6800). The specific binding of [^{3}H]DHA is defined as the difference between the total binding and the amount bound in the presence of 1 mM alprenolol. The average ratio of the specific bound vs the nonspecific bound was about 2.8. The dissociation constants (K_d) and the receptor number of the binding sites (B_{max}) were determined using Scatchard plots (33) which were compiled from the specific binding data, with lines being fitted by linear-regression analysis.

($Na^+ + K^+$)ATPase activity was determined in homogenates and membranes by measuring the release of inorganic phosphate after incubation of the membranes (20–50 μg protein) in the presence of 3 mM Tris-ATP, 130 mM NaCl, 20 mM KCl and 3 mM $MgCl_2$, with and without 1 mM ouabain. Ouabain-sensitive activity was calculated by subtracting the amount of Pi released in the presence of 1 mM ouabain (Mg^{2+}-ATPase activity) from the total activity measured in the absence of ouabain. Homogenates or membranes were preincubated in the presence of all other reagents for 5 min at 37°C. Then Tris-ATP was added and the incubation carried out for 10 min. The reaction was stopped by adding 0.2 ml of 50% cold TCA. The protein precipitate was removed by centrifugation at 4000 × *g* for 10 min at 4°C. The supernatant was used for measuring the concentration of Pi by colorimetric assay, which was a modification (27) of the method of Fisk and Subbarow (34). The reaction was linear with respect to the time of incubation and the concentration of the protein.

The characteristics of digitalis receptor were studied by measuring the binding of [^{3}H]ouabain to the cardiac membranes prepared with 0.1% Na-deoxycholate, according to the method of Lin and Akera (35). Assays were initiated by adding 100–300 μg of the membrane protein to an incubation mixture containing 1 mmol Tris-inorganic phosphate (orthophosphoric acid titrated to pH 7.4 with Tris-base), 1 mM $MgCl_2$, 10 mM Tris-HCl buffer (pH 7.4) and [^{3}H]ouabain in a final volume of 500 μl. Nonspecific binding was measured in the presence of 6 mM nonlabeled ouabain. All samples were assayed in duplicate or triplicate. The incubation was carried out for 5 min at 37°C with gentle shaking and terminated by immersing the reaction mixture in ice-water followed by rapid vacuum-filtration of the contents through GF/A glass fiber filters. The filters were washed 3 times with the 4-ml portions of the ice-cold incubation buffer, dried and counted. The dissociation constants and the maximum number of the binding sites were calculated by Scatchard analysis, as described above for DHA binding.

Protein was measured by the method of Lowry et al. (36) using bovine serum albumin as a standard.

Lipids were extracted from aliquots of homogenates and membranes with chloroform-methanol by the method of Bligh and Dyer (37). Phospholipids were separated from the neutral lipids by column chromatography on Bio-Sil A columns. Aliquots of total lipids and phospholipids were transesterified with boron trifluoride methanol (38), and the fatty acid composition of the methyl esters was determined by argentation-thin layer chromatography (A-TLC) and gas chromatography (GC) as described (21). Fatty acid methyl esters (FAME) were identified by comparison with the retention times of known standards using 2 different columns, a packed column, 15% Silar 10 C on Gas chrom Q (12 ft × 2.0 mm i.d.) and a megabore capillary column, DBWAX (12 m × 0.53 mm i.d., J and W Scientific, Folsom, CA). The former was run at 220 and 230°C, whereas the latter was temperature programmed from 60–220°C at 8°C/min and held at this temperature for 20 min.

RESULTS

Body weight gains of rats fed the diet containing 20% PHSBO were lower than those fed the control diet, 20% corn oil. The feeding of 2% corn oil with 18% PHSBO

restored most of the weight gain to the control levels. These data have been reported (23).

There was a 6-8-fold enrichment in 5′-nucleotidase activity in the membranes over that of the homogenates. There was no significant difference in the relative purity of the membranes among the 3 dietary groups. No mitochondrial contamination was detected, as shown by the absence of succinic dehydrogenase activity. The microsomal contamination, as evaluated by glucose 6-phosphatase activity, was 3-4%. Similarly, there was no difference in the yield of membrane protein among the 3 dietary groups.

The feeding of diets containing PHSBO resulted in the incorporation of *trans*-octadecenoate, the main geometric isomer in PHSBO, in the cardiac membrane phospholipids (Table 1). This fatty acid constituted 9-11% of the total fatty acids in the total phospholipids of membranes. A similar degree of incorporation of t-18:1 was observed in total lipids of the membranes (data not shown).

There were changes in the fatty acid patterns of total phospholipids of cardiac membranes of rats fed 20% PHSBO and of those fed 20% corn oil. Increases in the levels of 16:1 and *cis* 18:1 and decreases in the levels of 16:0 DMA, 16:0, 18:0 and 18:2 were generally observed in cardiac membranes of rats fed 20% PHSBO compared to those fed 20% corn oil. Also, 20:3 ω9 constituted 4.4% of the total fatty acids in membranes of the 20% PHSBO group, whereas it made up less than 1% of the total fatty acids in the other 2 groups. The inclusion of 2% corn oil with the 18% PHSBO diet resulted in restoring most of the fatty acid levels to those of the control group fed 20% corn oil. The levels of 20:4 were higher in cardiac membranes of rats fed 18% PHSBO + 2% corn oil than the other two groups. The double bond index of the total fatty acids in the membrane phospholipids was not significantly different among the 3 dietary groups.

TABLE 1

Fatty Acid Composition of Total Phospholipids of Cardiac Membranes of Rats Fed Diets Containing PHSBO and Corn Oil

Fatty acid	20% Corn oil	20% PHSBO	18% PHSBO + 2% corn oil
16:0 DMA	2.7 ± 0.4[a]	1.5 ± 0.3[b]	1.9 ± 0.1[a,b]
16:0	17.4 ± 1.4[a]	10.4 ± 0.6[b]	12.2 ± 0.7[b]
16:1	0.7 ± 0.1[a]	1.7 ± 0.3[b]	1.9 ± 0.2[b]
18:0 DMA	1.6 ± 0.3[a]	1.5 ± 0.3[a]	2.1 ± 0.1[a]
18:0	21.8 ± 0.6[a]	18.5 ± 1.2[b]	17.8 ± 0.7[b]
c-18:1	7.4 ± 0.7[a]	19.2 ± 1.0[b]	12.7 ± 0.8[c]
t-18:1	—[a]	9.4 ± 0.6[b]	10.8 ± 0.7[b]
18:2	19.5 ± 0.6[a]	14.1 ± 0.7[b]	13.5 ± 0.5[b]
20:2	0.5 ± 0.2[a]	0.2 ± 0.1[a]	0.3 ± 0.2[a]
20:3 ω9	0.7 ± 0.1[a]	4.4 ± 0.5[b]	0.9 ± 0.2[a]
20:3 ω6	0.2 ± 0.1[a]	0.7 ± 0.2[a]	0.4 ± 0.2[a]
20:4	14.7 ± 0.5[a]	14.0 ± 0.8[a]	19.3 ± 1.1[b]
22:4	1.1 ± 0.1[a]	0.8 ± 0.3[a]	0.7 ± 0.3[a]
22:5	2.2 ± 0.3[a]	1.5 ± 0.4[a]	1.8 ± 0.1[a]
22:6	2.1 ± 0.3[a]	0.8 ± 0.1[a]	1.6 ± 0.4[a]
Double bond index	136.6 ± 4.8[a] (136.6 ± 4.8)[a]	135.3 ± 5.4[a] (144.7 ± 5.4)[a]	144.6 ± 8.0[a] (155.4 ± 8.0)[a]

Values are in area percentage, mean ± SE of 3 analyses/group; — indicates trace. Values with different superscripts in the same row are significantly different ($P < 0.05$) from each other using Newman-Keul's test.

Double bond index = sum of (% fatty acid × number of double bonds). Values in parentheses designate double bond index calculated with t-18:1 as an unsaturated fatty acid.

DMA, dimethylacetal.

The activities of ouabain-sensitive $(Na^+ + K^+)$ATPase, Mg^{2+}-ATPase and 5′-nucleotidase in cardiac homogenates and membranes of rats fed the 3 diets are shown in Table 2. $(Na^+ + K^+)$ATPase activity in homogenates and membranes of rats fed 20% PHSBO was only 35-40% of that in the control group fed 20% corn oil. The feeding of 2% corn oil with 18% PHSBO resulted in partial restoration of the enzyme activity. A similar trend, but to a lesser degree, was also observed in Mg^{2+}-ATPase activity in cardiac homogenates. An opposite trend in Mg^{2+}-ATPase activity was observed in membranes of rats fed 20% corn oil vs those fed PHSBO. Due to a large variation, however, these differences in Mg^{2+}-ATPase activities were not significant. There was no significant difference among the 3 dietary groups in 5′-nucleotidase activity in homogenates or membranes. Similar differences were observed in $(Na^+ + K^+)$ATPase and Mg^{2+}-ATPase activities of cardiac homogenates and membranes prepared in the presence of 0.1% Na-deoxycholate (data not shown).

The results of receptor-binding studies are shown in Table 3. There was no significant difference in binding affinity of [^{3}H]ouabain in the cardiac membranes among the 3 groups. The maximum number of the binding sites, however, was the lowest in membranes of rats fed 20% PHSBO. The feeding of 2% corn oil with 18% PHSBO resulted in partial restoration of the B_{max} to that of the control group. There was a decrease in the density of β-receptors as evaluated by [^{3}H]DHA binding in membranes of rats fed 20% PHSBO compared with those fed 20% corn oil. The inclusion of 2% corn oil with the PHSBO diet resulted in partial restoration of the β-adrenergic receptor concentration to that of the control levels. Due to large variations in B_{max} from one experiment to another, the results of the combined experiments do not show a significant difference among the 3 dietary groups. However, in each experiment, the B_{max} was lower in the membranes of rats fed 20% PHSBO than those fed 20% corn oil. If the value for B_{max} in the control (20% corn oil) group is set at 100%, the comparative values for the 20% PHSBO group were 59 ± 9%, and those for 18% PHSBO + 2% corn oil group were 88 ± 7%. This type of consistent trend was not observed in the affinity of the binding sites.

Data on adenylate cyclase activity are presented in Table 4. There was no significant difference in the basal activity. However, fluoride-, isoproterenol- and forskolin-stimulated adenylate cyclase activity was generally lower in cardiac membranes of rats fed 20% PHSBO than those fed 20% corn oil. Unlike the $(Na^+ + K^+)$ATPase activity, however, the feeding of 2% corn oil with the 18% PHSBO did not restore the adenylate cyclase activity, which was still lower than that in cardiac membranes of the control rats fed 20% corn oil.

DISCUSSION

The incorporation of *trans* fatty acids into animal and human tissues (39), their metabolism, biological and nutritional effects have been extensively studied (40). Most of the studies show that *trans* fatty acids, which are

TABLE 2

ATPase and 5′-Nucleotidase Activities in Cardiac Homogenates and Membranes of Rats Fed Diets Containing Corn Oil and PHSBO

	$(Na^+ + K^+)$ATPase[a]		Mg^{2+}-ATPase		5′-Nucleotidase	
Diet fed	Homogenate	Membrane	Homogenate	Membrane	Homogenate	Membrane
20% Corn oil	3.5 ±0.5	25.6 ±2.4	34.8 ±0.4	103.1 ±17.4	2.3 ±0.1	14.3 ±1.4
20% PHSBO	1.4[b] ±0.4	9.1[c] ±3.0	31.4[b] ±0.7	168.6 ±36.0	2.0 ±0.1	16.0 ±0.7
18% PHSBO + 2% corn oil	2.4 ±0.3	18.8 ±3.0	32.9[b] ±0.2	143.6 ±27.6	2.2 ±0.1	16.4 ±2.4

[a]Ouabain-sensitive activity which represents the difference between the total activity (measured without ouabain) and the activity measured in the presence of 1 mM ouabain. PHSBO, partially hydrogenated soybean oil.

Values are mean ± SEM of 4 enzyme assays/group; each assay was done in duplicate. Values with superscripts are significantly different from the control group, 20% corn oil, [b]$P < 0.05$, [c]$P < 0.01$, using Student's t test. The specific activities are given as μmol Pi/mg protein/hr.

TABLE 3

Effect of Feeding Diets Containing Corn Oil and PHSBO on the Characteristics of [^{3}H]Ouabain Binding and [^{3}H]DHA Binding Sites in Rat Heart

	K_D		B_{max}	
Diet fed	Ouabain (nM)	DHA (nM)	Ouabain (pmol/mg protein)	DHA (fmol/mg protein)
20% Corn oil	36.7 ± 2.2[a]	5.0 ± 0.7[a]	1.22 ± 0.08[a]	203 ± 31[a]
20% PHSBO	35.4 ± 5.1[a]	3.4 ± 0.7[a]	0.77 ± 0.05[b]	124 ± 32[a]
18% PHSBO + 2% corn oil	33.2 ± 7.0[a]	5.0 ± 1.5[a]	0.99 ± 0.12[a,b]	178 ± 28[a]

Values are mean ± SEM of 4 preparations/group; each was assayed in duplicate or triplicate. Values with different superscripts in the same column are significantly different ($P < 0.05$) using analysis of variance, Newman-Keul's test. PHSBO, partially hydrogenated soybean oil.

TABLE 4

Adenylate Cyclase Activities in Cardiac Membranes of Rats Fed Diets Containing Corn Oil and PHSBO

Diet fed	Basal	+Fluoride	+Isoproterenol	+Forskolin
20% corn oil	23 +1.0	200 ± 6.6	55 ± 2.3	248 ± 22
20% PHSBO	25 ± 1.0	154 ± 5.8[b]	44 ± 2.3[a]	212 ± 10
18% PHSBO + 2% corn oil	20 ± 1.3	161 ± 12.5[a]	45 ± 3.3[a]	178 ± 17[a]

The enzyme activity is shown as pmol cAMP/mg protein/min. The values are mean ± SE of 4 experiments, each done in triplicate. Values with superscripts are significantly different ([a]$P < 0.05$, [b]$P < 0.01$ using Student t test) from the corresponding values in the control group (20% corn oil). The basal activity was measured in the absence of any exogenous activator. Stimulated activity was measured in the presence of 15 mM NaF, 10 μM isoproterenol or 33 μM forskolin. PHSBO, partially hydrogenated soybean oil.

commonly present in foods, do not have any adverse effects on human health, provided the diet contains sufficient amounts of the essential fatty acids. *Trans* fatty acids, like their naturally occurring *cis* isomers, can be incorporated into cell membrane lipids. Therefore, the focus of our study was to determine if some of the membrane properties, such as the activities of membrane-associated enzymes and receptor characteristics, are altered as a result of the incorporation of *trans* fatty acids. $(Na^+ + K^+)$ATPase was chosen in view of its role in cardiac muscle as a receptor for digitalis. Similarly, β-adrenergic-stimulated adenylate cyclase system in heart regulates a variety of physiological processes crucial for heart function.

The feeding of a diet containing 20% PHSBO may have caused a marginal EFA deficiency as shown by an accumulation of 20:3 ω9 and 20:3 ω9/20:4, which was 0.3. A ratio higher than 0.4 is a commonly used biochemical index of an EFA deficiency (41). There was no biochemical evidence of an EFA deficiency in terms of fatty acid patterns in cardiac membranes of rats fed 18% PHSBO + 2% corn oil.

Because the double bond index of total fatty acids in membrane phospholipids was the same in the 3 dietary

groups, the decrease observed in $(Na^+ + K^+)$ATPase and adenylate cyclase activity in cardiac membranes of rats fed diets containing *trans* fatty acids cannot be attributed to the membrane fluidity. We have observed a similar lack of correlation between membrane fluidity and cardiac adenylate cyclase activity, both in EFA deficiency (19) and by feeding diets containing different oils (20). In other tissues, such as salivary glands (23) and lacrimal glands (22), we found that membrane fluidity was decreased by feeding diets containing 20% PHSBO. It appears, therefore, that the heart differs from other tissues in terms of having a greater ability to regulate the fluidity of its membranes. There is also some evidence that arachidonic acid levels in the heart, as opposed to the kidney, were not significantly decreased in EFA deficiency (42), which is also consistent with our findings in a marginal EFA deficiency (19) and in the present study by feeding a 20% PHSBO-containing diet.

The decrease in $(Na^+ + K^+)$ATPase activity which was observed in cardiac homogenates and membranes of rats fed 20% PHSBO may be attributed partly to a reduced number of the enzyme molecules as shown by lower B_{max} values for [^{3}H]ouabain-binding and no change in the affinity constants. It is known that, under these experimental conditions, the specific binding of ouabain represents the concentration of the enzyme (43). Whether the synthesis of $(Na^+ + K^+)$ATPase or its degradation was affected by feeding diets containing 20% PHSBO cannot be determined and will need further investigations.

There are two types of ouabain-binding sites, high affinity and low affinity, which have been shown to exist in the heart (44-46). In the present study, we have measured mainly the high affinity binding sites by using the rapid filtration method which has been used to measure [^{3}H]ouabain-binding sites in rat cardiac microsomes (45).

$(Na^+ + K^+)$ATPase is a lipid-dependent enzyme, and the nature of the acyl group, as well as the polar head groups, has been shown to alter its activity (9-14). Therefore, the question is whether the decrease in its activity in membranes of rats fed 20% PHSBO may be due to the diet-induced incorporation of the *trans* fatty acids into membrane phospholipids or by some other mechanism such as the changes in the concentration of the enzyme. Our data on receptor binding using [^{3}H]ouabain would suggest that the number of enzyme units was decreased. Abeywardina et al. (47) have shown that different dietary lipids induced changes in the fatty acid composition of cardiac membranes. However, the specific activity nor the Arrhenius plots for $(Na^+ + K^+)$ATPase were changed. The characteristics of [^{3}H]ouabain-binding sites were not measured in this study. Modulation of ATPase (1) and other membrane-associated enzymes (5) in rat cardiac mitochondria has been reported by feeding diets containing different types and levels of lipids. Changes in enzyme activities were correlated with the changes in membrane phospholipid composition. A decrease in 5′-nucleotidase, phosphodiesterase and p-nitrophenylphosphatase activity in cardiac sarcolemma of rats fed for 4 wk, diets enriched with coconut oil, has been reported (2). The changes in enzyme activities were associated with fatty acid compositional changes in membrane phospholipids. The effects of feeding diets containing *trans* fatty acids on $(Na^+ + K^+)$ATPase have not been studied previously.

Adenylate cyclase system consists of the receptor (R), guanine nucleotide-binding regulatory proteins (G_s and G_i) and the catalytic unit of the enzyme (C). Diet-induced changes in any of these components may theoretically account for the decrease in adenylate cyclase activity which was observed in cardiac membranes of rats fed 20% PHSBO or 18% PHSBO + 2% corn oil, as compared with the control group fed 20% corn oil. The decrease in isoproterenol-stimulated activity in membranes of rats fed PHSBO may be due to the lower density of the β-adrenergic receptor as compared to the control group. However, the fluoride- and forskolin-stimulated activities were also lower in membranes of rats fed PHSBO-containing diets. Because fluoride and forskolin activation of adenylate cyclase does not involve the β-receptor, it is possible that other components of the adenylate cyclase system such as the levels of G proteins (G_s and G_i) or the catalytic unit itself may be altered by the feeding of diets containing PHSBO. Although we did not measure the characteristics of the catalytic unit in this study, in a previous study (20) we found that the number of the catalytic units of the enzyme were increased and the sensitivity of adenylate cyclase to forskolin, which directly binds to the catalytic unit (48-50), was higher in cardiac membranes of rats fed 10% menhaden oil compared with those fed 10% corn oil or 8% coconut oil + 2% corn oil. Our finding of lower adenylate cyclase activity and B_{max} for [^{3}H]DHA in membranes of rats fed 20% PHSBO is consistent with the results of our previous study (19) in which we found that a marginal or severe EFA deficiency was associated with lower adenylate cyclase activity and lower concentrations of the β-adrenergic receptor in rat heart. Also, it lends further support to the notion that the observed decrease in adenylate cyclase activity and in β-receptor concentration in heart of rats fed 20% PHSBO may be due to a concomitant EFA deficiency. However, our finding that in the 18% PHSBO + 2% corn oil group which did not cause an EFA deficiency, the adenylate cyclase activity was significantly lower than in the control group fed 20% corn oil, suggests that some of the effects of PHSBO may be due to *trans* fatty acids. This is an important observation because *trans* fatty acids are present in several foods, especially in partially hydrogenated vegetable oils. To maximize their effects, we used rather high levels of *trans* fatty acids (17-19% of the total calories). Therefore, it would be important to study the effects of feeding lower levels of *trans* fatty acids—4-5% of total calories, which is more common in the western diets—on cardiac adenylate cyclase system. It would also be interesting to study the effects of individual *trans* fatty acids. Because the PHSBO contained about 2% of the total fatty acids as c,t-(t,c or t,t) 18:2 (23), it is conceivable that some of the effects of diets containing PHSBO may be due to these isomeric fatty acids.

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Effect of Dietary n-3 Polyunsaturated Fatty Acids on Cholesterol Synthesis and Degradation in Rats of Different Ages

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Male Sprague-Dawley rats four weeks or eight months of age were fed purified diets containing 10% fat, either as a blend of safflower oil and palm olein (polyunsaturated fatty acids, PUFA, 34%), a blend of linseed oil and palm olein (PUFA, 33%) or sardine oil (PUFA, 33%) for four weeks. In other trials, sterol contents were made equivalent by supplementing cholesterol to a blend of corn oil and palm olein (PUFA, 30%) or phytosterol to sardine oil (PUFA, 30%). Fish oil was hypolipidemic in rats of different ages, but it tended to increase liver cholesterol in adult animals and this was not improved by the addition of phytosterol. The age-dependent increase in liver cholesterol was not duplicated in rats fed a vegetable fat blend supplemented with cholesterol. At both ages, liver 3-hydroxy-3-methylglutaryl coenzyme A reductase activity was lower in the sardine oil than in the other groups. There were no significant age- or diet-related differences in the activity of liver cholesterol 7α-hydroxylase. Fecal steroid excretion was comparable in age-matched rats fed diets supplemented either with cholesterol or phytosterol. Sardine oil reduced the Δ6-desaturase activity markedly as compared with linseed oil, and age-dependent reduction of the desaturase activity was observed in all dietary groups examined. Thus, the results showed a specific effect of fish oil on lipid metabolism.
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Evidence for the beneficial effect of fish oils rich in n-3 polyunsaturated fatty acids (PUFA), compared with vegetable oils, in the prevention of hyperlipidemia has been accumulating in experimental animals as well as humans, although it is still controversial (1-3).

An elevation of the serum cholesterol level with age is one of the major risk factors to atherosclerotic coronary heart disease in aged animals including humans (4,5). In this context, fish oil may be of value in preventing an age-dependent increase in serum lipid levels. Nevertheless, the effect of dietary fish oil on the cholesterol homeostasis in aged animals is not fully understood (6). Previously, we observed that the response of the hepatic cholesterol homeostatic mechanism to dietary cholesterol diminishes with age, whereas the susceptibility to dietary fat type is enhanced (7,8). In order to compare the possible effect of fish oil on lipid metabolism in aged rats, the difference in the fatty acid composition was confined in the present study to that of PUFA.

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Abbreviations: CPO, a blend of corn oil and palm olein (4:6); HMG-CoA reductase, 3-hydroxy-3-methylglutaryl coenzyme A reductase; LDL, low density lipoprotein; LPO, a blend of linseed oil and palm olein (3:7); PUFA, polyunsaturated fatty acid; SDO, sardine oil; SPO, a blend of safflower oil and palm olein (3:7); GLC, gas liquid chromatography; TLC, thin layer chromatography.

MATERIALS AND METHODS

Animals. Young (3 wk old) and adult (8 mo old) male Sprague-Dawley rats obtained from Japan Charles River (Kanagawa, Japan) were housed individually in a room with controlled temperature and light (20-23°C and 0800 to 2000 hr). After acclimatization for one week, rats were divided into groups of eight each and fed purified experimental diets ad libitum. After 4 wk, rats were decapitated at night (0200 hr). Feces were collected for 2 days, beginning 5 days before killing, and lyophilized.

Experimental diets were prepared according to the recommendation of the American Institute of Nutrition (9): casein, 20; fat, 10; mineral mixture, 3.5; vitamin mixture, 1.0; choline bitartrate, 0.2; DL-methionine, 0.3; cellulose, 5.0; corn starch, 15; and sucrose, 45.0 (in wt %). Vitamin and mineral mixtures were purchased from Oriental Yeast Co. (Tokyo, Japan). To prevent the oxidation of fats, experimental diets were prepared weekly and were packed in a pouch containing an oxygen absorbent (Ageless S-200, Mitsubishi Gas Chemical Co., Tokyo) and stored at 4°C (5). Diets served were changed every day. Safflower oil and linseed oil were products of the Nissin Oil Co. (Tokyo, Japan). Sardine oil (SDO) and palm olein were provided by the Nippon Oil & Fat Co. (Tokyo, Japan) and the Fuji Oil Co. (Osaka, Japan), respectively. Corn oil was the product of the Ajinomoto Co. (Tokyo, Japan). Safflower oil, linseed oil or corn oil were blended with palm olein (3:7 by weight for SPO [safflower oil + palm olein] and LPO [linseed oil + palm olein]), and 4:6 by weight for CPO (corn oil + palm olein) in order to make the total PUFA content equal to that of sardine oil. In Experiment 1 the sterol content was not adjusted, in order to learn the effect of the fat itself. In Experiment 2, in order to exclude the possible effect of sterols on lipid metabolism, cholesterol was added to CPO and plant sterols (β-sitosterol from soybean) to SDO, so that plant and animal sterol contents were similar in each diet. The crystalline sterols were dissolved in fat at 37°C. By gas liquid chromatographic (GLC) analysis, the content of plant sterols was 0.16% (campesterol, 20; β-sitosterol, 58; and stigmasterol, 22%), 0.22% (campesterol, 25; β-sitosterol, 49; and stigmasterol, 26%) and 0.52% (campesterol, 26; β-sitosterol, 73; and stigmasterol, 1%) for SPO, LPO and CPO, respectively. The cholesterol content of SDO was 0.31 and 0.59% in Experiments 1 and 2, respectively. β-Sitosterol (Sigma Chemical Co., St. Louis, MO) added in SDO was composed of 37% campesterol and 61% β-sitosterol. Fatty acid compositions and sterol contents of dietary fats are shown in Table 1.

Materials. DL-3-Hydroxy[3-^{14}C]methylglutaric (HMG) acid (52 mCi/mmol) and [1-^{14}C]linoleic acid (59 mCi/mmol) were obtained from Amersham International plc. [4-^{14}C]Cholesterol (57.5 mCi/mmol) was purchased from New England Nuclear (Boston, MA). Radioactive HMG-CoA (5,000 dpm/nmol) was prepared as described elsewhere (10). Radioactive cholesterol and linoleic acid were purified by thin layer chromatography (TLC) prior to use (11). All reagents used were analytical grade.

TABLE 1

Fatty Acid Compositions of Dietary Fats

	Fatty acid (wt %)												
	14:0	16:0	16:1 n-7	18:0	18:1 n-9	18:2 n-6	18:3 n-3	18:4 n-3	20:1	20:5 n-3	22:1	22:5 n-3	22:6 n-3
Experiment 1													
SPO	0.7	24.0	0.3	2.9	38.0	33.1	0.6	—	—	—	—	—	—
LPO	0.7	23.0	0.3	3.1	38.9	14.8	18.5	—	—	—	—	—	—
SDO	6.1	16.0	9.3	3.2	14.7	3.0	1.1	3.2	8.3	14.2	6.5	1.9	9.7
Experiment 2													
CPO	0.6	23.5	0.1	2.6	42.3	29.1	1.2	—	—	—	—	—	—
SDO	7.5	17.2	7.1	2.3	14.2	1.3	0.9	3.4	8.1	13.5	6.0	1.6	9.4

Experiment 1: SPO, safflower oil + palm olein (3:7 by weight) containing 0.16% phytosterol; LPO, linseed oil + palm olein (3:7 by weight) containing 0.22% phytosterol; and SDO, sardine oil containing 0.31% cholesterol. The sterol content was not adjusted in Experiment 1. Experiment 2: CPO, corn oil + palm olein (4:6 by weight) containing 0.52% phytosterol and SDO, sardine oil containing 0.59% cholesterol. The sterol content of each fat was adjusted in Experiment 2.

Analyses. The activities of HMG-CoA reductase, cholesterol 7α-hydroxylase and Δ6-desaturase in liver microsomes were measured as described previously (7,8). Serum, liver and microsomal lipids were extracted, and cholesterol, triglyceride and phospholipid were measured (10). The fatty acid composition of microsomal phosphatidylcholine was analyzed by GLC (8). Fecal neutral (12) and acidic steroids (13,14) were analyzed by GLC using 5α-cholestane (Nakarai Chemicals, Kyoto, Japan) and 23-nor-deoxycholic acid (Steraroids Inc., Wilton, NH) as an internal standard, respectively. Sterol contents in dietary fats were also analyzed by GLC (12). Microsomal cholesterol was determined enzymatically (8) and protein by the method of Lowry et al. (15).

Data were analyzed by Student's t-test to examine the age-effect between the same dietary group or a one-way analysis of variance followed by Duncan's multiple range test to inspect all differences within the same age group (16).

RESULTS

In both experiments, food intake and weight gain were comparable in age-matched rats. Relative liver weight (g/100 g body weight) was also comparable in Experiment 1, although in Experiment 2, it was slightly (but significantly) higher in the SDO groups of young, but not adult, rats (Table 2).

Table 3 shows concentrations of serum lipids. No significant age-dependent increase in the lipid level was found in both experiments, except for serum cholesterol in rats fed SDO in Experiment 2. In Experiment 1, the concentration of serum cholesterol was lower in the SDO than in the SPO and LPO groups regardless of age. The effect of fat type on the triglyceride level was more marked in adult than in young rats; it was significantly higher in adult rats fed fat rich in n-6 PUFA than in those fed fats rich in n-3 PUFA. A similar response was observed in phospholipid. These trends were essentially duplicated even when sterol contents were made equivalent (Experiment 2).

Table 4 shows concentrations of liver lipids. In Experiment 1, the liver cholesterol level was comparable among the groups of young rats, whereas it was considerably higher in the SDO than in the SPO and LPO groups among adult rats. In Experiment 2, the liver cholesterol level was significantly lower in young rats fed SDO than in those fed CPO, whereas no fat-dependent difference was found in adult rats due to an age-dependent increase in the former. The increase in liver cholesterol was attributable mainly to that of esterified cholesterol. In Experiment 1, there was no significant fat- or age-effect in esterification of liver cholesterol. However, in Experiment 2, a significant fat-dependent response was observed in esterification of liver cholesterol in young rats (young, 56.3 $\pm$ 3.2 and 34.2 $\pm$ 3.8 and adult, 49.5 $\pm$ 2.7 and 42 $\pm$ 3.6% of total liver cholesterol, for CPO and SDO, respectively). The concentration of triglyceride, especially in young rats, was low in the SDO group. An age-dependent increase in liver triglyceride was marked in rats fed n-3 PUFA fats in both experiments. There was a slight but significant difference in the concentration of phospholipid due to dietary fats. A significant age-related reduction of phospholipid was observed only in the SDO group in Experiment 1.

Table 5 shows the specific activity of HMG-CoA reductase, cholesterol 7α-hydroxylase and Δ6-desaturase of liver microsomes. In Experiment 1, there was a trend toward decreasing the HMG-CoA reductase activity with age, although the difference was not significant. In both ages, the reductase activity tended to be lower in the SDO than in the other groups. Cholesterol 7α-hydroxylase activity was comparable regardless of age or diet. There was a significant age-dependent decrease in the activity of Δ6-desaturase in all groups of rats. The desaturase activity was markedly higher in the LPO than in the SDO and SPO groups in both ages, that of the latter two groups being comparable. The microsomal cholesterol content was not influenced either by diet or age. In Experiment 2, the activity of HMG-CoA reductase tended to be higher in the CPO than the SDO groups, but no age-associated change was observed. Cholesterol 7α-hydroxylase activity was again comparable regardless of diet or age.

Table 6 shows the fatty acid composition of phosphatidylcholine of liver microsomes in Experiment 1. There was a detectable difference in the composition of PUFA

TABLE 2

Body Growth and Food Intake in Young and Adult Rats Fed Different Fats

		Initial body wt (g)	Weight gain (g/4 wk)	Food intake (g/day)	Liver weight (g/100 g BW)
Experiment 1					
Young	SPO	81 + 1	257 ± 10	21.4 ± 0.5	4.36 ± 0.13
	LPO	81 ± 1	219 ± 16	21.7 ± 0.3	4.43 ± 0.32
	SDO	81 ± 1	241 ± 14	19.8 ± 0.7	4.36 ± 0.07
Adult	SPO	586 ± 29	104 ± 14	30.8 ± 1.3*	2.98 ± 0.11*
	LPO	577 ± 19	79 ± 12	27.9 ± 1.2*	2.97 ± 0.11*
	SDO	584 ± 25	89 ± 15	26.7 ± 0.9*	3.10 ± 0.05*
Experiment 2					
Young	CPO	76 ± 1	217 ± 9	19.2 ± 0.8	4.81 ± 0.10[a]
	SDO	75 ± 1	217 ± 11	17.5 ± 0.7	4.41 ± 0.06[b]
Adult	CPO	578 ± 30	78 ± 13	26.0 ± 0.5*	3.20 ± 0.10*
	SDO	590 ± 19	79 ± 8	24.4 ± 0.7*	2.99 ± 0.10*

Values are mean ± SE of eight animals.

[a,b]In each experiment, values in the same column without common superscript letters denote significant difference ($p < 0.05$).

*Significantly different ($p < 0.05$) than the corresponding young rats. See Table 1 for abbreviations.

TABLE 3

Serum Lipid Concentrations in Young and Adult Rats Fed Different Fats

	Young			Adult		
	TCH	TG	PL	TCH	TG	PL
	mg/100 ml serum					
Experiment 1						
SPO	101.1 ± 6.3[a]	157 ± 22	218 ± 15[a]	101.4 ± 8.6[a]	188 ± 14[a]	195 ± 14[a]
LPO	89.2 ± 7.0[a]	142 ± 18	158 ± 18[b]	80.2 ± 5.5[a,b]	133 ± 15[b]	159 ± 7[a]
SDO	66.9 ± 2.7[b]	122 ± 4	142 ± 5[b]	61.3 ± 6.2[b]	116 ± 10[b]	107 ± 8[b]
Experiment 2						
CPO	96.4 ± 5.4[a]	207 ± 28[a]	190 ± 9[a]	105.2 ± 6.1[a]	221 ± 20[a]	195 ± 12[a]
SDO	59.8 ± 3.2[b]	131 ± 5[b]	123 ± 6[b]	77.4 ± 6.6[b,*]	141 ± 11[b]	132 ± 6[b]

Values are mean ± SE of eight rats.

[a,b,c]In each experiment, values in the same column without common superscript letters denote significant difference ($p < 0.05$).

*Significantly different ($p < 0.05$) than the corresponding young rats.

TCH, total cholesterol; TG, triglyceride; PL, phospholipid. See Table 1 for abbreviations.

TABLE 4

Liver Lipid Levels in Young and Adult Rats Fed Different Fats

	Young				Adult			
	TCH	ECH	TG	PL	TCH	ECH	TG	PL
	mg/g liver							
Experiment 1								
SPO	3.63 ±0.21	1.23 ±0.21	33.1[a] ±3.2	32.5[a] ±0.5	3.84[a] ±0.18	1.62[a] ±0.19	43.6 ±8.1	31.1 ±0.8
LPO	3.73 ±0.22	1.58 ±0.25	25.4[a,b] ±3.8	33.8[a] ±0.7	4.00[a,b] ±0.22	1.78[a,b] ±0.20	39.5* ±4.0	32.8 ±0.7
SDO	3.82 ±0.24	1.52 ±0.30	20.6[b] ±1.1	36.0[b] ±0.3	4.67[b,*] ±0.26	2.48[b,*] ±0.30	30.2* ±1.3	32.9* ±0.8
Experiment 2								
CPO	7.04[a] ±0.55	3.99[a] ±0.44	74.8[a] ±14.1	29.9[a] ±1.9	6.68 ±0.75	3.38 ±0.49	73.6 ±12.9	29.8[a] ±1.6
SDO	3.81[b] ±0.16	1.30[b] ±0.15	27.6[b] ±3.7	35.2[b] ±0.4	5.33* ±0.79	2.39 ±0.52	41.6 ±8.1	34.6[b] ±0.9

Values are mean ± SE of eight rats.

[a,b]In each experiment, values in the same column without common superscript letters denote significant difference ($p < 0.05$).

*Significantly different ($p < 0.05$) than the corresponding young rats.

ECH, esterified cholesterol; see Tables 1 and 3 for other abbreviations.

TABLE 5

The Activities of Liver Microsomal Enzymes in Young and Adult Rats Fed Different Fats

		HMG-CoA reductase	Cholesterol 7α-hydroxylase	Δ6-Desaturase	Microsomal cholesterol
		pmol/min/mg microsomal protein			μg/mg protein
Experiment 1					
Young	SPO	235 ± 41[a]	23.5 ± 1.8	309 ± 41[a]	20.2 ± 0.9
	LPO	161 ± 35[a,b]	25.1 ± 2.0	526 ± 85[b]	22.6 ± 0.9
	SDO	98.9 ± 18.5[b]	22.5 ± 0.9	222 ± 21[a]	22.1 ± 0.7
Adult	SPO	142 ± 34[a]	23.0 ± 1.2	156 ± 23[a,*]	22.1 ± 1.1
	LPO	133 ± 16[a]	23.8 ± 2.2	271 ± 16[b,*]	21.5 ± 1.3
	SDO	60.3 ± 11.7[b]	23.0 ± 1.9	122 ± 21[a,*]	24.8 ± 1.3
Experiment 2					
Young	CPO	91.7 ± 14.4	20.5 ± 2.6	ND	27.1 ± 0.8
	SDO	75.1 ± 9.5	18.8 ± 1.9	ND	25.5 ± 0.5
Adult	CPO	121 ± 17[a]	23.5 ± 0.7	ND	25.5 ± 0.8
	SDO	59.0 ± 6.1[b]	19.5 ± 2.2	ND	26.9 ± 0.8

Values are mean ± SE of eight rats.

[a,b]In each experiment, values in the same column without common superscript letters denote significant difference ($p < 0.05$).

*Significantly different ($p < 0.05$) than the corresponding young rats.

ND, not determined. See Table 1 for abbreviations.

TABLE 6

Fatty Acid Compositions of Liver Microsomal Phosphatidylcholine in Young and Adult Rats Fed Different Fats (Experiment 1)

	Young			Adult		
Weight %	SPO	LPO	SDO	SPO	LPO	SDO
16:0	16.2 ± 0.5[a]	17.6 ± 0.6[a]	22.3 ± 0.6[b]	16.3 ± 0.3[a]	17.2 ± 0.3[a]	22.6 ± 0.7[b]
16:1	0.6 ± 0.1[a]	0.6 ± 0.0[a]	2.3 ± 0.1[b]	0.9 ± 0.1[a]	1.0 ± 0.1[a]	3.1 ± 0.3[b]
18:0	27.2 ± 0.4[a]	26.6 ± 0.5[a]	20.7 ± 0.5[b]	26.1 ± 0.5[a]	25.4 ± 0.8[a]	20.3 ± 0.4[b]
18:1	6.3 ± 0.3[a]	6.5 ± 0.2[a]	8.6 ± 0.2[b]	6.9 ± 0.2[a]	7.7 ± 0.3[a,b,*]	9.1 ± 0.3[c]
18:2n-6	5.7 ± 0.3[a]	9.5 ± 0.3[b]	1.7 ± 0.1[c]	8.0 ± 0.4[a,*]	10.0 ± 0.4[b]	4.0 ± 0.3[c,*]
20:3n-6	0.6 ± 0.0[a]	2.3 ± 0.1[b]	0.8 ± 0.0[a]	1.3 ± 0.1[a,*]	2.1 ± 0.1[b,*]	1.0 ± 0.1[a]
20:4n-6	31.6 ± 0.6[a]	19.2 ± 0.6[b]	8.6 ± 0.3[c]	30.8 ± 0.6[a]	22.0 ± 0.8[b,*]	11.6 ± 0.6[c,*]
20:5n-3	0.5 ± 0.1[a]	3.4 ± 0.8[b]	12.2 ± 0.4[c]	0.6 ± 0.1[a]	3.3 ± 0.3[b]	10.7 ± 0.4[c,*]
22:5n-6	5.7 ± 0.2[a]	0.2 ± 0.1[b]	0.3 ± 0.1[b]	1.1 ± 0.1[a,*]	0.1 ± 0.0[b]	0.2 ± 0.0[b]
22:5n-3	0.3 ± 0.0[a]	1.5 ± 0.1[b]	4.4 ± 0.1[c]	0.5 ± 0.0[a]	1.6 ± 0.1[b]	3.2 ± 0.2[c,*]
22:6n-3	2.9 ± 0.1[a]	10.7 ± 0.4[b]	15.0 ± 0.4[c]	5.6 ± 0.3[a,*]	7.5 ± 0.3[b,*]	10.9 ± 0.7[c,*]
18:2 Metabolites/18:2	7.0 ± 0.4[a]	2.7 ± 0.2[b]	5.7 ± 0.3[c]	4.3 ± 0.2[a,*]	2.5 ± 0.1[b]	3.3 ± 0.3[c,*]

Values are mean ± SE of eight rats. Fatty acids less than 1% are excluded.

[a,b,c]In age-matched rats, values in the same line without common superscript letters denote significant difference ($p < 0.05$).

*Significantly different ($p < 0.05$) than the corresponding young rats. 18:2 metabolites include 20:3, 20:4 and 22:5n-6. See Table 1 for abbreviations.

due to the source of dietary fats, both the ratio of n-3 to n-6 PUFA and of the linoleate metabolites to linoleate being markedly different. In the SDO group percentages of n-6 PUFA increased, whereas those of n-3 PUFA decreased with age. A similar tendency was observed in the LPO group. On the contrary, in the SPO group, the percentage of 18:2n-6 and 22:6n-3 increased and that of 22:5n-6 decreased in relation to age. The ratio of linoleate metabolites to linoleate decreased significantly with age in rats fed SPO or SDO, but not in those fed LPO.

As Table 7 shows, the weight of dried feces was higher in the CPO than in the SDO groups, especially in young rats. Fecal weight increased with age, reflecting the amount of the diet ingested. Although there was no dietary fat-dependent difference in fecal excretion of neutral steroids, it tended to decrease with age, especially in rats fed a CPO diet. There was no significant difference in the excretion of phytosterol due to diet or age (young, 9.11 ± 0.46 and 7.42 ± 0.93 and adult, 9.61 ± 1.12 and 8.48 ± 0.60 mg/day, for CPO and SDO, respectively). Acidic steroid excretion per day was markedly larger in adults than in young rats, and in the former, it was significantly higher in the CPO than the SDO groups. However, when acidic steroid excretion was expressed as

TABLE 7

Fecal Steroid Excretion in Young and Adult Rats Fed Different Fats (Experiment 2)

	Young		Adult	
(mg/day)	CPO	SDO	CPO	SDO
Fecal weight (g/day)	1.71 ± 0.08[a]	1.40 ± 0.09[b]	2.29 ± 0.05*	1.99 ± 0.1*
Neutral steroids	10.0 ± 0.8	9.95 ± 0.65	8.05 ± 0.37*	7.63 ± 0.86
Acidic steroids	3.60 ± 0.32	3.86 ± 0.39	9.66 ± 0.64[a,]*	6.86 ± 0.31[b,]*
Total steroids	13.6 ± 0.7	13.2 ± 1.1	17.7 ± 0.9*	14.5 ± 1.5

Values are mean ± SE of eight rats.

[a,b]In age-matched rats, values in the same line without common superscript letters denote significant difference ($p < 0.05$).

*Significantly different ($p < 0.05$) than the corresponding young rats.

See Table 1 for abbreviations.

mg/day/kg body weight, there was no significant age- or fat-effect (young, 12.1 ± 1.1 and 13.0 ± 1.6 and adult, 13.9 ± 0.9 and 10.8 ± 1.6 mg/day/kg body weight for CPO and SDO, respectively).

DISCUSSION

Numerous studies have shown a hypocholesterolemic effect of fish oil compared with vegetable oil in rats (1,6, 17–19). Recently, Roach et al. (19) showed that there was no significant difference in the low density lipoprotein (LDL) receptor activity in the liver between rats fed fish oil and safflower oil, and suggested that reduced production rather than reduced uptake from the plasma of LDL cholesterol is responsible for the cholesterol-lowering effect.

In the present study, SDO reduced the serum cholesterol level both in young and adult rats even when compared with a linseed oil blend containing α-linolenic acid. In Experiment 1, aging did not influence the concentration of serum cholesterol in all groups, whereas in Experiment 2 it increased significantly in rats fed SDO. The age-dependent increase might be attributed in part to a higher cholesterol content of fish oil used in this trial than in the preceding experiment. It has been reported that cholesterol homeostatic mechanism in response to dietary cholesterol operates efficiently in young but not in adult rats (7). Although the serum triglyceride level increased with age (4), the triglyceride-lowering effect of SDO seemed to be more efficient in adult than in young rats. LPO exerted an intermediate effect on the serum lipid profile, in agreement with the observation by Suzuki et al. (6), but not with our previous study (8). These results at least indicate an importance of the dietary level of α-linolenic acid in manifesting a desirable effect, especially in adult rats (20).

Conflicting data are available with respect to the concentration of liver cholesterol of young rats fed fish oil (17,18). In the present study, the liver cholesterol level in young rats was comparable among dietary groups in Experiment 1, although SDO contained a small amount of cholesterol. In Experiment 2, in which the dietary cholesterol level was adjusted, the concentration of liver cholesterol remained normal in young rats fed SDO, but increased in young rats fed CPO. In adult rats, however, the liver cholesterol level was comparable in two groups due to the rise in the SDO group with age. These results indicated that the liver cholesterol-lowering ability of SDO is age-dependent. The effect of plant oils was not influenced by aging. Therefore, it is evident that dietary fat and aging interact each other in regulation of hepatic cholesterol homeostasis. Although there was no significant dietary fat- or age-effect in esterification of liver cholesterol (Experiment 1), n-6 fatty acid supplemented with cholesterol provoked a higher esterification of liver cholesterol than did fish oil (Experiment 2), suggesting that n-6 PUFA supplemented with cholesterol increases esterification of liver cholesterol more than fish oil increases it. The low liver triglyceride level in rats fed fish oil may, in part, be attributable either to the low absorption rate (21), reduced lipogenesis (22) or an increased β-oxidation (23), regardless of a low serum triglyceride level.

Fecal steroid excretion appeared to be independent of fat type and age (Table 6). N-3 as well as n-6 PUFA have been reported to enhance biliary steroid excretion compared with saturated fatty acids (8,24). The hypocholesterolemic effect of fish oil, compared to n-6 PUFA, appears at least to be not relevant to the change in fecal steroid excretion (17). Although the age-dependent increase in the bile acid excretion did not parallel the change in the activity of liver cholesterol 7α-hydroxylase, no age-effect was observed on the bile acid excretion when expressed per kg body weight.

Fatty acids of n-3 series, particularly those in fish oil, reduced the activity of liver HMG-CoA reductase compared with the n-6 counterparts irrespective of the addition of cholesterol. The results are consistent with the observation reported by Roach et al. (19). When the diets contained cholesterol, age-related reduction of the reductase activity was not observed, probably due to the feedback regulation by dietary cholesterol. The results suggest the possibility that the hypocholesterolemic effect of fish oil may at least be manifested by the decrease in cholesterol synthesis without largely influencing steroid excretion. It has been reported that there was no close relationship between HMG-CoA reductase activity and bile acid metabolism in rats fed different fats (25).

The significant dependence of Δ6-desaturase on dietary fat type or age was consistent with other reports (8,20,26). The desaturase activity in rats fed a SDO diet was lower

than in those fed diets rich in linoleic or linolenic acid. It has been reported recently that in rats 20:5n-3 and/or 22:6n-3 may act as an analogue of 20:4n-6 in inhibiting Δ6-desaturation by a feedback mechanism (27). The higher desaturase activity in rats fed α-linolenic acid than in those fed linoleic acid may reflect the preference of this type of n-3 PUFA as a substrate (8,20). In fact, a significant proportion of n-3 PUFA such as 20:5, 22:5 and 22:6 was detected in rats fed LPO.

The fatty acid profile of microsomal phosphatidylcholine indicated an alteration of the desaturation systems depending on fat type. Although the ratio of linoleate metabolites to linoleate might reflect the various desaturation systems, Δ6-desaturation seems to be primarily responsible for the change in the ratio (8). Fish oil inhibits liver Δ6- and Δ5-desaturation of n-6 PUFA (28). In the present study, the change in the proportion of 22:5n-6 and 22:6n-3 in the various groups might also imply an inhibition of Δ4-desaturation of both the linoleate and linolenate series, thus suggesting an inhibition of overall desaturation of PUFA.

In summary, the hypolipidemic effect of n-3 PUFA, particularly those in sardine oil, was confirmed even in aged rats, but fish oil exaggerated an accumulation of liver cholesterol with age. Cholesterol occurring in fish oil appeared to influence its metabolic effect. PUFA of the n-3 series inhibited the overall desaturation of n-6 PUFA. These results suggest an interplay among dietary fat, cholesterol and age in maintaining the cholesterol homeostasis.

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Mechanisms of Linoleic Acid Uptake by Rabbit Small Intestinal Brush Border Membrane Vesicles

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We examined the initial transport of a long-chain unsaturated fatty acid, linoleic acid, by brush border membrane vesicles isolated from rabbit small intestine. This preparation allowed us to examine the transport of linoleic acid across the brush border membrane without the effect of the unstirred water layer or cytosol binding proteins. Linoleic acid was solubilized in a 2 mM taurocholate solution which did not compromise the functional integrity of the vesicles. Linoleic acid uptake in the range of 1 to 100 μM followed passive diffusion kinetics. Time course study showed that linoleic acid uptake reached maximal levels during the initial 15 seconds. Although the amount of linoleic acid accumulated in the vesicles diminished over the next 30 minutes, the molar quantity was still twentyfold higher than that of D-glucose (6.5 vs 0.33 nmol/mg protein). Uptake of D-glucose by the vesicles demonstrated typical osmotic responsiveness. We found no osmotic effect on linoleic acid uptake. Hypotonic lysis of membrane vesicles loaded with linoleic acid released 40% of the fatty acid. We concluded that a major portion of the accumulated fatty acid was bound to or incorporated into the membrane itself while ca. 40% did traverse the membrane and accumulated in the intravesicular space as nonmicellar aggregates. The known inhibitors of anion transport, diisothiocyanatostilbene and isothiocyanatostilbene did not change the transport of linoleic acid. We conclude that, in the absence of an unstirred layer or cytosol proteins, linoleic acid transport at up to 100 μM concentration is passive with rapid accumulation both by the cell membrane and the lumen of vesicles.
Lipids 24, 51-55 (1989).

Linoleic acid is a major dietary essential fatty acid. Aside from being incorporated into cellular and membrane phospholipids (1), linoleic acid is an important source of energy and is the molecular precursor of dihomo-gamma-linoleic acid and arachidonic acid, which are precursors of prostaglandins synthesis (2,3). Because of the importance of prostanoids in normal physiological functions, the consequences of dietary deficiency or malabsorption of linoleic acid can involve many organs and systems (3). Prostanoid metabolites of linoleic acid may also play a major role in the prevention of peptic disease as seen in the recent decline in incidence and virulence of peptic ulcer disease which has been linked to the marked increase in consumption of linoleic acid-containing vegetable oils in the western world (4).

Very limited information is available regarding the initial mechanism of linoleic acid transport across the lumenal brush border membrane of the small intestine. Most previous studies of intestinal transport of fatty acids in vivo and in vitro have demonstrated that simple diffusion is the main mechanism of absorption which is modified by the resistance of the unstirred water layer and a number of other lumenal factors (5-9). Chow and Hollander (10,11) have shown that the absorption of linoleic acid by either everted gut sacs or perfused small intestinal segments is a concentration-dependent dual transport mechanism. That is, carrier-mediated diffusion and simple diffusion are predominant at low and high lumenal concentration of the substrate, respectively. To bypass the interference of the unstirred water layer, cytosol binding proteins and other physiological variables, Proulx and coworkers have isolated brush border membranes from small intestinal segments to study the uptake of fatty acids, cholesterols and phospholipids in vitro (12-15). They found that lipids were taken up by simple diffusion and were either bound to or incorporated into the membrane bilayers with little accumulation in the vesicular lumen.

In the present study, we used an established membrane vesicle filtration technique (16) to examine the initial transport of linoleic acid across intestinal brush borders using brush border membrane vesicles isolated from rabbit small intestine.

MATERIALS AND METHODS

Chemicals. 1-[^{3}H]-D-glucose was purchased from ICN Radiochemicals (Irvine, CA), and [^{14}C]linoleic acid was purchased from NEN (Boston, MA). Phloridzin, phloretin, 4,4′-diisothiocyananostilbene-2,2′-disulfonic acid (DIDS), 4-acetamido-4′-isothiocyanatostilbene-2,2-disulfonic acid (SITS) and all chemicals for various enzyme assays were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals used in the present study were reagent grade.

Isolation of brush border membrane vesicles (BBMV). BBMV were prepared from frozen jejunal and ileal mucosal scrapings of New Zealand white rabbits by a calcium precipitation method described by Stevens et al. (17) with some modifications developed in the laboratory of E. M. Wright (personal communication). All steps were performed at 4°C. Briefly, each gram of thawed mucosal aliquot was suspended in 20 vol of an isolation buffer I (300 mM D-mannitol in 10 mM Tris-HCl buffer, pH 7.0) and homogenized with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) set at #9 for 30 sec. The mucosal homogenates were centrifuged at 500 g for 5 min. The supernatant was aspirated and saved. Each of the pellets was homogenized again at the same setting for 15 sec in 20 ml of fresh buffer I. After centrifugation at 500 *g* for 5 min, the supernatant was aspirated and pooled with that collected from the previous step. The combined supernatant was then homogenized in a loose-fitting glass/Teflon homogenizer using a Stirrer RZR1 (Wiarton, Ont., Canada) set at #2 for 5 strokes. The pooled

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Abbreviations: DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; LLA, linoleic acid; SITS, 4-acetamido-4′-isothiocyanatostilbene-2,2-disulfonic acid; BBMV, brush border membrane vesicles.

homogenate was then stirred vigorously, while a sufficient amount of 0.1 M CaCl was added to give a final concentration of 10 mM. After continuous stirring for 45 min, the turbid mixture was centrifuged at 4,000 *g* for 5 min. Most organelles except a good portion of the brush border members were discarded in the pellets. The supernatant was then subjected to another centrifugation at 7,500 *g* for 20 min. The supernatant that contained brush border membranes was recentrifuged at 28,000 *g* for 45 min. The pellets were resuspended in an isolation buffer II (300 mM D-mannitol in 10 mM Hepes-Tris, pH 7.4). The suspension was homogenized in a tight-fitting glass/Teflon homogenizer set at #2 for 10 strokes. The brush border homogenate was centrifuged at 30,000 *g* for 60 min. The final BBMV pellets were resuspended in buffer II at a final concentration of 10–12 mg protein per ml. These BBMV were used fresh or stored in 1 ml aliquots in liquid nitrogen (18). The D-glucose and linoleic acid transport activities of these BBMV changed little upon storage.

The purity of BBMV was routinely monitored by marker enzyme assays. Brush border alkaline phosphatase and lysosomal acid phosphatase were determined using *p*-nitrophenyl phosphate as substrate (19). Basolateral membrane Na^+/K^+ ATPase (20) and mitochondrial succinate dehydrogenase (21) were determined according to standard methods. Protein was determined by a modification of Lowry et al. (22) using bovine serum albumin as standards. The BBMV preparation was consistently enriched in alkaline phosphatase, at activity of 10- to 13-fold over that of the mucosal homogenate. It was relatively free of all other subcellular organelles, except a 2- to 5-fold contamination of lysosomal acid phosphatase of that of the mucosal homogenate.

Preparation of linoleic acid incubation media. For the present studies, we solubilized linoleic acid in a taurocholate solution by sonication, as described by Proulx et al. (12), with some modifications. Briefly, the amount of unlabeled linoleic acid that will give a final concentration of 0.5 mM in a 10 ml volume was sonicated with 1 ml of transport buffer (100 mM NaCl, 100 mM D-Mannitol, 1 mM $MgCl_2$, 2 mM $CaCl_2$ and 10 mM Hepes-Tris, pH 7.4) for 2 min in a water-bath sonicator (Laboratory Supplies Co. Inc., Hicksville, NY). The turbid solution cleared up when enough powdered sodium taurocholate was added to give a final concentration of 10 mM in the final volume of 10 ml. After adding another 9 ml of transport buffer, the mixture was sonicated for another 2 min. This sonicated preparation stayed in a clear micellar solution for at least 4 hr and was used as the unlabeled linoleic acid-taurocholate stock solution. The [^{14}C]labeled linoleic acid in ethanol was dried under N_2 and sonicated with an appropriate amount of the unlabeled linoleic acid-taurocholate stock stolution to formulate the final incubation medium. When transport of D-glucose was measured simultaneously, appropriate amounts of [^{3}H]-D-glucose and unlabeled D-glucose were also included.

Transport assays. The uptake of substrate by BBMV at 20°C was determined by the filtration technique described by Hopfer et al. (16). To measure Na^+-dependent D-glucose uptake in the absence of linoleic acid, the transport buffer described in the above section was used. To measure Na^+-dependent D-glucose uptake in the presence of taurocholate, the transport buffer plus specified concentration of the bile acid was used. Uptake assay was initiated by the addition of 100 μl of the radioisotope-labeled incubation medium to 10 μl of BBMV. Incubation was stopped with 2 ml of ice-cold stopping buffer (0.15 M KCl, 2 mM Hepes. Tris, pH 7.4) and 0.6 mM phloridzin for D-glucose uptake (16) and 0.2 mM phloretin for linoleic acid uptake (23). The mixture was rapidly filtered through an HA nitrocellulose 0.45 μm filter (Millipore Corp., Bedford, MA). The filter was rinsed twice with 2 ml of the stopping buffer. The radioactivity associated with the filter was measured in Ultrafluor (National Diagnostic, Manville, NJ) using a Beckman LS9000 liquid scintillation counter (Beckman Instruments, Inc., Palo Alto, CA). Each data point presented in this report was the average value of duplicate samples. The value of each sample was the net radioactivity after subtraction of the nonspecific linoleic acid binding to the filter.

To study the effect of sodium ion on transport activities, either KCl or choline chloride were used in place of NaCl. To study the effect of osmolarity on the accumulation of D-glucose or linoleic acid by BBMV, we added various concentrations of D-mannitol to the final incubation medium. The data presented in the figures represented individual values from a typical experiment as some minor variations do exist between preparations of membrane vesicles.

RESULTS

Effect of taurocholate on D-glucose uptake in BBMV. In the intestinal lumen, dietary fatty acids are present either in mixed bile acids micelles or as free monomers. To mimic these physiological conditions and to ensure that linoleic acid in the incubation medium was in solution, we used taurocholate as the micellarizing agent at a concentration that is well below the intralumenal bile salt concentration of 10–40 mM. Taurocholate is the main component of rat bile and, by comparison with other bile acids, it has been shown to exert the mildest damaging effect on brush border membranes (14).

The ability of BBMV to accumulate D-glucose in the presence of a Na^+-gradient deteriorated with time as the concentration of taurocholate was increased (Fig. 1). For 1 min incubation, taurocholate at 2 mM did not have a

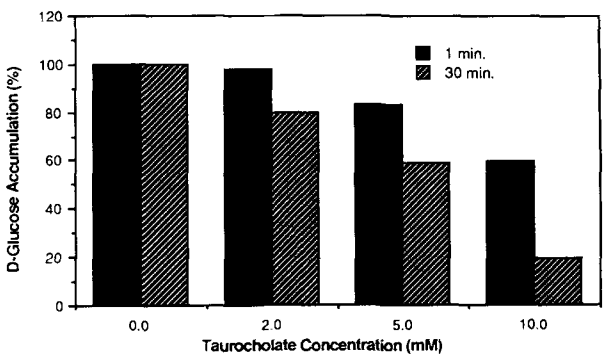

FIG. 1. Effect of taurocholate on D-glucose uptake. BBMV were preloaded with 300 mM D-mannitol in 10 mM Hepes-Tris buffer, pH 7.4. They were incubated at 21°C in a transport medium containing 0.1 mM D-glucose (unlabeled and [^{3}H]labeled), 100 mM NaSCN, 100 mM D-mannitol, 1 mM $MgCl_2$ and 10 mM Hepes-Tris buffer, pH 7.4, plus various concentrations of traurocholate.

detectable effect on D-glucose up to 40%. A 30 min incubation in 2 mM and 10 mM taurocholate caused a decrease of 20% and 80% in D-glucose accumulation in BBMV. These results indicate that taurocholate does affect brush border functions, at least D-glucose transport in a time- and dose-dependent way. We, therefore, limited our studies to linoleic acid concentrations that produced optically clear solutions in a 2 mM taurocholate solution. We also found that the addition of linoleic acid at a concentration of 100 μM had no effect on the uptake of D-glucose by BBMV in the presence of 2 mM taurocholate.

Time course of linoleic acid and D-glucose uptake. When isolated membrane vesicles were incubated simultaneously with labeled linoleic acid and D-glucose, the familiar "overshoot" of D-glucose uptake by brush border membrane vesicles in the presence of sodium gradient was observed (Fig. 2). There was a rapid uptake of D-glucose during the first 90 sec, followed by efflux of D-glucose, which eventually came to equilibrium across the membrane. The initial uptake of linoleic acid was extremely fast and the amount of fatty acid accumulated was much greater than that of D-glucose (Fig. 2). Over the 30 min incubation period, the amount of linoleic acid accumulated in the vesicles diminished, but the value was still 25- to 30-fold greater than the D-glucose accumulation. When the sodium ion in the incubation medium was replaced by potassium or choline, the Na^+-dependent D-glucose overshoot disappeared, but linoleic acid uptake remained unchanged (data not shown). Also, lowering the incubation temperature from 21° to 4°C wiped out D-glucose overshoot and yet did not affect linoleic acid transport.

Uptake of linoleic acid and D-glucose under different osmotic conditions. To determine if the accumulated linoleic acid is bound to the membrane or is being transported into the vesicular lumen or both, two types of experiments were performed. In one, uptake of linoleic acid and D-glucose was measured in incubation media with increasing osmolarity. The uptake of D-glucose after 30 min of incubation decreased linearly with the increase in osmolarity from 200 mOsm to 820 mOsm (Fig. 3A). Extrapolation of the data to conditions of infinite osmolarity demonstrated that little of the accumulation represented binding to the membranes. The amount of D-glucose accumulated changed linearly with the osmolarity in the medium demonstrating that the BBMV we used were sealed and osmotically reactive. Therefore, the D-glucose accumulation can be used for calculating the intravesicular volume by using the concentration of D-glucose in the incubation medium as a reference (24). Based on an accumulation of 329 pmol/mg protein at an initial D-glucose concentration of 0.1 mM after a 30 min incubation, the BBMV have an intravesicular volume of 3.3 μl/mg protein (Fig. 2). This value is well within the range of vesicle size reported for transport studies. Uptake of linoleic acid after 10 min incubation under similar conditions (240 mOsm to 820 mOsm) did not vary with the osmolarity (Fig. 3B). Furthermore, if we used the amount of linoleic acid accumulation after a 30 min incubation (Fig. 2) to calculate the theoretical intravesicular volume, the calculated value was 65 μl/mg protein, a value which is far too large. These results suggest that the observed linoleic acid accumulation was to a large degree due to binding to the membrane rather than intravesicular lumenal accumulation.

In the second experiment, membrane vesicles incubated with linoleic acid were deliberately lysed with ice-cold distilled water (25). Lysis of the vesicles releases the linoleic

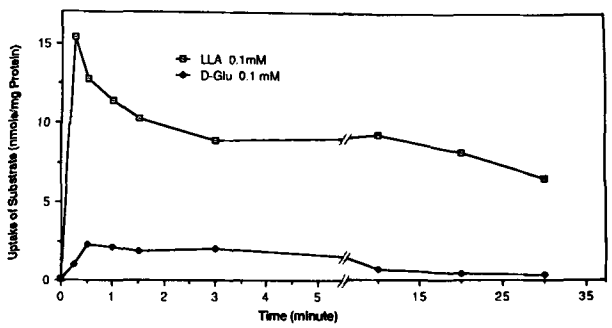

FIG. 2. Time course of D-glucose and linoleic acid (LLA) uptake. BBMV were preloaded with 300 mM D-mannitol in 10 mM Hepes-Tris buffer, pH 7.4. They were incubated at 21°C in transport medium containing 0.1 mM D-glucose (unlabeled and [^{3}H]labeled), 0.1 mM linoleic acid (unlabeled and [^{14}C]labeled), 100 mM NaSCN, 100 mM D-mannitol, 1 MM $MgCl_2$, 2 mM $CaCl_2$, 2 mM taurocholate and 10 mM Hepes-Tris buffer, pH 7.4.

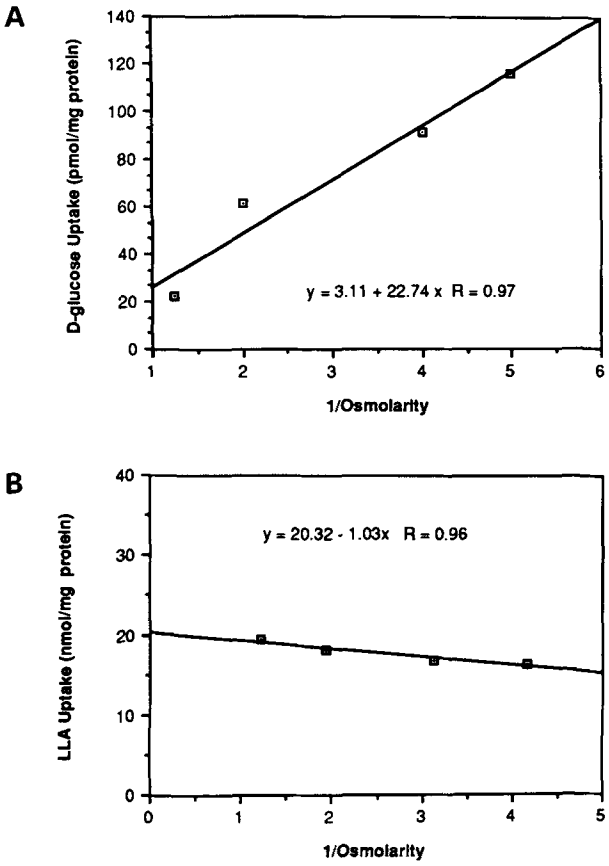

FIG. 3. Effect of increasing osmolarity on the uptake of D-glucose (panel A) and linoleic acid (panel B). BBMV were preloaded with 300 mM D-mannitol in 10 mM Hepes-Tris buffer, pH 7.4. The membrane vesicles were incubated for 30 min at 21°C in uptake media containing either 0.1 mM D-glucose (unlabeled and ^{3}H labeled) or 0.1 mM linoleic acid (unlabeled and ^{14}C labeled) in 100 mM NaSCN, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 2 mM taurocholate, 10 mM Hepes-Tris buffer, pH 7.4, with an appropriate amount of D-mannitol to give the indicated osmolarity.

acid which had accumulated in the lumen of the vesicles. In these experiments, we found a loss of ca. 40% of the accumulated linoleic acid, indicating that 60% of the total linoleic acid is firmly attached to the brush border membranes. Such attachment could be due to tight adsorption to the outer or inner surfaces of the membrane or incorporation into the membrane itself.

Kinetics of uptake of linoleic acid. The rate of linoleic acid uptake increased linearly with increasing concentrations from 10 to 100 μM (Fig. 4). Thus, transport at this range of concentrations was compatible with simple passive diffusion. Although linoleic acid uptake at 25 μM and 100 μM was not affected by DIDS or SITS at inhibitor concentrations up to 5 mM, it was inhibited by 23%, following the addition of 0.2 mM phloretin to the incubation medium.

DISCUSSION

We investigated the initial absorption of linoleic acid by intestinal BBMV isolated from rabbit small intestine. The linear relationship between linoleic acid concentrations and its transport rates (Fig. 4) suggests that linoleic acid absorption in the range of concentrations we studied is a simple diffusion process. This conclusion is further supported by our observation that lowering the incubation temperature had no effect on the transport rate, nor did the inhibitors DIDS and SITS.

Chow and Hollander (10,11) previously demonstrated saturation kinetics of linoleic acid transport in vivo and in vitro, and recently, Stremmel (26) reported a saturable oleate transport in isolated rat jejunal mucosal cells. The study of linoleic acid absorption in everted small intestinal sacs had a reported Km of 0.967 mM (10) in the presence of 10 mM taurocholate. Accordingly, it is only reasonable that we did not observe saturation kinetics in the present experiments, because the highest concentration of linoleic acid we were able to solubilize in the taurocholate solution is much lower than the apparent affinity constant of linoleic acid transport. Others have used 7.2 mM taurocholate in their membrane vesicle uptake study of cholesterol and fatty acids (15). At 7.2 mM, taurocholate caused membrane solubilization and the loss of 5% membrane proteins in 20 min (14,15). In our studies, we found that taurocholate at concentrations greater than 2 mM is damaging to the membrane, as reflected by its effect on glucose transport (Fig. 1). Therefore, we restricted the concentration of taurocholate to 2 mM, which is well below the normal intralumenal concentration of bile acid (10–40 mM) or the critical micellar concentration (2.5 mM), and studied only the concentrations of linoleic acid that can be solubilized in it. If it were not for this limitation in the concentrations of taurocholate and linoleic acid, we perhaps could have also demonstrated saturation kinetics in our present experiments. It is possible that taurocholate could inhibit a carrier-mediated transport system for linoleic acid. Furthermore, inhibition of a possible linoleic acid carrier-mediated transport system could also occur by the incorporation of linoleic acid within the lipid bilayer of the membrane. We found that the addition of 0.2 mM phloretin to the uptake medium inhibited linoleic acid uptake by 20–25%. Phloretin has been shown to inhibit specific membrane transport proteins (27). The partial inhibition of linoleic acid uptake by phloretin suggests that membrane protein could play some role in the initial uptake of linoleic acid, in addition to passive diffusion of the fatty acid across the lipid cell membrane. Thus, the absorption of linoleic acid in vivo could be partly due to a carrier-mediated, facilitated transport mechanism.

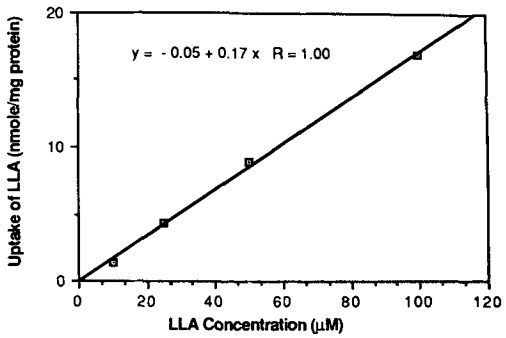

FIG. 4. Effect of linoleic acid concentration on its uptake. BBMV were preloaded with 300 mM D-mannitol in 10 mM Hepes-Tris buffer, pH 7.4. The incubation media were 100 mM NaSCN, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 2 mM taurocholate, 10 mM Hepes-Tris buffer, pH 7.4, plus the specified concentration of linoleic acid (unlabeled and [^{14}C]labeled) and a sufficient amount of D-mannitol to give a final osmolarity of 330 mOsm.

Using D-glucose accumulation in the BBMV as a reference, we calculated that our vesicles have an intravesicular volume of 3.3 μl/mg protein, which is in the same range of vesicle sizes reported in other uptake studies. But using linoleic acid accumulation values gave an apparent vesicular volume of 65 μl/mg protein. This calculation suggests that the majority of the linoleic acid could not have accumulated in the lumen of the vesicles at the same concentration as in the incubation mixture and must have been partly bound to the membrane of the vesicles. The results in Figure 3B showing the lack of response of linoleic acid uptake to changes in osmolarities in the incubation medium also support the idea that a great deal of the linoleic acid is bound to the vesicular membrane.

Although it is not impossible for fatty acids to bind only to the outside surface of membrane vesicles, a variety of fatty acids and lipids had been demonstrated to be incorporated into the lipid bilayer itself (12,13,15,28). Fatty acid transport occurs very rapidly (29). Thus, rapid intercalation of linoleic acid into the membrane, itself, could be substantial enough to compenstate for any reduction in intravesicular accumulation and, hence, account for the lack of measurable osmotic response in total vesicular accumulation of linoleic acid (Fig. 4).

In order to determine how much of the linoleic acid actually accumulates in the vesicular lumen vs how much is bound or incorporated into the membrane, we lysed the vesicles at the end of incubation with ice-cold distilled water to release any fatty acid that did accumulate in the vesicular lumen (25). We found at least 40% of the linoleic acid was released when the vesicles were lysed. These data indicate that 40% of the linoleic acid must have traversed the lipid bilayer of the brush border membrane and accumulated in the vesicular lumen. Because the average intravesicular volume can only accommodate 5–10% of the accumulated linoleic acid if its concentration in the intravesicular compartment had remained the same as in

the incubation medium, the quantity of linoleic acid we found in the vesicular lumen is much higher than expected. One possible explanation for this finding is the formation of linoleic acid aggregates within the vesicular lumen. Taurocholate has a relatively low apparent affinity constant (0.59–0.66 mM) for brush border membranes and is transported by a relatively slow Na^+-dependent active transport mechanism (30). Therefore, even at very high exogenous concentrations, a very limited quantity of taurocholate will traverse across the membrane. The fact that we did not observe any differences in the uptake of linoleic acid whether sodium or potassium ion was present in the incubation medium indicates that, even in the presence of Na^+, the amount of taurocholate entering the vesicular space would not be sufficient to solubilize the linoleic acid accumulated in the vesicular lumen. Therefore, we propose that, once linoleic acid penetrates across the membrane into the vesicular lumen, it does not stay in solution but could precipitate as aggregates in the presence of calcium which accumulated in the lumen (31). The possibility that the vesicular linoleic acid is entrapped as nonsoluble aggregates is in accord with the report by Proulx et al. (15) and Merrill et al. (28) that fatty acids transported into BBMV are not exchangeable with exogenous fatty acids.

By using BBMV, we were able to study the initial uptake of linoleic acid by the cell membrane. This preparation is devoid of unstirred water layer effects and cytosolic proteins that can bind fatty acids. Previously, we demonstrated that fatty acids are absorbed in vivo by a saturable process compatible with a facilitated transport mechanism (10,11). In our in vivo experiments, we were unable to conclude whether saturation was due to unstirred layer effects or, perhaps, due to cytosolic binding proteins. The present experiments allow us to propose that the considerable degree of linoleic acid binding to the brush border membrane could be due in part to binding to membrane proteins. Further studies are needed in order to explore the possibility that brush border membrane proteins could play a role in the absorption of lipids.

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Effect of Alcohols on the Oxidation of the Vitamin E Model Compound, 2,2,5,7,8-Pentamethyl-6-chromanol

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The vitamin E model compound, 2,2,5,7,8-pentamethyl-6-chromanol, has been oxidized with *t*-butyl hydroperoxide in chloroform in order to simulate in vivo oxidations due to lipid hydroperoxides. In the presence of a variety of alcohols, ranging from methanol to cholesterol, the corresponding 5-alkoxymethyl-2,2,7,8-tetramethyl-6-chromanols were formed in fair to good yield and were the major products in each reaction.
***Lipids* 24, 56-60 (1989).**

Recent work has shown that, when α-tocopherol (**1a**) (Scheme 1) and its model compound, 2,2,5,7,8-pentamethyl-6-chromanol (**1b**) are oxidized by *t*-butyl hydroperoxide in chloroform to which a small amount of ethanol has been added, the major products are 5-ethoxymethyl-7,8-dimethyltocol (**7a**; R′ = CH_3-CH_2-) and 5-ethoxymethyl-2,2,7,8-tetramethyl-6-chromanol (**7b**; R′ = CH_3-CH_2-), respectively (1). It appears that these products are formed by the oxidation of **1a** and **1b** to a quinone methide intermediate which then adds ethanol. The aim of the present work was to determine whether this reaction was general for all alcohols.

MATERIALS AND METHODS

IR spectra were determined on a Perkin Elmer 580B spectrometer, UV spectra on a Perkin Elmer 124 double beam spectrophotometer, ^{1}H NMR spectra on a Bruker AM 500 spectrometer and electron impact mass spectra on an A.E.I MS 12 mass spectrometer. NMR spectra were taken in $CDCl_3$ and are reported in parts per million downfield from tetramethylsilane as internal standard.

Chloroform was purified by washing with 18 M sulfuric acid, distilled water until the washings were neutral, drying (Na_2SO_4) and distilling immediately before use.

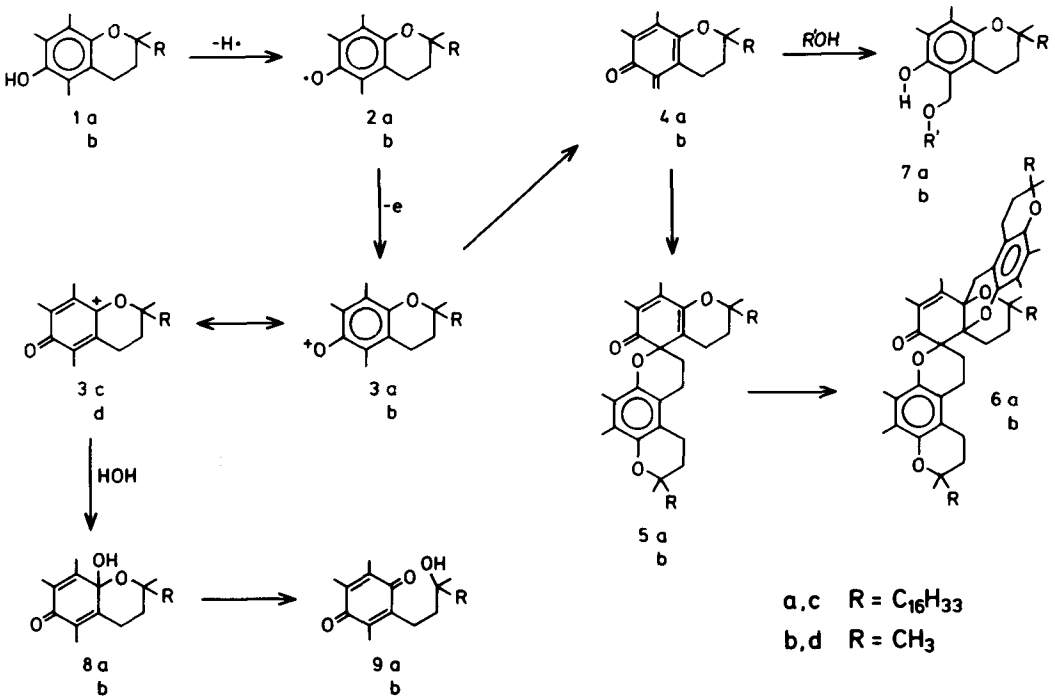
Phytol (Sigma Chemical Co., St. Louis, MO) was used without purification.

t-Butyl hydroperoxide (70%, EGA CHEMIE, Steinheim, West Germany) was purified by the sodium salt method (2). Purity (iodometrically) was 95%.

Cholesterol (E. Merck, Darmstadt, West Germany) was purified by a bromination-debromination procedure (3).

Compound **1b** together with its spirodimer (**5b**) and spirotrimer (**6b**) and 2-(3-hydroxy-3-methylbutyl)-3,5,6-trimethyl-1,4-benzoquinone (**9b**) were prepared as reference compounds by known methods (4-7).

Oxidation of **1b** *by* t-*butyl hydroperoxide.* To **1b** (220 mg, 1.0 mmol) in chloroform (24 ml, containing the respective alcohol (6.0 g) (Table 1) was added *t*-butyl hydroperoxide (91 mg, 1.0 mmol) and the solution refluxed for 3 hr. The solution was then washed with 5% ferrous sulfate solution (2 × 30 ml), distilled water (3 × 30 ml), dried (Na_2SO_4) and the solvent removed in vacuo. The residue was chromatographed on thin layers of Silica Gel GF_{254} (solvent: light petroleum (bp 60-80°C)/ethyl



SCHEME 1

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TABLE 1

Oxidation of 2,2,5,7,8-Pentamethyl-6-chromanol (1b) by *t*-Butyl Hydroperoxide in the Presence of Various Alcohols and Formation of 5-Alkoxymethyl Adducts

5-Alkoxymethyl adduct of		Yield mol %	R_f	m.p. °C	Elemental analysis
Methanol		44	0.47	67-68	calc. C, 72.00; H, 8.80 found C, 72.09; H, 9.09
Ethanol		55	0.55	93-93.5	calc. C, 72.73; H, 9.09 found C, 72.51; H, 9.32
Propanol-1		39	0.59	80-81.5	calc. C, 73.38; H, 9.35 found C, 73.48; H, 9.58
Butanol-1		59	0.61	65-66.5	calc. C, 73.97; H, 9.59 found C, 73.85; H, 9.78
2-Methyl propanol-1	*	68	0.62	69.5-71	calc. C, 73.97; H, 9.59 found C, 73.99; H, 9.82
2-Methyl propanol-2	*	54	0.61	104.5-105.5	calc. C, 73.97; H, 9.59 found C, 73.80; H, 9.83
Heptanol-1	*	61	0.64	49-50	calc. C, 75.45; H, 10.18 found C, 75.15; H, 10.43
Undecanol-1	*	52	0.66	35-36	calc. C, 76.92; H, 10.77 found C, 76.82; H, 11.04
Phytol	*	38	0.67		calc. C, 79.38; H, 11.28 found C, 79.87; H, 11.12
Cholesterol	*	11	0.68	169-170	calc. C, 81.46; H, 10.59 found C, 81.12; H, 10.86

The reaction mixtures containing 2,2,5,7,8-pentamethyl-6-chromanol (220 mg; 1.0 mmol), *t*-butyl hydroperoxide (91 mg; 1.0 mmol), chloroform (24 ml) and respective alcohol (6 g), were heated at 60°C for 3 hr, washed with 5% ferrous sulfate to destroy unreacted hydroperoxide (2 × 30 ml), water (3 × 30 ml), dried (Na_2SO_4) and the solvent removed in vacuo. The residue from each reaction was chromatographed on thin layers of Silica Gel GF_{254} (solvent: light petroleum (bp 60-80°C)/ethyl acetate (9:1), the products located under UV light, eluted with chloroform, and the solvent removed under a stream of nitrogen and weighed.

*These reactions were performed on half of the above scale.

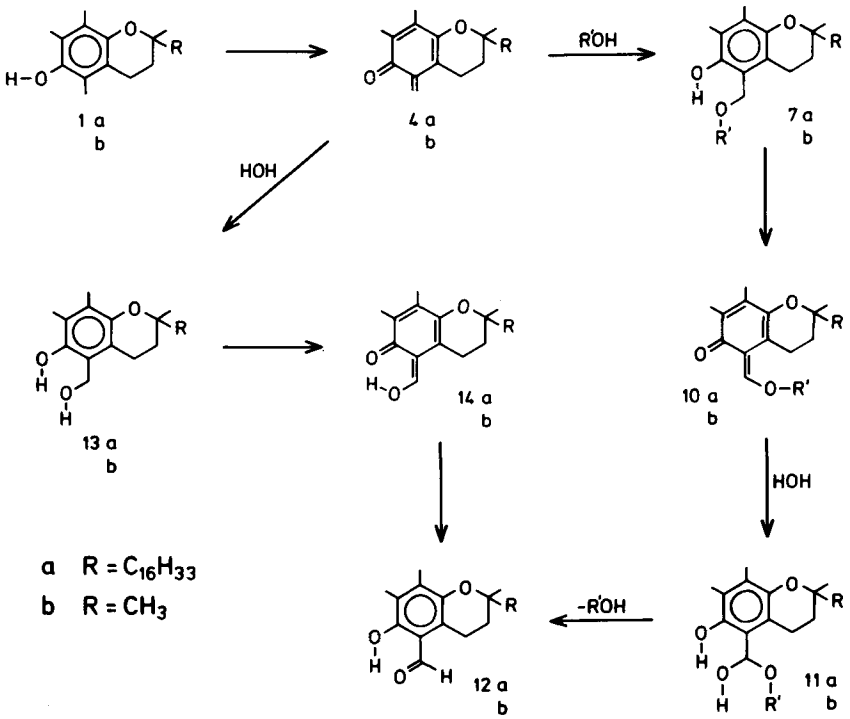
acetate (9:1)), the products located under ultraviolet light, eluted with purified chloroform, the solvent removed under a stream of nitrogen and weighed. The following products were identified by comparison of their ultraviolet and ^1H NMR spectra with those of the prepared reference compounds: **1b** R_f 0.44; **9b** R_f 0.08.

5-Formyl-2,2,7,8-tetramethyl-6-chromanol (**12b**) (Scheme 2) R_f 0.68 was identified by comparison of its UV and ^1H NMR spectra with literature values for the authentic compound (8,9). An additional band was noted at R_f 0.90, which is still under investigation. The following 5-alkoxymethyl adducts of **1b** were identified (Table 1).

5-Methoxymethyl-2,2,7,8-tetramethyl-6-chromanol had UV λ_{max} (hexane) 303 nm (log 3.70); IR (KBr) 3313 (OH), 2982, 2933 (CH_3), 1624, 1455, 1387, 1368, 1276, 1230, 1175, 1132, 1099 (C-O-C), 938, 902, 854, 678 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.28 (*s*, 6H, 2 × CH_3), 1.77 (*t*, 2H, 3-CH_2, J = 6.9 Hz), 2.11 (*s*, 3H, Ar-CH_3), 2.15 (*s*, 3H, Ar-CH_3), 2.62 (*t*, 2H, 4-CH_2, J = 6.9 Hz), 3.43 (*s*, 3H, CH_3-O-), 4.66 (*s*, 2H, Ar-CH_2-O-), 7.42 (*s*, 1H, OH) ppm; irradiation of the singlet at 4.66 ppm gave a Nuclear Overhauser Enhancement (NOE) on the singlet at 7.42, on the singlet at 3.43 and on the triplet at 2.62 ppm, thus proving that the methoxy group was on position 5 of the chroman ring; MS (ei) *m/z* (rel intensity) 250 $[M]^+$ (34), 218 (100), 203 (75), 189 (24), 175 (78).

5-Propoxymethyl-2,2,7,8-tetramethyl-6-chromanol had UV λ_{max} (hexane) 303 nm (log 3.71); IR (KBr) 3325 (OH), 2974, 2942 (CH_3), 1623, 1463, 1386, 1370, 1277, 1231, 1172, 1130, 1104 (C-O-C), 973, 944, 903, 854, 652 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.96 (*t*, 3H, $\underline{CH_3}$-CH_2-, J = 7.4 Hz), 1.28 (*s*, 6H, 2 × CH_3), 1.67 (m, 2H, CH_3-$\underline{CH_2}$-CH_2-), 1.77 (*t*, 2H, 3-$\underline{CH_2}$, J = 6.9 Hz), 2.11 (*s*, 3H, Ar-CH_3), 2.15 (*s*, 3H, Ar-CH_3), 2.61 (*t*, 2H, 4-CH_2, J = 6.9 Hz), 3.51 (*t*, 2H, -CH_2-$\underline{CH_2}$-O-, J = 6.6 Hz), 4.70 (*s*, 2H, Ar-$\underline{CH_2}$-O-), 7.73 (*s*, 1H, OH) ppm; irradiation of the singlet at 4.70 ppm gave an NOE on the singlet at 7.73, on the triplet at 3.51 and on the triplet at 2.61 ppm, thus proving that the propoxy group was on the 5-position of the chroman ring; MS (ei) *m/z* (rel intensity) 278 $[M]^+$ (22), 218 (100), 203 (63), 189 (18), 175 (61).

5-n-Butoxymethyl-2,2,7,8-tetramethyl-6-chromanol had UV λ_{max} (hexane) 303 nm (log 3.71); IR (KBr) 3320) (OH), 2960, 2925 (CH_3), 1620, 1455, 1380, 1360, 1270, 1225, 1160, 1120, 1095 (C-O-C), 935, 895, 855 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.93 (*t*, 3H, $\underline{CH_3}$-CH_2-, J = 7.4 Hz), 1.28 (*s*, 6H, 2 × CH_3), 1.41 (m, 2H, CH_3-$\underline{CH_2}$-CH_2-), 1.63 (m, 2H, CH_2-$\underline{CH_2}$-CH_2-), 1.77 (*t*, 2H, 3-CH_2, J = 6.9 Hz), 2.11 (*s*, 3H, Ar-CH_3), 2.15 (*s*, 3H, Ar-CH_3), 2.61 (*t*, 2H, 4-$\underline{CH_2}$, J = 6.9 Hz), 3.55 (*t*, 2H, -CH_2-$\underline{CH_2}$-O-, J = 6.5 Hz), 4.70 (*s*, 2H, Ar-$\underline{CH_2}$-O-), 7.73 (*s*, 1H, OH) ppm; irradiation of the singlet at 4.70 ppm gave an NOE on the singlet at 7.73, on the triplet at 3.55 and on the triplet



SCHEME 2

at 2.61 ppm, thus proving that the butoxy group was on the 5-position of the chroman ring; MS (ei) *m/z* (rel intensity) 292 $[M]^+$ (14), 218 (100), 203 (64), 189 (19), 175 (60).

5-(2-Methyl-1-propoxy)methyl-2,2,7,8-tetramethyl-6-chromanol had UV λ_{max} (hexane) 303 nm (log 3.71); IR (KBr) 3330 (OH), 2974, 2945 (CH_3), 1623, 1464, 1387, 1370, 1277, 1231, 1173, 1131, 1101 (C-O-C), 1024, 945, 903, 855, 653 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.95 (d, 6H, 2 × CH_3, J = 6.7 Hz), 1.28 (*s*, 6H, 2 × CH_3), 1.77 (*t*, 2H, 3-CH_2, J = 6.9 Hz), 1.94 (m, 1H, CH), 2.11 (*s*, 3H, Ar-CH_3), 2.15 (*s*, 3H, Ar-CH_3), 2.60 (*t*, 2H, 4-CH_2, J = 6.9 Hz), 3.32 (d, 2H, -CH-$\underline{CH}_2$-O-, J = 6.5 Hz), 4.70 (*s*, 2H, Ar-$\underline{CH}_2$-O-), 7.75 (*s*, 1H, OH) ppm; irradiation of the singlet at 4.70 ppm gave an NOE on the singlet at 7.75, on the doublet at 3.32 and on the triplet at 2.60 ppm, thus proving that the 2-methyl-1-propoxy group was on the 5-position of the chroman ring; MS (ei) *m/z* (rel intensity) 292 $[M]^+$ (20), 218 (100), 203 (48), 189 (14), 175 (48).

5-(2-Methyl-2-propoxy)methyl-2,2,7,8-tetramethyl-6-chromanol had UV λ_{max} (hexane) 303 nm (log 3.75); IR (KBr) 3298 (OH), 2980, 2942 (CH_3), 1639, 1456, 1367, 1270, 1230, 1198, 1169, 1129, 1094, 1044, 999, 931, 894, 858, 820 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.28 (*s*, 6H, 2 × CH_3), 1.33 (*s*, 9H, 3 × CH_3), 1.76 (*t*, 2H, 3 - $\underline{CH}_2$, J = 6.9 Hz), 2.09 (*s*, 3H, Ar-CH_3), 2.14 (*s*, 3H, Ar-CH_3), 2.58 (*t*, 2H, 4 - $\underline{CH}_2$, J = 6.9 Hz), 4.66 (*s*, 2H, Ar-CH_2-O-), 8.30 (*s*, 1H, OH) ppm; irradiation of the singlet at 4.66 ppm gave an NOE on the singlet at 8.30 and on the triplet at 2.58 ppm, thus proving that the 2-methyl-2-propoxy group was on the 5-position of the chroman ring; MS (ei) *m/z* (rel intensity) 292 $[M]^+$ (16), 218 (100), 203 (48), 189 (14), 175 (48).

5-n-Heptoxymethyl-2,2,7,8-tetramethyl-6-chromanol had UV λ_{max} (hexane) 303 nm (log 3.71); IR (KBr) 3322 (OH), 2972, 2935 (CH_3), 1623, 1464, 1387, 1370, 1279, 1232, 1171, 1130, 1104 (C-O-C), 1024, 945, 903, 854, 650 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.88 (*t*, 3H, $\underline{CH}_3$-CH_2-, J = 6.8 Hz), 1.28 (*s*, 6H, 2 × CH_3), 1.22 – 1.38 (broad m, 8H), 1.63 (m, 2H, -CH_2-$\underline{CH}_2$-CH_2-O-), 1.76 (*t*, 2H, 3 - $\underline{CH}_2$, J = 6.9 Hz), 2.10 (*s*, 3H, Ar-CH_3), 2.15 (*s*, 3H, Ar-CH_3), 2.60 (*t*, 2H, 4 - $\underline{CH}_2$, J = 6.9 Hz), 3.54 (*t*, 2H, -CH_2-$\underline{CH}_2$-O-, J = 6.6 Hz), 4.69 (*s*, 2H, Ar-CH_2-O-), 7.73 (*s*, 1H, OH) ppm; irradiation of the singlet at 4.69 ppm gave an NOE on the singlet at 7.73, on the triplet at 3.54 and on the triplet at 2.60 ppm, thus proving that the *n*-heptoxy group was on the 5-position of the chroman ring; MS (ei) *m/z* (rel intensity) 334 $[M]^+$ (10), 218 (100), 203 (37), 189 (10), 175 (35).

5-n-Undecoxymethyl-2,2,7,8-tetramethyl-6-chromanol had UV λ_{max} (hexane) 303 nm (log 3.70); IR (KBr) 3317 (OH), 2973, 2961, 2929 (CH_3), 1623, 1463, 1387, 1370, 1278, 1232, 1172, 1130, 1104 (C-O-C), 1013, 946, 902, 855, 653 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.88 (*t*, 3H, $\underline{CH}_3$-CH_2-, J = 6.9 Hz), 1.28 (*s*, 6H, 2 × CH_3), 1.22 – 1.38 (broad m, 16H), 1.63 (m, 2H, -CH_2-$\underline{CH}_2$-CH_2-O-), 1.77 (*t*, 2H, 3-CH_2, J = 6.9 Hz), 2.11 (*s*, 3H, Ar-CH_3), 2.15 (*s*, 3H, Ar-CH_3), 2.60 (*t*, 2H, 4-CH_2, J = 6.9 Hz), 3.54 (*t*, 2H, -CH_2-$\underline{CH}_2$-O-, J = 6.6 Hz), 4.70 (*s*, 2H, Ar-CH_2-O-), 7.73 (*s*, 1H, OH) ppm; irradiation of the singlet at 4.70 ppm gave an NOE on the singlet at 7.73, on the triplet at 3.54 and on the triplet at 2.60 ppm, thus proving that the undecoxy group was on the 5-position of the chroman ring; MS (ei) *m/z* (rel intensity) 390 $[M]^+$ (7), 218 (100), 203 (22), 189 (7), 175 (22).

5-Phytoxymethyl-2,2,7,8-tetramethyl-6-chromanol had UV λ_{max} (hexane) 303 nm (log 3.68); IR (KBr) 3377 (OH), 2954, 2931 (CH_3), 1747, 1674, 1622, 1462, 1384, 1370, 1274, 1231, 1172, 1127, 1095 (C-O-C), 1051, 1016, 935, 903, 852 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.87 (m, 12H, 4 × CH_3), 1.29 (*s*, 6H, 2 × CH_3), 1.03 – 1.57 (broad m, 19H),

1.65 (*s*, 3H, =C-CH_3), 1.77 (*t*, 2H, 3-CH_2, J = 6.9 Hz), 2.02 (m, 2H, =C-CH_2-), 2.11 (*s*, 3H, Ar-CH_3), 2.16 (*s*, 3H, Ar-CH_3), 2.61 (*t*, 2H, 4-CH_2, J = 6.9 Hz), 4.09 (d, 2H, =CH-$\underline{C}\underline{H}_2$-O-, J = 6.9 Hz), 4.70 (*s*, 2H, Ar-CH_2-O-), 5.39 (*t*, 1H, =CH-, J = 6.8 Hz), 7.68 (*s*, 1H, OH) ppm; irradiation of the singlet at 4.70 ppm gave an NOE on the singlet at 7.68, on the doublet at 4.09 and on the triplet at 2.61 ppm, thus proving that the phytoxy group was on the 5-position of the chroman ring; MS (ei) *m/z* (rel intensity) 514 $[M]^+$ (7), 296 (8), 294 (30), 278 (27), 264 (28), 236 (31), 218 (100), 203 (67), 189 (23), 175 (82).

5-Cholesteroxymethyl-2,2,7,8-tetramethyl-6-chromanol had UV λ_{max} (hexane) 303 nm (log 3.69); IR (KBr) 3382 (OH), 2940 (CH_3), 1623, 1457, 1384, 1370, 1274, 1231, 1171, 1127, 1095 (C-O-C), 1074, 1058, 1021, 943, 931, 903, 854, 803 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.68 (*s*, 3H, CH_3), 0.86 (d, 3H, CH_3-CH-, J = 6.6 Hz), 0.866 (d, 3H, CH_3-CH, J = 6.6 Hz), 0.91 (d, 3H, CH_3-CH, J = 6.5 Hz), 1.00 (*s*, 3H, CH_3), 1.27 (*s*, 6H, 2 × CH_3), 0.95 – 1.20 (broad m, 9H), 1.22 – 1.42 (broad m, 4H), 1.42 – 1.61 (broad m, 8H), 1.76 (*t*, 2H, 3-CH_2, J = 6.9 Hz), 1.8 – 1.9 (m, 2H), 2.00 (m, 3H), 2.10 (*s*, 3H, Ar-CH_3), 2.14 (*s*, 3H, Ar-CH_3), 2.30 (m, 1H), 2.44 (m, 1H), 2.60 (*t*, 2H, 4-CH_2, J = 6.9 Hz), 3.34 (m, 1H, -CH-O-), 4.75 (*s*, 2H, Ar-CH_2-O-), 5.35 (m, 1H, =CH-), 7.86 (*s*, 1H, OH) ppm; irradiation of the singlet at 4.75 ppm gave an NOE on the singlet at 7.86, on the multiplet at 3.34 and on the triplet at 2.60 ppm, thus proving that the cholesteroxy group was on the 5-position of the chroman ring; MS (ei) *m/z* (rel intensity) 604 $[M]^+$ (5), 602 (8), 386 (74), 368 (51), 353 (37), 301 (42), 275 (53), 255 (31), 247 (18), 231 (31), 219 (97), 218 (100), 213 (43), 203 (64), 189 (29), 175 (82).

RESULTS AND DISCUSSION

Oxidation of **1a** and **1b** is believed to proceed in two single electron steps leading first to the tocopheroxyl (**2a**) and chromanoxyl radicals (**2b**) (10-12) and, subsequently, to the phenoxylium (protonated quinone methide, **3a, 3b-3c, 3d**) or quinone methide (**4a,4b**) (13,14) species (Scheme 1). In the absence of other reactants, the quinone methide can dimerize to **5a,5b** and trimerize to **6a,6b** (15,5,6) in reactions typical of the species (16,17). Good yields of **5a**, in particular, are obtained in these reactions (5,15). Oxidations of simpler phenols have given very good yields of trimer (17). However, the present work shows that, if a nucleophile is present, an alcohol in this case, 1-4 addition to the quinone methide to form 5-alkoxymethyl derivatives (**7b**) is clearly preferred to polymerization. Indeed, only traces of the dimer **5b** and trimer **6b** were observed in these reactions.

Table 1 shows that the 5-alkoxymethyl derivatives of **1b** are formed in moderate to good yield in all cases except that of cholesterol and are, in each case, the major products of reaction. The reason for the lower yield of the cholesterol adduct was probably due to steric hindrance of the secondary alcohol group of cholesterol and to the much lower molar ratio of alcohol to chroman used in this reaction than in the other reactions. However, steric hindrance can be overcome by a sufficient concentration of alcohol as was the case with the more hindered, but much more compact 2-methylpropanol-2. NOE studies revealed that, in all cases, the alcohol had added exclusively to the 5-position of the chroman ring. No sign of substitution in other positions of the chroman ring was observed.

The second most plentiful product in almost all reactions was **12b** (Scheme 2). It has been demonstrated previously that 5-ethoxymethyl-7,8-dimethyltocol (**7a**; R′ = CH_3-CH_2-) may be oxidized to 5-formyl-7,8-dimethyltocol (**12a**), probably by formation of an intermediate quinone methide (**10a**), addition of water to form a hemiacetal (**11a**) and loss of ethanol (18). It seems likely that further oxidation of all 5-alkoxymethyl derivatives (**7b**) to **12b** may occur in the same manner. An alternative way in which **12b** may arise is by the addition of water to the first formed quinone methide (**4b**) to form 5-hydroxymethyl-2,2,7,8-tetramethyl-6-chromanol (**13b**) (C. Suarna and P.T. Southwell-Keely, unpublished observations) followed by oxidation to another quinone methide (**14b**) which is the enol of **12b**.

α-Tocoquinone (**9a**) and **9b** (Scheme 1) were the first oxidation products of **1a** and **1b**, respectively, to be characterized (7). These compounds were formed in very high yield by oxidation of the parent materials with silver nitrate or ferric chloride in alcoholic solvents. Their route of formation is believed to involve an intermediate phenoxylium species (**3a-3d**), evidence for which exists (19,20), followed by addition of water to form the quinone hemiketal (**8a,8b**) (21,22) and then ring opening to the quinone. In the present reactions, which were performed in chloroform, **9b**, although always present, was formed only in small amounts.

This, it may be inferred that, when oxidations are carried out in nonpolar solvents such as chloroform or light petroleum, the neutral intermediate quinone methide (**4a,4b**) determines the products. However, when oxidations are performed in polar media such as alcohols, the positively charged phenoxylium ion is the favored intermediate, due to solvent stabilization of the charged species, and determines the products.

Both **9b** and **7b** are formed by 2 electron oxidation of the starting material. However, **9b** has no antioxidant activity, whereas such 5-alkoxymethyl-2,2,7,8-tetramethyl-6-chromanols, as have been tested, have quite good antioxidant activity (23). In view of the relative ease with which the 5-alkoxymethyl adducts form, it is tempting to speculate that, if such reactions occurred in membranes (with cholesterol, phosphatidylinositol, phosphatidylglycerol, cardiolipin, etc., serving as the alcohol), they would extend the antioxidant activity of α-tocopherol by forming oxidation products which also contained this activity. If, as has been suggested earlier, the 5-alkoxymethyl adducts are further oxidized to **12b**, this would represent an additional means of retaining antioxidant activity (23).

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Incorporation of *trans*-8- and *cis*-8-Octadecenoic Acid Isomers in Human Plasma and Lipoprotein Lipids

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Mixtures of deuterium-labeled *trans*-8-, *cis*-8- and *cis*-9-octadecenoic acids (8t-18:1, 8c-18:1, 9c-18:1) were fed as triglycerides (TG) to two adult male subjects. Blood samples were collected sequentially over a 48-hour period. Plasma and lipoprotein lipids were separated by thin layer chromatography and analyzed by gas chromatography–mass spectroscopy. Results indicate (i) absorption of the 8t- and 8c-18:1 isomers were similar to 9c-18:1; (ii) the 8t-18:1 isomer was cleared approximately 30% faster than 9c-18:1 from plasma TG; (iii) cholesterol ester samples contained 8.4 times less 8t-18:1 than 9c-18:1; (iv) incorporation at the 1-acyl phosphatidylcholine (PC) position was higher for 8t-18:1 and 8c-18:1 (2.2 and 1.7 times) than for 9c-18:1; and (v) discrimination at the 2-acyl PC position was 4.6-fold against 8t-18:1 and 1.3-fold against 8c-18:1 compared with 9c-18:1. Discrimination against uptake of the Δ-8 isomers in both neutral and phospholipid classes suggests that both 8t- and 8c-18:1 may be preferentially oxidized relative to 9c-18:1. Except for triglycerides, data for each of the lipid classes from total plasma and individual lipoprotein samples were similar. These data indicate that differences for incorporation and turnover of the 8t- and 8c-18:1 isomers relative to 9c-18:1 are not substantially influenced by the lipoprotein classes. The maximum isotopic enrichment detected in the chylomicron triglycerides fractions was 60%, which indicates that a substantial amount of endogenous triglycerides was mobilized during absorption of the deuterated fats.

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Isomers of *cis* and *trans* octadecenoic acid with double bonds in the 8 to 14 positions have been identified in a variety of human tissues (1,2). The main dietary sources of these isomeric structures are partially hydrogenated vegetable oils, which supply a significant portion (ca. 6.5%) of the total dietary fat consumed in the U.S. (3).

A number of human studies have been conducted to investigate the effect of diets containing hydrogenated oils on serum cholesterol and triglyceride (TG) levels (4,5). In general, the results of these studies indicate that hydrogenated oils do not increase TG or cholesterol levels sufficiently to merit major concern regarding their nutritional impact in healthy individuals. Because these human studies provide no actual metabolic data for individual 18:1 isomers the effect of isomeric fatty acids on health problems is still questioned. Except for human tissue composition data, existing biochemical data for the 8t- and 8c-18:1 isomers are based on in vitro studies and animal data.

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Abbreviations: CE, cholesterol ester; CHYLO, chylomicron; FFA, free fatty acid; HDL, high density lipoprotein; LCAT, lecithin:cholesterol acyltransferase; LDL, low density lipoprotein; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; TG, triglyceride; VLDL, very low density lipoprotein; GC–MS, gas chromatography–mass spectroscopy; TLC, thin layer chromatography.

The main objective of this study was to compare the metabolism of the 8t- and 8c-18:1 isomers to 9c-18:1 in "normal" young adult male subjects in order to determine if there is a biochemical basis for suspecting that the Δ-8 isomers might have adverse nutritional or physiological impact on health. A second objective was to validate in human subjects the results reported for in vitro and animal studies. A third objective was to gain further insight into the absorption and transport of dietary fat in man.

EXPERIMENTAL

Preparation of the deuterium-labeled fatty acids and sampling schedule. The composition of the deuterium-labeled fatty acids in the mixtures of monoacid triglyceride (TG) fed are summarized in Table 1. The number of deuterium atoms used to label the 8t- and 8c-18:1 isomers was reversed in the two studies to permit detection of unanticipated systematic analytical errors and isotope effects. Synthesis of the labeled fatty acids (8t-18:1-17,18-$^2H_2$, 8t-18:1-12,12,13,13-$^2H_4$, 8c-18:1-17,18-$^2H_2$, 8c-18:1-12,12,13,13-$^2H_4$ and 9c-18:1-14,14,15,15,17,18-$^2H_6$) and their monoacid triglycerides has been described previously (6,7).

The mixture of deuterated TG (27.5 g, subject 1, and 24.0 g, subject 2) was heated to ca. 70°C and emulsified in a blender with 30 g calcium-sodium caseinate, 30 g dextrose and 15 g sucrose in 200 ml of water, which had also been heated to 70°C. The mixture was fed at 8 a.m., in place of the subject's normal breakfast, following a 10–12-hr fast. The subjects ate a light lunch at ca. 12:30 p.m. and a normal evening meal at ca. 6:30 p.m.

Blood samples (ca. 22 ml each) were obtained by venipuncture at 0, 2, 4, 6, 8, 12, 15, 24 and 48 hr for plasma lipid class fatty acid analysis. Samples (ca. 30 ml) were collected at 2, 4, 6, 8, 12, 15 and 24 hr for lipoprotein lipid class analysis. Standard preparative ultracentrifuge

TABLE 1

Deuterium-labeled Fatty Acids Used in Triglyceride Mixtures Fed

Fatty acids in mixture	Melting point of acid	Wt (g)	Wt %	Ratio by MS	
				8t/9c-18:1	8c/9c-18:1
Subject 1					
8t-18:1-$^2H_2$	53.3	9.4	34.2	1.09	
8c-18:1-$^2H_4$	27.4	9.3	33.8		1.08
9c-18:1-$^2H_6$	−6.4	8.8	32.0		
	Total fed	27.5			
Subject 2					
8t-18:1-$^2H_4$	52.8	7.9	32.3	0.99	
8c-18:1-$^2H_2$	26.9	8.3	34.6		1.10
9c-18:1-$^2H_6$	−6.4	7.8	32.5		
	Total fed	24.0			

methods were used to separate chylomicron (CHYLO), very low density (VLDL), low density (LDL) and high density (HDL) lipoprotein fractions (8). Samples were analyzed by electrophoresis to confirm the purities of the lipoprotein fractions.

Subjects. The subjects were two Caucasian males, ages 26 and 23. Medical histories, physical examinations and clinical blood profile data indicated that the subjects were in excellent health, had no history of congenital ailments and had not taken any medication for at least 3 wk before the study. Dietary histories confirmed that food selection was typical of American diets reported in the Hanes and USDA surveys (9,10). The subjects' height/weight ratios (195 cm/88.5 kg and 178 cm/70.4 kg), blood pressure (120/75 and 120/80), and serum cholesterol (133 mg/dl and 171 mg/dl), and fasting TG (65 mg/dl and 83 mg/dl) were within normal ranges.

The subjects were requested to follow, for 1 wk prior to feeding, the standard diet for diabetics recommended by the American Diabetic Association. The purpose was to help the subjects select diets of ca. 40% fat, 40% carbohydrate and 20% protein. No significant changes in the subjects' weights were observed during this period, which indicated a stable energy balance. The subjects fasted for 12 hr before the experimental meals were fed. This protocol was approved by the ARS/USDA Human Studies committee, and informed consent was obtained prior to feeding the mixture of deuterium-labeled fats.

Lipid class separation. Total lipids were extracted with 2:1 chloroform/methanol (11). Known weights of triheptadecanoin, cholesteryl heptadecanoate, heptadecanoic acid (17:0) and diheptadecanyl-L-α-phosphatidylcholine were added as internal standards to the total lipid extract to allow calculation of the concentrations (mg/ml) of the deuterated fatty acids in these plasma lipid classes. The concentrations of the other plasma lipid classes were calculated from known weights of the free acid of 17:0 added after their isolation by thin layer chromatography (TLC). Internal standards were not added to the chylomicron extracts from subject 1.

Preparative TLC was used for isolation of TG, cholesterol ester (CE), free fatty acid (FFA), phosphatidylethanolamine (PE), phosphatidylcholine (PC) and phosphatidylserine (PS) lipids (12,13). Methyl esters of the isolated lipids were prepared by heating with a 5% HCl-methanol solution (14). To determine the distribution of the deuterium-labeled fatty acids in the 1- and 2-acyl position of PC, a portion of the isolated PC was treated with phospholipase A_2 (15), and the reaction products were separated by TLC and esterified.

Analytical methods. Quantitation of deuterium-labeled fatty acids incorporated into plasma and lipoprotein lipids was achieved by gas chromatography-mass spectroscopy (GC-MS) analysis of their methyl esters. Fatty acid percentages and concentrations for both labeled and unlabeled fatty acid esters were obtained by analysis with a Finnigan model 4500 quadrupole mass spectrometer operated in a chemical ionization mode with isobutane as the ionization reagent. The gas chromatograph-mass spectrometer was equipped with a Supelcowax 10 fused silica column (30 m × 0.32 mm; Supelco Inc., Bellefonte, PA) and was temperature programmed from 165°C to 265°C at 5°C/min with a 20-min final hold. The MS methodology included selected-ion monitoring of each GC peak, followed by integration of the peak areas at appropriate mass numbers. The specific operating conditions and computer-assisted storage and processing of the MS data have been described previously (16).

Quantitation and absolute weight data for both the isotope-labeled and nonlabeled fatty acid methyl esters in the plasma lipid samples were based on the known weight of heptadecenoic acid added as an internal standard prior to conversion of the lipid classes to their methyl esters. Response factors were determined by analysis of standard mixtures containing weighted amounts of pure fatty acid methyl esters purchased from Nu-Chek Prep Inc. (Elysian, MN) and Applied Science (State College, PA). The accuracy of the GC-MS data was estimated at 2% relative standard deviation from analysis of various standard mixtures of known composition that simulated the composition of actual samples.

A Varian model 3400 gas chromatograph, equipped with a 100-m × 0.25-mm SP2560 fused silica capillary column (Supelco Inc., Bellefonte, PA) and a flame ionization detector, was used to analyze many of the samples to confirm the quantitation of the methyl ester data obtained by GC-MS. Operating conditions were: split ratio, 1:100; linear velocity of helium, 21 cm/sec; detector and injection temperature, 235°C. The column oven temperature was programmed from 165 to 220°C at 3 c/min with an initial hold of 15 min and a final hold of 30 min. Methyl ester GC peaks were identified and quantitation was confirmed by analysis of authentic standards and mixtures of known composition and by MS data.

Selectivity values. Selectivity values were calculated by dividing the experimental ratio by fed ratio where the experimental ratio equals the ratio of labeled 8t- or 8c-18:1 to labeled 9c-18:1 in the sample and the fed ratio equals the ratio of the corresponding fatty acids in the fed mixture. If the value obtained for the selectivity value is less than 1.0, the reciprocal is calculated and the value given a negative sign. Because calculation of values by this procedure results in values either greater than 1 or less than −1, the values were adjusted to a zero basis by adding or subtracting −1 as appropriate. The use of the reciprocal for selectivity values less than 1.0 avoids the problem of compressing negative values between 0.0 and 1.0, which facilitates comparison of negative values with positive values. Thus, a selectivity value less than 0.0 denotes discrimination against incorporation of the deuterium-labeled fatty acid in the numerator, and a value greater than 0.0 indicates preferential incorporation. Expression of the data as selectivity values compensates for the small differences in the percentage of labeled fatty acids in the fed mixtures.

Area selectivity values are calculated by the same procedure as for selectivity values for individual samples, except the experimental ratio is obtained from the area under the curves after the data is plotted, for example, as in Figure 1. Thus, area selectivity values are a "weighted" average of all the samples analyzed for an individual plasma or lipoprotein lipid class.

RESULTS

Digestion. Absorption and clearance of the deuterium-labeled 8t-, 8c- and 9c-18:1 acids are reflected in the plasma and CHYLO TG data plotted in Figure 1. The

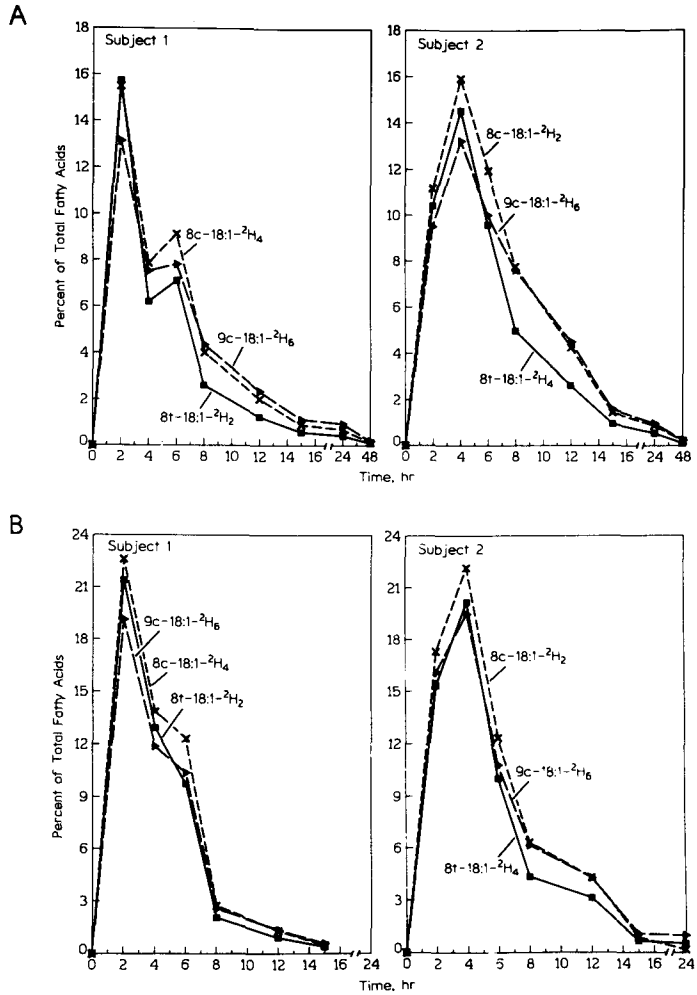

FIG. 1. Incorporation and turnover of deuterium-labeled 8t-, 8c- and 9c-18:1 in (A) plasma triglycerides and (B) chylomicron triglycerides.

profiles of the CHYLO and plasma TG curves are similar because the CHYLO TG contribute the majority of deuterated TG in the plasma TG samples. For example, the distribution of the total deuterated fatty acids in the 6-hr lipoprotein sample from subject 2 was CHYLO TG, 161 μg/ml (67.7%); VLDL TG, 34.2 μg/ml (14.4%); LDL TG, 29.7 μg/ml (12.5%); and HDL TG, 12.8 μg/ml (5.3%). The percentages for each of the deuterated fatty acids incorporated into the lipoprotein TG fractions collected over a 24-hr period from subject 2 are plotted in Figure 1B and Figure 2. The VLDL and HDL TG samples (Fig. 2) contain slightly smaller percentages for 8t-18:1-$^2H_4$, but the 6- to 15-hr LDL TG samples contain about 25% less 8t-18:1 than 8c- and 9c-18:1. This difference probably reflects a higher rate of removal of 8t-18:1 during formation of LDL from VLDL.

Comparison of the shape of the curves for 9c-18:1 to the curves for 8t- and 8c-18:1 (CHYLO TG data, Fig. 1B) indicates similar absorption of both Δ8-18:1 isomers and 9c-18:1. Calculated selectivity values for the plasma TG fraction show the absorption of the Δ8-18:1 isomers varied from 8% higher to 12% less than 9c-18:1. The maximum percent of total deuterated fatty acid in the CHYLO TG samples was similar for both subjects. The only major difference between CHYLO TG data from the two subjects was the 2-hr offset for the time of maximum absorption.

Incorporation in plasma and lipoprotein lipids. Uptake and turnover of the labeled fatty acids in plasma CE samples are illustrated in Figure 3. These data show large differences between the percentages of 8c-, 8t- and 9c-18:1 incorporated into plasma CE samples. The area selectivity values (Table 2) indicate that esterification of cholesterol with 9c-18:1 was 35–50% greater than for 8c-18:1 and about 8.5 times higher than for 8t-18:1.

Plots of the plasma PC data are shown in Figure 4. Variation between the curves for the two subjects is due to a high deuterated fatty acid content in the 8-hr sample from subject 1 and to a small general discrimination against 8c-18:1 for samples from subject 2. Determination of the deuterated fatty acid distribution at the 1- and 2-acyl positions of PC shows strong preferential incorporation of 8t-18:1 at the 1-acyl position, which is balanced by a preferential incorporation of 9c-18:1 at the 2-acyl position. Maximum percentage deuterated fatty acids incorporated, time of maximum incorporation and selectivity values for the plasma lipids are presented in

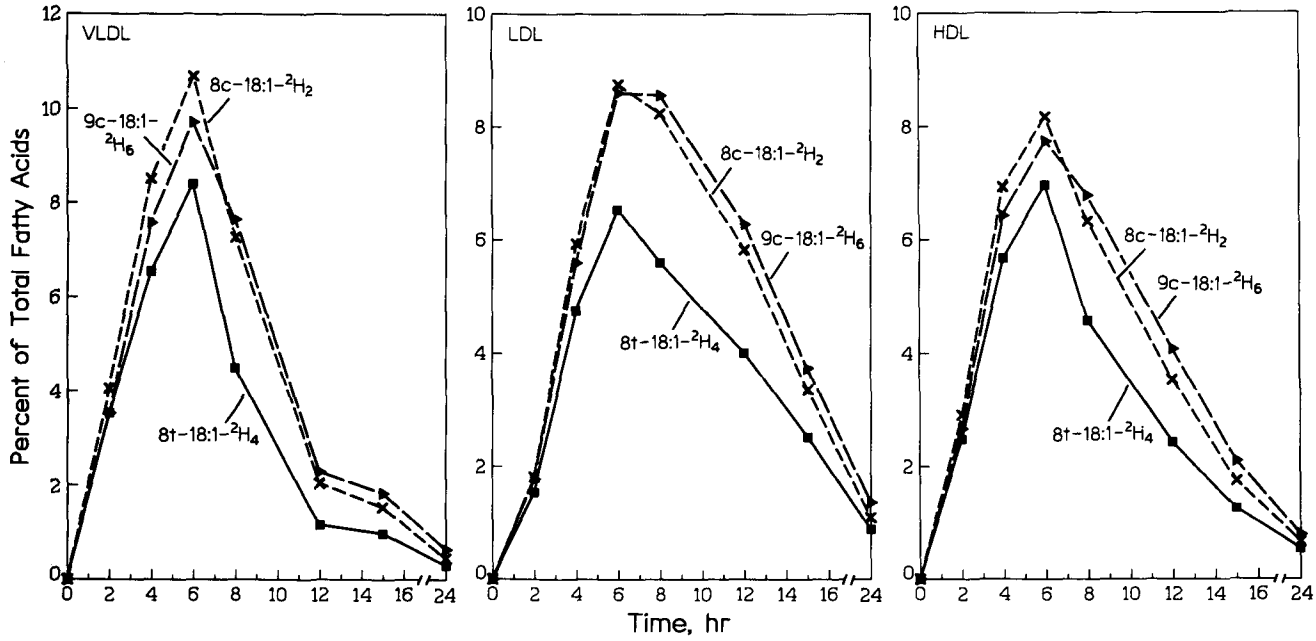

FIG. 2. Incorporation and turnover of deuterium-labeled 8t-, 8c- and 9c-18:1 in very low, low and high density lipoprotein triglycerides from subject 2.

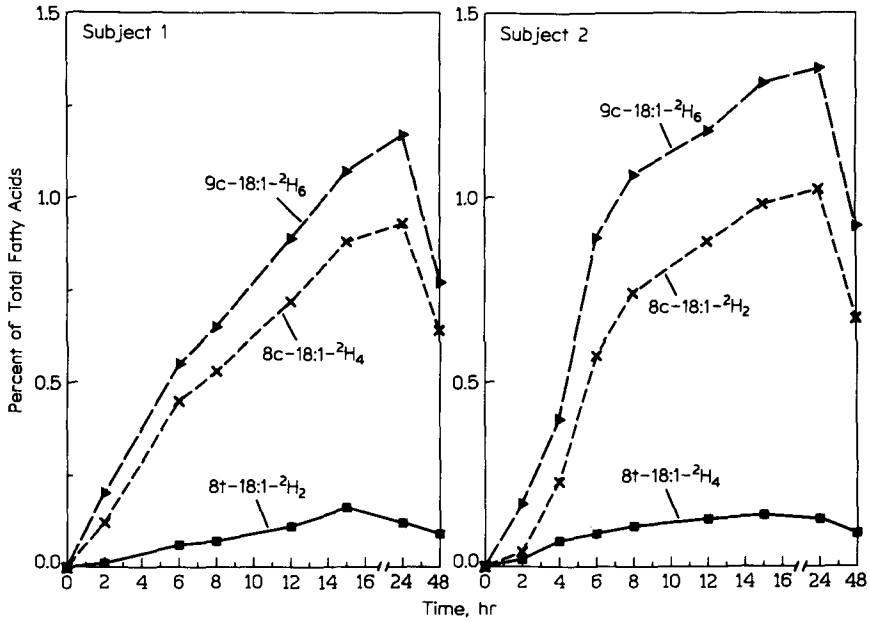

FIG. 3. Uptake and turnover of deuterium-labeled 8t-, 8c- and 9c-18:1 in plasma cholesterol ester samples.

Table 2. The selectivity values are negative for nearly all the lipid fractions. The exception is the positive selectivity value for the 1-acyl PC fraction.

Area selectivity values and the maximum percentages for the deuterated fatty acids incorporated into lipoprotein TG, CE, PE and PC fractions are summarized in Table 3. The selectivity values for CE, PE and PC followed the same trends in both the plasma and lipoprotein samples. The negative selectivity values for VLDL, LDL and HDL TG samples indicate discrimination against 8t- and 8c-18:1 incorporation. These values probably reflect reduced amounts of the 8c- and 8t-18:1 isomers available for incorporation into these fractions due to more rapid removal of these isomers rather than differences in enzyme specificities involved in TG synthesis.

Chain-shortened and elongated fatty acids. Chain-shortened deuterium-labeled 16:1 fatty acids were detected in the TG, CE, FFA and PC plasma lipid classes with the

TABLE 2

Plasma Lipids: Distribution of Deuterated 8t-, 8c- and 9c-18:1 at Maximum Enrichment and Area Selectivity Values

Plasma lipid class	Time, hr		Percent of total lipid[a]						Area selectivity values					
			8t-18:1-^{2}H		8c-18:1-^{2}H		9c-18:1-^{2}H		8c/9c-18:1		8t/9c-18:1			
	Subject		Subject		Subject		Subject		Subject		Subject		18:0	18:2/
	1	2	1	2	1	2	1	2	1	2	1	2	9c-18:1[b]	9c-18:1[b]
TG	2	4	15.70	14.54	15.50	15.93	13.10	13.23	−0.09	−0.03	−0.37	−0.21	−0.7	−0.1
FFA	2	4	10.30	7.29	11.30	7.97	9.30	6.97	0.06	−0.03	−0.17	−0.13	−0.3	−0.2
CE	24	24	0.10	0.13	0.90	1.02	1.17	1.35	−0.34	−0.50	−8.44	−8.44	−100.0	9.6
PE	8	6	0.30	0.85	0.30	0.72	0.40	0.98	−0.70	−0.66	−0.60	−0.15	0.3	1.0
PS	8	8	2.04	0.61	0.78	0.31	1.19	0.65	−0.49	−0.58	0.76	−0.03	0.4	.5
LPC	12	6	0.40	0.64	0.79	0.96	1.08	1.20	−0.49	−0.38	−1.38	−0.45	—	—
SM	12	12	0.49	0.81	0.64	0.79	0.91	0.97	−0.39	−0.35	0.14	−0.27	—	—
PC	8	8	2.20	1.88	2.26	1.62	2.86	1.91	−0.55	−0.39	−0.36	0.06	5.5	10.0
PC-1	12	12	0.55	2.76	0.29	1.54	0.17	0.07	0.63	1.80	1.57	2.90	11.5	−1.6
PC-2	8	12	0.24	0.57	0.57	1.11	1.14	2.28	−1.32	−1.24	−5.64	−3.59	−0.9	9.0

[a]Weight percent of deuterium-labeled fatty acid in total lipid class.

[b]Area selectivity values are the average of two subjects, data from Ref. 23.

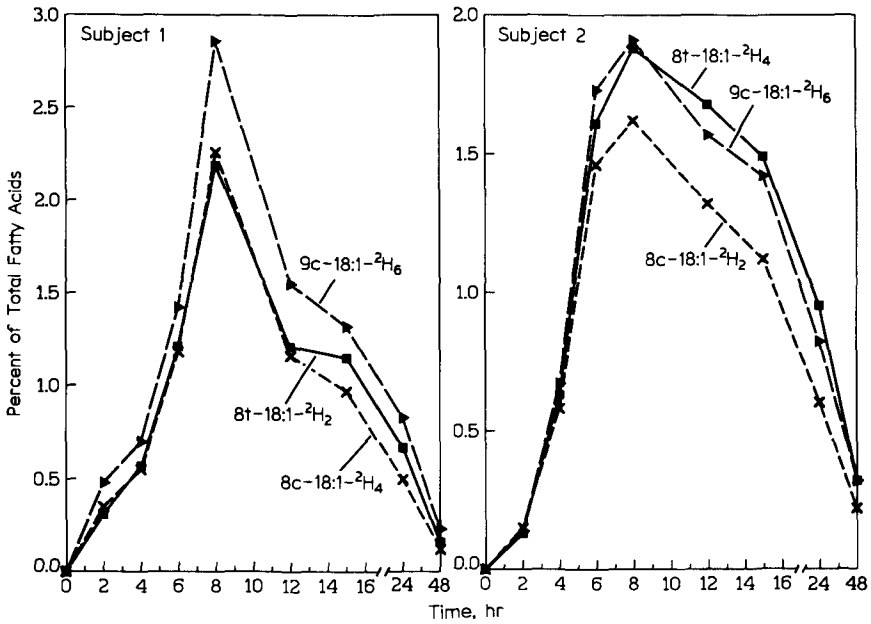

FIG. 4. Uptake and turnover of deuterium-labeled 8t-, 8c- and 9c-18:1 in plasma phosphatidylcholine samples.

TG fractions containing the highest amounts. The maximum percentages for deuterium-labeled 16:1 acids in the plasma TG and FFA fractions (based on total fatty acid content) were: TG (0.95-0.86% 6t-16:1; 0.12-0.17% 6c-16:1; 0.07-0.06% 7c-16:1) and FFA (0.29-0.17% 6t-16:1; 0.07-0.06% 6c-16:1, 0.0-0.03% 7c-16:1. Percentages for deuterium-labeled 16:1 in the VLDL, LDL and HDL TG samples are plotted in Figure 5. The amount of 6t-16:1 chain-shortened product from 8t-18:1 was 5 to 6 times higher than the 6c-16:1 product from 8c-18:1, and chain-shortened 9c-18:1 was detected at very low levels. Deuterated 10t-20:1, 10c-20:1 and 11c-20:1 were found in trace (.01 to .05%) amounts in only the TG fractions.

DISCUSSION

The variability between the results from each subject was evaluated on the basis of total percentage deuterated fatty acid incorporated, mg/ml of deuterated fatty acid incorporated, selectivity values, overall curve shape or profile and time of maximum deuterated fat incorporated for each lipid class. Based on these five criteria, the overall variability between results from each subject was small and indicates that the results were not influenced by the lack of rigorous control of the diet before or after the deuterated fat mixtures were fed.

The percentages of deuterated 9c-18:1 incorporated into

TABLE 3

Lipoprotein Lipids: Distribution of Deuterated 8t-, 8c- and 9c-18:1 at Maximum Enrichment and Area Selectivity Values

			Percent of total lipid[a]						Area selectivity values			
	Time, hr		8t-18:1-^{2}H		8c-18:1-^{2}H		9c-18:1-^{2}H		8c/9c-18:1		8t/9c-18:1	
	Subject		Subject		Subject		Subject		Subject		Subject	
Lipid class	1	2	1	2	1	2	1	2	1	2	1	2
Triglyceride												
CHYLO	2	4	21.40	20.16	22.62	22.14	19.14	19.48	0.02	−0.04	−0.07	−0.10
VLDL	2	6	8.41	8.40	8.96	10.69	7.55	9.72	−0.08	−0.07	−0.36	−0.34
LDL	8	6	2.49	6.54	4.07	8.76	4.44	8.61	−0.17	−0.16	−0.68	−0.42
HDL	6	6	3.65	6.96	4.63	8.17	4.59	7.74	−0.19	−0.16	−0.47	−0.34
Cholesterol ester												
CHYLO	24	24	0.11	0.11	0.81	0.97	1.07	1.25	−0.42	−0.48	−9.93	−10.29
VLDL	12	8	0.14	0.10	0.83	0.81	1.05	1.04	−0.42	−0.48	−14.06	−9.66
LDL	24	24	0.07	0.13	1.19	1.07	1.54	1.43	−0.44	−0.58	−15.98	−9.76
HDL	12	8	0.11	0.19	1.02	1.37	1.23	1.52	−0.34	−0.33	−11.17	−8.13
Phosphatidylcholine												
CHYLO	8	8	1.50	1.89	1.51	1.60	1.81	1.94	−0.44	−0.35	−0.37	0.03
VLDL	8	8	0.96	1.03	0.98	0.91	1.20	1.14	−0.45	−0.45	−0.51	−0.05
LDL	12	8	1.13	1.84	0.99	1.50	1.46	1.88	−0.61	−0.45	−0.37	0.00
HDL	15	8	1.68	2.01	1.21	1.72	1.81	1.98	−0.54	−0.32	−0.26	0.08
Phosphatidylethanolamine												
CHYLO	6	6	1.50	1.35	1.70	1.25	1.95	1.59	−0.43	−0.62	−0.61	−0.24
VLDL	12	6	0.28	0.22	0.29	0.30	0.37	0.26	−0.44	−0.22	−0.48	−1.26
LDL	6	6	0.48	0.61	0.50	0.63	0.69	0.73	−0.66	−0.29	−0.82	−0.25
HDL	6	6	0.81	0.73	0.78	0.69	1.16	0.94	−0.82	−0.65	−0.68	−0.21

[a]Weight percent of deuterium-labeled fatty acid in total lipid fraction.

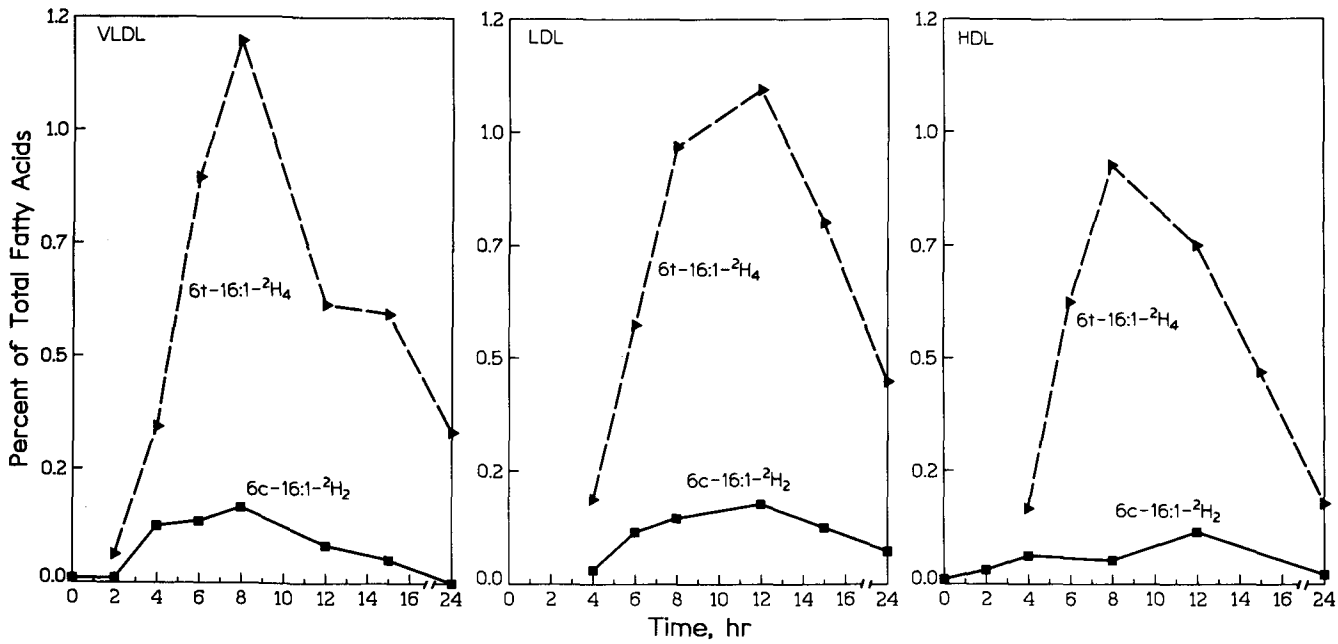

FIG. 5. Percentage of deuterium-labeled 6t- and 6c-16:1 incorporated into plasma triglycerides.

the various lipid classes from subjects used in this study were consistent with 9c-18:1 data from 11 subjects fed deuterated 9c-18:1 as a control in previous studies (17-21). Medical histories did not identify any health problems, and clinical data and fatty acid composition of individual lipid classes were within ranges reported as normal for the U.S. population. Based on these combined data, the metabolism of the Δ8-18:1 fatty acids in these subjects

TABLE 4

Composition of Unlabeled Fatty Acids in Chylomicron Triglycerides[a]

Subject	Time (hr)	Percent fatty acid 16:0	18:0	18:1	18:2	Total weight μg/ml
1	2	38.6	12.7	34.8	13.9	—
	4	44.8	10.6	35.4	9.2	—
	6	38.0	10.0	38.2	12.8	—
	8	40.0	15.6	35.4	9.0	—
	12	39.2	16.2	36.0	8.6	—
2	2	29.4	7.2	48.4	14.1	133
	4	28.7	7.5	49.5	14.3	149
	6	26.2	10.7	52.5	10.6	327
	8	27.8	10.2	50.9	11.1	330
	12	32.8	7.7	49.1	10.5	187

[a]The percentages for the 4 fatty acids listed represented about 95% of the total unlabeled fatty acids. Data normalized to 100%.

is considered to be representative of normal young adult males.

Absorption. The plasma and CHYLO TG data in Figure 1 and the TG selectivity values in Tables 2 and 3 are clear evidence that absorption of the 8t- and 8c-18:1 isomers was similar to 9c-18:1, which is about 95% absorbed by normal subjects. These data are consistent with the nonselectivity of pancreatic lipase for positional 18:1 isomers (22). The maximum total isotopic enrichment in the CHYLO TG samples was ca. 60%. The presence of ca. 40% unlabeled TG in these fractions is a very consistent feature that we have observed in previous human studies (18,19,21,23).

The fatty acid composition of the four major unlabeled fatty acids present in the 2- to 12-hr CHYLO TG fractions (Table 4) was relatively constant. The increase in total weight of unlabeled fatty acids (subject 2) in the 6- and 8-hr fraction is likely due to fat consumed at lunch. The consistency in both the unlabeled fatty acid composition of the CHYLO TG and the isotopic enrichment data suggests that mobilization of stored fat provides a portion of the fatty acids incorporated into CHYLO particles during digestion of dietary fat. This suggestion for mobilization of a pool of stored fat is based on the fact that subjects who fasted for 12 hr have low levels of CHYLO TG, and that dietary fat from previous meals has been removed from the digestive system and the maximum isotopic enrichment of the fatty acids in the CHYLO-TG samples is about 60%. This combination of experimental data indicates some source of unlabeled endogenous fat must have been incorporated during formation of the CHYLO TG particles, because none is present in the experimental mixture fed. In fact, the unlabeled fatty acids in the 6- and 8-hr CHYLO TG samples, which should contain fatty acids from the noon meal, have a composition that is similar to the unlabeled fatty acids in the 2- and 4 hr-samples. These data suggest that the body has a mechanism for attempting to maintain the CHYLO TG fatty acid composition within certain limits by mobilization of an endogenous pool of stored fat.

Based on the isotopic enrichment of the CHYLO TG samples, we calculate that ca. 16.1 g (subject 1) and 14.8 g (subject 2) of unlabeled fat was incorporated into the CHYLO TG during absorption of the labeled fats. These data agree with results from rat studies which reported that endogenous fat supplied up to 50% of the fat in CHYLO particles (24). The source of the endogenous fat in the chylomicrons is unclear. The liver parenchymal and fat-storing cells (25) are possible sources of endogenous fat because of their high TG content. The amount of residual TG and FFA in the plasma and intestinal cells, following a 10- to 12-hr fast, is too small to supply 15–16 g of endogenous fat. The turnover rate for CHYLO TG was calculated from the weight of the total labeled fatty acids fed plus the calculated weight (16.1 g and 14.8 g) of unlabeled endogenous fatty acids incorporated into the CHYLO TG during absorption of the labeled fatty acids. The calculated turnover rates for CHYLO TG were 4.1 and 4.6 mg/min/kg of body weight for subjects 1 and 2, respectively. These data are consistent with values of 3.9 and 4.7 mg/min/kg for mixtures containing the 11c and 11t-18:1 isomers and 5.2 mg/min/kg for a mixture of 10c-, 10t- and 9c-18:1 (18,19).

Oxidation. Plasma TG data indicate that the 8t- and 8c-18:1 isomers are removed slightly more rapidly than 9c-18:1, but neither isomer is selectively incorporated into PC or CE fractions (Figs. 3 and 4, Tables 2 and 3). Thus, the 8t- and 8c-18:1 isomers must be either selectively oxidized or deposited into tissue lipids. Because analysis of tissue lipids does not provide evidence for preferential incorporation of the Δ8-18:1 isomers (1,2), a higher rate of β-oxidation is left as the most plausible explanation for the plasma TG data. This explanation is consistent with in vitro rat and human data which have shown that the *trans* isomers are oxidized more rapidly than 9c-18:1 (26–28) and with the higher conversion of 8t- and 8c-18:1 to 16:1 isomers (see following section). It does not agree with in vitro rat liver studies which have reported that the 8t- and 8c-18:1 isomers are oxidized more slowly than 9c-18:1 (29) and with rat liver peroxisome studies that found similar rates for the oxidation of *cis* and *trans* fatty acids (30).

Interconversion. The 8t- and 8c-18:1 isomers have been reported to be retroconverted to 14:1 (31), desaturated to 6c,8t-18:2 (32,33) and elongated to 20:1 (34). Retroconversion of deuterated 8t- and 8c-18:1 to deuterated 6t- and 6c-16:1 (but not 14:1) and elongation to deuterated 10t- and 10c-20:1 (trace amounts) were observed. The higher percentage of 6t-16:1 compared with 6c-16:1 (Fig. 5) was similar to the pattern for chain-shortened products from the Δ 10 and 11 *cis*- and *trans*-18:1 isomers (18,19). Desaturation of 8t- and 8c-18:1 to 18:2 isomers was not detected.

Transfer times. Lipoprotein LDL and HDL TG data (Fig. 2 and Table 3) indicate maximum isotope enrichment occurred 6 hr after feeding. The times of maximum enrichment indicate that about a 2-hr period is necessary for transfer of fatty acids from CHYLO TG to LDL and HDL TG. The time of maximum enrichment for lipoprotein and plasma PC (Fig. 4 and Table 3) is 4–6 hr after maximum CHYLO-TG enrichment. These data suggest cell uptake and excretion of a portion of the deuterated fatty acids occur before they are incorporated into TG and PC associated with lipoprotein fractions. Much of the 2- to 4-hr time delay observed for the incorporation of deuterated chain-shortened 16:1 fatty acids into TG (Fig. 5) is

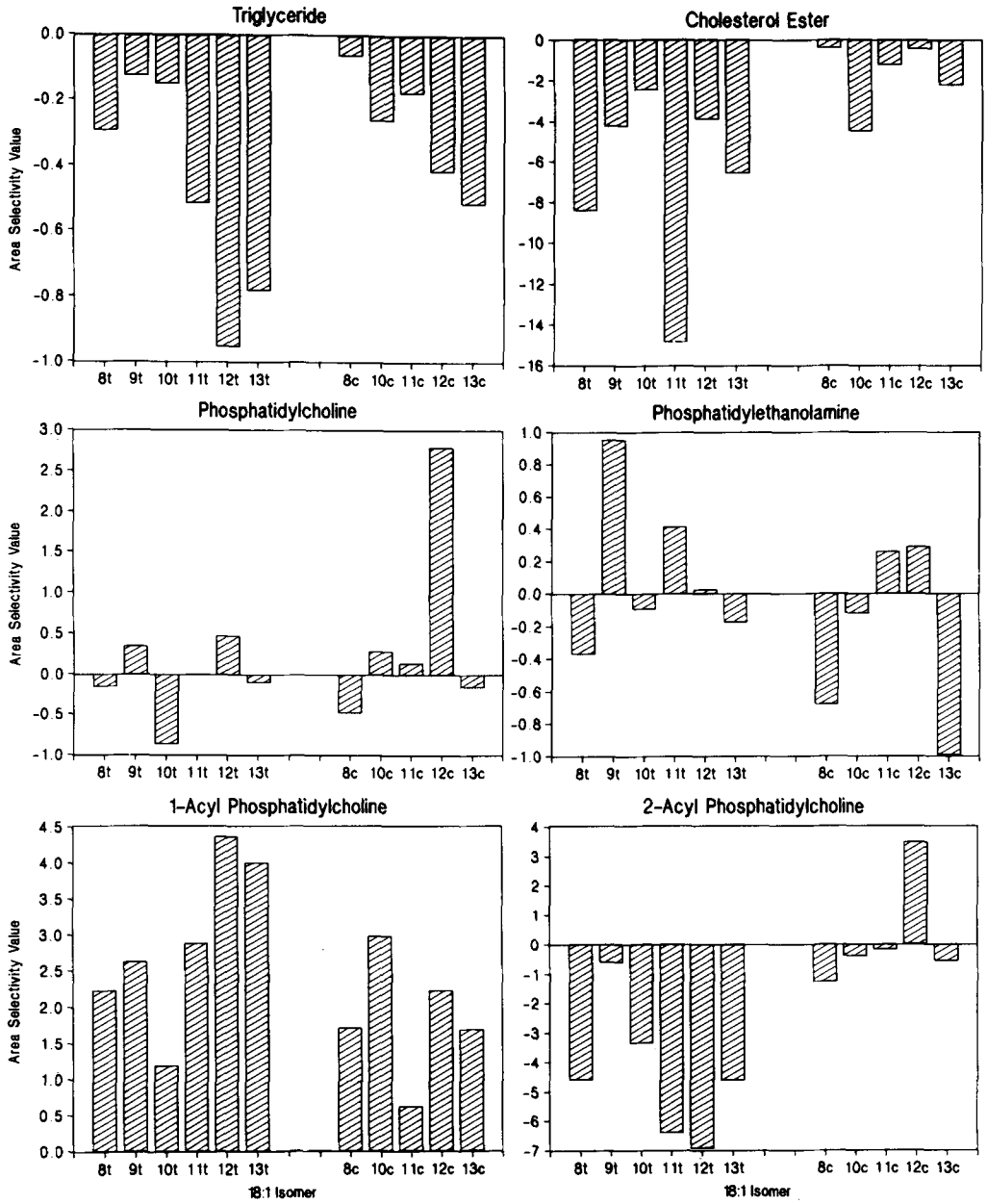

FIG. 6. Comparison of plasma triglyceride, cholesterol ester, phosphatidylcholine, phosphatidylethanolamine, 1-acyl phosphatidylcholine and 2-acyl phosphatidylcholine area selectivity values for the 8 through 13 *trans*- and *cis*-18:1 positional isomers. All area selectivity values are relative to 9c-18:1. Data for isomers other than 8t- and 8c-18:1 are from references 17–21.

probably due to the time required for the fatty acids to be internalized into the liver cells and then excreted into the plasma. Liver cells are probably responsible for much of the PC synthesized, but cells other than liver cells may be involved in TG synthesis. The maximum enrichment for the 24-hr LDL CE sample is more difficult to understand. The LDL CE data suggests the possibility that transfer of deuterated fatty acids from the 2-acyl position of PC is responsible for most of the deuterated LDL CE and that the newly synthesized CE remains associated with the LDL particles for a comparatively long time period.

Enzyme specificity. The deuterated fatty acid content and selectivity values for the plasma CE and PC-2 fractions (Table 2) indicates the 8t-18:1 isomer is a poor substrate for lecithin:cholesterol acyltransferase (LCAT) and 2-acyl phosphatidylcholine acyltransferase. The PC-1 data indicates that 8t-18:1 is preferred over 9c-18:1 for acylation of the 1 position of phosphatidylcholine. This selectivity for incorporation of 8t-18:1 at the 1-acyl position is balanced by discrimination against incorporation of 8t-18:1 at the 2-acyl PC position and is similar to results reported for rat liver PC (35). The PC-1 and PC-2 selectivity values for the 8c-18:1 isomer were intermediate between 8t- and 9c-18:1.

The specificities of LCAT and acyltransferase enzymes for 8t- and 8c-18:1 resemble the pattern reported for 18:0

vs 9c-18:1 (Table 2), but the 18:0/9c-18:1 CE- and PC-selectivity values (23) are much larger than the 8t- and 8c-18:1/9c-18:1 values. The relative incorporation of the 8t- and 8c-18:1 isomers into plasma TG, CE and PC are compared with data for other 18:1 isomers in Figure 6. These data indicate the Δ-8 bond position does not have a unique effect on metabolism when compared with other positional 18:1 isomers. The data compared in Figure 6 are from similar designed studies, and 9c-18:1 was used as an internal control in all experiments. Possible trends in selectivity values within lipid classes appear to be associated with the position of the double bond as it is moved away from the center of the fatty acid chain or toward the methyl end of the chain. There are no consistent correlations with known physical properties. Each 18:1 isomer has its own "individuality," but the difference between the metabolism of the *cis*- and *trans*-positional 18:1 isomers and 9c-18:1 is much less than the differences between 9c-18:1 and 18:0, 16:0 or 9c,12c-18:2 (23). Thus, the 18:1 isomers should not be regarded as equivalent to saturated fatty acids or oleic acid. In general, human plasma lipid selectivity values validate in many many of the results reported for various tissue lipids from rat (36,37), laying hen (38,39) and in vivo studies (40-42). Given the wide variation in diets, species and experimental designs, the similarity between results from different species is closer than expected. The structural features of the 8t- and 8c-18:1 isomers obviously influence the binding constants of some of the enzymes involved with fatty acid metabolism, but it is unknown if this sensitivity to the structure of these isomers extends to other enzymes and alters cell metabolism.

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Eskimo Plasma Constituents, Dihomo-γ-linolenic Acid, Eicosapentaenoic Acid and Docosahexaenoic Acid Inhibit the Release of Atherogenic Mitogens

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Studies in man and laboratory animals suggest that ω3 polyunsaturated fatty acid constituents of fish oils have antiatherosclerotic properties. We have studied the effects of several such polyunsaturated fatty acids for ability to modify the in vitro release of mitogens from human platelets. Such mitogens may produce the fibroproliferative component of atherosclerotic plaques. Both 5,8,11,14,17-eicosapentaenoic acid (20:5ω3) and 4,7,10,13,-16,19-docosahexaenoic acid (22:6ω3), major constituents of fish oils, inhibited adenosine diphosphate-induced aggregation of platelets and the accompanying release of mitogens. These effects are dose dependent. Linolenic acid (18:3ω3), the biosynthetic precursor of eicosapentaenoic acid, also inhibited platelet aggregation and mitogen release. Eicosapentaenoic acid also inhibited mitogen release from human monocyte-derived macrophages, which, in vivo, are an additional source of mitogens during atherogenesis.

Potent inhibition of human platelet aggregation and mitogen release was also seen with dihomo-γ-linolenic acid (8,11,14-eicosatrienoic acid 20:3ω6), whose levels are reportedly elevated in Eskimos subsisting on marine diets.

We conclude that diets that elevate plasma and/or tissue levels of eicosapentaenoic acid, docosahexaenoic acid and dihomo-γ-linolenic acid precursor γ-linolenic acid (18:3ω6) may exert antiatherosclerotic effects by inhibiting the release of mitogens from platelets and other cells. *Lipids 24,* **70-75 (1989).**

Marine lipid diets rich in 5,8,11,14,17-eicosapentaenoic acid and 4,7,10,13,16,19-docosahexaenoic acid seem to exert antiatherosclerotic effects in both man and experimental animals (1-12). However, there is no definitive description of the mechanisms involved. There is a mild cholesterol-lowering effect and marked triglyceride-lowering effect of such diets (4-6). However, these effects, alone, may not be sufficient to inhibit atherosclerosis.

The principal feature of human atherosclerosis is the development of fibroproliferative plaque, probably in response to mitogens released from platelets, macrophages and other cells resident in or adhering to the vascular wall (13,14). By contrast, lipid accumulation per se may result in development of fatty streaks composed of only foam cells. In previous studies, we have shown that some of the eicosanoids derived from polyunsaturated fatty acids can inhibit some or all of these processes. For example, prostacyclin (PGI_2; derived from arachidonic acid), some stable prostacyclin mimetics and prostaglandin E_1 (PGE_1; derived from dihomo-γ-linolenic acid) inhibit not only platelet aggregation, but also release of mitogens from platelets and macrophages and accumulation of cholesterol in macrophage foam cells (13,15,16). In the present study, we examined effects of dihomo-γ-linolenic, eicosapentaenoic and docosahexaenoic acids on platelet aggregation and mitogen release and compared them with those of several fatty acids that have varying degrees and positions of unsaturation.

METHODS

Materials. Arachidonic acid, dihomo-γ-linolenic acid, α-linolenic acid, γ-linolenic acid, eicosapentaenoic acid, docosahexaenoic acid, 11,14,17-eicosatrienoic acid, Ficoll (histopaque-1077) and adenosine diphosphate (ADP) were all obtained from Sigma Chemical Company (St. Louis, MO). Adrenic acid was from NuChek Prep (Elysian, MN). Mead acid was supplied by Dr. Howard Sprecher (Ohio State University). Toluene (reagent grade; Mallinckrodt, Paris, KY) was redistilled before use. All fatty acids were dissolved as stock solutions of 10 mg/ml in toluene and stored at −20°C under nitrogen for not more than two months. Hank's balanced salts solution, Dulbecco's phosphate buffered saline (PBS), Dulbecco's modified Eagle's medium (DMEM), L-glutamine, sodium pyruvate, HEPES buffer and trypsin-EDTA (0.5 mg/ml trypsin, 0.2 mg/ml EDTA) were from Gibco (Grand Island, NY). Fetal calf serum was purchased from Irvine Scientific (Santa Ana, CA). Pooled human hyperlipemic serum (>250 mg/dl cholesterol) was provided by the Lipid Research Laboratory at Stanford University Medical School. All serum was heat inactivated (56°C, 30 min). Kits for radioimmunoassay of platelet factor 4 (PF4) were from Abbott Labs (N. Chicago, IL). A Diff-Quik differential staining kit was obtained from American Scientific Products (McGaw Park, IL).

Cell cultures. Human preputial skin fibroblasts were originally obtained from Dr. C. Fielding (University of California, San Francisco, CA) and maintained at 37°C (in an atmosphere containing 5% CO_2, 95% air) in culture medium (DMEM with 25 mM HEPES buffer, pH 7.3), L-glutamine and sodium pyruvate (Gibco #380-2320) supplemented with 100 μg/ml gentamicin and 10% fetal calf serum).

Human monocytes were prepared by modification of previously described methodology (17) from 100 ml of human blood from one male volunteer, age 37, or one female volunteer, age 25. In both cases, no drugs had been ingested during the previous 14 days. The blood was collected into 20 ml vacutainer tubes containing 1 ml heparin (final concentration 10 Units/ml), mixed with an equal volume of PBS; and 20 ml were carefully layered on 15 ml of Ficoll-sodium metrizoate (density = 1.077 g/l) in 50 ml conical polypropylene centrifuge tubes. This preparation was centrifuged for 30 min at 400 × *g* at room temperature. After aspiration of the top (diluted plasma) layer,

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Abbreviations: ADP, adenosine diphosphate; PRP, platelet-rich plasma; PF4, platelet factor 4; PFP, plataelet-free plasma; PFPDS, platelet-free plasma-derived serum; DMEM, Dulbecco's minimum essential medium; PBS, Dulbecco's phosphate-buffered saline.

mononuclear cells at the interface were collected, washed twice in PBS, and resuspended and cultured in DMEM with autologous serum (10%), as described by Fogelman et al. (18), except that insulin was not present in the medium. Cells were plated at approximately 10^6 cells per well into 16-mm diameter culture wells of 24-well plates and allowed to adhere for 2 hr at 37°C and 5% CO_2. Nonadherent lymphocytes were then removed, by aspirating supernatant after gentle rocking of the plates, and the adherent monocytes were washed and incubated in fresh culture medium with 10% autologous serum. The adherent cells were kept in culture for seven days (and the medium changed every two days), allowing the monocytes to become macrophage-like cells (18).

Monocyte purity (absence of lymphocytes) of adherent cells was verified by standard differential staining methodology, using a Diff-Quik differential staining set.

Platelet aggregation and release of mitogens. Human blood (50–100 ml) from each of six healthy, drug-free volunteers of either sex, and in an age range of 21–50 years, was withdrawn by venipuncture via a 19-gauge butterfly infusion set into a plastic syringe containing 0.1 volume of 3.8% sodium citrate. Platelet-rich plasma (PRP) was then prepared by centrifugation of 10 ml aliquots at 1,500 × *g* for 4 min at room temperature, ca. 25°C. Fifteen microliters of a stock solution of arachidonic acid in toluene vehicle, or toluene vehicle alone, were dried under nitrogen gas at room temperature in a siliconized aggregation cuvette (0.312 in. diam.). A stirring bar was inserted, followed by 600 μl of human PRP. Mean platelet count (± SEM) was 551,100 ± 47,900/μl. Contents were stirred (1,000 rpm at 37°C) for a minimum of 3 min, while aggregation was recorded as maximal increase in light transmission. Control response to ADP (1.6 μg/ml; 3.75 μM) was also measured for comparison. Aggregation response to arachidonic acid was expressed as percentage of the response to ADP.

When inhibitory effects of fatty acids were examined, 15 μl of the appropriate stock solution or toluene vehicle was added to the cuvette, the toluene evaporated (as previously described), and the PRP was added and stirred (1,000 rpm at 37°C) for 20 min, to allow the fatty acid to dissolve and exert maximal effects on the platelet response. Aggregation was then induced by addition of 6 μl of a solution containing ADP (1.6 μg/ml, 3.75 μM, final concentration). Aggregation was recorded for 3 min, as change in light transmission of the PRP, and response reported as percentage inhibition of maximum amplitude from the control (without fatty acid).

Immediately after each sample of PRP had aggregated for 3 min, platelets were removed by centrifuging at 15,000 × *g* for one min, and supernatants again centrifuged under the same conditions. A portion of the resulting platelet-free plasma (PFP) was clotted by an adaptation of the method of Weiss et al. (19), i.e., 0.1 volume of 0.5 M $CaCl_2$ was added and samples were incubated at 37°C for 1 hr. This procedure eliminated clotting of the plasma during the subsequent mitogenesis assay, which sometimes interfered with accurate cell counting. Clots were removed with a sterile plastic rod, and the resulting platelet-free plasma-derived serum (PFPDS) was frozen at −20°C (or used immediately) for platelet mitogen assay. It was essential to remove all platelets before freezing, to avoid the artifact of freeze-thaw release of mitogens. Background samples (with no induced release of platelet mitogens) were prepared in an identical manner, except that PFP samples were used in place of PRP samples. When measured, PF4 was determined by competitive radioimmunoassay using a commercial kit (see Materials section).

Because there was a remote possibility that mitogens and/or PF4 might be trapped within the matrices of the clot, PFP and PFPDS were tested to see if they would give equivalent results in the mitogenesis assay. Mitogens in the serum produced a net increase from initial cell number (mean ± SEM) of 12,500 ± 1340 cells for PFP and 13,200 ± 1660 cells for PFPDS ($p > 0.1$, $n = 18$ in each case). Similar data were obtained in measurements of PF4: Values of 3.07 ± 0.16 μg/ml for PFP and 2.95 ± 0.16 μg/ml for PFPDS (mean ± SEM) were obtained ($p > 0.1$, $n = 7$ in each case). It is concluded that the use of PFPDS is both convenient and valid in the mitogenesis assay.

Mitogenesis assay. PFPDS, prepared during aggregation studies, was examined for its ability to support growth of human fibroblasts in culture. Cells were seeded at 8,000 cells/well into 16-mm diameter culture wells of 24-well plates. After 24 hr, culture medium was replaced with serum-free culture medium containing thawed, sterile-filtered (0.2 μm pore size) PFPDS, at a final concentration of 10% PFPDS. Cells were incubated at 37°C, 5% CO_2 for two days. The medium was then removed and the cells released with trypsin-EDTA. Cells were counted at appropriate settings in a Coulter counter (model ZBI, Coulter Instruments, Hialeah, FL), and percentage inhibition of growth was determined from counts corrected for PFP background samples (typically 35–65% of control counts).

Direct effects on cell proliferation. Fifteen microliters of a stock solution containing the appropriate fatty acid in toluene vehicle, or toluene alone, were added to tubes and the toluene evaporated under nitrogen gas. Fetal calf serum was added to a final concentration of 250 μg/ml fatty acid in serum. Mixtures were subjected to identical conditions to those used to prepare postaggregation samples for mitogen release. These samples were then diluted to 10% fetal calf serum with culture medium for proliferation assay using human fibroblasts (see Mitogenesis Assay). In this assay, final concentration of fatty acid in the cell incubation medium was thus 25 μg/ml; this is equivalent to a mitogenesis assay, starting with 250 μg/ml of fatty acid in the platelet-rich plasma. After correction for seed number, results were expressed as percentage inhibition from the control (without fatty acid).

Release and estimation of macrophage-derived growth factors. Five microliters of a solution containing eicosapentaenoic acid in toluene vehicle, or toluene alone, were added to tubes and the toluene evaporated under nitrogen gas. Then 500 μl of human hyperlipemic serum were added to stimulate conversion of the macrophages into foam cells that released mitogens. The tubes were gently mixed for 10 min, followed by addition of 4.5 ml of DMEM. The medium of the macrophage-like cells (see Cell Cultures) was replaced with this new medium and the incubation continued for another three days. The medium was then decanted, sterile-filtered (0.2-μm pore size), and examined for ability to support growth of human fibroblasts in culture, as follows: Fibroblast cells were seeded at 8,000

cells/well into 16-mm-diameter culture wells of 24-well plates. After 24 hr, culture medium was replaced with 1 ml of the filtered macrophage-conditioned medium per well. Cells were then incubated at 37°C, 5% CO_2 for two days, the medium removed, and the cells released and counted as described earlier (Mitogenesis Assay). After correction for seed number, results were expressed as percentage inhibition from the control (without fatty acid).

Statistical analysis. Values in the text and figures are expressed as the mean ± SEM. Because of variability in platelet sensitivity of different donors, comparisons among platelet-inhibitory polyunsaturated fatty acids were made within the same group of donors. Overall comparisons of data among inhibitory fatty acids (Fig. 1 and text) were determined by one-way analysis of variance, and comparisons with control group were determined using the Duncan multiple range test. For arachidonic acid (Fig. 2 and text), significance was determined by t-test for a single mean or between groups by two-tailed unpaired Student's t-test. For other studies (Figs. 3 and 4), comparisons with control were determined using two-tailed unpaired Student's t-test. In all cases, statistical significance was assumed at $p < 0.05$.

RESULTS

Eicosapentaenoic acid (20:5ω3), docosahexaenoic acid (22:6ω3), docosahexaenoic acid (22:6ω3) and EPA precursor α-linolenic acid (18:3ω3) inhibited both platelet aggregation and mitogen release at 250 μg/ml (Fig. 1). By contrast, arachidonic acid at 250 μg/ml induced platelet aggregation (percentage transmission = 42.8 ± 12.2) to ca. 79% of that induced by ADP at 1.6 μg/ml examined in direct comparison (Fig. 2a), with a corresponding stimulation of platelet mitogen release (Fig. 2b). Part of the inhibitory effects on mitogen release seen with docosahexaenoic and α-linolenic acids may have resulted from direct antiproliferative effects (hatched bars, Fig. 1b). Significant direct antiproliferative effects were not seen with eicosapentaenoic acid (Fig. 1b) or arachidonic acid (19.0 ± 8.4%, p vs control >0.1, n = 8) under these conditions.

All of the polyunsaturated fatty acids tested at 50 μg/ml were either less active than at 250 μg/ml or inactive in both assays (Fig. 1). Although not shown in Fig. 1, adrenic acid (22:4ω6), examined only at 250 μg/ml (5 donors), inhibited aggregation 30.8 ± 2.6% ($p < 0.05$): Platelet mitogen release was also lower by 28.6 ± 24.0%, but this change was not statistically significant.

Mead acid (5,8,11-eicosatrienoic acid; 20:3ω9) is elevated in many atherosclerotic individuals and in many who are at risk for atherosclerosis (13,20); and low concentrations of Mead acid potentiate the in vitro platelet aggregatory effects of other agents (21). Accumulation of Mead acid is thought to be a hallmark of essential fatty acid deficiency resulting from insufficient dietary intake of ω6 and ω3 fatty acids, as would occur with a high saturated-fat diet (22). By contrast with the strong inhibitory effects of the trienoic ω6 fatty acid dihomo-γ-linolenic acid (8,11,14-eicosatrienoic acid; 20:3ω6) (Fig. 1), Mead acid (not shown in Fig. 1) had little effect on platelet aggregation or mitogen release. In addition to inhibiting the maximum amplitude of ADP-induced platelet aggregation, dihomo-γ-linolenic acid also significantly ($p < 0.05$)

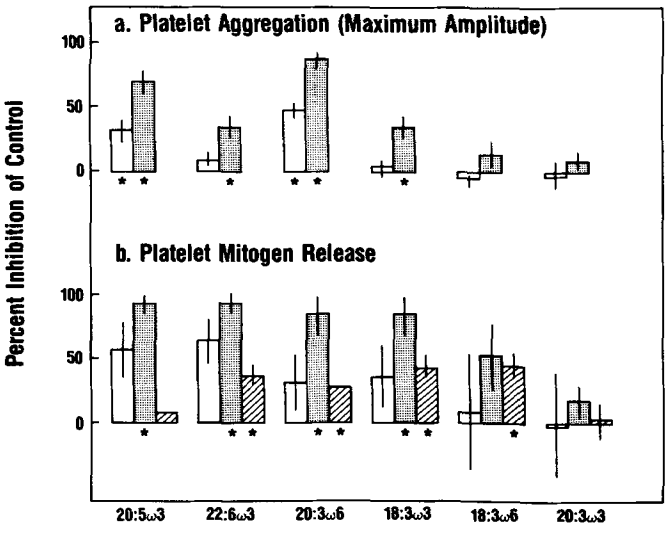

FIG. 1. Effects of several polyunsaturated fatty acids on (a) maximum amplitude of ADP-induced platelet aggregation and (b) mitogen release, at 50 μg/ml (plain bars) and 250 μg/ml (stippled bars). 20:5ω3, eicosapentaenoic acid; 22:6ω3, docosahexaenoic acid; 20:3ω6, dihomo-γ-linolenic acid; 18:3ω3, α-linolenic acid; 18:3ω6, γ-linolenic acid; 20:3ω3, 11,14,17-eicosatrienoic acid. Results are expressed as percentage inhibition of fatty acid-free control adenosine diphosphate-induced aggregation or mitogen release. Cell proliferation results (hatched bars) at 250 μg/ml in serum are expressed as percentage inhibition of fatty acid-free control proliferation. Aggregation and mitogen release values are reported as means ± standard error of 4–6 donors. Proliferation values are reported as means ± standard error of 4–8 incubation wells. *p vs control <0.05.

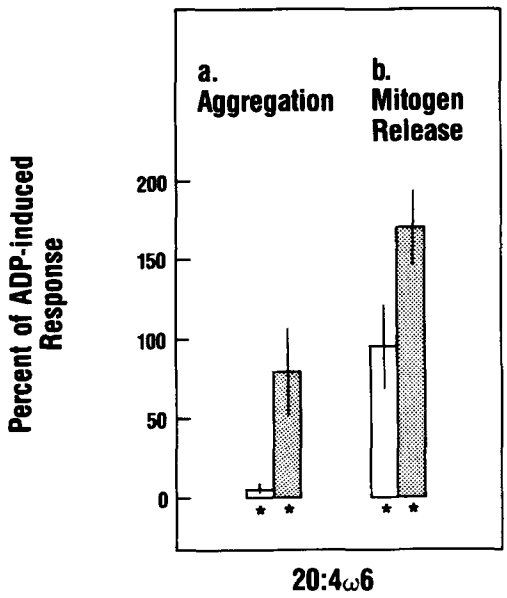

FIG. 2. Effect of arachidonic acid (20:4ω6) on (a) maximum amplitude of platelet aggregation and (b) mitogen release at 50 μg/ml (plain bars) and 250 μg/ml (stippled bars). Results are expressed as percentage of adenosine diphosphate-induced aggregation or mitogen release response. These effects were opposite in direction to those in Fig. 1. Aggregation and mitogen release values are reported as means ± standard error of 6 donors. *p vs control <0.05.

inhibited the initial rate of ADP-induced platelet aggregation at 250 μg/ml (87.0 ± 4.8% inhibition) and at 50 μg/ml (32.1 ± 3.3% inhibition). Significant inhibition of initial rate was not seen with the other fatty acids examined. Values of percentage inhibition of initial rate at 250 μg/ml and 50 μg/ml, respectively, were: 35.0 ± 16.5 and −9.3 ± 5.2 for eicosapentaenoic acid; 9.1 ± 4.3 and −2.3 ± 5.7 for docosahexaenoic acid; 4.9 ± 5.6 and −0.3 ± 4.5 for α-linolenic acid; 2.0 ± 5.3 and −18.1 ± 6.0 (significant stimulation, $p < 0.05$) for γ-linolenic acid; and 7.3 ± 9.9 and −6.1 ± 4.6 for 11,14,17-eicosatrienoic acid.

The dose-response curve for eicosapentaenoic acid on platelet aggregation and mitogen release showed inhibitory ED_{50} values of 45 μg/ml and 30 μg/ml, respectively (Fig. 3). Corresponding ED_{50} values for docosahexaenoic acid were 480 μg/ml and 160 μg/ml, respectively (not shown in Fig. 3).

Eicosapentaenoic acid also inhibited release of mitogens from human monocyte-derived macrophages: ED_{50} = 60 μg/ml (Fig. 4).

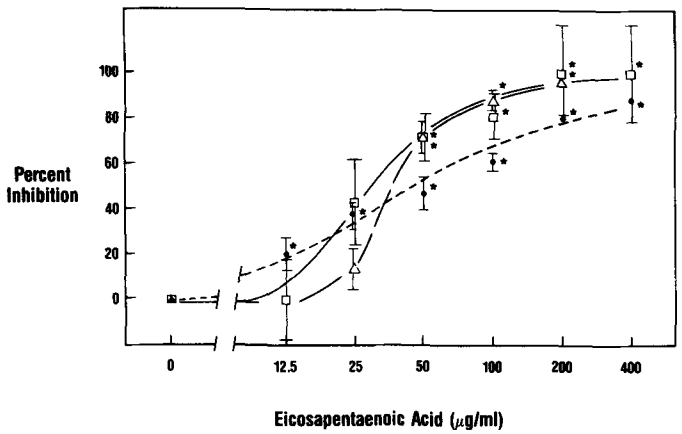

FIG. 3. Platelet effects of eicosapentaenoic acid. (●), Aggregation; (□), Mitogen release; (△), Platelet-factor-4 release. Means ± standard error; n = 6–12 adenosine diphosphate incubations (from 2 donors). *p vs control <0.05. Mean ED_{50} values between donors were within 20% of each other.

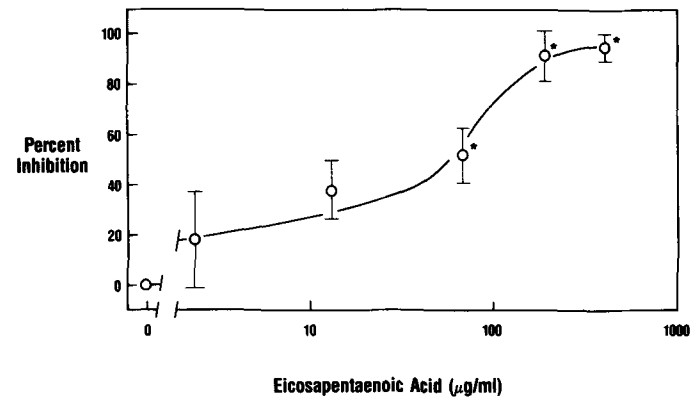

FIG. 4. Effect of eicosapentaenoic acid on release of macrophage-derived growth factors (MDGF). Means ± standard error of ≥5 macrophage incubation wells (from 2 donors). *p vs control <0.05. Mean ED_{50} values between donors were within 20% of each other.

DISCUSSION

Our findings provide the first report on the effects of purified fatty acid eicosanoid precursors on release of platelet or macrophage mitogens.

Unsaturated fatty acids are present in the diet as components of phospholipids, cholesteryl esters and (mainly, in the case of fish oils) as triglycerides. The fatty acid is digestively cleaved from these more complex lipids and then absorbed via the gastrointestinal tract by an apparent active transport mechanism (13,23,24). It is reesterified into triglycerides that are acted upon by lipoprotein lipase to release fatty acids that are then reesterified into adipose tissue or into membrane phospholipids (see ref. 13).

From these sources, the free fatty acid forms of eicosanoid precursors (e.g., arachidonic acid, dihomo-γ-linolenic acid, and eicosapentaenoic acid) are released. This is accomplished by the actions of hydrolytic enzymes including phospholipases, and the free acids are rapidly converted to eicosanoids.

During in vitro cell biology studies, one attempts to mimic the latter part of the above sequence of events by addition of the unsaturated fatty acid in free acid form (The esterified form in foods probably cannot be digested, as in the gastrointestinal tract.). However, sufficiently large concentrations have to be added in order to surmount binding to plasma albumin and allow penetration into appropriate cellular compartments. The relevance of this approach is clearly documented in the case of arachidonic acid. Although, as seen here, high concentrations (ca. 250 μg/ml) of arachidonic acid have to be used to surmount binding to plasma albumin and produce aggregation of the platelets (25) (Fig. 2), concentrations about tenfold lower produce similar effects in the absence of plasma (26). Furthermore, there is little doubt as to the in vivo relevance of in vitro platelet studies with exogenous arachidonic acid. Thus, oral administration of purified arachidonate to human volunteers can markedly increase ex vivo aggregation of platelets (27). Also, the mode of action of aspirin-like drugs via cyclooxygenase (28,29) is attributable to inhibition of conversion of intracellularly released arachidonate to endoperoxides and thromboxanes (13).

In the present studies, addition of exogenous arachidonic acid to PRP induced release of atherogenic platelet mitogens at a concentration (50 μg/ml) that did not induce significant aggregation. This result further underscores our suggestion that dietary arachidonate from meat and dairy constituents of the diet are proatherosclerotic (30).

By contrast to findings with arachidonic acid, we have shown here that eicosapentaenoic acid (≥50 μg/ml), docosahexaenoic acid (250 μg/ml) and dihomo-γ-linolenic acid (250 μg/ml) inhibited both platelet aggregation and mitogen release.The inhibition of platelet mitogen release by eicosapentaenoic acid and docosahexaenoic acid clearly has direct relevance to the antiatherosclerotic potential of marine lipid (Eskimo) diets that are rich in these fatty acids. The similar activity of dihomo-γ-linolenic acid is of considerable interest. Although it is an ω6 fatty acid and is not present in marine lipids to any extent, it is reportedly elevated in Eskimos subsisting on such diets (31) and is the precursor for prostaglandin E_1 that inhibits

platelet function (32). This elevation may be a consequence of impaired Δ^5 desaturation of dihomo-γ-linolenic acid (itself derived from linoleic acid, 18:2ω6) to arachidonic acid. This may be of genetic origin (33) or due to interference by fatty acids of the ω3 series (34).

In people consuming large amounts of fish oil, epidemiological (1,2,35,36) and most (37-45), but not all (46,47), clinical evidence points to reduced platelet aggregation to ADP and collagen, prolonged bleeding times and reduced platelet retention to glass beads (5,48). However, there have been few studies showing direct effects of purified ω3 fatty acids on platelet function either in vivo or in vitro. Early work showed that eicosapentaenoic acid slightly inhibited the second wave of aggregation produced by ADP (2). Oral administration, by humans, of highly purified eicosapentaenoic acid inhibits platelet aggregation induced by epinephrine, collagen or ADP and inhibits ex vivo platelet retention to glass beads (49). Docosahexaenoic acid, an inhibitor of cyclooxygenase (50), has been shown to reduce in vitro aggregation induced by thrombin, epinephrine and collagen (51,52). Also, dihomo-γ-linolenic acid (an ω6 fatty acid) markedly inhibits platelet aggregation when administered in vivo or in vitro (53-57). By contrast, as described earlier, arachidonic acid has the potential prothrombotic effect of inducing platelet aggregation even upon repeated oral administration of arachidonate in human volunteers (27).

Plasma and platelet levels of these complex ω3 fatty acids are barely detectable in people consuming little or no fish oil (38). However, they become markedly elevated (largely at the expense of arachidonic acid) in people consuming marine diets rich in ω3 fatty acids (35,37,58).

In our in vitro studies, analogy with arachidonic acid indicates that the amount of the fatty acids we used was of physiological significance. In confirmation of this assumption, fish oil administration in dogs reduces clotting-induced release of mitogenic activity from platelets (59), and there is a corresponding reduction in experimental atherosclerosis induced by arteriovenous grafting and cholesterol feeding. These recent findings extend earlier in vivo evidence for antiatherosclerotic effects of diets rich in marine oils—i.e., epidemiological evidence showing lower rates of cardiovascular disease in man (1-5,9,35, 60,61) apparently confirmed in prospective studies (62). Antiatherosclerotic effects of fish oils have also been shown in animal models (7,8,10,11).

Macrophage-derived foam cells may be an early source of mitogenic material inducing proliferative lesions (63,64). The ability of eicosapentaenoic acid to suppress mitogen release from both platelets and macrophages, thus, may be important in potential antiatherosclerotic properties of marine lipids.

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METHODS

Simple High Performance Liquid Chromatography Methods for Monitoring Lipase Reactions

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This paper describes three simple high performance liquid chromatography methods to separate mixtures of free fatty acids, mixtures of different triglycerides and mixtures of all fat classes (monoglycerides, diglycerides, triglycerides and free fatty acids). It is possible with our methods to identify and quantify each peak of the chromatogram. These methods have been designed to monitor lipase reactions. Using a first set of conditions, we have been able to separate five fatty acids: linolenic, linoleic, palmitic, oleic and stearic, without any specific preparation of the samples. With a second set of conditions, we showed that the same mobile phase and the same column could separate both triglyceride species and fat classes. However, in the latter case, a flow gradient was used. *Lipids 24,* 76–78 (1989).

Modification of fats and oils is a new area for enzyme technology. Not only are triglycerides being hydrolyzed or broken down, they are also rearranged and synthesized. These reactions are carried out with lipases. There are several compounds that have to be measured as a result of these reactions. Very often it is sufficient to follow the appearance or disappearance of free fatty acids (FFA). However, sometimes it is necessary to monitor the levels of monoglycerides (MG), diglycerides (DG) and triglycerides (TG). In the case of flavor development, it may be necessary to profile all of these. Sometimes after a lipolysis treatment, the amounts of all lipid species or classes in the reaction mix are requested, i.e., in addition to the total FFA, the amounts of MG, DG and TG have to be determined. Measuring the content of glyceride compounds such as MG and TG is of interest in reactions other than lipolysis. Examples of these reactions are the synthesis of MG via glycerolysis or the synthesis of cocoa butter substitute from palm oil via transesterification. Sometimes it is desirable to know how much of each FFA or other lipid is in the sample. The many individual components can be resolved using the methods described in this paper to give what is known as a "profile" of a modified fat or oil.

Methods for monitoring lipid enzyme reactions are still being developed. Many of the present methods are time-consuming and are based on procedures used by chemists to characterize fats and oils, e.g., titration or gas chromatography (GC). They involve solvent extractions, sample transfers and, sometimes, chemical modifications. FFA can be monitored using the pH-stat methods or by direct titration. GC can be used provided that FFA are first converted to fatty acid methyl esters (FAME) (1) or acidified with sulfuric mixed with silicic acid and then eluted with 1% butanol in petroleum ether (2). Lipid class profiles can be found by thin layer chromatography (TLC) (3) or high performance liquid chromatography (HPLC), provided that the sample is modified in order to convert the FFA into their respective methyl esters (4) or that a transport-flame ionization detector (T-FID) is used (5). Triglyceride profiles are easily obtainable through HPLC (6,7) and high temperature GC (5).

In this paper, we describe simple HPLC methods in order to easily obtain FFA profiles, TG profiles and lipid class profiles without any previous modification or preparation of the sample. Those methods differ from each other as little as possible, so that it is relatively easy to switch from one type of analysis to another on the same sample.

EXPERIMENTAL PROCEDURES

Reagents. TG standards (POP, POS, SOS) of the highest purity grade were purchased from Supelco Canada (Oakville, Ont.). Free fatty acids, esters of fatty acids, triolein, diolein, monoolein, lipase L-0382 from porcine pancreas, and lipase L-4384 from *Rhizopus arrhizus* were purchased from Sigma Chemical Co. (St. Louis, MO). HPLC-grade acetonitrile, acetone and tetrahydrofuran (THF) were obtained from Fisher Scientific (Pittsburgh, PA). Cocoa butter was donated by General Foods (Montreal, Canada). Lipozyme was a gift from Novo (Lachine, Québec, Canada).

Equipment. HPLC was carried out using the following equipment: Waters M590 solvent delivery systems, WISP 710B sample processor, Waters M481 LC spectrophotometer (Waters Assoc., Milford, MA); refractive index detector 1037A (Hewlett-Packard, Palo Alto, CA) and Professional 350 computer (Digital Equipment Corporation, Concord, MA).

Columns. TG profiles and lipid class profiles. TG, as well as lipid classes, are resolved by a HPLC system equipped with a reverse phase column: CSC-S, Spherisorb ODS-2, 5 μm (25 cm × 4.6 mm).

FFA profiles. The column used for FFA separation was a Supelcosil (octyl bonded spherical silica) LC-8, 3 μm (15 cm × 4.6 mm) to which a guard column Supelcosil LC-8, 5 μm (2 cm × 4.6 mm) was connected.

Standards preparation. FFA. Twenty-five mg of each FFA (linolenic, linoleic, palmitic, oleic and stearic acids) were dissolved in 1 ml of acetonitrile. Three to four drops of THF were added when solubilization was incomplete. More acetonitrile was then added after solubilization, up to a final volume of 5 ml.

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Abbreviations: DG, diglycerides; FAME, fatty acid methyl esters; FFA, free fatty acids; MG, monoglycerides; OOO, triolein; POP, *rac*-glyceryl-1,3-palmitate-2-oleate; POS, *rac*-glyceryl-1-palmitate-2-oleate-3-stearate; RI, refractive index; SOS, *rac*-glyceryl-1,3-stearate-2-oleate; TG, triglyceride; THF, tetrahydrofuran; GC, gas chromatography; HPLC, high performance liquid chroamtography; T-FID, transport-flame ionization detector; TLC, thin layer chromatography; CSC, Chromatography Sciences Co., Canada.

TG. Ten mg of POP, POS and SOS were dissolved in 1 ml of acetone. Cocoa butter was prepared at a concentration of 10% in acetone.

Lipid classes. A mixture containing 10 mg of triolein, 10 mg of diolein, 10 mg of monoolein and 10 mg of oleic acid was dissolved in 1 ml of acetone. No pretreatment was required for any of these standards before injection in the column.

Lipase reaction and samples preparation. Two types of reaction were performed with lipase enzymes and monitored by HPLC methods.

Hydrolysis. Triolein (0.5 g) was mixed in an Eppendorf tube with 0.5 ml of 10 mM sodium phosphate buffer, pH 5.8, containing 0.2 mg of lipase from porcine pancreas (10,800 U). The mixture was shaken vigorously at 34°C on a vortex shaker.

Synthesis. Oleic acid (0.5 g) was mixed in an Eppendorf tube with 0.05 g of glycerol in the presence of 5 μl of Lipozyme (10,000 LU/g). The mixture was shaken vigorously at 34°C on a vortex shaker.

For both types of experiments, 5 μl aliquots were taken at preset intervals and diluted in 200 μl of acetone. No pretreatment was necessary before injecting those samples into the HPLC system. Oleic acid, monoolein, diolein and triolein amounts were calculated in each aliquot as percentages of the initial amount of oleic acid, in oleic acid equivalents, taking into account that diolein and triolein contain 2 and 3 mol of oleic acid, respectively.

RESULTS AND DISCUSSION

FFA. The mobile phase is acetonitrile/tetrahydrofuran/0.1% H_3PO_4 (50.4:21.6:28, v/v/v). The flow rate is 1 ml/min. Twenty-five μl are injected and the column is thermostated at 35°C. Figure 1 represents the FFA separation with a refractive index (RI) detector. The response factors are more similar with the RI detector than with the UV detector. A good separation is achieved with the LC-8 column and an isocratic run.

TG. The mobile phase used here is acetone/acetonitrile (50:50, v/v). The flow rate is 2.5 ml/min. Fifteen μl are injected, and the column is thermostated at 35°C. Figure 2 represents the chromatogram of cocoa butter obtained using a UV detector at 220 nm.

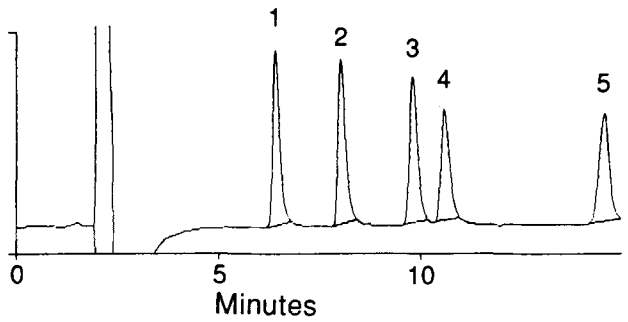

FIG. 1. Chromatogram of FFA (RI detector). 1, linolenic acid; 2, linoleic acid; 3, palmitic acid; 4, oleic acid; and 5, stearic acid. Column: Supelcosil (octyl bonded spherical silica) LC-8, 3 μm, (15 cm × 4.6 mm) + guard column Supelcosil LC-8, 5 μm, (2 cm × 4.6 mm). Mobile phase: acetonitrile/tetrahydrofuran/0.1% H_3PO_4 (50.4:21.6:28). Flow rate: 1 ml/min, T = 35°C, injected volume 25 μl, standard concentrations 5 mg/ml of acetonitrile.

Lipid classes. The mobile phase is the same one as previously described for TG analysis. However, a flow gradient is used: 0.8 ml/min for 6 min, increased in 4 min up to 4 ml/min, held at 4 ml/min for 11 min, decreased back to 0.8 ml/min in 4 min and held for another 5 min at 0.8 ml/min before the next injection. Fifteen μl are injected, and the column is thermostated at 35°C. Figure 3A is the UV chromatogram at 206 nm of a mixture containing 10 mg of monoolein, diolein, triolein and oleic acid/ml of acetone.

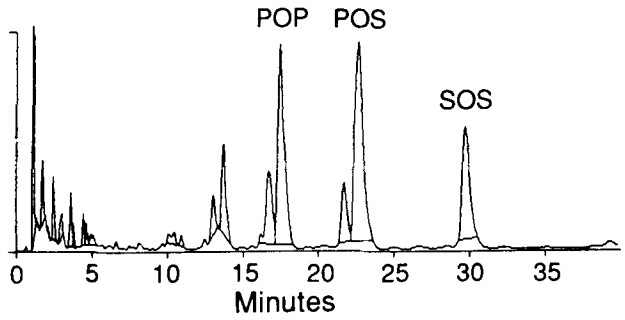

FIG. 2. Chromatogram of the TG of cocoa butter (UV detector 220 nm). P, palmitic acid; O, oleic acid; S, stearic acid. Column: CSC-S, Spherisorb ODS-2, 5 μm (25 cm × 4.6 mm). Mobile phase: acetone/acetonitrile (50:50). Flow rate: 2.5 ml/min, T = 35°C, injected volume 15 μl, sample concentration 10% in acetone.

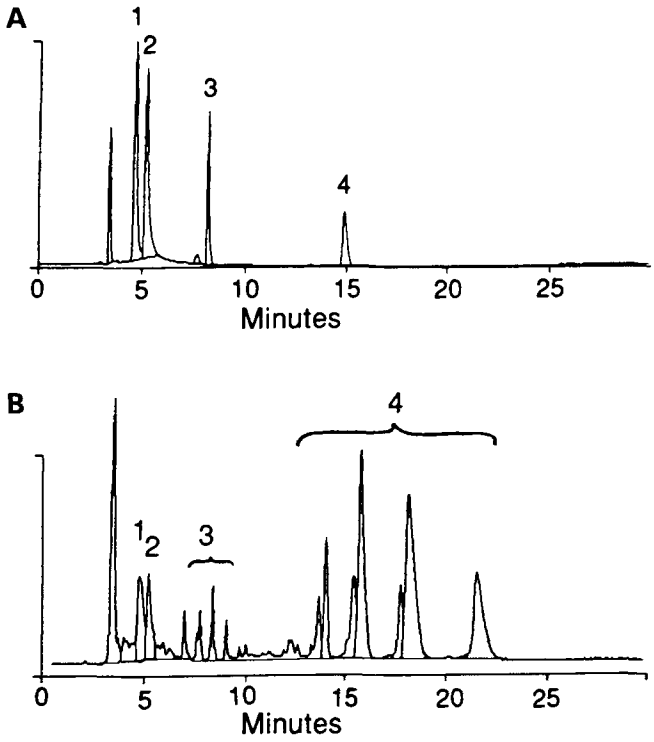

FIG. 3. (A) Chromatogram of lipid classes (UV detector at 206 nm). 1, monoolein; 2, oleic acid; 3, diolein; and 4, triolein. Column: CSC-S, Spherisorb ODS-2, 5 μm (25 cm × 4.6 mm). Mobile phase: acetone/acetonitrile (50:50). Flow rate: see text, T = 35°C, injected volume 15 μl, standard concentrations 10 mg/ml of acetone. (B) Chromatogram of lipid classes contained in cocoa butter (UV detector 206 nm). 1, MG; 2, FFA; 3, DG; 4, TG. Chromatographic conditions as in Figure 3A.

It is possible to separate 1,2-diolein from 1,3-diolein. Actually, the diolein standard used contained 85% of the 1,3-isomer and 15% of the 1,2-isomer. Our lipid class profile shows the separation between the two diolein isomers when using the UV detector.

If the methods have general utility, they must be applicable to real samples. The previous analyses deal only with lipid classes containing a single fatty acid (oleic acid). One problem in applying reversed phase chromatography to lipid class separations is that components containing different fatty acids are also resolved. We show here that the resolution is still adequate when applied to a cocoa butter sample (Fig. 3B). This chromatogram (UV, 206 nm) shows one single peak for MG, another peak for FFA, a series of peaks for DG and another distinct series for TG. DG and TG species containing different FA are resolved; however, there is no overlapping between the different classes.

Lipase reactions. Figures 4A and 4B show two examples of the use of previously described methods as process monitors. Hydrolysis (Fig. 4A) and synthesis (Fig. 4B) of triolein have been followed through HPLC. Both modes of detection have been compared for each type of analysis, and the best one is shown in the figures. In the case of FFA, the UV detector gives very different response factors with the different fatty acids. For this reason, the RI detector is preferred for FFA analysis. For all the other compounds and types of analysis, the sensitivity of the UV detector (at 206 nm) is better than that of the RI detector. Therefore, UV detection is preferred for TG analyses and fat classes analyses.

We have shown in this paper that HPLC techniques are suitable for many analyses of fats without previous modification and treatment of the sample. With only two different columns and two different mobile phases, it has been possible to monitor lipase reactions, not only for substrates and products of the reaction, but also for intermediate products of the reaction. This paper supports the view that HPLC can provide a relatively rapid and reproducible method for monitoring bench scale and pilot-plant scale reactions involving hydrolysis and synthesis of fats using lipases.

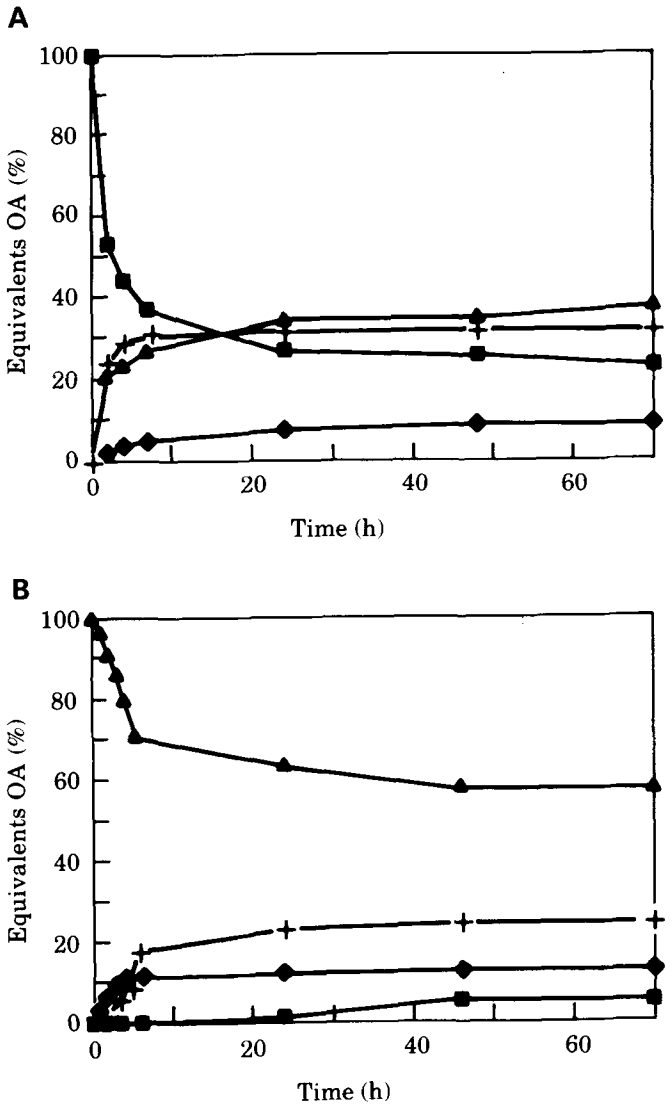

FIG. 4. (A) Hydrolysis of triolein with a lipase from porcine pancreas. The HPLC method described in Fig. 3A was used to monitor the reaction. ■, Triolein, + diolein, ◆ monoolein and ▲oleic acid. (B) Synthesis of glycerides (mono-, di- and tri-) from oleic acid and glycerol using a lipase from *Mucor miehei* (Lipozyme). The HPLC method described in Fig. 3A was used to monitor the reaction. Symbols are similar to those used in Fig. 4A.

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Location of Double Bonds in Fatty Acids of Fish Oil and Rat Testis Lipids. Gas Chromatography-Mass Spectrometry of the Oxazoline Derivatives[1]

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A new approach to the derivatization and analysis of long chain polyunsaturated fatty acids is described. The method is based on the formation of 2-alkenyl-4,4-dimethyloxazolines by condensation of the starting material with 2-amino-2-methylpropanol. The derivatization method is rapid, efficient and specific with respect to the chain feature of the parent acids. Volatility, comparable with that of the corresponding simple esters, and improved gas chromatographic separation are achieved without difficulty. The derivatives exhibit clear and regular fragmentation patterns that allow easy discrimination of positional isomers and assignment of double bond location in the chain.
***Lipids* 24, 79-83 (1989).**

Determining the location of double bonds in fatty acids (FA) has been a topic of continuing interest in analytical biochemistry. Gas chromatography-mass spectrometry (GC-MS) techniques approach this problem in two different ways, each consisting of a key step involving chemical modification prior to analysis. Derivatization at the double bond ("on-site" modification [2-4]) to oxygenated compounds (e.g., mixtures of carbonyl compounds and vicinal diols) leads to more distinctive fragmentation patterns that allow final determination of the unsaturated points. This approach is often associated with the formation of more polar derivatives and an increase in molecular weights, and is therefore unsuited for the analysis of polyunsaturated fatty acids (PUFA). Another technique, first proposed by Vetter et al. in 1971 (5), is based on the derivatization of the terminal carboxylic acid group ("remote site" modification [2-4]), whereby fatty acids are converted to amides or esters such as N-acylpyrrolidides (6) and picolinyl esters (7) that easily stabilize the ions containing the double bonds. In consequence, spectra are recorded that are frequently characteristic of particular unsaturated fatty acids (UFA).

Pyrrolidides have been studied most often for the analysis of natural samples (8-14). Although distinctive modes of fragmentation can be obtained for UFA, the interpretation of spectra becomes substantially more difficult if the number of double bonds is greater than four (6). A superficially attractive method has been suggested that involves specific deuteration of the double bonds in PUFA using nascent deuteriodiimide (N_2D_4), followed by conversion to pyrrolidides and mass spectrometric analysis of the resulting derivatives (15,16). Although correct assignment was obtained for up to six double bonds under certain circumstances, the results were found variable with different types of instruments (16).

Detailed study of the fragmentation of isomeric unsaturated FA has been accomplished by Christie et al. (17-19) via derivatization to picolinyl esters. It has been demonstrated, in a comparative study using PUFA-rich natural samples, that picolinyl esters are preferred over pyrrolidides because they give more abundant diagnostic ions (20). Furthermore, the picolinyl ester derivatives can be separated by high performance liquid chromatography (HPLC) in the reversed-phase mode before GC-MS analysis. Such a procedure is of value for the detection of minor components in natural samples (21-22).

A somewhat different strategy to increase the power and versatility, so that unusual fatty acids can be studied, would be to develop a method of direct analysis of FA mixtures without prior chromatographic separation. Such analysis is indeed possible by fast atom bombardment (FAB) in combination with tandem mass spectrometry (MS-MS). Gross and coworkers (23) have reported that free FA can be readily desorbed either as anions $(M-H)^-$ or as alkali metal-cationized species $(M + Li)^+$ or $(M - H + 2Li)^+$ (24) and collisionally activated (CA). This technique has been used for the analysis of picolinyl esters of UFA samples (25). Usually, the determination of chain structure based on FAB-CA-MS-MS of these derivatives is possible for PUFA with up to three ethylenic linkages, but becomes more difficult for molecules with higher degrees of unsaturation.

The preparation of 2-substituted 4,4-dimethyloxazolines (DMOX) by condensation of the FA with 2-amino-2-methylpropanol (AMP) and subsequent measurement of their electron impact (EI) mass spectra represents a useful alternative technique for the structure determination of UFA (26). Being different from the commonly used simple esters and amides, such derivatives with their terminal carboxylic acid group incorporated into a heterocycle show improved MS and GC properties due to the enhanced charge stabilization and the reduced tendency of hydrogen bonding in the resulting molecules. DMOX has proved to exhibit clear-cut and regular fragmentation patterns that precisely indicate the double bond position in the chain. To establish the usefulness of this method, the GC-MS of PUFA mixtures from fish oil and rat testis lipids have been studied, and the results are reported in this paper.

MATERIALS AND METHODS

Materials. 2-Amino-2-methylpropanol (AMP) and dicyclohexylcarbodiimide (DCC), with purity over 99% were purchased from Aldrich Chemical Co. (Milwaukee, WI). Fish oil (from *Navodon septentrionalis*) was obtained

[1]Chemical Modification in Mass Spectrometry, Part 8. For preceding paper, see reference 1.

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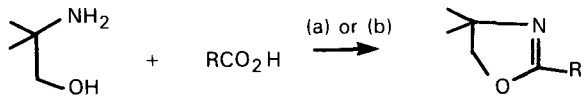
Abbreviations: AMP, 2-amino-2-methylpropanol; DCC, dicyclohexylcarbodiimide; DMOX, 2-alkenyl-4,4-dimethyloxazoline; FA, fatty acid; PUFA, polyunsaturated fatty acid; UFA, unsaturated fatty acid; CA, collisionally activated; EI, electron impact; FAB, fast atom bombardment; GC-MS, gas chromatography-mass spectrometry; HPLC, high performance liquid chromatography; MS-MS, tandem mass spectrometry; TIC, total ion current.

from a local fish-products factory. Rat testes were collected from freshly killed laboratory animals and the lipids were extracted with chloroform/methanol (2:1, v/v). FA were prepared by saponification with aqueous methanolic potassium hydroxide solution.

Derivatization. DMOX were prepared (in μg to mg scale) according to the procedures described earlier (26,27):



(a) The FA fraction was heated with a 2-fold excess of AMP at 170°C for 1–2 hr, or (b) the FA fraction was first allowed to react with equal amount of DCC (in CH_2Cl_2, room temperature, 2 hr) followed by treating with $SOCl_2$ (0°C, 0.5 hr). The conversion (estimated by GC) was better than 90% by either method. To obtain high quality spectra, a critical step is to allow the reaction product to pass through a small column of silica gel, followed by eluting with minimum amount of ethyl acetate before analysis.

GC-MS. All GC-MS analyses were carried out on a Finnigan-MAT 44S gas chromatographer-mass spectrometer equipped with a 18 m × 0.28 mm glass capillary column coated with SE-54 (crosslinked and bonded). Helium was the carrier gas at a flow rate of about 1 ml/min, split ratio 1:10. The samples were injected onto the column at 250°C; oven temperature was programmed at 4°C/min from 150 to 240°C. Spectra were recorded at an ionization energy of 70 eV. Ion source was maintained at 250°C during the analysis. Quantities of individual compounds were determined by percentages of total integrated areas in total ion current (TIC) tracing.

RESULTS AND DISCUSSION

Fish oil and rat testis lipids were chosen as model samples in this study because both were known to contain a number of mono- and polyunsaturated fatty acids. Good separation was developed with the DMOX of each sample. The DMOX were eluted at column temperatures below 240°C with negligible column bleedings from a cross-linked SE-54 glass capillary column. The TIC tracings of FA of fish oil derivatized as methyl esters and DMOX are presented in Figure 1.

Both derivatives provide similar chromatographic profiles, although slightly broader peaks are observed in the latter. With a few exceptions, most of the components derivatized as DMOX were separated with sufficient resolution. Well-interpretable mass spectra were recorded for each peak. Peaks which were not fully resolved such as peaks #9, #10 and #11 in Figure 1b yielded respective mass spectra (Fig. 2) that were still clear enough to permit correct identification of the "unknowns." Under the conditions described above, UFA derivatives are eluted before the saturated ones, whereas monoenoic acid derivatives with double bonds near the carboxylic group emerge before those having a double bond remote from the polar end. This is also the case of UFA derivatives from rat testis lipids, and the chromatogram of the latter is presented in Figure 3.

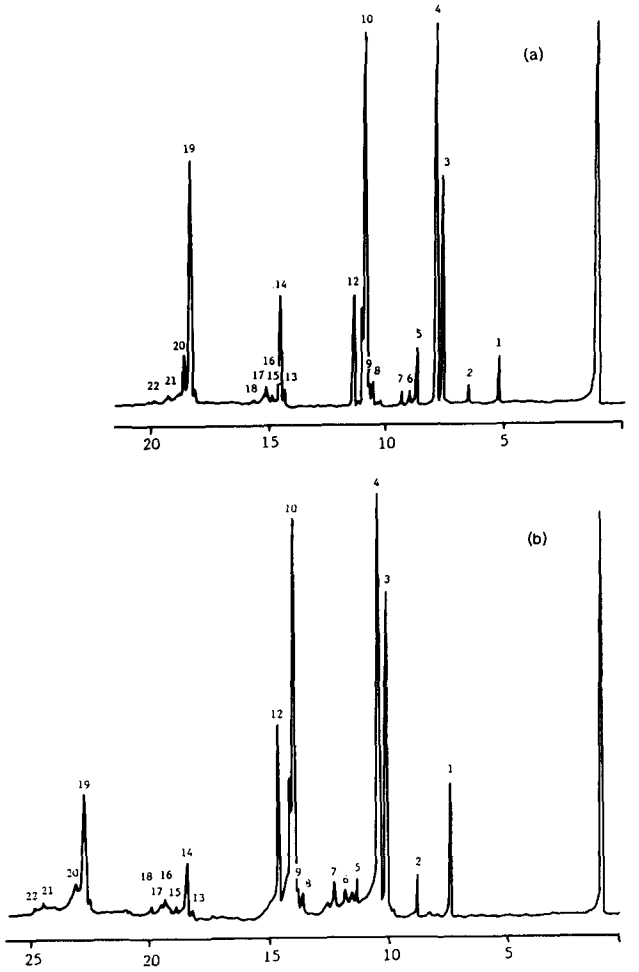
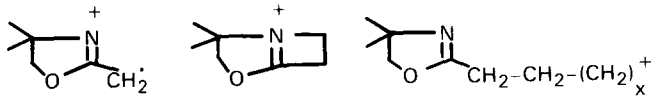


FIG. 1. Gas chromatograms of fatty acid derivatives from fish oil: (a) methyl esters and (b) DMOX. For GC conditions, see Experimental.

Component fatty acids and characteristic ions of DMOX obtained from the two natural samples are listed in Table 1. The assignment is consistent with the results obtained by other methods (28,29), with small differences in certain minor constituents for species-dependent reasons.

Close resemblance is observed between the mass spectra of DMOX and those of the corresponding N-acyl pyrrolidides in the region above *m/z* 100. One important advantage of the present method is that DMOX furnish much more prominent fragment ions (especially in the high mass end), whereas the interference arising from peaks composed of pure "hydrocarbons" is minimum. The fragmentation is simple. Only three types of ions are present in the spectra—the McLafferty rearrangement product (*m/z* 113, usually as the base peak for 2-unsubstituted FA series), the ion due to cyclization-displacement (*m/z* 126 usually as the second large peak in the spectrum) and a series of homologous ions containing the heterocyclic ring (*m/z* 126 + 14x, x = 1,2,3, ...):



m/z 113 *m/z* 126 *m/z* 126 + 14x

The double bond position can be deduced easily by using the empirical rule formulated in our previous work (26). Considering only the most pronounced ion in each cluster of FA derivatives, an even-mass ion series starting from *m/z* 126 is distributed regularly at intervals of 14 mass units. It turns out that the presence of a double bond at carbon n of the fatty acid is indicated by a gap of 12 mass units in the homologous series instead of the normal 14 units between ions containing n-1 and n carbon atoms of the original acid moiety. For monoenoic acids, additional information is provided by two more intense peaks corresponding to fragments containing n − 2 and n + 2 carbon atoms due to allylic cleavage on both sides of the double bond, hence greatly facilitating the correct determination of this particular site of unsaturation (Fig. 4).

TABLE 1

DMOX of Fatty Acids of Rat Testis Lipids and Fish Oil

Peak no.	Fatty acid component	Retention time (min)	Wt (%)	Mol. ion *m/z* (rel. abund.)	Diagnostic ions *m/z* (rel. abund.)
Rat testis lipids					
1	12:0	3.8	0.3	253 (2.9)	
2	14:0	7.1	2.0	281 (0.9)	
3	15:0	8.8	0.6	295 (1.3)	
4	16:1 (9)	10.3	8.8	307 (5.6)	196 (3.2), 208 (3.1)
5	16:0	10.9	39.7	309 (0.8)	
6	17:1 (9)	12.0	0.6	321 (2.8)	196 (2.8), 208 (2.2)
7	17:0	12.6	0.4	323 (1.0)	
8	18:1 (9)	14.0	36.9	335 (4.8)	196 (4.2), 208 (3.2)
9	18:0	14.6	6.9	337 (0.8)	
10	20:4 (5,8,11, 14)	16.7	1.0	357 (1.6)	153 (19.1), 180 (3.5), 192 (3.2), 220 (2.7), 232 (1.6), 260 (1.3), 272 (0.8)
11	20:3 (5,11,14)	17.6	0.3	359 (1.3)	153 (2.0), 222 (2.6), 234 (0.6), 262 (0.6), 274 (1.3)
12	20:2 (5,14)	17.9	0.3	361 (1.1)	153 (3.4), 264 (4.5), 276 (3.4)
13	22:5 (4,7,10, 13,16)	20.2	0.6	383 (1.9)	139 (9.1), 166 (4.3), 178 (4.8), 206 (4.3), 218 (4.3), 246 (3.8), 258 (2.4), 286 (1.0), 298 (1.0)
14	22:4 (4,10,13, 16)	20.3	0.1	385 (2.3)	139 (8.7), 208 (5.5), 220 (3.3), 248 (4.4), 260 (2.1), 288 (1.0), 300 (0.4)
Fish oil					
1	14:0	7.4	5.5	281 (3.5)	
2	15:0	8.8	1.7	295 (1.9)	
3	16:1 (9)	10.1	16.0	307 (5.2)	196 (3.8), 208 (4.2)
4	16:0	10.3	21.0	309 (2.2)	
5	17:1 (5)	11.4	1.0	321 (4.1)	
6	17:1 (9)	12.0	0.5	321 (2.1)	196 (2.3), 208 (4.0)
7	17:0	12.4	1.0	323 (1.2)	
8	18:4 (6,9,12, 15)	13.0	0.9	329 (1.9)	167 (14.7), 194 (5.4), 206 (4.3), 234 (3.5), 246 (1.9), 274 (0.9), 286 (0.7)
9	18:2 (9,12)	14.0	1.0	333 (2.8)	196 (2.5), 208 (2.5), 236 (3.7), 248 (2.0),
10	18:1 (9)	14.1	19.5	335 (0.9)	
11	18:1 (11)	14.3	5.5	335 (3.4)	224 (1.8), 236 (2.3)
12	18:0	14.7	7.9	337 (3.6)	
13	20:4 (5,8,11, 14)	18.4	0.4	357 (1.6)	153 (29.6), 180 (4.9), 192 (3.7), 220 (4.1), 232 (2.0), 260 (0.8), 272 (0.4)
14	20:5 (5,8,11, 14,17)	18.6	3.0	355 (1.9)	153 (18.5), 180 (3.8), 192 (2.0), 220 (4.0), 232 (1.9), 260 (1.2), 272 (0.7), 300 (0.5), 312 (0.5)
15	20:4 (8,11,14, 17)	19.1	0.2	357 (3.3)	182 (6.3), 194 (2.9), 222 (4.8), 234 (2.4), 262 (4.8), 274 (1.8), 302 (1.1), 314 (0.9),
16	20:1 (11)	19.5	0.9	363 (3.6)	214 (2.2), 226 (1.8)
17	20:1 (13)	19.7	0.6	363 (5.5)	252 (2.0), 264 (3.7)
18	20:0	20.0	0.4	365 (2.1)	
19	22:6 (4,7,10, 13,16,19)	22.9	7.0	381 (1.6)	139 (5.1), 166 (8.9), 178 (4.1), 206 (2.0), 218 (1.2), 246 (1.6), 258 (1.4), 286 (0.9), 298 (1.2), 326 (1.0), 338 (0.8)
20	22:5 (7,10,13, 16,19)	23.3	1.7	383 (3.7)	140 (7.6), 168 (6.2), 180 (10.9), 208 (5.6), 220 (4.6), 248 (7.7), 260 (3.5), 288 (3.7), 300 (3.1), 328 (2.6), 340 (2.8)
21	22:1 (11)	24.7	0.5	391 (3.8)	224 (1.5), 236 (2.3)
22	22:1 (13)	24.9	0.2	391 (3.8)	252 (1.6), 264 (1.8)

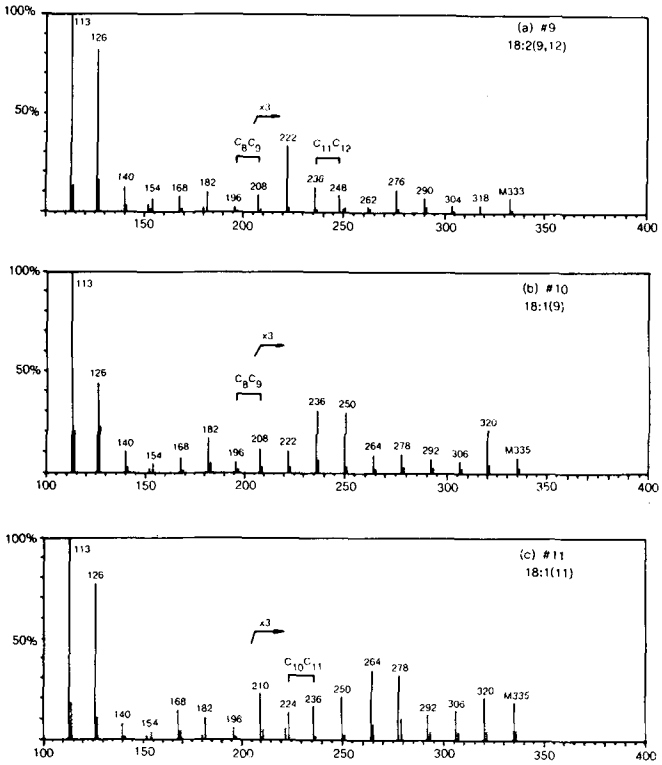


FIG. 2. Mass spectra recorded during GC-MS of DMOX of fish oil products: (a) #9, (b) #10 and (c) #11.

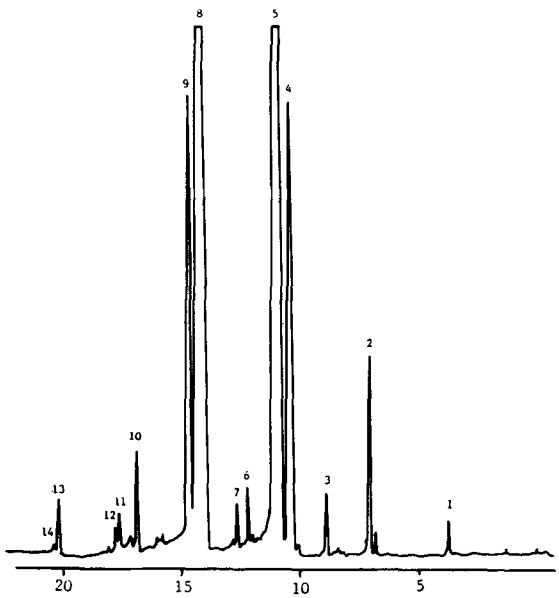


FIG. 3. Gas chromatogram of DMOX derived from fatty acids of rat testis lipids. For GC conditions, see Experimental.

Spectra of PUFA derivatives are depicted in Figure 5, which reveals typical fragmentation for methylene-interrupted acids: the "abnormal" interval of 12 amu (Δ^n) is flanked by two higher homologous peaks (fragments with C_{n-2} and C_{n+1}), thus forming a number of easily

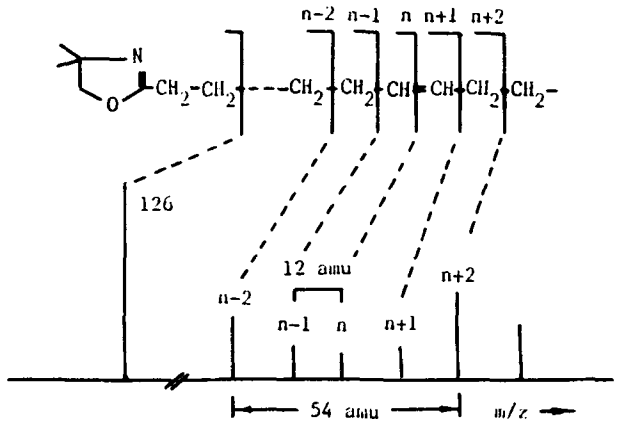


FIG. 4. Schematic presentation of the mass spectral characteristics of monoenoic acid derivatives.

recognizable markers separated by 40 amu in the spectrum profile of the compound in question. As confirmed on model compounds (26), peaks indicating the first double bond in spectra of PUFA derivatives at C_4 (*m/z* 138), C_5 (*m/z* 152) and C_6 (*m/z* 166) are accompanied by a strong odd-mass ion at *m/z* 139, 153 and 167, respectively. Mechanistic aspects of their formation remain unexplored. The empirical rule works well for all compounds in this study.

In summary, this study provides a useful method for simultaneous separation and structure elucidation of UFA. With conversions higher than 90%, the derivatization is easy. It does not require a complicated cleanup procedure, and the GC-MS analysis is usually completed within 30 min. Although the GC of pyrrolidide or picolinyl derivatives necessitates a column temperature approximately 50°C higher than that used for the methyl esters (10,20), it suffices for DMOX to maintain the column temperature only 10°C higher, compared with those of methyl esters (26). This procedure gives more specific results regarding the double bond position and the chain feature as well. Therefore, the DMOX method is potentially of value for the GC-MS of naturally occurring long or very long chain (>22C) PUFA. The encouraging results of extended application to branched acids (30), cyclopropanoid acids (31), cyclopentenoid acids (32) and oxygenated acids (33,34) will be reported elsewhere.

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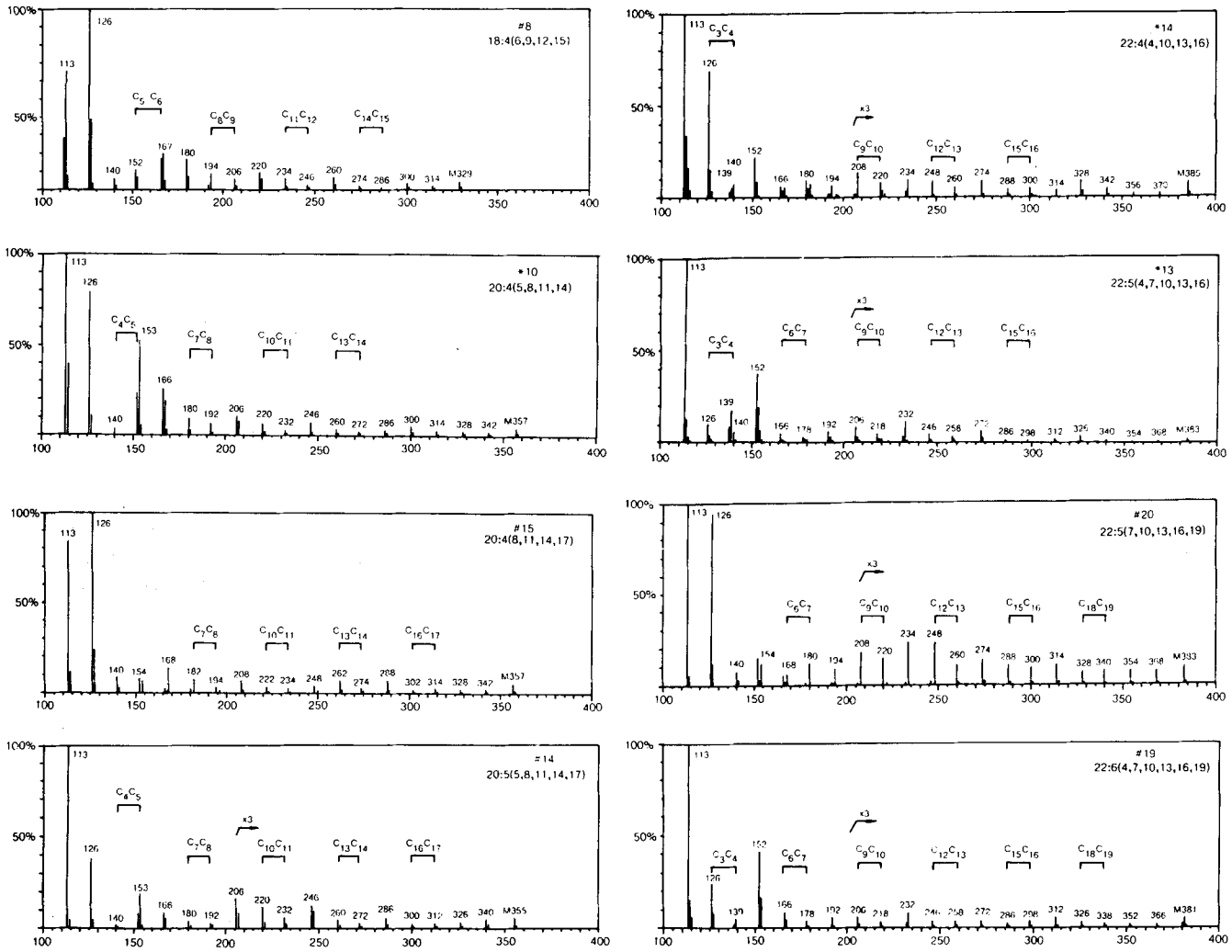

FIG. 5. Mass spectra of DMOX of PUFAs from fish oil (#) and rat testis lipids (*).

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COMMUNICATIONS

Biosynthesis of (^{14}C)Arachidonic Acid From (^{14}C)Linoleate in Primary Cultures of Rat Sertoli Cells

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The conversion of [^{14}C]linoleate to [^{14}C]arachidonate by rat Sertoli cells was established by use of primary cultures. Most of the ^{14}C from [1-^{14}C]linoleate was located in C-3 of the synthesized arachidonate, indicating that the labeled tetraene had originated largely by elongation and desaturation of the intact labeled substrate rather than by mere addition of ^{14}C-acetate generated by bio-oxidation of the radioactive substrate to an already existing 18-carbon precursor. Although a relatively small amount of ^{14}C was present in 18:3ω6 and a relatively large amount of ^{14}C was present in 20:2, it was not possible from these data to establish the relative importance of 20:2 in the biosynthesis of arachidonic acid in rat Sertoli cells.
Lipids 24, 84-85 (1989).

Arachidonic acid is a major component of phospholipids in testes of rats and most other animals. Its synthesis from linoleate in intact rat testis has been shown (1), but the testicular cell type(s) responsible for the synthesis is not known.

Sertoli cells are thought to be responsible for supplying nutritive substances to the developing germinal cells. Thus, there is a possibility that rat Sertoli cells utilize dietary linoleate for the synthesis of arachidonate, which can then be transferred to the developing germinal cells or utilized for further conversion to the 22-carbon pentaene which accumulates in rat spermatids and spermatozoa. Intratesticular injection of [^{14}C]linoleate in rats led to significant labeling of arachidonic acid in Sertoli cells separated from other testicular cells at 3-48 hr after injection (2). However, from these in vivo studies, it could not be determined if the Sertoli cells had incorporated the ^{14}C into arachidonic acid or if the labeled arachidonate originated in some other cell type and subsequently was transferred to the Sertoli cell.

In this communication, we report the use of primary cultures of rat Sertoli cells to establish the conversion of [^{14}C]linoleate to [^{14}C]arachidonate in this cell type.

MATERIALS AND METHODS

Primary cultures of Sertoli cells of Sprague-Dawley rats, 20-25 days old, were prepared according to the method of Tung et al. (3). Testes from 5 or 6 animals yielded sufficient cells to plate 4 dishes (10 ml medium per dish). About 5 million cells per dish were harvested (counted only in one experiment). On the fifth day of the culture, 1 μCi [1-^{14}C]linoleic acid (Sp. act. 55.6 mCi/mmole; New England Nuclear Corp., Boston, MA) and 0.50 mg ^{12}C-linoleic acid were provided as the albumin complex (prepared in a 4:1 molar ratio of salt to fatty acid-poor albumin) to each dish of culture in 10 ml medium. The cells were maintained at 31°C for 48 hr, after which they were harvested and digested with KOH using hydroquinone as antioxidant and a nitrogen atmosphere. After acidification, the fatty acids were extracted with petroleum ether. About 2-4 mg carrier, nonradioactive fatty acids (obtained from testes of nonradioactive rats) were added to the extracted ^{14}C-fatty acids before methylation. The preparation of methyl esters and the radio gas chromatographic analysis were done as reported previously (4). About 15-25 μg fatty acid methyl esters were used per radio gas chromatographic analysis. The location of the ^{14}C in the isolated pure arachidonic acid molecule was done by Dauben degradation (5) of the saturated 20-carbon acid obtained by hydrogenation of the polyene.

In some experiments, 10 μCi [1-^{14}C]sodium acetate (Sp. act. 57.7 mCi/mmole; New England Nuclear Corp.) and 0.2 mg ^{12}C-sodium acetate per dish were used as substrate.

RESULTS AND DISCUSSION

Sertoli cells in primary cultures incorporated 7.2% (SD = 1.8, n = 4) of the linoleate-^{14}C substrate into long chain fatty acids in 48 hr. Of the long chain fatty acid ^{14}C in the harvested cells 39% (SD = 5.1) was present as linoleic acid. The distribution of the remaining ^{14}C is shown in Table 1. Over 60% of the total fatty acid ^{14}C (not counting linoleate-^{14}C) was in 20-carbon metabolites (20:2,

TABLE 1

Distribution of ^{14}C from [1-^{14}C]Linoleate or [1-^{14}C]Acetate in Fatty Acids of Sertoli Cells Harvested After 48 hr Incubation With the ^{14}C-Substrate

Fatty acid fraction	[1-^{14}C]Linoleate[a]		[1-^{14}C]Acetate[b]	
	Mean	S.D.	Mean	S.D.
<16:0	2.6	1.7	7.4	1.8
16:0,16:1	16.0	8.3	59.3	1.7
18:0,18:1	2.1	0.6	24.9	3.8
18:2	—	—	1.1	0.31
18:3-20:1	1.1	0.2	1.6	0.16
20:2	17.2	4.2	0.6	0.23
20:3	34.4	10.3	0.8	0.72
20:4	11.8	1.1	0.5	0.26
20:5-22:2	2.6	2.3	} 0.2	0.22
22:3	2.5	0.9		
22:4	6.8	1.1	2.6	0.98
22:5	1.6	0.9	0.2	0.21
>22:5	1.4	0.7	—	—

[a] Results are reported as % of ^{14}C in total fatty acids (less linoleate ^{14}C) and standard deviation; n = 4.

[b] Results are reported as % of ^{14}C in total fatty acids and standard deviation; n = 3.

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20:3, 20:4). The collected fraction identified in Table 1 as 18:3-20:1 from each of the four experiments was pooled into one sample and further purified by preparative gas chromatography. Radio gas chromatographic analysis of the purified fraction showed that only about 5% of the ^{14}C was in the 18:3ω6.

Chemical degradation of the labeled arachidonic acid showed that over 90% of the ^{14}C was in carbon 3 (carboxyl carbon is number 1). The ratio of the specific activity of C-1:average carbon in the fatty acid chain (C1:average carbon) was 3.0 and that of C-3:average carbon in the fatty acid chain (C3:average chain) was 17.0. A ratio of 2.0 indicates complete de novo synthesis, and a ratio of 20 (for a 20-carbon fatty acid) indicates elongation of the immediate precursor. The palmitic acid fraction was also isolated and degraded. The ratio of C1:average carbon was 2.3 and that of C-3 was 3.5, indicating that the palmitate had been synthesized mostly by de novo synthesis using the ^{14}C-acetate generated by cellular oxidation of the radioactive linoleate.

When [^{14}C]acetate was used as substrate, 2.7% (SD = 1.05, n = 3) of the administered dose was incorporated into long chain fatty acids. The distribution of the ^{14}C in individual fatty acids is shown in Table 1. Over 90% of the incorporated ^{14}C was in saturated fatty acids. With the exception of 22:4, which had about 2% of the total ^{14}C, each of the polyenoic acids had less than 1% of the total ^{14}C activity.

Conversion of [^{14}C]linoleate to [^{14}C]arachidonate in these experiments is firm evidence that rat Sertoli cells can synthesize the tetraene from the dietary precursor, linoleic acid. It had previously been shown that rat testes could synthesize arachidonate from linoleate, but it was not determined in which cell type(s) the biosynthesis occurred (1). Because linoleate was metabolized to acetate by the cells in culture, it was of interest to determine whether the ^{14}C-tetraene had been synthesized by mere addition of ^{14}C-acetyl CoA (generated by oxidation of the labeled linoleate) to small amounts of an existing appropriate precursor (18:4) or by utilization of the ^{14}C-linoleate substrate. The results of Dauben degradation of the isolated pure 20:4 clearly demonstrate that the ^{14}C-20:4 had been produced mainly by elongation and further desaturation of the ^{14}C-linoleate substrate. The rather low incorporation of ^{14}C into 20:4, when [1-^{14}C]-acetate was used as substrate, is in agreement with these results. The incorporation of [^{14}C]acetate into arachidonic acid in isolated incubated germinal cells has been reported (6).

The rather large amount of ^{14}C in 20:2 is of interest in that Δ^8 desaturation would provide 20:3, the precursor of arachidonic acid. Although, in most organs, Δ8-desaturase is not present and desaturation of 20:2 yields 5,11,14-20:3 (a dead-end product), there is an active Δ8-desaturase in rat testes (7). However, it remains to be established whether or not this desaturase is active in Sertoli cells. Although the small amount of ^{14}C in the 18:3ω6 fraction may indicate a minor role for this metabolite in arachidonate biosynthesis in Sertoli cells, it is also possible that a high rate of turnover of 18:3 to 20:3 kept the ^{14}C 18:3 from accumulating. Therefore, it is not possible from these data to evaluate the relative importance of these two metabolic pathways in the biosynthesis of arachidonic acid in rat Sertoli cells.

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Plasma Esterase-1 (ES-1) Activity is Increased in Rats Fed High-Fat Diets

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The question addressed is whether the amount and type of dietary fat affects esterases in plasma. Rats were fed semipurified diets containing 2.0 to 19.4% (w/w) of fat in the form of coconut fat or corn oil. Fat was added to the diets at the expense of isocaloric amounts of carbohydrates. Plasma total esterase activities measured with 4-nitrophenylacetate as substrate were slightly increased with increasing fat intakes. However, an increase in fat concentration of the diet was associated with a pronounced increase in the activity of the so-called ES-1 isozyme in plasma. ES-1, which represents very little plasma total esterase activity, was quantified densitometrically as the high-mobility, anodal esterase band on polyacrylamide gel electrophoresis. The positive association between amount of dietary fat and ES-1 activity was identical for coconut fat and corn oil.
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The plasma of vertebrate animals contains various enzymes that can hydrolyze artificial fatty acid esters of aromatic alcohols. Although the physiological function of these esterases is obscure, there is some evidence that they are involved in lipid metabolism. The addition of olive oil (5%, w/w), cholesterol (2%) and cholate (0.5%) to the diet of rats and mice caused an increase in plasma total esterase activity, as measured with β-naphthylpropionate as substrate (1,2). The increase in plasma total esterase activity was associated with an increase in the activity of an anodal, fast-moving esterase zone in the plasma zymogram. Lewis and Hunter (3) reported that injection of fat into the stomach produced an increase in the activity of plasma esterases of high electrophoretic mobility. These studies prompted us to investigate whether, under more physiological conditions, the amount and/or type of fat in the diet would affect plasma esterase activities in rats. For this purpose, rats were fed diets with increasing levels of either coconut fat or corn oil, the fats being added at the expense of isocaloric amounts of carbohydrates.

MATERIALS AND METHODS

Male rats, aged 4 weeks, of an outbred Wistar colony (Cpb/WU) were used. The animals were housed individually exactly as described previously (4). On day 0 of the experiment, the rats were divided into 8 dietary groups of 6 animals each. The groups had similar distributions of serum cholesterol concentration and body weight; the mean values were 2.69 mM and 118 g. The groups were fed semipurified diets in meal form which contained either coconut fat or corn oil at various concentrations. The fat concentrations expressed as percentage of weight (and energy) were: 2.0 (5.5), 4.1 (11.0), 8.6 (22.0) and 19.4 (44.0). The low-fat diets contained (g/100 g): casein, 16; corn starch, 25.585; dextrose, 25.585; molasses, 10; corn oil, 2 (or corn oil, 1 plus coconut fat, 1); cellulose, 15; dicalcium phosphate, 0.61; calcium carbonate, 0.62; magnesium carbonate, 0.07; magnesium oxide, 0.03; potassium bicarbonate, 1.8; sodium chloride, 0.5; vitamin premix, 1.2, and mineral premix, 1.0. The composition of the vitamin and mineral premix has been described (5). All diets contained at least 2.75% of energy as corn oil so as to provide sufficient linoleic acid. This implies that the coconut fat diets actually contained 2.75% of energy less in the form of coconut fat than indicated. Extra fat was added to the diets at the expense of isocaloric amounts of corn starch and dextrose in a 1:1 (w/w) ratio. Food and tap water were provided ad libitum.

At the end of the experiment (day 58), the rats were anesthetized in the nonfasting state (between 10.00 and 13.00 hr) by the intraperitoneal administration of 15 mg of pentobarbital (Nembutal, Sanofi Sante Animale SA, Paris, France). Blood was taken by aortic puncture, and 4.5 ml was mixed with 0.5 ml of distilled water containing 3.8% (w/w) of sodium citrate. Plasma was collected by low speed centrifugation.

Plasma cholesterol was measured enzymatically using the kit (Monotest) supplied by Boehringer-Mannheim GmbH (Mannheim, FRG). Plasma triglycerides were determined enzymatically as described (6). Total esterase activity in plasma, which had been stored for 3 days at −20°C, was measured with 4-nitrophenylacetate as substrate. The final reaction mixture was incubated at 37°C and consisted of 2.5 mM 4-nitrophenylacetate, 0.5% (w/w) Triton X-100 and 0.5% (w/w) acetone in 50 mM Tris buffer (pH 7.1). The release of product, 4-nitrophenol, was monitored spectrophotometrically at 410 nm. The absorption of a solution of 0.1 mM 4-nitrophenol (dissolved in the same buffer as the substrate) served as a standard. Reaction conditions were chosen so that the amount of 4-nitrophenol formed was linear with time and enzyme concentration. Enzyme activity was calculated after correction for spontaneous hydrolysis of the substrate.

Plasma esterase patterns were determined by vertical 7.6–15.0% (w/v) polyacrylamide gradient slab gel electrophoresis using a multiphasic buffer system. The gel buffer was a 37.5 mM Tris buffer (pH 8.9). The electrode buffer consisted of 5 mM Tris and 38.5 mM glycine (pH 8.3). To each slot of the slab gels, 25 μl of a mixture containing 67.5% (v/v) of plasma, 0.285% (w/w) sodium citrate and 10% (w/w) glycerol was added. After electrophoresis, the gels were stained for esterase activity with α-naphthylacetate, α-naphthylpropionate and Fast Blue BB (Merck, Darmstadt, FRG). Of both esters, 100 mg was dissolved in 2 ml of Triton X-100/acetone (1:1, w/w), and this solution was mixed with 25 ml of 50 mM Tris buffer (pH 7.3). Immediately before use, this mixture was added to 175 ml of 50 mM Tris buffer (pH 7.3) containing 100 mg of Fast Blue BB. The gels were

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TABLE 1

Effect of Dietary Fat on Lipid Concentrations and Total Esterase Activities in Plasma of Rats

Measure	Type of fat	Amount of fat in the diet (%, w/w) 2.0	4.1	8.6	19.4	Sign.[a]
Body wt (g)	Coconut fat	365 ± 9	349 ± 12	370 ± 21	382 ± 12	
	Corn oil	341 ± 10	375 ± 10	349 ± 15	364 ± 16	
Feed intake (g/d)	Coconut fat	26.4 ± 0.5	24.4 ± 0.9	23.9 ± 0.7	22.5 ± 0.5	A
	Corn oil	25.2 ± 0.5	25.3 ± 0.4	22.3 ± 0.8	20.6 ± 1.0	
Plasma cholesterol (mM)	Coconut fat	2.76 ± 0.09	2.52 ± 0.19	2.54 ± 0.18	2.65 ± 0.09	
	Corn oil	2.79 ± 0.06	2.84 ± 0.07	2.72 ± 0.13	2.75 ± 0.22	
Plasma triglycerides (mM)	Coconut fat	1.21 ± 0.12	1.19 ± 0.20	1.67 ± 0.27	2.20 ± 0.36	T,AXT
	Corn oil	1.74 ± 0.41	1.52 ± 0.18	0.98 ± 0.18	0.72 ± 0.12	
Plasma esterase activity (μmol/min/ml)	Coconut fat	1.42 ± 0.05	1.83 ± 0.23	1.63 ± 0.08	1.91 ± 0.11	A
	Corn oil	1.57 ± 0.07	1.55 ± 0.14	1.73 ± 0.11	1.83 ± 0.09	

The diets were fed for 58 days. Results expressed as means ± SE for 6 animals/group, except for the group on the diet containing 2% coconut fat, which consisted of 5 animals (on day 30, one animal in this group died).

[a]Significance ($P < 0.05$) was calculated by analysis of variance. A, effect of amount of fat; T, effect of type of fat; AXT, effect of interaction.

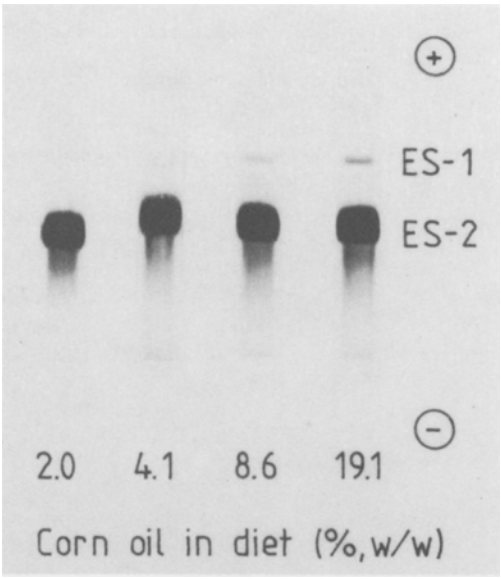

FIG. 1. Electrophoresis on polyacrylamide gradient gels of esterases in plasma of individual rats fed diets containing various levels of corn oil.

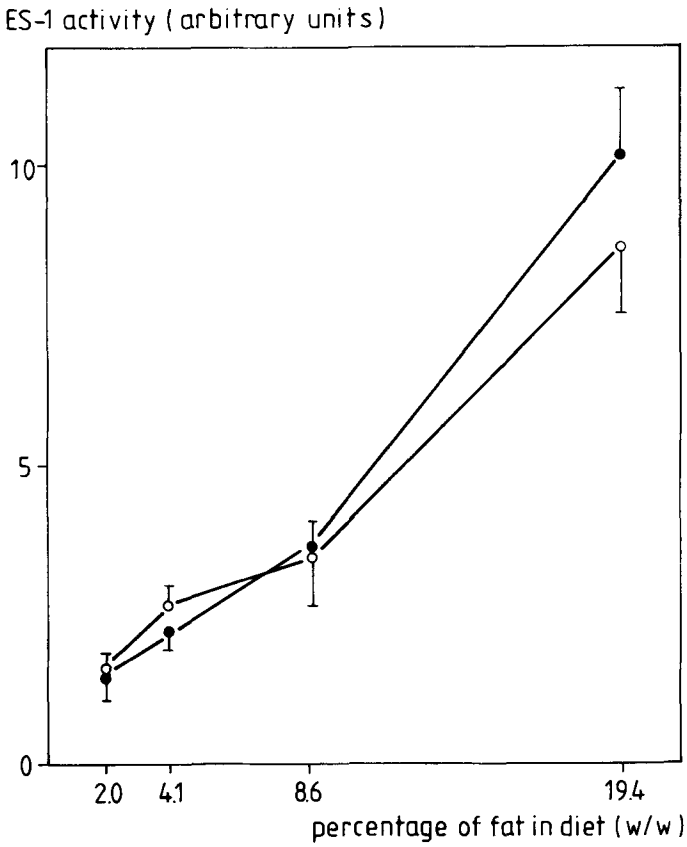

FIG. 2. Relationship between the activity of ES-1 and amount of fat in the diet. Results, expressed as means ± SE; ○ = corn oil; ● = coconut fat. There was a significant ($P < 0.05$, analysis of variance) effect of amount of dietary fat, but no effect of fat type and no interaction.

incubated with this solution at room temperature, under slow shaking, for exactly 10 min. After staining, the gels were fixed in 5% (w/v) trichloric acid and photographed. The intensity of the ES-1 band was measured by densitometric scanning of the stained gels at 530 nm.

RESULTS AND DISCUSSION

Dietary coconut fat vs corn oil did not systematically affect body weight of the rats (Table 1). Likewise, the amount of fat in the diet did not clearly influence body weight. This is explained by the significant reduction of feed intake with increasing fat concentrations. In this way caloric intake remained essentially similar in rats fed the various experimental diets.

There was no significant effect of the amount and type of fat on plasma cholesterol levels. However, at dietary fat levels higher than 2.0% (w/w), corn oil produced higher group mean plasma cholesterol concentrations than did coconut fat. This corroborates earlier work using rats fed cholesterol-free, semipurified diets (7,8). Above dietary fat levels of 4.1% (w/w), plasma triglyceride concentrations increased with increasing amounts of coconut fat in the diet, but with corn oil the opposite was seen (Table 1). Thus, there was an interaction of amount and type of fat. The triglyceride lowering action of corn oil, when compared with coconut fat, agrees with other investigations (8,9).

The amount of fat in the diet significantly influenced plasma total esterase activities. However, this effect was relatively small (Table 1). Plasma total esterase activities represent the sum of the activities of a number of different esterases. In rats, the so-called ES-2 isozyme is most abundant. This is illustrated by the separation of these enzymes on polyacrylamide gradient gel electrophoresis and visualized with α-naphthylacetate and α-naphthylpropionate as substrates (Fig. 1). It is also clear that an increase in dietary corn oil caused an increase in the intensity of the most anodal plasma esterase zone (ES-1). This is supported by the data in Figure 2, showing the relationship between the amount of fat in the diet and the activity of ES-1 which was assessed densitometrically. The relationship was almost identical for corn oil and coconut fat.

This study shows that ES-1 in plasma of rats is positively associated with the concentration of fat in the diet. Dietary corn oil and coconut fat had identical effects on ES-1 but influenced plasma cholesterol and triglyceride concentrations differentially. Thus, ES-1 would appear not to be related to these plasma lipids. As to the physiological function of ES-1, we can only speculate. Possibly, this esterase isozyme is involved in fat absorption and released from the intestine during this process. Administration of fat to rats by stomach tube resulted in an increase in the activity of esterases of high electrophoretic mobility in the intestinal lymph (3).

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On the Isolation of 2-Hydroxydocosanoic and 2-Hydroxytricosanoic Acids From the Marine Sponge *Amphimedon compressa*

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The first α-hydroxy fatty acids from a marine sponge, namely 2-hydroxydocosanoic and 2-hydroxytricosanoic, were identified in the Caribbean sponge *Amphimedon compressa*. These acids were found to occur in phosphatidylethanolamine and phosphatidylserine and constituted 52% of the total fatty acid mixture of this sponge. The long chain fatty acids 5,9,23-nonacosatrienoic (29:3) and 5,9,23-tricontatrienoic (30:3), as well as a new tetratricontatetraenoic (34:4) acid, were also found in *A. compressa*.
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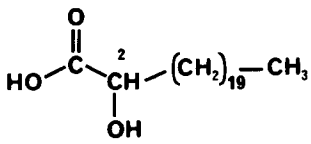
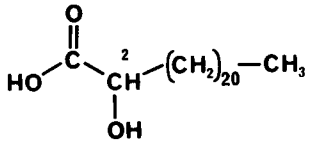
Sponges have attracted in recent years the attention of marine natural products chemists because they have proved to be rich sources of bioactive and novel secondary metabolites. Unusual and unprecedented phospholipid fatty acids with no terrestrial counterpart have been isolated and characterized from dozens of marine sponges (1). However, one important group of phospholipid fatty acids, the 2-hydroxy acids, has not been recognized to exist in the phospholipids of marine sponges. One piece of work by Ayanoglu et al. (2), in which the phospholipid fatty acids of the marine sponge *Higginsia tethyoides* were analyzed, describes the isolation of a series of closely related 2-methoxy fatty acids, the only example to date of 2-oxa-substituted fatty acids in marine sponges. Unique to the latter work was the observation that these fatty acids were shown to be present in conventional phospholipids like phosphatidylethanolamine and phosphatidylserine (2), when the normal expectation is for these acids to be present in sphingolipids. Four kinds of fatty acid occur in sphingolipids, the saturated long-chain fatty acids like palmitic (16:0), very long chain saturated fatty acids like behenic (22:0), monoenoic fatty acids such as oleic (18:1) and α-hydroxy very long chain fatty acids such as cerebronic (h 24:0). Important in this context is to mention that polyunsaturated fatty acids are conspicuously absent from these lipids.

In this paper we wish to report, for the first time, the isolation of the important fatty acids 2-hydroxydocosanoic (Scheme 1) and 2-hydroxytricosanoic (Scheme 2) from the phospholipids of the marine sponge *Amphimedon compressa*. This is the first time, to our knowledge, that these 2-hydroxy fatty acids have been isolated from conventional sponge phospholipids. Such hydroxylated acids usually are encountered in brain cerebrosides, sphingomyelins and lecithins (3) and in smaller concentrations in the kidney (4).



SCHEME 1



SCHEME 2

EXPERIMENTAL PROCEDURES

Amphimedon compressa was collected May 3, 1988, near La Parguera, Puerto Rico. The sponge was washed in sea water, carefully cleaned of all nonsponge debris and cut into small pieces. Immediate extraction with chloroform/methanol (1:1, v/v) yielded the total lipids. The neutral lipids, glycolipids and phospholipids were separated by column chromatography using the procedure of Privett et al. (5). The phospholipid classes were investigated by thin layer chromatography (TLC) using silica gel and chloroform-methanol-water (75:25:4, v/v/v) as solvent. The ^{31}P-NMR of the phospholipids was performed at 22°C on a GN 300 FT-NMR spectrometer at 121.6 MHz. For the acquisition, 16K data points were used and ca. 1000 accumulations were obtained before Fourier transformation of the free induction decay. In a typical run, phospholipids (20-30 mg) were dissolved in 3 ml of deuterated chloroform-methanol (2:1, v/v) containing, as internal reference, triphenylphosphine (ϕ_3P). The fatty acyl components of the phospholipids were obtained as their methyl esters by reaction of the phospholipids with methanolic hydrogen chloride as described before (6). The resulting methyl esters were analyzed by gas chromatography-mass spectrometry (GC-MS) using a Hewlett-Packard 5995 A gas chromatograph-mass spectrometer equipped with a 30-m × 0.32-mm fused silica column coated with SE-54. 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 (1 hr at 100°C) followed by ethereal extraction from the acidified solution and purification by preparative layer chromatography (PLC). Hydrogenations were carried out in 10 ml of absolute methanol and catalytic amounts of platinum oxide (PtO_2). Mass spectral data of key fatty acids for this discussion are presented below.

2-Hydroxydocosanoic acid methyl ester. MS *m/z* (rel intensity): 370 (M^+, 88), 348 (6), 338 (M^+ − MeOH, 5), 312 (13), 311 (M^+ − COOMe, 56), 309 (9), 292 (9), 293 (2), 159 (6), 145 (10), 127 (15), 125 (14), 111 (27), 103 (14), 97 (48), 90 (38), 83 (50), 71 (38), 69 (52), 57 (100), 55 (81).

2-Hydroxytricosanoic acid methyl ester. MS *m/z* (rel intensity): 384 (M^+, 80), 370 (4), 352 (M^+ − MeOH, 6), 338 (5), 326 (15), 325 (M^+ − COOMe, 62), 323 (17), 306 (13), 280 (3), 159 (9), 145 (13), 127 (22), 125 (18), 103 (14), 90 (31), 83 (49), 69 (49), 59 (27), 57 (100), 55 (84).

5,9,23-Nonacosatrienoic acid methyl ester. MS *m/z* (rel intensity): 446 (M^+, 7), 414 (2), 345 (5), 221 (4), 208 (4),

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Abbreviations: ϕ_3P, triphenylphosphine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; ECL, equivalent chain length; FT-NMR, Fourier transform nuclear magnetic resonance; GC-MS, gas chromatography-mass spectrometry; PLC, preparative layer chromatography; ^{31}P-NMR, phosphorus nuclear magnetic resonance; TLC, thin layer chromatography.

207 (5), 194 (16), 180 (23), 163 (10), 150 (17), 149 (19), 141 (29), 140 (14), 136 (17), 135 (21), 123 (12), 121 (22), 109 (61), 108 (17), 95 (35), 81 (100), 74 (21), 69 (47), 67 (64).

5,9,23-Tricontatrienoic acid methyl ester. MS *m/z* (rel intensity): 460 (M^+, 6), 429 (2), 359 (4), 345 (6), 221 (3), 208 (5), 207 (5), 194 (18), 180 (28), 163 (9), 150 (17), 149 (17), 141 (35), 140 (18), 136 (17), 135 (24), 123 (13), 121 (23), 109 (57), 108 (17), 95 (36), 81 (100), 74 (19), 69 (51), 67 (69).

Tetratricontatetraenoic acid methyl ester. MS *m/z* (rel intensity): 514 (M^+, 8), 347 (2), 346 (6), 330 (2), 329 (8), 328 (2), 273 (2), 241 (3), 223 (2), 180 (2), 179 (8), 178 (28), 161 (16), 143 (30), 132 (17), 127 (5), 113 (7), 105 (11), 97 (13), 89 (16), 83 (14), 73 (16), 71 (19), 70 (14), 69 (25), 57 (56), 56 (23), 55 (100).

RESULTS

Our results are presented in Table 1 where the fatty acid composition of *A. compressa* is presented. The sponge contained, among others, a series of saturated long chain fatty acids, the most abundant being hexadecanoic (16:0), octadecanoic (18:0), behenic (22:0) and tricosanoic (23:0), which together accounted for 20% of the total fatty acid composition of this sponge. In the fatty acid mixture, the most striking result was the presence of a very long chain fatty acid in an impressive 42% abundance. The mass spectrum of the methyl ester of this acid exhibited a molecular ion peak at *m/z* 370 (88%, $C_{23}H_{46}O_3$) with a higher than usual abundance. The existence of a $M^+ - COOCH_3$ fragment ion at *m/z* 311 with an abundance of 56% strongly suggested α-substitution that was further confirmed by other diagnostic peaks at *m/z* 338 ($M^+ - CH_3OH$) and at *m/z* 292. Lack of peaks at *m/z* 74 and 104, but presence of fragmentation ions at *m/z* 90 and *m/z* 103 arising from the characteristic McLafferty rearrangement (7), suggested immediately the presence of α-hydroxy substitution instead of, for example, α-methoxy. In fact, after a literature comparison (7), it became obvious that we were dealing with 2-hydroxydocosanoic acid (Scheme 1), which is unprecedented in marine sponges.

A second very long chain fatty acid that was also present in the mixture with an abundance of 11.5% presented a very characteristic MS spectrum with a molecular ion peak at *m/z* 384 (80%, $C_{24}H_{48}O_3$). The existence of a $M^+ - COOCH_3$ fragment ion at *m/z* 325 (62%) also strongly suggested α-substitution. The other diagnostic peaks were found at *m/z* 352 ($M^+ - CH_3OH$) and at *m/z* 306, also consistent with α-substitution. Lack of peaks at *m/z* 74 and 104, but presence of fragmentation ions at *m/z* 90 and *m/z* 103 from the characteristic McLafferty rearrangement confirmed again the presence of α-hydroxy substitution (7). In fact, after a detailed literature comparison (7), it became obvious that we were dealing with still another α-hydroxy fatty acid, the 2-hydroxytricosanoic acid (Scheme 2), which is also unprecedented in marine sponges. These two α-hydroxy fatty acids accounted for ca. 52% of the total phospholipid fatty acid composition of this sponge, a rather unusual finding.

Capillary GC and GC-MS analysis of the mixture also revealed the presence of two very long chain phospholipid fatty acids displaying base peaks at *m/z* 81, a value diagnostic of acids possessing the Δ5,9 unsaturation pattern typical of "demospongic acids" (8). On the basis of capillary GC retention times and equivalent chain length (ECL) values, together with mass spectral comparisons, these acids were characterized as 5,9,23-nonacosatrienoic ($\Delta^{5,9,23}$-29:3) and 5,9,23-tricontatrienoic ($\Delta^{5,9,23}$-30:3), which were present in 8 and 15%, respectively. The latter acids already have been reported to occur in marine sponges (8). A third polyunsaturated very long chain phospholipid fatty acid was also detected in trace amounts in this sponge which, by means of GC-MS analysis of its methyl ester, had a molecular weight of 514. A careful analysis of the mass spectrum of the methyl ester of this acid and comparison of ECL values suggested that we were dealing with tetratricontatetraenoic acid methyl ester (34:4), an extremely long chain fatty acid, unprecedented in marine sponges. Unfortunately, the trace amounts of this acid prevented us from locating the double bond positions.

The phospholipids from *A. compressa* were separated and analyzed by preparative thin layer chromatography (TLC), and the acids reported in this work are major constituents of phosphatidylethanolamine (PE) and phosphatidylserine (PS). To our surprise, not even traces of phosphatidylcholine (PC) or sphingomyelin were observed in the phospholipid mixture of *A. compressa.* In order to verify our TLC results, we decided to run a ^{31}P-NMR spectrum of the total phospholipids from *A. compressa* (Fig. 1). It can be seen that only two phospholipids, namely PE and PS, comprise the total phospholipid mixture of this sponge.

TABLE 1

Identified Phospholipid Fatty Acids From *Amphimedon compressa*

Fatty acid	Abundance (%)
Hexadecanoic (16:0)	2.3
Octadecanoic (18:0)	7.0
Nonadecanoic (19:0)	0.8
Eicosanoic (20:0)	0.3
Heneicosanoic (21:0)	0.9
Docosanoic (22:0)	8.5
Tricosanoic (23:0)	3.1
2-Hydroxydocosanoic (22:0)	42
2-Hydroxytricosanoic (23:0)	11.5
5,9,23-Nonacosatrienoic (29:3)	8.2
5,9,23-Tricontatrienoic (30:3)	15.4
Tetratricontatetraenoic (34:4)	traces

DISCUSSION

The results presented here are interesting because, to the best of our knowledge, this is the first time that 2-hydroxy fatty acids have been isolated from a marine sponge. The high abundance of these acids (53% of the total phospholipid fatty acids) practically excludes any possible microbial symbiont source. These fatty acids have been found before in brain cerebrosides, sphingomyelins, lecithins arising from the cerebral cortex, cerebral white matter, diencephalon and midbrain in amounts ranging between 10–15% (9). The two α-hydroxy acids isolated in this work have also been detected in monohexosylceramides, lactosylceramides and oligohexosylceramides from A_2-Asia and A_2-England Influenza Virus (10). From the above reports, it is assumed that these α-hydroxy fatty acids occur exclusively in sphingolipids.

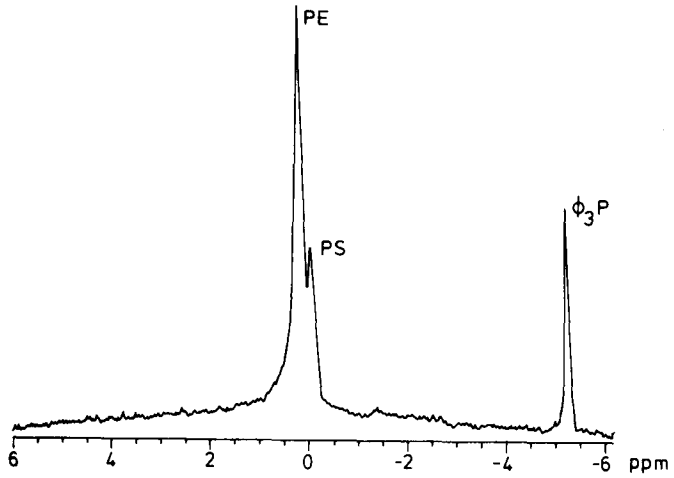

FIG. 1. The complete ^{13}P-NMR spectrum of the total phospholipids from *A. compressa*. The abbreviations are PE, phosphatidylethanolamine; PS, phosphatidylserine; ϕ_3P, triphenylphosphine.

This probably implies that there are mechanisms of compartmentalizing the pools of these fatty acids, or that α-hydroxylation occurs after the regular chain length fatty acids have been introduced into a sphingolipid or some specialized precursor. Naturally occurring 2-hydroxy fatty acids are known to possess the R configuration (11), and we believe that the acids isolated in this work also possess the R configuration at carbon 2.

Our findings with the sponge *Amphimedon compressa* suggest that these 2-hydroxy fatty acids are not only restricted to sphingolipids, but that they can be found, at least in marine sponges, in other phospholipids like PE and PS. Our results are also compatible with previous findings by Ayanoglu et al. (12) that α-methoxy fatty acids from the marine sponge *Higginsia tethyoides* are present in common phospholipids like PE and PS. Interesting to point out is the observation that in these sponges the α-substituted fatty acids seem to occur concomitantly with the very long chain fatty acid 5,9,23-tricontatrienoic (30:3). It will be very interesting to find out if this 30:3 acid and the α-hydroxy fatty acids are related in their biosynthetic origin or if they play special membrane functions, because in sphingolipids polyunsaturated acids are normally absent.

The origin of the α-hydroxy fatty acids presented in this work is worthy of investigation. One good possibility is α-oxidation of the long chain saturated fatty acids docosanoic (22:0) and tricosanoic (23:0), because these are found in relatively large amounts in *A. compressa*. This possibility has been demonstrated in the case of brain sphingolipids by Mead and Levis (13), who demonstrated that α-oxidation is the preferred pathway for the biosynthesis of 2-hydroxytetracosanoic acid.

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Microsomal Δ5 Desaturation of Eicosa-8,11,14-trienoic Acid is Activated By a Cytosolic Fraction

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Δ5 Desaturation of eicosa-8,11,14-trienoic acid to arachidonic acid was studied in rat liver microsomes. It was shown that Δ5 desaturation of fatty acids in vitro requires the participation of a peripheral component of cytosolic origin. Desaturation of 20:3n-6 to 20:4n-6 decreases in washed microsomes as they lose an adsorbed cytosolic fraction (CF), but the enzymatic activity can be recovered as a function of CF concentration in the incubation medium. Albumin does not substitute for CF. Δ5 Desaturation of 20:3n-6 is inhibited by arachidonic acid by a product inhibition effect, but CF prevents retroinhibition of Δ5-desaturase by 20:4n-6. This ability of CF is eliminated by preincubation of CF with 20:4n-6, but not with γ-18:3n-6, the product of Δ6 desaturation of 18:2n-6, thus indicating that CF impairs the retroinhibitory effect of arachidonic acid on Δ5-desaturase in a specific manner. Δ6 Desaturation of linoleic acid to γ-18:3n-6 is also activated by CF and retroinhibited by γ-18:3n-6. CF activity on Δ6 desaturation is retained after preincubation with 20:4n-6, but it is lost after preincubation with γ-18:3n-6. Activation of Δ6-desaturase by CF is associated with the removal of the reaction product in a specific manner. Chromatography of CF by Sephacryl S-200 separates two major subfractions which show different efficiency in reactivating Δ5- and Δ6-desaturase activities in washed microsomes. Therefore, CF may contain subfractions that can prevent Δ5- and Δ6-desaturase retroinhibition by apparently binding their respective reaction products specifically.
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Fatty acid desaturase systems involve three integral microsomal components: the desaturase, the NADH cytochrome-b_5 reductase and cytochrome-b_5 (1-11). The requirement of a soluble factor for Δ6 desaturation of fatty acids was first recognized in our laboratory (10,12,13). Some of its characteristics and its cytosolic origin have been described (14,15), as well as the mechanism of its participation in the Δ6-desaturation process (16). It primarily prevents the retroinhibition of the enzyme by apparently binding the reaction product, 18:3n-6, as it is formed. The factor is different from the one described by Jeffcoat et al. (17).

The requirement of a soluble factor by the Δ5-desaturase system has also been proposed by us (18). The importance of the Δ5-desaturase lies in its catalyzing effect in the conversion of eicosa-8,11,14-trienoic acid to arachidonic acid, eicosa-8,11,14,17-tetraenoic acid to eicosa-5,8,11,14,17-pentaenoic acid and, then, in eicosanoid production. The participation of soluble cytosolic fractions in fatty acid Δ5 desaturation could be related to a fine-tuned regulation of arachidonic and eicosa-5,8,11,14,17-pentaenoic acid biosynthesis.

In the present work, we studied the participation of a cytosolic fraction in Δ5 desaturation of 20:3n-6 to 20:4n-6. It was found that washed rat-liver microsomes largely lost their Δ5-desaturase activity, but this activity was recovered by the addition of a cytosolic fraction. The inhibition of Δ5-desaturase by arachidonic acid was eliminated by the addition of the cytosolic fraction. Cross experiments performed with Δ5- and Δ6-desaturases and the addition of the fraction preincubated with either 18:3n-6 or 20:4n-6 indicated that, apparently, definite sites exist that bind 18:3n-6 and 20:4n-6.

EXPERIMENTAL PROCEDURES

Materials. [1-^{14}C]Linoleic (58 mCi/mmol) and [1-^{14}C]-eicosa-8,11,14-trienoic acids (55 mCi/mol) were provided by New England Nuclear (Boston, MA). Linoleic and eicosatrienoic acids (99% pure) were provided by Nu-Chek Prep Inc. (Elysian, MN). ATP, CoA, NADH, N-acetylcysteine were provided by Boehringer Argentina (Buenos Aires, Argentina). Sephacryl S-200 was supplied by Pharmacia Fine Chemicals (Uppsala, Sweden). All other chemicals and solvents, reagent grade, were provided by Carlo Erba (Buenos Aires, Argentina).

Microsomes. Wistar rats of 150-200 g reared on Purina Chow were used to obtain liver microsomes (13). Unwashed microsomes and cytosol were separated by centrifugation at 105,000 *g* for 60 min. The microsomes were then washed at 0-4°C with a low ionic strength solution of 0.25 M sucrose, 0.15 M NaCl, 0.04 M buffer phosphate (pH 7.4), 0.3 mM nicotinamide and 1.5 mM N-acetylcysteine. After centrifugation at 105,000 *g* for 60 min, the extracted microsomes were separated from the adsorbed cytosolic fractions.

Preparation of the cytosolic fraction. NaCl (0.254 g/ml) was added to 0.6 ml of cytosol. This mixture was then placed on top of 3.5 ml NaBr solution (0.44 g/ml) in a 4.2-ml tube and centrifuged at 220,000 *g* for 24 hr at 14°C in a Kontron Centrifuge. The saline gradient formed was monitored by a refractive index of 0.3 ml fractions collected in control tubes where cytosol was replaced by the low ionic strength solution. The fraction isolated from cytosol at density 1.26 g/ml was dialyzed against 0.15 M NaCl, flushed with nitrogen and kept frozen at −80°C until used. The fraction was called cytosolic fraction (CF).

Sephacryl S-200 fractionation. A 56 cm × 1.6 cm Sephacryl S-200 column was used. The cytosolic fraction (0.6 ml) containing ca. 12 mg of protein was applied to a column equilibrated with 0.02 M phosphate buffer (pH 7.4), 0.1 mM EDTA. Fractions of 1.2 ml were collected and their absorption was analyzed at 280 nm in a Zeiss PMQII spectrophotometer (Carl Zeiss, Oberlochen, Württemberg, FRG). Tentative molecular weights were estimated by using different proteins like lactic dehydrogenase, bovine serum albumin and trypsin.

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Fatty acid desaturating assay. Δ5- and Δ6-desaturase activities were determined in microsomes by measuring the conversion of 34 μM [1-^{14}C]eicosatrienoic and [1-^{14}C]linoleic acids to arachidonic and γ-linolenic acids, respectively, at 35°C for 15 min in the presence of 2.5 mg (unwashed microsomes) or 2 mg (washed microsomes) of protein in a final volume of 1.5 ml. When the assay was run for the reconstituted system, 0.5 mg of cytosolic fraction or subfractions of Sephacryl S-200 were added to 2 mg of washed microsomes in order to complete 2.5 mg equivalent to whole microsomes. The incubation solution contained 0.25 M sucrose, 0.15 M KCl, 0.04 mM phosphate buffer (pH 7.4), 0.70 mM N-acetyl cysteine, 0.04 M NaF, 1.3 mM ATP, 0.06 mM coenzyme A, 0.87 mM NADH, 5 mM, $MgCl_2$, 0.33 mM nicotinamide. After the incubation time, the reaction was stopped and the products were saponified with 2 ml 10% methanolic KOH, extracted with petroleum ether (boiling point 30–60°C) and methylated. The methyl esters of the obtained fatty acids were analyzed by radiochromatography in an Acromat CG-100 apparatus equipped with a proportional counter.

Analytical procedures. Protein concentration was determined by the method of Lowry et al. (19). Binding of fatty acids to the cytosolic fraction was achieved by incubating either linoleic or eicosatrienoic acid (120 nmol/mg protein) with CF at 25°C for 15 min.

RESULTS

Effect of cytosolic fraction on Δ5 desaturation of eicosatrienoic acid. Unwashed microsomes (M) desaturated eicosatrienoic to arachidonic acid at a rate of 230.0 pmol × min^{-1} × mg^{-1}. When microsomes were washed and centrifuged (Me), the enzymatic activity decreased to ca. 127.0 pmol × min^{-1} × mg^{-1}. Addition of the cytosolic fraction (CF, d = 1.26 g/ml) made it possible to recover Me-desaturating activity (Fig. 1). The addition of increasing amounts of CF to Me brought about a dose-dependent recovery of the desaturation. This recovery was not observed when CF was substituted by bovine serum albumin (Fig. 2). Arachidonic acid, the product of Δ5 desaturation of eicosatrienoic acid, inhibited the reaction (Fig. 3), but free CF was able to revert the inhibition when added to the microsomes. The preincubation of CF with arachidonic acid impaired its ability to reactivate the retroinhibited microsomes (Fig. 3).

On the other hand, Δ6-desaturase activity diminished after washing rat liver microsomes, but it was recovered by the addition of the cytosolic fraction CF (Fig. 1). The product of Δ6 desaturation of linoleic acid (18:3n-6) inhibited the enzyme (Fig. 3), but free CF eliminated the inhibition when added to the incubation medium. Moreover, the preincubation of CF with 18:3n-6 impaired the ability of the factor to reactivate the retroinhibited microsomes.

Therefore, the effect produced by CF on both Δ5- and Δ6-desaturases in the presence of the corresponding reaction products is similar. The crude cytosolic fraction separated by gradient centrifugation at 1.26 g/ml density

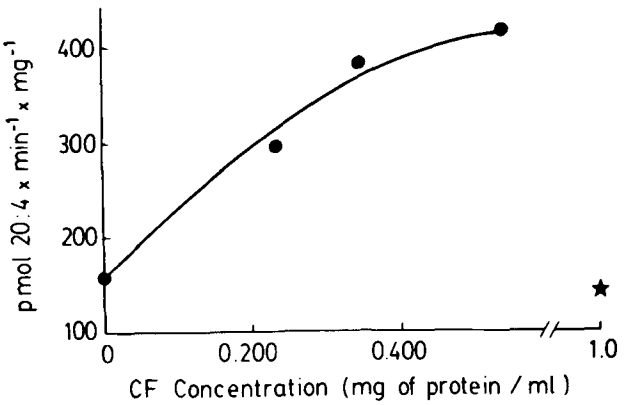

FIG. 2. Δ5-Desaturase activity in washed microsomes (Me) as a function of CF protein concentration in the medium. ★, Effect of 1 mg of albumin.

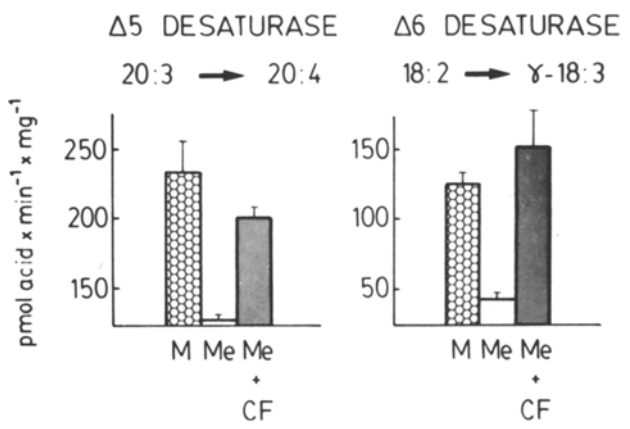

FIG. 1. Δ5-Desaturase activity [20:3n-6 → 20:4n-6] in M, Me and Me + CF; and Δ6-desaturase activity [18:2n-6 → 18:3n-6] in M, Me and Me + CF. The assays were carried out as described in Experimental Procedures.

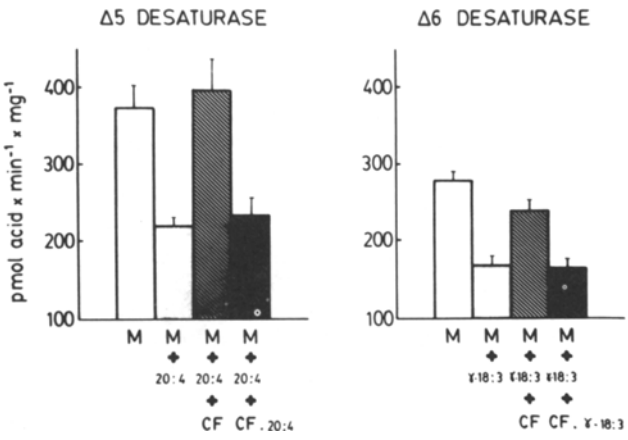

FIG. 3. Δ5-Desaturase activity in M and M plus 20:4n-6; and Δ6-desaturase activity in M and M plus 18:3n-6. 17 nmol of inhibitor acid were added to 0.75 mg of M protein (second bars). 34 nmol of inhibitor acid were added to 0.75 mg M protein simultaneously with 0.25 mg of CF protein (third bars). 34 nmol of inhibitor acid were added to 0.75 mg of M protein simultaneously with 0.25 mg of CF protein preincubated with 30 nmol of inhibitor acid (fourth bars). Final volume was 0.5 ml and the assays were carried out as described in Experimental Procedures.

was able to reactivate the two desaturases eliminating the inhibitions evoked by those products.

Differences in the participation of the cytosolic fraction on Δ5 desaturation of eicosatrienoic acid and Δ6 desaturation of linoleic acid. In another series of experiments, we found that whole microsomes desaturated eicosatrienoic acid to arachidonic acid at a rate of 374.0 pmol × min^{-1} × mg^{-1}. In washed microsomes, that activity decreased to 163.1 pmol × min^{-1} × mg^{-1}. The addition of CF to Me recovered the activity, not only when it was added free, but also after preincubation with 18:3n-6. However, CF preincubated with arachidonic acid did not increase Me Δ5-desaturation rates over 150.0 pmol × min^{-1} × mg^{-1} (Table 1).

Similarly, unwashed microsomes desaturated linoleic acid to γ-linolenic acid at a rate of 284.9 pmol × min^{-1} × mg^{-1} and the Δ6-desaturase activity in Me decreased to 121.2 pmol × min^{-1} × mg^{-1}. CF increased the desaturation activity when it was added free or, in this case, preincubated with 20:4n-6. Conversely, CF preincubated with 18:3n-6 did not increase Me Δ6-desaturation rates over 136.2 pmol × min^{-1} × mg^{-1} (Table 1). Therefore, the mechanism of CF activation of Δ6- and Δ5-desaturases in vitro would be specific for each enzyme and evoked by the binding of the corresponding products to CF eliminating the product inhibition.

TABLE 1

Effect of CF Free or Preincubated With 18:3n-6 and 20:4n-6 Acids and of Sephacryl Subfractions on Δ5- and Δ6-Desaturase Activities

	Δ5-Desaturase pmol 20:4n-6 × min^{-1} × mg^{-1}	Δ6-Desaturase pmol 18:3n-6 × min^{-1} × mg^{-1}
M	374.0 ± 44.2*	284.9 ± 47.1*
Me	163.1 ± 18.0*	121.2 ± 2.0*
Me + CF	326.2 ± 29.0*	262.1 ± 9.0*
Me + CF 18:3n-6	277.0-397.0**	127.2-145.2**
Me + CF 20:4n-6	133.0-167.0**	230.0-234.0**
Me + CF_I	323.4-381.2**	267.0-333.3**
Me + CF_{II}	183.9-201.1**	215.8-263.8**

The conditions were described in Experimental Procedures.

*Mean values of five determinations ± SD.

**Values of two determinations.

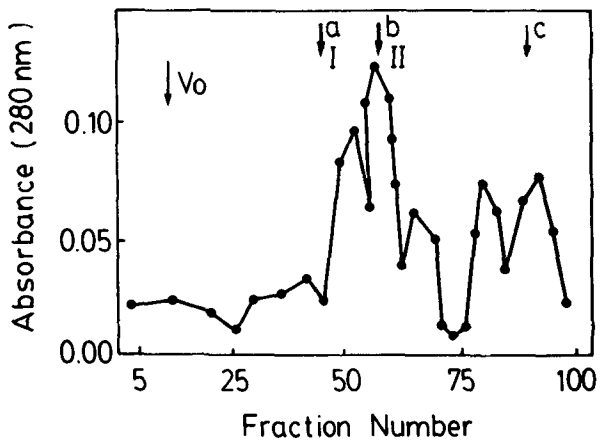

FIG. 4. Sephacryl S-200 chromatographic profile of d = 1.26 g/ml cytosolic fraction. V_o, exclusion volume; (a) elution volume of lactic dehydrogenase, MW: 142,000 daltons; (b) elution volume of bovine serum albumin, MW: 68,000 daltons; (c) elution volume of trypsin, MW: 23,800 daltons; I, CF subfraction of estimated MW = 130,000; and II, CF subfraction of estimated MW = 68,000. The conditions were described in Experimental Procedures.

Cytosolic fraction chromatography by Sephacryl S-200. The differences found in the effects of CF on Δ5- and Δ6-desaturases led us to attempt its further subfractionation by Sephacryl S-200. The CF floating at d = 1.26 g/ml was applied to a 56 cm × 1.6 cm column, and fractions of 1.2 ml were collected. Two main subfractions were obtained (Fig. 4) and added to Me in order to measure Δ5 and Δ6 desaturation. The subfraction CF_I, corresponding to an estimated molecular weight of 130,000 daltons, reactivated Me Δ5-desaturase from 163.1 to 352.0 pmol × min^{-1} × mg^{-1} (Table 1), and recovered Me Δ6-desaturase activity from 121.2 to 303.0 pmol × min^{-1} × mg^{-1}. The subfraction CF_{II}, corresponding to an estimated molecular weight of 68,000 daltons, produced little reactivation of Δ5-desaturase and reactivated Δ6-desaturase of Me from 121.2 to 240.0 pmol × min^{-1} × mg^{-1} (Table 1).

DISCUSSION

The present experiments show that in vitro rat-liver microsomal Δ5 desaturation of 20:3n-6 to 20:4n-6 requires the participation of a cytosolic fraction to reach full activity. This result is similar to that found for microsomal Δ6 desaturation of linoleic acid (16). In both cases, the CF fraction loosely binds to microsomes. The mechanism proposed for CF participation in Δ6 desaturation relates to the specific uptake of the reaction product 18:3n-6 as it is formed and, in this way, avoids its inhibitory effect (16). In the case of Δ5-desaturase, CF reactivates the diminished Me activity in a dose-dependent fashion (Fig. 2). The ability of the fraction to restore the maximal Δ5-desaturase activity could be explained by a similar mechanism associated with a particular interaction with an inhibitory compound. In fact, arachidonic acid produced by Δ5-desaturase from 20:3n-6 inhibits the enzyme, and CF impairs that inhibition in whole M (Fig. 2). To produce this effect, CF must be free, because the preincubation of CF with arachidonic acid impairs its anti-inhibitory ability, as if specific sites were no longer available for the 20:4 produced by Δ5-desaturase (Fig. 3, Table 1). However, CF preincubation with 18:3n-6 does not deactivate the factor, thus, suggesting that CF would apparently bind arachidonic acid in sites different than those for γ-linolenic acid.

Δ6-Desaturase is inhibited by 18:3n-6, as shown in present and previous experiments (16,21-23). When the enzymatic activity was measured in Me, it was lower and the addition of CF, either free or preincubated with 20:4n-6, increased the values up to those of M, whereas the preincubation of CF with γ-linolenic acid impaired its ability to reactivate the enzyme (16) (Fig. 1). Therefore, as in the case of the Δ5-desaturase system, the existence of specific sites for the location of the Δ6-desaturase product in CF, 18:3n-6, can be envisioned. Cross experiments (Table 1) support the idea of the presence of specific sites in CF for Δ5- and Δ6-desaturase products. Moreover, the results suggest that these sites would be different for 20:4

and 18:3n-6. Even more significant are the results obtained by employing the Sephacryl S-200 subfractions of CF. Although there is some overlapping, two partially resolved fractions having different estimated molecular weights and the ability to reactivate Δ5- and Δ6-desaturases were detected. This would imply the existence of two independent cytosolic subfractions able to interact with each enzyme. The aforementioned proteins would apparently bind the corresponding reaction products, thus, avoiding the respective retroinhibition of Δ5 and Δ6 desaturases in a specific mode. CF_{II} would concentrate a fraction specific for Δ6-desaturase reactivation, whereas CF_I would be specific for Δ5-desaturase, but with some overlapping and contamination with CF_{II} activity. Because the subfractions here employed were not pure, further purification will prove useful in defining their true identity.

It has been suggested that catalase would stimulate stearoyl-CoA desaturase (18). However, we have already demonstrated (14) that it is not the catalase activity present in cytosolic fractions that is responsible for the Δ6-desaturase reactivating ability. Moreover, in our experience, albumin does not substitute for CF in desaturase reactions.

Despite the present conclusions, we must take into consideration that desaturases were tested under in vitro conditions, in which these enzymes (but not the elongases) were active. In this case, the fate of the desaturated products is not exactly the same as in the in vivo system, because they are released into the incubation medium instead of being available to the elongating system.

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Cholesterol Metabolism in Frog (*Rana esculenta*) Liver: Seasonal and Sex-related Variations

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Many aspects of lipid metabolism have been studied in amphibians, but seasonal lipid modulation in male and female frogs has not been investigated. We describe here the yearlong patterns of hepatic lipid content and enzyme activities related to cholesterol homeostasis, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase activity and acyl coenzyme A:cholesterol acyltransferase (ACAT) activity in liver of the male and female frog, *Rana esculenta*. Lipid storage follows distinct seasonal patterns, with an increase in June that is more pronounced in the female than in the male frog. Cholesterol content and cholesterol storage as cholesteryl ester in male liver are consistent with the activity of HMG-CoA reductase and of ACAT enzymes. HMG-CoA reductase activity of the female frog shows an extra peak in fall unrelated to cholesterol storage and probably related to the production of essential compound for oogenesis.
***Lipids 24,* 105-108 (1989).**

The frog, as a poikilothermic vertebrate, shows seasonal variations in physiological activities closely reflecting environmental changes. Oxygen consumption, CO_2 production and respiratory quotient vary during the year (1) with fluctuations greater than those normally found in warm blooded animals. The variations suggest a differential utilization of energy reserves depending on the season, viz. glycogen in winter and lipids in summer.

This view is supported by the occurrence of marked changes in weight, composition (2-6) and enzymatic activities (2,7,8) in the liver of these animals. Such changes could be related to the fluctuations of blood hormone levels detectable during the year; actually, glucagon and insulin peak in March and in October (6), respectively, whereas sex hormones show maximal levels between fall and winter (9,10). Sex-related changes in blood sugar level (11), liver and fat body weight and composition (2), and liver content of dolichol phosphate (12) have been reported.

As lipid metabolism is related to membranogenesis and gametogenesis and no data are available on the annual or sex-dependent cholesterol metabolism of the frog, it is of interest to determine whether seasonal behavior and sex-related physiological activities affect lipid content in the liver and production or utilization of cholesterol. For this purpose, lipid and cholesterol content of male and female frogs *(Rana esculenta)* were compared, on an annual basis, to the activity of two enzymes involved in hepatic cholesterol homeostasis: the key enzyme of biosynthesis, 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, and the enzyme of cholesterol esterification, acyl coenzyme A:cholesterol acyltransferase (ACAT).

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Abbreviations: ACAT, acyl coenzyme A:cholesterol acyltransferase; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; TLC, thin-layer chromatography.

EXPERIMENTAL

Animals. Male and female frogs *Rana esculenta* (23.2 ± 5.5 g average body weight) kept in an outdoor terrarium were collected at random every month for one year. Animals were killed after anesthesia with 0.2% MS222 (tricaine methane sulphonate). Livers were removed rapidly and weighed. Chemical and biochemical assays were performed in duplicate on liver from 7-9 frogs for each sex.

Lipid and cholesterol content. Lipids were extracted in chlorophorm/methanol/water (0.8:2:1, v/v/v) according to the method of Bligh and Dyer (13), in the presence of 50 μg/ml BHT (2,6-di-tert-butyl-*p*-cresol) in order to avoid unsaturated fatty acid oxidation. An aliquot of chloroform phase was evaporated to dryness under a stream of N_2 and vacuum desiccated to constant weight over KOH for determination of total lipid content. Total and free cholesterol content in liver lipid extracts was determined on aliquots from the chloroform phase by the cholesterol esterase and cholesterol oxidase method using monotest High Performance and Test Combination reagent kits, supplied by Boehringer (Mannheim, Federal Republic of Germany). A sterol recovery of 82-90% was obtained, as determined by the addition of a known amount of specific compounds during the extraction procedure.

Preparation of microsomes. Liver tissue was homogenized in 5 volumes (w/v) of microsomal buffer (0.1 M sucrose/0.01 M KCl/0.03 M EDTA/0.004 M KH_2PO_4, pH 7.4) with a glass-Teflon Potter-Elvejhem homogenizer (3 strokes at moderate speed). The subcellular fractionation was carried out according to the method described by Erickson et al. (14) for rat liver. Briefly, the homogenate was centrifuged twice for 10 min at 12,000 × *g* and the supernatant centrifuged for 60 min at 105,000 × *g*. The microsomal pellet was resuspended in 1 ml of buffer by homogenization with a loose-fitting Teflon pestle.

Enzyme assays. HMG-CoA reductase was assayed by the radioisotope method reported by Erickson et al. (14), following the formation of mevalonic acid from [^{14}C]-3-hydroxy-3-methylglutaryl-CoA. Essentially, 80-120 μg frog liver microsomes were incubated in 0.2 ml microsomal buffer containing 10 mM dithiothreitol, 30 mM EDTA, 70 mM NaCl, 3 mM NADP and 1 enzyme unit of glucose-6-phosphate dehydrogenase. The mixture was preincubated 7 min at 37°C; 10 nmol [^{14}C]HMG-CoA (24,000 dpm/nmol) was added and the incubation continued for another 20 min. After alkaline hydrolysis of residual [^{14}C]HMG-CoA and addition of [^{3}H]mevalonate as the internal standard (10,000 dpm), labeled mevalonic acid formed was separated by Bio Rad AG1-X8 formate ion-exchange column chromatography (15). Recovery was never less than 60%. ACAT activity of aliquots of microsomes preincubated 90 min at 37°C was assayed by the radioisotope method of Dietschy et al. (16). Labeled cholesteryl oleate formed from 1-^{14}C-oleoyl-CoA (sp. act. 2700 dpm/nmol) during a 4-min incubation at 37°C was

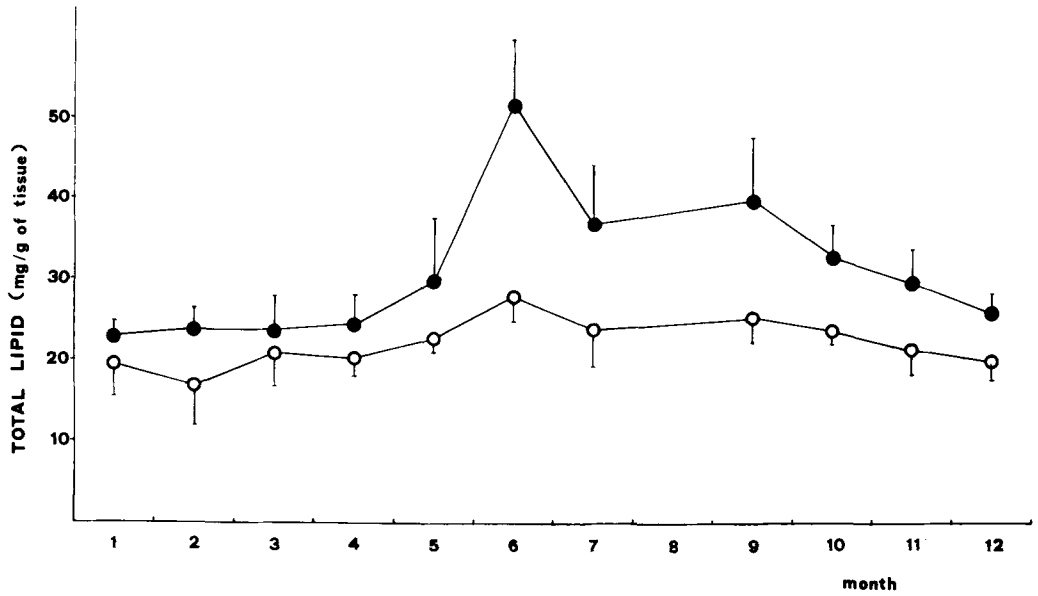

FIG. 1. Yearlong variations of total lipid content of male (○) and female (●) frog liver. Results are expressed as mg of lipids/g of liver wet weight, ± SD. Statistical details in the text.

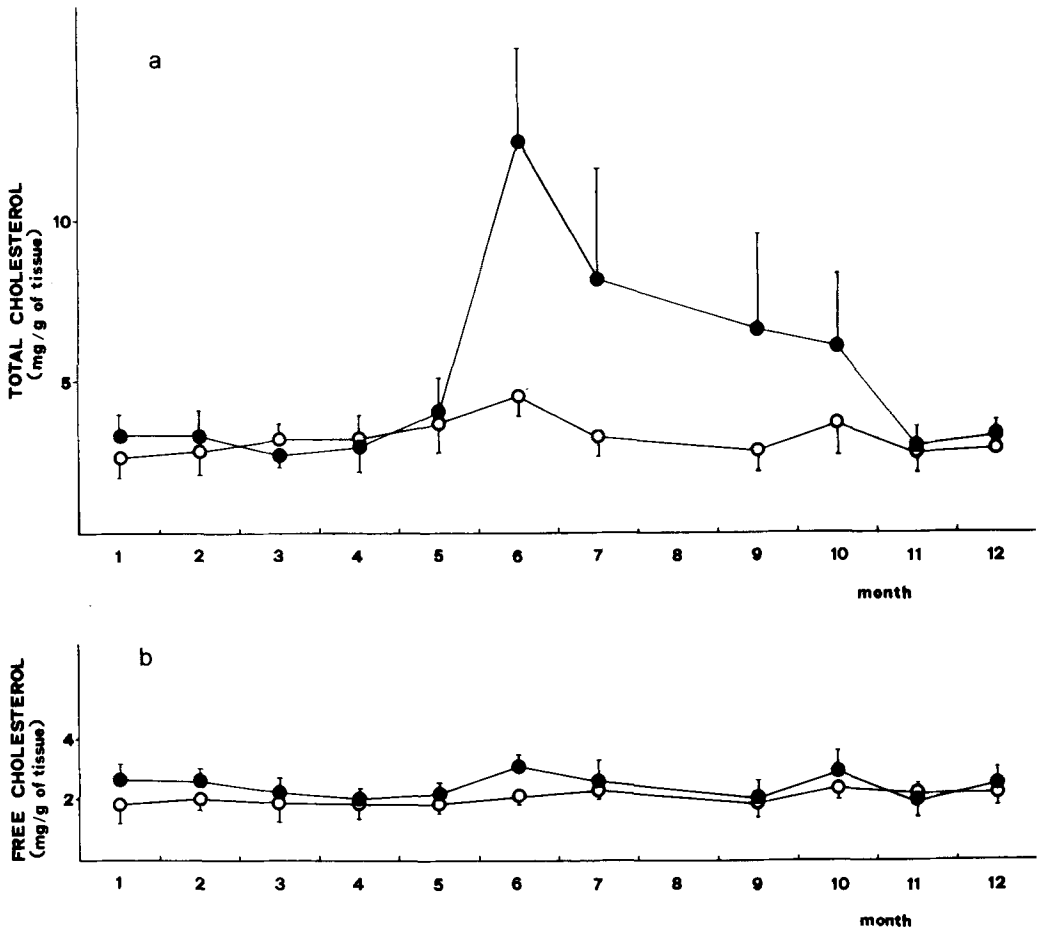

FIG. 2. Yearlong variations of total cholesterol (a) and free cholesterol (b) of male (○) and female (●) frog liver. Results are expressed as mg/g of liver wet weight, ± SD. Statistical details in the text.

extracted in chlorophorm/methanol (2:1, v/v) and separated by thin-layer chromatography in hexane/ethyl acetate (90:10, v/v). [^{3}H]Cholesterol was used as internal standard (8,000 dpm). Recovery was never less than 60%.

Radioactive compounds were obtained from Amersham.

Other assays. Proteins were measured according to Lowry et al. (17) using bovine serum albumin as standard.

Statistical analysis. Significance of data was determined by analysis of variance and Student's t test.

RESULTS AND DISCUSSION

Total lipid content of liver (Fig. 1) varied significantly throughout the year both in male and female frogs ($P < 0.005$ from the analysis of variance); furthermore, the females showed a significantly higher lipid content than males ($P < 0.05$ at least). The maximum lipid content was reached in June with 2.5- and 1.5-fold increases in females and males, respectively.

Total cholesterol content varied in a pattern comparable to that represented by total lipids ($P < 0.005$ from the analysis of variance in both sexes). From June to October, females showed a significantly higher total cholesterol content than did males (Fig. 2a). In contrast, free cholesterol was constant throughout the year in both sexes (Fig. 2b).

To establish whether fluctuations of cholesterol content during the year could be caused by a modified biosynthetic rate, the activity of HMG-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis, was assayed in liver microsomes on a monthly basis (Fig. 3). HMG-CoA reductase activity showed minimal fluctuations in male frogs, generally reflecting cholesterol content. HMG-CoA reductase activity in females seemed to vary more sharply with seasonal changes ($P < 0.05$ from the analysis of variance). Actually, its level increased in June, slightly decreased during summer, and showed another much higher peak in November. The fluctuation of HMG-CoA reductase activity/g of liver was confirmed by a similar profile of the specific activity (nmol/mg membrane protein) (Fig. 3, inset).

Whereas the June peak was in good correlation with the modification of liver cholesterol content, the later peak seemed unassociated with cholesterol storage.

The activity of the ACAT enzyme that catalyzes

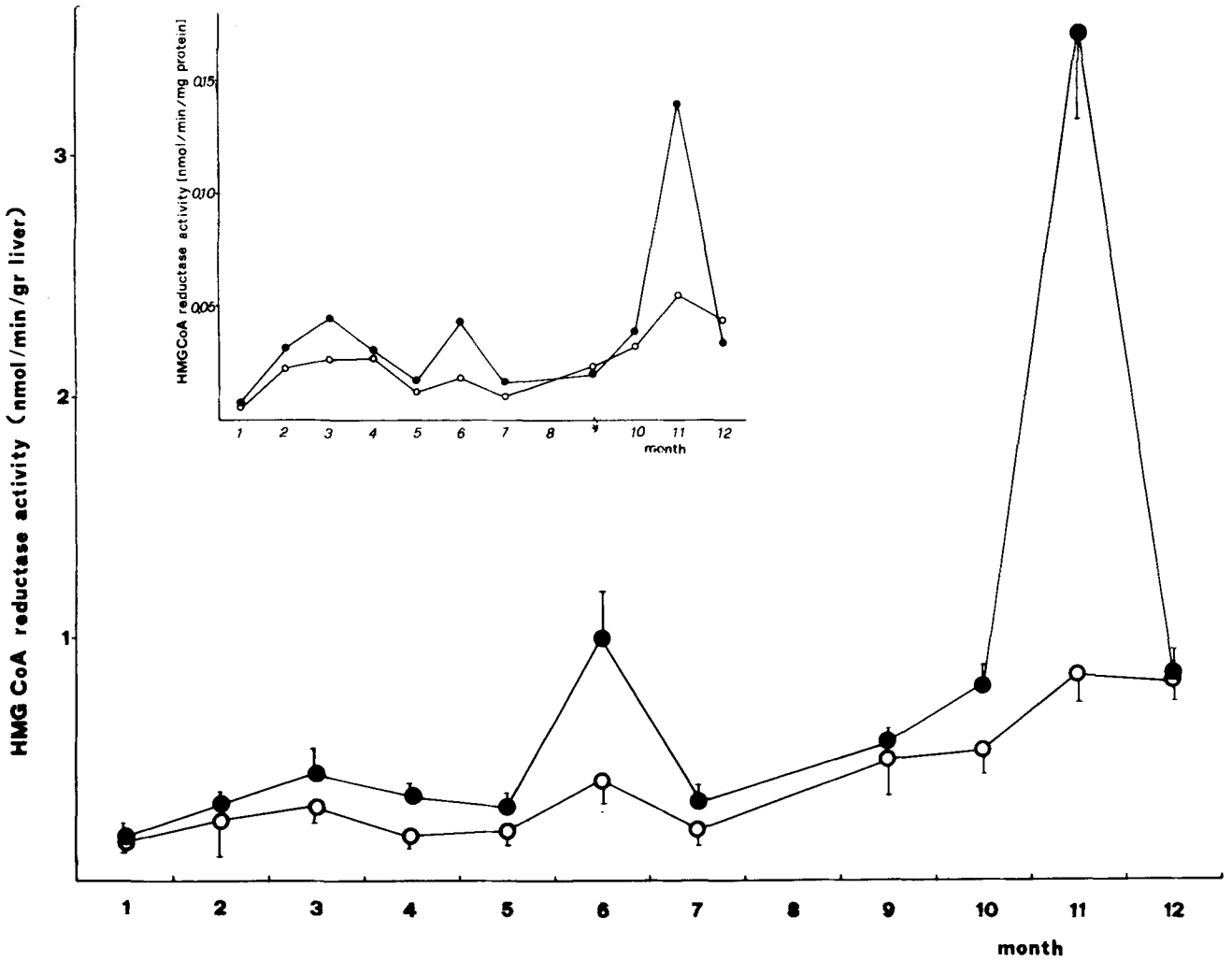

FIG. 3. Yearlong variations of HMG-CoA reductase activity in male (○) and female (●) frog liver. The results (the average of 4 separate preparations) are expressed as nmol mevalonate/min/g of liver wet weight, ±SD. In the inset, specific activity of the enzyme (nmol mevalonate/min/mg membrane protein) is shown. Statistical details in the text.

TABLE 1

ACAT Activity Measured in Male and Female Frog Liver in February, June and July

	Enzyme activity (nmol/min/g liver)	
Month	Female	Male
February	0.35	0.30
June	1.21	0.86
July	0.35	0.51

The results are expressed as nmol cholesteryl oleate/min/g of tissue (representative experiment).

cholesterol esterification, which was assayed during the three months in which cholesteryl ester content changed, showed different values for male and female frogs (Table 1). The activity was higher in females, but the pattern was similar in both. As cholesteryl ester content increased, ACAT showed greater activity.

Annual observations of sex differences have rarely been reported in poikilothermic vertebrates (18), and generally have been related to reproductive function (10).

This study found marked differences between male and female frogs in cholesterol content, biosynthesis and esterification activities during the one-year period of observation.

In male liver, the variations of these parameters were comparable, pointing out an increased synthesis of cholesterol stored as cholesteryl ester in summer. In females, on the other hand, fluctuations of cholesterol metabolism were more complex and two phases could be distinguished. In the first part of the year, cholesterol content, biosynthesis and esterification seemed interrelated, whereas in winter and particularly in November, such correlation was lost.

The high activity of HMG-CoA reductase detected in females in November could be related to the higher concentration of estrogens (10), hormones known to stimulate this HMG-CoA reductase activity (19). The higher reductase activity not accompanied by a high cholesterol content would suggest that the enzyme is producing cholesterol for export. The enhanced liver production of total RNA observed in the female *Rana esculenta* by Brachet et al. (7) in the same period of the year supports the idea that cholesterol is exported from the liver by some newly synthesized protein.

Because estrogens stimulate hepatic synthesis of essential products for oogenesis, such as the lipoprotein vitellogenin (20-22), we suggest that the extra peak of reductase activity could be related to the production of these compounds.

ACKNOWLEDGMENT

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Very Low Density Lipoprotein Secretion by Cultured Hepatocytes of Rabbits Fed Purified or Autoxidized Cholesterol

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The main objectives of this study were to compare the effects of dietary commercial cholesterol (containing 5% of oxidized cholesterol derivatives) and purified cholesterol on the secretion rate of very low density lipoprotein apolipoproteins and lipids by cultured rabbit hepatocytes and to verify the hypothesis that products of cholesterol autoxidation stimulate the rapid development of hypercholesterolemia. Rabbits fed dietary (old) commercial cholesterol for six weeks showed a fivefold increase in the serum concentration of cholesterol compared with that in purified cholesterol-fed rabbits. The secretion rates of very low density lipoprotein total protein and very low density lipoprotein [^{3}H]apolipoproteins were similar for the hepatocytes of these two cholesterol-fed groups of animals and were two- and threefold greater, respectively, than for cells from control rabbits. Cholesteryl ester content of the hepatocytes from dietary (old) commercial cholesterol-fed rabbits was dramatically increased in comparison with hepatocytes from control and purified cholesterol-fed rabbits. The elevated intracellular cholesteryl ester content is assumed to account for such an increase of very low density lipoprotein-cholesteryl ester secretion by cells prepared from dietary (old) commercial cholesterol-fed rabbits. These effects appear to be caused by activation of cholesterol esterification by oxidized cholesterol derivatives. The rapid development of hypercholesterolemia induced by dietary (old) commercial cholesterol is associated, at least in part, with the stimulated production of hepatic very low density lipoprotein apolipoproteins and cholesteryl esters.
Lipids 24, 109–115 (1989).

Cholesterol-rich diet induces in rabbits a marked elevation in plasma cholesterol level, particularly for cholesteryl esters that are associated with β-very low density lipoproteins (VLDL), which are also enriched in apo-E and apo-B (1). The accumulation of these particles is explained by the combination of a decrease of hepatic clearance (2,3) and hepatic and/or intestinal overproduction (4–7). It has been shown previously that cholesterol-feeding raises the cholesterol pool in the liver (8). The accumulation of hepatic cholesterol leads to a suppression of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity, thus blocking cholesterol synthesis, and to suppressed production of low density lipoprotein (LDL) receptors (3). Recent studies have shown that a cholesterol diet also increases the rate of apo-E synthesis in rabbit liver (9) and that cholesteryl-ester content of VLDL secreted by perfused liver may be increased significantly as well (7).

These hepatic effects of dietary cholesterol are not conclusive evidence that it acts directly as a regulatory molecule (10,11). Oxidized cholesterol derivatives (OCD) may arise from spontaneous, nonenzymatic oxidation of dietary cholesterol (12,13) or may be of natural origin, occurring as a product of hepatic cholesterol catabolism (14–17). Feeding rabbits with purified cholesterol (PCh) caused a substantial delay (1–1.5 months) in the development of high hypercholesterolemia as compared with animals that received an equal dose of cholesterol that contained OCD (18).

Clarification of the mechanism of the development of hypercholesterolemia induced by oxysterol-rich cholesterol is very important, because the isolation of these substances from a variety of foods has been documented (13), and their presence in cholesterol-rich diet has been linked to the aetiology of atheromata (12,19).

In the present study, the secretion rates of lipids and apolipoproteins in the VLDL fraction by hepatocytes from rabbits fed OCh or cholesterol purified from its autoxidation products were compared to estimate the extent to which hepatic VLDL production is responsible for highly elevated cholesterol plasma concentration.

MATERIALS AND METHODS

Materials. All chemicals used were reagent grade. Minimal essential medium (MEM) with Earle's salts, fetal calf serum (FCS), MEM nonessential amino acids, kanamycin, L-glutamine and 35- to 100-mm culture dishes were purchased from Flow Laboratories, Inc. (Zwanenburg, The Netherlands). Collagenase, type IV, was a product of Sigma Chemical Co. (St. Louis, MO). L-[4,5-^{3}H]Leucine (sp. act. 135 Ci/mmol), L-[^{35}S]methionine (sp. act. >800 Ci/mmol) and [1-^{14}C]acetic acid, sodium salt (sp. act. 59 mCi/mmol) were purchased from Amersham International (Amersham, Buckinghamshire, U.K.). PCh was obtained from old USP cholesterol by two crystallizations from ethanol. USP cholesterol was purchased from Sigma Chemical Co. (St. Louis, MO) and stored at room temperature for ca. 5 years. Cholesterol purity was checked by high performance liquid chromatography (HPLC) using a DuPont 8800 (Du Pont Instruments, Wilmington, DE) liquid chromatograph equipped with ultraviolet (209 nm) and refractive index ERC-7510 (Erma Optical Works, Tokyo, Japan) detectors. A reversed-phase column (Altex, Berkeley, CA, Ultrasphere-ODS, 250 × 4.6 mm, 5 μ particles) and an isocratic method with eluting system of 5% H_2O/95% MeOH were used. PCh was stored at −20°C. The purity of samples checked by HPLC before and after each experiment was greater than 99%. Dietary commercial cholesterol (OCh) contained about 5% OCD, the majority being 7α- and 7β-hydroxycholesterol, 7-ketocholesterol, cholestane-3β, 5α, 6β-triol.

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Abbreviations: ACAT, acyl coenzyme A cholesterol-acyltransferase; EDTA, ethylenediamine tetraacetic acid; FCS, fetal calf serum; OCD, oxidized cholesterol derivatives; OCh, dietary (old) commercial cholesterol; PCh, purified cholesterol; SDS, sodium dodecylsulphate; (V)LDL, (very) low density lipoprotein; HPLC, high performance liquid chromatography; HPTLC, high performance thin layer chromatography; MEM, minimal essential medium.

PCh and OCh were solubilized in olive oil with a stainless steel paddle for 10 min at room temperature.

Animals. Male rabbits of the Chinchilla breed, weighing 2.5-3 kg, were used. Control animals were maintained on a laboratory rabbit chow and, in addition, received olive oil (0.5 ml/kg body wt) orally each day. One experimental group received a suspension of OCh in olive oil (0.2 g cholesterol/kg body weight); the other experimental group received an analogous suspension of PCh (0.2 g cholesterol/kg). After 1.5 months, the animals were killed and blood was collected on EDTA (0.1%). Plasma was separated immediately by centrifugation.

Cultured hepatocytes. Rabbit hepatocytes were prepared and plated after collagenase digestion by methods described in detail elsewhere (20). The cells were plated in 35-mm dishes at 2×10^5 cells/cm^2 in ME medium containing FCS, kanamycin (100 μg/ml) and nonessential amino acids (1%) and were maintained at 38°C in 95% air/5% CO_2 atmosphere. The cells were incubated for 24 hr under these conditions for measurement of VLDL secretion over the following 18 hr. A percentage viability of hepatocyte monolayer was determined by addition of 0.075 ml 0.4% (w/v) Trypan Blue in 1 ml of cultured medium. Monolayers were incubated for 5 min at room temperature, then Trypan Blue was removed and percentage viability was assessed.

Analytical methods. For studies of secretion rates of de novo synthesized VLDL apolipoproteins, cells were incubated with leucine-free or methionine-free ME media containing 1% of nonessential amino acids and [^{3}H]-leucine (40 μCi/ml) or [^{35}S]methionine (50 μCi/ml), respectively, for 18 hr. Then the medium was removed for analysis. Plasma VLDL and VLDL fractions secreted by rabbit hepatocytes were isolated by preparative ultracentrifugation of the plasma and media in cellulose propionate tubes (Beckman Instruments, Palo Alto, CA) at 40,000 rpm in a LP-42 Ti rotor (Beckman Instruments) for 2.5 hr at 18°C. VLDL were isolated from the centrifugation tubes at d > 1.006 (50 μl from the top) and were recentrifugated. Protein was determined by the method of Lowry et al. (21) using bovine albumin as a reference standard. For sodium dodecylsulphate (SDS)-polyacrylamide gel electrophoresis, aliquots of VLDL fractions were prepared as previously described (22). They were subjected to electrophoresis on polyacrylamide gel that contained 0.1% SDS (3-20% gradient polyacrylamide) and were stained with Coomassie Blue. The relative quantity of VLDL apolipoproteins in two cholesterol-fed groups as compared with control was estimated by scanning using an LKB 2202 Ultrascan laser densitometer. The isotope incorporation into apolipoprotein band was estimated by counting the gel slices. The gels were cut into 2-3 mm slices, placed into separate vials containing 30% H_2O_2 (w/w), incubated overnight at 70°C and 10 ml of scintillation mixture (Ready-Solv EP, Beckman Instruments) were added to the vials. The radioactivity was determined in a scintillation counter Rackbeta 11 1215 (LKB Wallac, Turku, Finland).

For studies of de novo lipid biosynthesis, cells were incubated with ME medium containing [^{14}C]acetate (5 μCi/ml, 0.1 mM) for 18 hr. Sonicated cells and VLDL fractions were extracted twice with 5 ml of a mixture of chloroform/methanol (2:1, v/v) (23). The organic phase was washed 3 times with water to remove ca. 99% of [^{14}C]-acetate and then dried under reduced pressure. In the preliminary experiments, the extraction recovery of [^{14}C]triglyceride added as a standard was 94%. Lipids in an aliquot of the chloroform/methanol extract were separated into classes by high performance thin layer chromatography (HPTLC) using silica gel plates (0.2 mm Merck, Darmstadt, FRG) developed in hexane/diethyl ether/acetic acid (70:30:2, v/v/v). Quantitative determination of neutral lipids was performed by a densitometry of HPTLC plates (24) with an automated Camag HPTLC/TLC Scanner (Muttens, Switzerland) connected with a recording integrator SP 4100 (Spectra Physics, Darmstadt, FRG) (25). Each determination was accompanied by a calibration using cholesteryl oleate, triolein and cholesterol as standards. The spots on silica gel plates containing the certain lipid species were scraped into counting vials and the radioactivity was assayed.

For the determination of a reuptake of secreted lipoproteins, cells were incubated with [^{35}S]methionine (250 μCi/100-mm culture dish) for 18 hr. The medium with labeled lipoproteins was dialyzed for 24 hr against an unlabeled medium and then was administered to hepatocytes or to 35-mm culture dishes without hepatocytes. After an 18 hr incubation, the medium taken from both groups was ultracentrifuged for the isolation of lipoproteins (d < 1.21 g/ml). Following the separation on SDS-polyacrylamide gel, the amount of ^{35}S in apolipoproteins B and E was quantified by a scintillation counting.

Plasma total cholesterol was assayed enzymatically using the Total Cholesterol Kit (Medix, Helsinki, Finland) by chemistry analyzer FP-901 (Labsystems, Oy, Finland). Ten μl of plasma or standard solution were combined with 1 ml of cholesterol enzymatic reagent and incubated at 37°C for 20 min. The absorbance at 500 nm was measured for each sample against a reagent blank.

Statistical analysis. Results were analysed with Student's t test and expressed as mean ±SD. Values of P < 0.05 (doubled-tailed) were considered significant.

RESULTS

Plasma cholesterol and VLDL apolipoprotein analysis. OCh-fed rabbits were characterized by a 15-fold increase of plasma cholesterol concentration (Table 1). On the other hand, PCh-fed rabbits manifested only a 3-fold augmentation of cholesterol level. These results agree well with those previously published (18).

Plasma VLDL apolipoproteins were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 1). The apolipoprotein qualitative composition of VLDL (d > 1.006 g/ml) for three experimental groups of animals was the same

TABLE 1

Plasma Cholesterol Concentration

	C	PCh	OCh
Cholesterol (mg/dl)	60 ± 8	180 ± 30[a]	900 ± 140[a,b]

[a] P < 0.01 vs control.

[b] P < 0.01 vs PCh.

C, Control; PCh, purified cholesterol; OCh, old commercial cholesterol. Values are means ±SD of 8 rabbits.

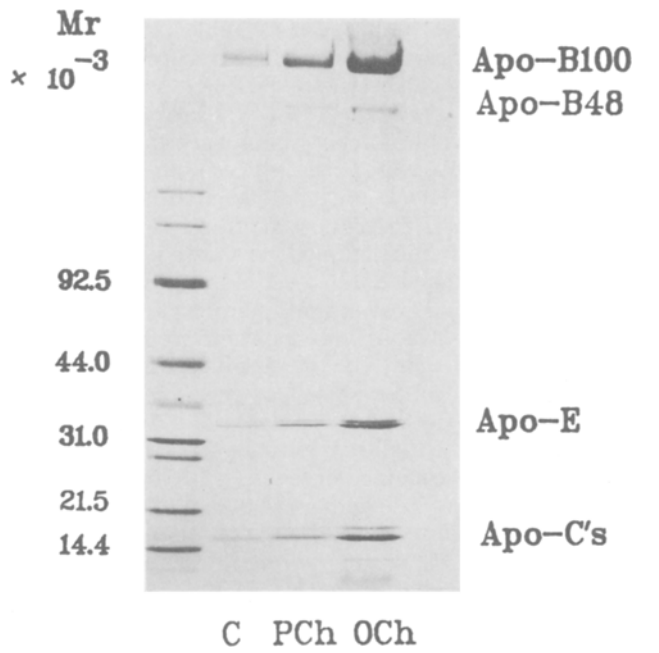

FIG. 1. SDS-polyacrylamide gel electrophoresis of plasma VLDL fractions obtained from control rabbits (C) and animals fed purified (PCh) and old commercial (OCh) cholesterol. The gel (3 to 20% gradient polyacrilamide) was loaded with VLDL (d < 1.006 g/ml) derived from an equal volume of plasma. It was stained with Coomassie Blue. The vertical axis indicates the molecular weights of the marker proteins.

TABLE 2

Plasma VLDL Apo-B-100 and Apo-E Concentrations

	Apo-B-100 (% from control)	Apo-E (% from control)
C	100 ± 12	100 ± 8
PCh	207 ± 15[a]	203 ± 32[a]
OCh	576 ± 104[a,b]	885 ± 127[a,b]

[a] $P < 0.01$, vs control.

[a,b] $P < 0.01$, vs PCh.

C, control; PCh, purified cholesterol; OCh, old commercial cholesterol. Values are means ±SD of 4 rabbits.

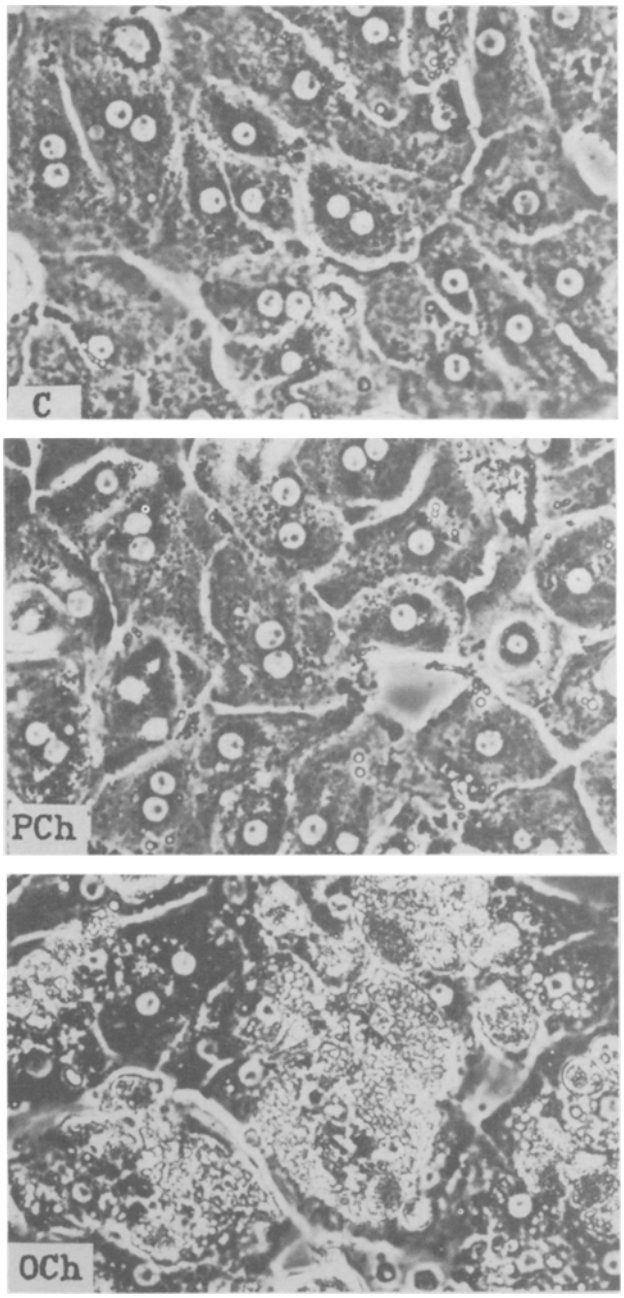

FIG. 2. Phase-contrast microscopy of hepatocytes obtained from control rabbits (C), animals fed old commercial cholesterol (OCh) and purified cholesterol (PCh) which have been cultured for 24 hr.

with respect to general apolipoproteins B-100, E and C. The analysis of polyacrylamide electrophoretic gel by densitometry showed a marked increase (6-to 10-fold, 4 experiments) in the levels of plasma VLDL apo-B-100 and apo-E of OCh-fed rabbits (Table 2). In contrast to this, plasma concentration of VLDL apolipoproteins of rabbits that received PCh was increased only by 2-fold (4 experiments) as compared with control.

Cultured hepatocytes and lipid content. There was no difference in the plating efficiency of the hepatocytes obtained from animals of three experimental groups (55 ± 7%, control; 50 ± 9%, OCh; 60 ± 9%, PCh; five different preparations in each group). The viability of hepatocyte monolayers after 24 hr in culture was greater than 90%. OCh-hepatocytes were loaded with lipids, which was clearly visible via phase-contrast microscopy (Fig. 2). The lipid inclusions in these cells were maintained throughout 5 days in primary culture. Cholesterol ester concentration (Table 3) was elevated in PCh-hepatocytes (230%, $p < 0.05$) and was dramatically increased (24-fold, $p < 0.05$) in OCh-hepatocytes. Triacylglycerol concentration had also risen in hepatocytes from both experimental groups (160%, $p < 0.05$; and 180%, $p < 0.05$).

Incorporation of [^{14}C]acetate into lipids. Cholesterol biosynthesis measured using [^{14}C]acetate was inhibited by 40–50% in hepatocytes from both groups of animals fed cholesterol (Table 4). Consistent with the immense 24-fold increase in cellular cholesteryl ester concentration

TABLE 3

Hepatocyte Cholesterol and Triacylglycerol Concentrations

Hepatocytes	Triacylglycerols	Cholesterol	Cholesterol esters
	μg lipid/mg cell protein		
C	35.4 ± 4.8	25.6 ± 3.1	39.2 ± 5.6
PCh	58.2 ± 6.1[a]	36.1 ± 6.1[a]	91.4 ± 12[a]
OCh	64.9 ± 7.2[a]	117 ± 18[a,b]	949 ± 120[a,b]

[a]P < 0.05, vs control.

[b]P < 0.05, vs PCh.

C, control; PCh, purified cholesterol; OCh, old commercial cholesterol. Values are means ± SD of 3 replicates for 4 experiments from each group.

TABLE 4

Effects of Dietary Purified and Commercial Cholesterol on the Incorporation of Sodium [^{14}C]Acetate into Lipids of Cultured Rabbit Hepatocytes

Hepatocytes	Triacylglycerols	Cholesterol	Cholesterol esters
	10^{-3} × cpm/mg cell protein		
C	340.4 ± 38.5	74.2 ± 7.6	4.1 ± 0.5
PCh	502.2 ± 69.8[a]	41.2 ± 5.2[a]	6.3 ± 0.7[a]
OCh	482.4 ± 64.4[a]	36.2 ± 5.4[a]	16.2 ± 3.1[a,b]

[a]P < 0.05, vs control.

[b]P < 0.05, vs PCh.

C, control; PCh, purified cholesterol; OCh, old commercial cholesterol. Values are means ± SD of 3 replicates for 3 experiments from each group.

(Table 3), OCh-hepatocytes revealed a 4-fold increase of [^{14}C]acetate incorporation (Table 4) into cholesteryl esters. In contrast, PCh-hepatocytes incorporated a relatively low (150% of control) amount of [^{14}C]acetate into cholesteryl ester. The incorporation of the labeled acetate into the triacylglycerol pool of cells obtained from two cholesterol-fed groups was also above control value (by 45%).

Secretion of VLDL lipids and apolipoproteins. The secretion rates of VLDL lipid and protein components were different for hepatocytes obtained from control and both cholesterol-fed rabbit groups (Table 5). PCh-hepatocytes secreted larger amounts of VLDL protein, triacylglycerol and free cholesterol (by 90, 90 and 30%, respectively), whereas cholesteryl ester secretion was not altered. Equal 2-fold increases have been demonstrated for VLDL total protein, triacylglycerol and cholesterol secreted by OCh-cells. Additionally, a 5.2-fold rise in content of released cholesteryl esters was found.

From Coomassie Blue-stained gel shown in Fig. 3 (left), it is readily seen that VLDL secreted by control cells contained apo-B, E and C as major apolipoproteins. Certain quantitative differences are apparent in the material secreted by cells from control animals and from two groups of cholesterol-fed animals. Secretion of all VLDL apolipoproteins by cells from cholesterol-fed animals was highly activated compared with control hepatocytes. Close results were obtained in several experiments. When [^{35}S]methionine or [^{3}H]leucine was added in medium and its incorporation into the various apolipoprotein bands was determined, it became evident that alteration in secretion of newly synthesized VLDL apolipoproteins actually took place (Fig. 3, right; Table 6). Figure 3 (right) shows the autoradiogram of SDS-polyacrylamide electrophoresis gel and the stimulation of [^{35}S]methionine incorporation into apo-B and apo-E for two experimental groups. The secretion rates of apo-B and apo-E as components of de novo synthesized VLDL were similar for PCh- and OCh-cells and surpassed those of control by ca. 3-fold (Table 6).

Reuptake of newly secreted apo-B and E. To examine the possibility that the results obtained may reflect certain changes in the reuptake of secreted VLDL, control and OCh-cells were incubated with [^{35}S]methionine-labeled secreted lipoproteins. After an 18 hr cultivation, the amount of [^{35}S]methionine-labeled apolipoproteins left in the medium was determined. The results shown in Table 7 demonstrate that cultured control and OCh-hepatocytes do not actively (at least not more than 10% of total apolipoproteins added) take up and degrade newly secreted apo-B and apo-E in the culture medium. Thus, the accumulation of VLDL in the medium of the primary culture of rabbit hepatocytes is purely the process of secretion.

DISCUSSION

Recently it was found that rabbits fed OCh during 1.5 months showed a marked (15-fold) elevation in plasma

TABLE 5

Rates of VLDL Protein and Lipid Secretion by Cultured Hepatocytes

Hepatocytes	Total protein	Triacylglycerol	Cholesterol	Cholesterol ester
	μg lipid/mg cell protein/18 hr			
C	2.01 ± 0.16	9.72 ± 1.08	1.98 ± 0.15	3.24 ± 0.66
PCh	3.82 ± 0.72[a]	18.14 ± 1.68[a]	2.85 ± 0.18[a]	3.36 ± 0.48
OCh	4.24 ± 0.81[a]	18.45 ± 2.45[a]	4.14 ± 0.69[a,b]	16.98 ± 1.77[a,b]

[a]P < 0.05, vs control.

[b]P < 0.05, vs PCh.

C, control; PCh, purified cholesterol; OCh, old commercial cholesterol. Values are means ± SD of 3 replicates for 4 experiments.

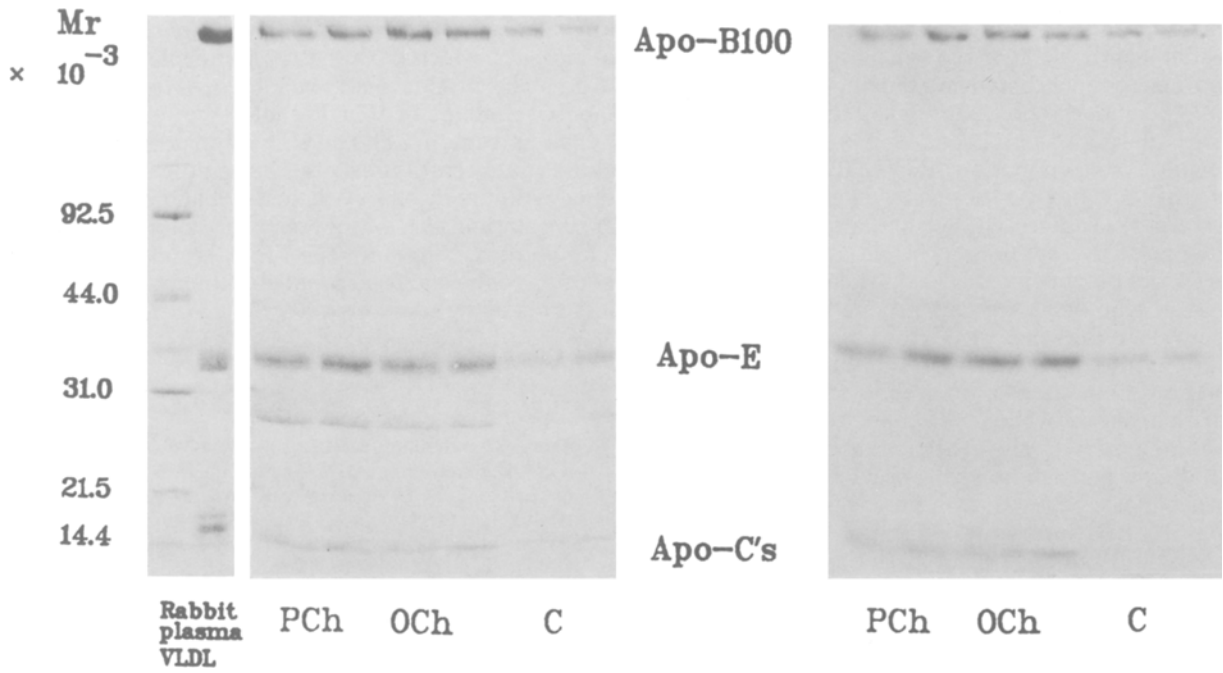

FIG. 3. (Left): SDS-polyacrylamide gel electrophoresis of VLDL fractions secreted by rabbit hepatocytes obtained from control rabbits (C), fed old commercial cholesterol (OCh) and purified cholesterol (PCh). The gel (3 to 20% gradient polyacrylamide) was loaded with VLDL fractions derived from an equal volume of medium (5 ml). This corresponded to VLDL secreted by 4.1 ± 0.4 mg cell protein. It was stained with Coomassie blue. The vertical axis indicates the molecular weights of the marker proteins. (Right): autoradiogram of newly synthesized and secreted VLDL apolipoproteins.

TABLE 6

Rates of VLDL Apolipoproteins Secretion by Cultured Hepatocytes

	Apo-B	Apo-E
	10^{-2} × cpm/mg cell protein	
C	36.7 ± 9.4	19.8 ± 2.8
PCh	95.8 ± 13.9[a]	57.5 ± 7.8[a]
OCh	117.4 ± 7.0[a]	56.2 ± 9.9[a]

[a]P < 0.05, vs control.

C, control; PCh, purified cholesterol; OCh, old commercial cholesterol. Values are means ± SD of 3 replicates for 5 experiments.

TABLE 7

Reuptake of Secreted Radiolabeled Apo-B and Apo-E by Control and OCh-Hepatocytes

	Apo-B	Apo-E
	cpm/ml culture medium	
A.		
Without cells	9816 ± 813	18460 ± 1640
Control cells	9112 ± 948	16856 ± 1240
B.		
Without cells	21636 ± 1426	46811 ± 2911
OCh-cells	19320 ± 912	41921 ± 3086

Values are means ± SD of 3 replicates.

cholesterol content, whereas the plasma cholesterol level of rabbits that received PCh was only 3-fold higher than in control animals (18). However, after a 3-month feeding of rabbits with PCh, their plasma level of cholesterol reached the same high values that were registered for OCh-fed animals. An addition of butylated hydroxytoluene to PCh inhibits the development of higher hypercholesterolemia (18). These results can be explained, considering the action of OCD that are present in OCh and might be formed during hepatic metabolism. Indeed, the liver has been shown to compensate for an increase of dietary cholesterol intake by suppressing its own cholesterol synthesis and by secreting more cholesterol in the bile (26). The presence of OCD in cholesterol-rich diet, which was found capable of blocking hepatic secretion of free cholesterol (27) and bile acid synthesis (28–31), can be responsible for the rapid increase in the hepatic cholesterol level and development of hypercholesterolemia in OCh-fed rabbits as compared with PCh-fed animals.

Data presented in this report demonstrate that, in OCh-fed rabbits the plasma concentrations of apo-B and E are increased by 3- to 4-fold as compared with those in PCh-fed animals. These results are in contrast to the changes found in primary cultures. In cultured hepatocytes prepared from PCh- and OCh-fed animals, the VLDL apolipoprotein accumulation rates were similar and elevated ca. 2-fold as compared with control animals. It is noteworthy that the accumulation of VLDL apo-B and E in a primary culture medium is due only to the process of secretion and is not due to a difference between the secretion and reuptake (Table 7). Earlier data procured for a primary culture of rat hepatocytes also demonstrated that only a small turnover of newly synthesized

lipoprotein components occurs and that a measurement of the rates of an accumulation in the medium does provide a reliable indication of the synthetic rates (32). The observed discrepancy between the in vivo and in vitro data could be understood taking into account that the in vivo accumulation of apo-B and E is not caused solely by the hepatic overproduction of β-VLDL, but also by the reduction of their uptake (2). It is well established that, in cholesterol-fed rabbits, the accumulation of cholesterol in the liver leads to a suppression of production of hepatic LDL receptors responsible for the uptake of β-VLDL (3). Although in this work we carried out no comparative studies on the uptake of β-VLDL by hepatocytes isolated from various dietary groups of animals, based on the published data (3), one can infer that hepatocytes overloaded with cholesterol should be characterized by a lower uptake compared with the other two groups of animals.

Our finding is consistent with a previous in vitro observation that dietary cholesterol increases rabbit hepatic secretion of VLDL protein (7). Earlier, Garcia et al. (9) (for rabbits) and Lin-Lee et al. (33) (for rats) have shown that cholesterol diet stimulates synthesis of apo-E in the liver. Such an increase in apo-E content in rats is mediated by an accumulation of translatable apo-E mRNA. Recently it has been reported that cholesterol feeding in rats also resulted in a several-fold increase in the level of apo-B mRNA in the liver (34). Thus, the overproduction of VLDL apolipoproteins may account for the augmentation in plasma VLDL apolipoprotein concentration caused by dietary PCh or OCh.

The significant increase in VLDL apolipoprotein secretion by hepatocytes prepared from OCh-fed rabbits compared with control cells interrelated with stimulation of VLDL neutral lipid secretion so that only an absolute increase in cholesteryl ester secretion occurs. An elevated level of VLDL cholesteryl ester was observed in the presence of the enhanced intracellular cholesteryl ester synthesis and dramatic accumulation of cholesteryl ester in cells. It has been previously reported that cholesterol feeding increases the hepatic acyl coenzyme A cholesterol-acyltransferase (ACAT) activity and stimulates VLDL cholesteryl ester secretion by perfused rabbit liver (7,35). Our data for the primary culture testify that the cholesteryl ester pool within hepatocytes is responsible for the stimulation of VLDL cholesteryl ester secretion. At the same time, we found no differences in the secretion rates of VLDL cholesteryl esters by hepatocytes of PCh-fed rabbits and those of control, although the cholesteryl ester pool in former hepatocytes was 2 to 3 times bigger than in control cells. It is possible that such unaltered cholesteryl ester secretion by these hepatocytes may be due to a relatively small intracellular cholesteryl ester pool and to the absence (under culture conditions) of additional input of free cholesterol derived in vivo from cholesterol-rich lipoproteins. In addition, intracellular cholesteryl ester pool can be utilized also for synthesis of bile acids and for secretion of free cholesterol (29). A profound rise in the rate of formation of cholesteryl esters and their rapid accumulation in hepatocytes from OCh-fed rabbits compared with cells from PCh-fed animals are probably associated with the increase in concentration of substrate cholesterol for ACAT and its stimulation by OCD (11). Earlier, it was shown that 25-hydroxycholesterol activated cholesteryl ester formation in isolated hepatocytes (36,37). The enhancement of this stimulatory effect was observed when cells were incubated with mevalonic acid, which is a substrate for cholesterol synthesis. Moreover, in this case, hepatocytes secreted an augmented amount of VLDL cholesteryl esters (36).

Taking together all the above considerations, we conclude that results presented here indicate that OCD induce rapid intracellular cholesterol and cholesteryl ester accumulation in hepatocytes of rabbits receiving OCh. This, in turn, stimulates the VLDL secretion that, at least partly, could evoke a rapid development of hypercholesterolemia.

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Analysis of Seed Oils Containing Cyclopentenyl Fatty Acids by Combined Chromatographic Procedures

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The fatty acids of seed oils of the Flacourtiaceae, *Hydnocarpus anthelmintica, Caloncoba echinata* and *Taraktogenus kurzii,* have been examined by a combination of capillary gas chromatography, silver ion high performance liquid chromatography and gas chromatography–mass spectrometry. In addition to the common range of cyclopentenyl fatty acids found in such oils, 13-cyclopent-2-enyltridec-4-enoic acid was a major component of *H. anthelmintica* and was identified by mass spectrometry as its picolinyl ester and dimethyldisulphide adduct. It has not previously been found in nature. In the other seed oils, the isolated double bond in the corresponding fatty acid was in position 6, as expected. Similarly, *cis*-4-hexadecenoic acid and C_{16} and C_{18} cyclopentyl fatty acids were identified for the first time in *H. anthelmintica. Iso-* and *anteiso*-methylbranched fatty acids were present in trace amounts.
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The seed oils of many species of plants of the family Flacourtiaceae are known to contain fatty acids with terminal cyclopent-2-enyl rings (1–3). They have been used for centuries in traditional medicine as a treatment for leprosy. The analysis of these oils by gas chromatography (GC) on packed columns, especially with polar liquid phases, is not straightforward, because cyclic and straight-chain components overlap, and it is necessary to use silver ion thin layer chromatography as a complementary technique to obtain adequate resolution of all the components (4,5). In this study, three seed oils have been examined and it is confirmed that GC with wall-coated open tubules (WCOT) columns of fused silica with a variety of different liquid phases resolves all the main components without difficulty (cf [3]). Silver ion high performance liquid chromatography (HPLC) of the methyl esters followed by GC–mass spectrometry (MS) of the picolinyl ester derivatives, a combination that has been proven to be of value with other natural lipid samples (6), permitted the identification of the individual fatty acid components including several that were hitherto not known.

MATERIALS AND METHODS

The authenticated seeds of *Hydnocarpus anthelmintica, Caloncoba echinata* and *Taraktogenus kurzii* were obtained from Dr. N. V. Bringi of Bombay and Dr. S. R. Shukla, Pharmacology Department, MLN Medical College, Allahabad, India. They were extracted with hexane.

Methyl ester derivatives of the fatty acids were prepared by sodium methoxide-catalyzed transesterification (7). A portion was hydrogenated with platinum oxide as catalyst (8).

For analytical purposes, a Carlo Erba Model 4130 capillary gas chromatograph (Erba Science, Swindon, U.K.), fitted with split/splitless injection and equipped with a capillary column (0.25 mm i.d. × 25 m in length) of fused silica coated with Silar 5CP (Chrompak Ltd, London, U.K.), was used with the methyl ester derivatives. After holding the temperature at 155°C for 3 min, it was temperature-programmed to 195°C at 4°C/min, then, was held at this point for a further 17 min. Hydrogen was the carrier gas. Components were quantified by electronic integration.

A Spectra-Physics Model 8700 solvent delivery system was used in separations by HPLC (Spectra-Physics Ltd., St. Albans, U.K.), together with an ACS Model 750/14 mass detector (Applied Chromatography Systems, Macclesfield, U.K.). A stream-splitter (ca. 10:1) was inserted between the column and the detector. A column (4.6 mm × 250 mm) of Partisil 10SCX (Hichrom Ltd, Reading, U.K.) was utilized in the silver ion form as described elsewhere (9). One to 2 mg of methyl esters were applied to the column in 10 μl of dichloroethane. Gradient elution was used and the solvent reservoirs contained dichloroethane/dichloromethane (1:1, v/v) (A) and dichloroethane/dichloromethane/acetonitrile/methanol (20:20:1:1, v/v/v/v) (B). The gradient was changed over 40 min from 100% A to 50% A − 50% B; the flow rate was 1 ml/min.

After analysis of the fractions by GC, they were hydrolyzed to the free acids before conversion to the picolinyl ester derivatives as described elsewhere (10). These were submitted to electron-impact GC–MS, as described earlier, on a capillary column coated with a 5% phenylmethylsilicone phase (except that the upper temperature of the column was 10°C lower) (11).

A portion of the final dienoic fraction from each sample was converted to the dimethyldisulphide adduct by reaction with dimethyldisulphide in the presence of iodine as catalyst as described by Francis (12). It was subjected to GC–MS under the same conditions as the picolinyl ester derivatives, although it eluted somewhat later. A further aliquot was subjected to permanganate-periodate oxidation (13), the products were esterified by acid-catalyzed methanolyis and analysed by GC–MS.

RESULTS

The methyl ester derivatives prepared from the seed oil of *H. anthelmintica* were initially examined by GC on a capillary column of fused silica coated with Silar 5CP and the peaks expected from the cyclopentenyl fatty acids were all well resolved from the normal straight-chain components, as shown in Figure 1. The C_{16} cyclopentenyl fatty acid, hydnocarpic acid, elutes before 18:0, whereas the C_{18} cyclopentenyl compounds, chaulmoogric and gorlic acids, elute after 18:2, but well before any C_{20} straight-chain fatty acids. Very similar results were obtained on a column of Carbowax 20M, as reported earlier

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Abbreviations: GC, gas chromatography; HPLC, high performance liquid chromatography; MS, mass spectrometry.

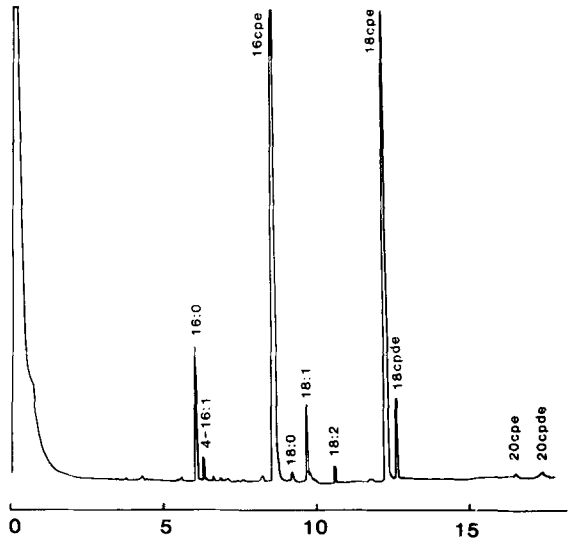

FIG. 1. Separation of the methyl ester derivatives of the fatty acids of *H. anthelmintica* on a WCOT column of Silar 5CP (see Experimental section for conditions). Abbreviations: 16cpe, 11-hendecylcyclopent-2-enoate; 18cpe, 13-tridecylcyclopent-2-enoate; 18cpde, 13-tridec-4-enylcyclopent-2-enoate; 20cpe, 15-pentadecylcyclopent-2-enoate; 20cpde, 15-pentadec-9-enylcyclopent-2-enoate.

by others (3). On a more polar phase, CP-Sil 84 (broadly equivalent in polarity to diethylglycolsuccinate polyester [DEGS]) hydnocarpic acid elutes just after 18:0, but is clearly separated from 18:1. There were a number of minor components (each less than 0.1%) which were not immediately identifiable. In contrast to previous experience with packed columns (4,14), it appears that cyclopentenyl seed oils are readily resolved on polar phases in capillary columns. The three seed oils each seemed to contain similar fatty acids, although the relative proportions in each were very different, and the results of the quantitative analyses are presented in Table 1.

In order to confirm the identities of the various components, the methyl ester derivatives from *H. anthelmintica* were first separated by silver ion HPLC, as shown in Figure 2, from which 5 fractions were obtained. These were each hydrolyzed and converted to the picolinyl ester derivatives for exmination by GC-MS. Prefractionation in this way ensures adequate resolution on the nonpolar GC phase required to elute picolinyl esters in a reasonable time (6). The saturated fraction consists mainly of 16:0, with smaller amounts of 14:0, 15:0, 17:0 and 18:0. In addition and rather surprisingly, small amounts of 13- and 12-methyl-14:0, 14-methyl-15:0 and 15- and 14-methyl-16:0, and two cyclopentyl fatty acids were found. The branched-chain components are rather easily identified as picolinyl ester derivatives, as described by Harvey (15), as a gap of 28 amu is seen in the regular series of ions for successive methylene groups for loss of the carbon containing the methyl group. The spectra compare well with those of authentic standards. 11-Cyclopentylhendecanoate and 13-cyclopentyltridecanoate were recognized from their mass spectra, which have good molecular ions, a gap of 69 amu for the loss of the cyclopentyl ring (together with a substantial ion at m/z = 69), and a regular series of ions 14 amu apart for fragmentations

TABLE 1

Fatty Acid Compositions (wt %) of Cyclopentene-containing Seed Oils

Fatty acid	*H. anthelmintica*	*C. echinata*	*T. kurtzii*
14:0	0.08		0.09
14i	tr		tr
14ai	tr		
15:0	0.20		0.11
15i	0.07		0.11
16:0	4.61	8.24	6.37
16i	0.13	0.12	0.13
16ai	0.11	0.18	0.20
16:1(n-12)	1.01		
16:1(n-7)	0.18	0.93	4.16
17:0	0.06		0.05
16cpa	0.06		tr
16cpe	59.60	1.24	33.86
18:0	0.42	0.17	0.31
18:1(n-9)	2.37	0.78	1.85
18:1(n-7)	tr		1.51
18:2(n-6)	0.82	1.33	1.14
18cpe	25.95	63.11	27.55
18cpde	3.37	23.27	21.18
20cpe	0.30	tr	0.11
20cpde	0.19	0.36	0.17

Abbreviations: i, iso-; ai, anteiso; cpa, cyclopentyl ring; cpe, cyclopent-2-enyl ring; cpde, cyclopent-2-enyl ring and a double bond in the aliphatic chain; tr, trace.

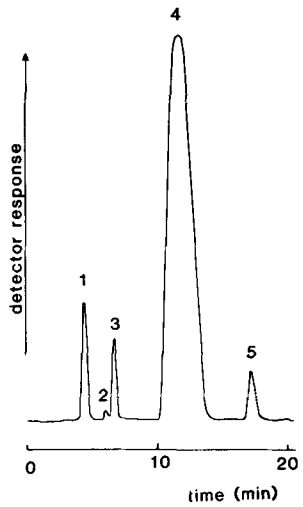

FIG. 2. Silver ion HPLC trace of the methyl esters of the fatty acids of *H. anthelmintica*. See Experimental section for conditions. Fraction 1 = saturated, 2 = 4-16:1, 3 = 9-18:1, 4 = cyclopentenoic fatty acids predominantly, 5 = as last, but with an additional double bond in the alkyl-chain.

between successive methylene groups in the chain. In addition, the spectra of the compounds and their GC retention times are identical to those of authentic derivatives prepared by hydrogenation of the oils.

The second fraction was a minor one and consisted mainly of 4-16:1. It has the *cis*-configuration from its retention time on the silver ion column. This particular isomer is distinguished from others by its mass spectrum

as the picolinyl ester in that it is the only one not to have readily identifiable fragmentations at the double bond and to have its base peak at m/z = 151; there is also a characteristic ion at m/z = 218 (16). The spectrum is very similar to that of authentic 4-18:1 and quite different from that of adjacent isomers. A further trace constituent in this fraction gave too noisy a spectrum for unequivocal identification, but it was consistent with that expected for 4-15:1.

The third fraction was mainly a 9-18:1 fatty acid, but there were also small amounts of 9-16:1, 9-17:1 and 9-19:1. Again, the mass spectral identification of the picolinyl esters is rather simple (15,16).

The fourth fraction contained predominantly cyclopentenyl fatty acids with some 18:2(n-6). The mass spectrum of 11-cyclopent-2-enyl-hendecanoate (hydnocarpate) is illustrated in Figure 3. There is an abundant molecular ion at m/z = 343, and the usual ions containing the pyridine ring at m/z = 92, 108, 151 and 164. The base ion at m/z = 67 presumably is the cyclopentenyl ring, and there is a significant complementary ion, representing $(M - 67)^+$ at m/z = 276. Again, there are ions 14 amu apart representing cleavage between the successive methylene groups. The C_{18} and C_{20} isomers have analogous spectra.

The mass spectrum of the main component of the fifth fraction is illustrated in Figure 4 and was not as expected for "gorlic acid," i.e., 13-cyclopent-2-enyltridec-6-enoate, but could be interpreted as having a double bond in position 4; the molecular ion is at m/z = 369, as expected, and the base peak is at m/z = 67, confirming the presence of the cyclopentenyl ring. There are no ions that can be readily assigned to fragmentation at a double bond in the aliphatic chain, although the ion at m/z = 151 is particularly prominent (60% of the base peak), as in the derivatives of fatty acids with an isolated double bond in position 4 (cf. the 16:1 straight-chain isomer). In contrast, the corresponding fractions from *C. echinata* and *T. kurzii* contained the true 6 isomer, and the mass spectrum of its picolinyl ester derivative is illustrated in Figure 5. Ions for fragmentation at the cyclopentenyl ring at m/z = 67 and 302 are as expected. In addition, there is a substantial ion at m/z = 246, and a gap of 26 amu between m/z = 192 and 218, all characteristic of an isolated double bond in position 6 (16). Similarly in all the oils, the analogous C_{20} homologue, "hormelic" acid, has the isolated double bond in position 9, because the mass spectra of the picolinyl ester derivatives all have a characteristic doublet of prominent ions at m/z = 274 and 288, together with ions expected for fragmentation on either side of the double bond (15,16).

In order to confirm the position of the isolated double bond in the C_{18} dienoic component from *H. anthelmintica*, the methyl ester derivative was converted to the *bis*-dimethyldisulphide adduct, as these are simple to prepare and have excellent mass spectral fragmentation properties (12). The reaction gave several products, when examined by GC-MS, but the last and most abundant component to elute had the correct molecular weight (m/z = 480) and the complete spectrum is illustrated in Figure 6. There is a major fragment (37%) at m/z = 147, representing cleavage between carbons 4 and 5 and containing the carboxyl group, and one at m/z = 115 (147 − methanol), as expected for an adduct of a double bond in position 4 (12). In the high mass range, there were ions at m/z = 432 (M-48, loss of CH_3SH presumably from the ring). Therefore, although an expected ion at m/z = 333 (containing carbon 5 and the remainder of the molecule) is not seen, there is one at m/z = 285 (333 − 48) (43%). If the molecule loses both sulphur moieties from the ring,

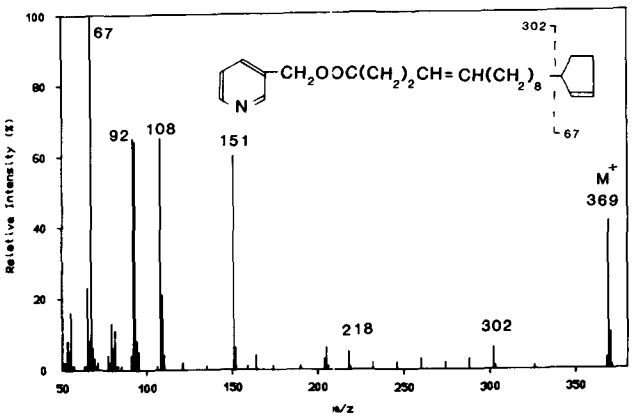

FIG. 4. Mass spectrum of the picolinyl ester derivative of 13-cyclopent-2-enyl-tridec-4-enoic acid.

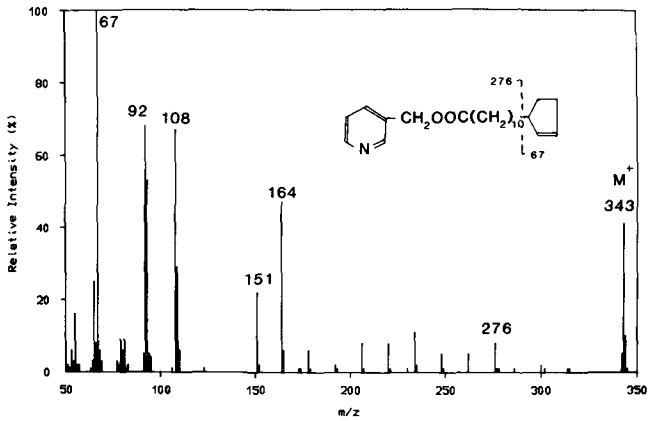

FIG. 3. Mass spectrum of the picolinyl ester derivative of hydnocarpic acid.

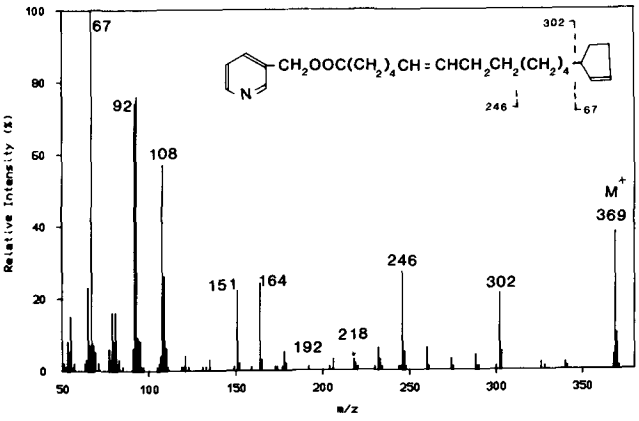

FIG. 5. Mass spectrum of the picolinyl ester derivative of 13-cyclopent-2-enyl-tridec-6-enoic acid (gorlic acid).

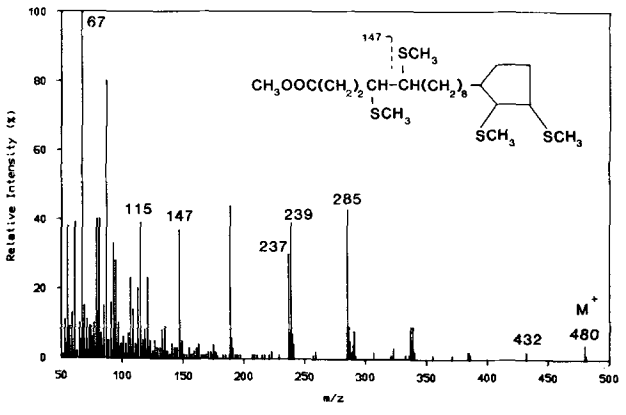

FIG. 6. Mass spectrum of the *bis*-dimethyldisulphide adduct of methyl 13-cyclopent-2-enyl-tridec-4-enoate.

i.e., to $m/z = 384$, loss of 147 gives an ion at $m/z = 237$, and this also is abundant (30%). If the two sulphur atoms are expelled together, an ion at $m/z = 239$ is expected and this is similarly prominent (39%). It is evident that the sulphur atoms are readily lost from the cyclopentenyl ring under electron impact, because the base peak is at $m/z = 67$, as in a nonadducted cyclopentenyl fatty acid derivative. These mass spectral assignments were confirmed by examining the mass spectrum of the dimethyldisulphide derivative of the 6-isomer from *T. kurzii*. In this the ion at $m/z = 147$ is shifted to 175 (37%), i.e., plus 28 amu, and that at 115 becomes 143 (85%). The ions containing the ring at $m/z = 285$ and 237, in the spectrum of the 4-isomer, are shifted to 257 (47%) and 209 (44%) respectively, i.e., a loss of 28 amu. An unidentified ion at $m/z = 189$, in the spectrum of the 4-isomer, is shifted to 161 in that of the 6-isomer, so is presumably derived from the ring portion of the molecule.

Permanganate-periodate oxidation of the diene from *H. anthelmintica* gave a compound with a mass spectrum consistent with that expected from the methyl ester derivative of a C_{14} keto dibasic acid, i.e., a molecular ion at $m/z = 300$ (5%) with $[M-1]^+$ also abundant (30%). There are also substantial ions at $m/z = 128$ (73%), 152 (35%), 160 (66%), 184 (45%), 207 (20%), 225 (20%) and 239 (20%). The spectrum of the corresponding compound from the cyclic diene of *T. kurzii* has a molecular ion at $m/z = 272$ (4%), with $[M-1]^+$ at 271 (26%) and substantial ions also at 128 and 160, among others. A keto dibasic acid can only be formed if the double bond in the ring is in position 2, although the complexities of the spectra are such that the position of the keto group cannot be immediately confirmed. The dibasic acid fragments, which should also be formed during the oxidation, were apparently lost on work-up and were not detected.

It, therefore, appears that the principal cyclic dienoic fatty acids in *H. anthelmintica* is cyclopent-2-enyltridec-4-enoic acid, not the 6-isomer as reported earlier (5).

DISCUSSION

The novelty of much of the data acquired in this study from samples that had been examined to some extent before (5) confirms the value of using silver ion HPLC to provide simplified fractions and GC-MS of the picolinyl esters to determine fatty acid structures. This approach had earlier proven its worth in analyses of lipids from marine invertebrates (6). In particular, it facilitates unequivocal identification of components present in samples at trace levels. Picolinyl esters per se have now been used for identification purposes with a number of natural samples as opposed to model compounds in GC-MS (10,11,17-19). Here, it is shown that they provide the essential structural details for a variety of different cyclopentenyl fatty acids. A double bond in position 4, as was the case in this study, is probably the least easy to locate, but access to authentic models eliminated much of the problem and the alternative approach of preparing chemical adducts provided final confirmation. Previously, mass spectra of methyl esters of cyclopentenyl fatty acids were shown to give valuable information on the nature of the cyclic moiety, but not of double bonds (20). Pyrrolidides gave information on both the position of the double bonds and the ring structure (21), but the spectra are not as easily interpreted as are those of picolinyl esters.

Cyclopent-2-enyltridec-4-enoate does not appear to have been found before in nature, although an analogous C_{16} fatty acid has been detected (5). Presumably there is a biosynthetic relationship to *cis*-4-hexadecenoic acid, with which it occurs in *H. anthelmintica*. This acid also does not appear to have been found in a seed oil, or elsewhere in nature, although C_{10} to C_{14} homologues were detected in seed oils of the Lauraceae (22). Although they were only present in small amounts, the C_{16} and C_{18} fatty acids containing a cyclopentyl (saturated) ring have not previously been reported from plant sources, nor are they known to occur naturally elsewhere, although they can be produced in bacteria supplied with suitable precursors (23,24). Trace levels of five branched-chain (*iso* and *anteiso*) were detected and have only been found in one seed oil previously (25). It is not impossible that the last could be products of bacterial contamination of the seed or fruit.

In *H. anthelmintica*, there is no evidence for a C_{18} monoene with a double bond in position 4, whereas the C_{20} cyclopentenyl fatty acid with the double bond in the aliphatic chain is the 9-enoic isomer ("hormelic" acid).

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Capillary Gas Chromatography and Tandem Mass Spectrometry of Paf-acether and Analogs: Absence of 1-*O*-Alkyl-2-propionyl-*sn*-glycero-3-phosphocholine in Human Polymorphonuclear Neutrophils

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Fast atom bombardment-tandem mass spectrometry was used to identify molecular species of paf-acether (paf) produced by human polymorphonuclear neutrophils. Using this biological material, normal phase high performance liquid chromatography was necessary prior to the fast atom bombardment-tandem mass spectrometry step. Gas liquid chromatography/electron capture detection after hydrolysis with phospholipase C and conversion to heptafluorobutyrate derivatives was used to confirm the results. The results indicated the presence of mainly 1-*O*-hexadecyl/octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine, acyl analogs of paf and only trace amounts of other alkyl analogs of paf. We did not detect the 2-propionyl analog of paf. Moreover, supplementation of human polymorphonuclear neutrophils with sodium propionate did not result in formation of the 2-propionyl analog of paf.
Lipids 24, 121-124 (1989).

Paf-acether (paf), a mediator of anaphylaxis and inflammation and an antihypertensive agent (1-4) has the following structure: 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (AAGPC) (5-7). It is synthesized by a variety of cells: macrophages, monocytes, polymorphonuclear leukocytes, platelets, exocrine secretory glands, endothelial cells and also in organs such as heart and kidney (2,3,8,9). Its biological activity requires an *O*-alkyl moiety at the *sn*-1 position of the molecule, a short acyl chain at the *sn*-2 position and a phosphocholine at the *sn*-3 position of glycerol (10-19). Advanced separation techniques have demonstrated that paf produced by human polymorphonuclear neutrophils (PMN) is composed of primarily (80%) 1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (16:0-paf), but also 1-*O*-octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (18:0-paf) (20%). A least 14 molecular species of phospholipids containing saturated or unsaturated alkyl or acyl chains have been described as paf analogs (7,20,21). Using synthetic compounds, several investigators indicated that the 2-propionyl analog of paf (APGPC) is as active as paf itself in inducing platelet activation (12,14,16-18), PMN degranulation (17) and hypotension (14), whereas the butyryl and hexanoyl analogs are much less active. In 1982, Ninio et al. (22) demonstrated that, when the acetyl-CoA substrate was replaced by propionyl-CoA in lysates from murine macrophages, an ether lipid was produced that was as potent an aggregating agent as paf. Lee (23) confirmed this result showing that the (^{3}H)propionyl-CoA was effectively incorporated into lyso paf in rat spleen microsomes. Tokumara et al. (24) have shown the presence in bovine brain of ten 1-acyl analogs of paf including species having an *sn*-2 propionyl/butyryl or acetyl group. But the presence of paf analogs such as 1-alkyl-2-propionyl/butyryl derivatives in biological systems has not yet been demonstrated.

The aim of this work was the identification of paf elicited from stimulated PMN, and the focus was upon the search for 2-propionyl analogs. Accordingly, direct mass spectrometry analysis of intact native molecular species of paf appeared essential. To eliminate matrix interferences, family members of paf derivatives were isolated by liquid chromatography. Application of fast atom bombardment-mass spectrometry (FAB-MS) resulted in limited fragmentation of compounds, but failed to identify paf derivatives mixed in the chromatographic fractions. The combination of FAB and tandem mass spectrometry (MS-MS) resulted in formation characteristic daughter ions generated from stable and metastable ions (parent ions), thus, increasing the specificity and allowing the identification. In parallel, paf was quantified by gas liquid chromatography-electron capture detection (GC-ECD), after hydrolysis with phospholipase C and conversion to the heptafluorobutyrate derivative (25), and by classical biological methods, as well (1,26).

MATERIALS AND METHODS

Reagents. 1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (16:0-paf), 1-*O*-octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (18:0-paf) and 1-*O*-octadecenyl-2-acetyl-*sn*-glycero-3-phosphocholine (18:1-AAGPC) were purchased from Novabiochem (Laùfelchingen, Switzerland). 1-*O*-hexadecyl-2-propionyl-*sn*-glycero-3-phosphocholine (16:0-APGPC) and 1-*O*-octadecyl-2-propionyl-*sn*-glycero-3-phosphocholine (18:0-APGPC) were purchased from Seratec (Epinay/Seine, France). Phospholipase C from *Clostridium welchii,* fatty acid-free bovine serum albumin (BSA), sodium propionate and acetate and heptafluorobutyric anhydride were obtained from Sigma Chemical Co. (St. Louis, MO). Ionophore A 23187 (Io) was purchased from Calbiochem (La Jolla, CA). Ficoll-Hypaque was obtained from Pharmacia (Uppsala, Sweden).

Preparation and stimulation of PMN. Human PMN were purified according to previously described procedures (27). Briefly, venous blood from healthy donors was collected into tubes containing citric acid-dextrose as anticoagulant and sedimented for 30 to 45 min in the presence of gelatin 0.3% final (Plasmagel). Twenty ml of

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Abbreviations: AAGPC, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; APGPC, 1-*O*-alkyl-2-propionyl-*sn*-glycero-3-phosphocholine; BSA, bovine serum albumin; HFB, heptafluorobutyric derivative; Io, Ionophore A 23187; paf, paf-acether; PMN, polymorphonuclear neutrophil(s); 16:0-paf, 1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine; 18:0-paf, 1-*O*-octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine; AMU, atomic mass unit; FAB-MS-MS, fast atom bombardment-tandem mass spectrometry; GC-ECD, gas liquid chromatography-electron capture detection; HPLC, high performance liquid chromatography.

supernatant were layered onto 10 ml Ficoll-Hypaque (density 1.077) and centrifuged at 400 × *g* for 20 min at 20°C. The cell pellet was resuspended in 500 μl of HEPES buffer (pH 7.4) containing: KCl (2.6 mM), NaCl (137 mM), glucose (5.6 mM) and HEPES (4.2 mM). Contaminating erythrocytes were lyzed by adding 3 volumes of distilled water to the cell suspension for 40 sec and then 1 volume of 3.5% (w/v) NaCl. The cells were then washed twice with HEPES buffer and resuspended at 1×10^6 cells/ml with the same buffer supplemented with 1.3 mM $CaCl_2$, 1 mM $MgCl_2$ and 0.25% (w/v) BSA. The cell suspension contained 98% of PMN as assessed by May-Grünvald Giemsa staining. Replicate tubes with 1×10^7 PMN in 10 ml of HEPES buffer, containing 0.25% BSA were stimulated for 15 min at 37°C with 6 μM ionophore A 23187 and compared with unstimulated controls. In some studies, PMN were preincubated with either 1 mM sodium propionate or 1 mM sodium acetate. All incubations were stopped by the addition of ethanol (to reach a final concentration of 80%) and rapid cooling at 4°C. Tubes were kept at 4°C overnight and were then centrifuged at 3000 × *g* for 10 min at 20°C. The supernatants were brought to dryness under a stream of air at 40°C and were stored at −20°C. In three separate experiments, the supernatants were brought to dryness under a stream of nitrogen at 40°C. The same results were obtained (data not shown), allowing us to consider the use of air as a sufficient method.

Purification and identification of paf by GC-ECD. Paf of the complex lipid fraction recovered in aqueous ethanol was purified by normal phase high performance liquid chromatography (HPLC) using a Microporasil column (3.9 mm i.d. × 300 mm long, Waters Associates, Milford, MA) with a mobile phase of dichloromethane/methanol/water (300:250:25, v/v/v). Samples obtained after HPLC separation were prepared for GC-ECD. Briefly, samples were hydrolyzed to the diglycerides with phospholipase C from *C. welchii,* followed by heptafluorobutyric (HFB) derivatization (25). GC was performed using a Girdel 30 instrument (Delsi, Suresnes, France) and an OV 1 fused silica capillary column (0.25 mm i.d. × 25 m long) maintained at 215°C. The temperature of the capillary injector was kept at 270°C. The capillary head pressure was set at 0.4 bar with helium as carrier gas. For ECD, nitrogen was used as a make-up gas at a flow rate of 30 ml/min and the 10 mCi ^{63}Ni detector was maintained at 300°C (25).

Identification by MS-MS. Lipids were separated by HPLC as described above. Aliquots of HPLC fractions were tested for platelet aggregation activity. Structural identification of these fractions was performed using a Nermag R 30-10 triple quadrupole mass spectrometer (Rueil Malmaison, France) equipped with an FAB source. Samples were introduced in a glycerol matrix with a direct insertion probe fitted with a copper sample stage. Measurements were made with a krypton pressure of approximately 1×10^{-5} Torr. The first quadrupole (Q1) scanned the 100-600 AMU range. The second (Q2) was used as a collision chamber, whereas the third (Q3) analyzed the common daughter ion (*m/z* = 184). When a current ion was measured in Q3, the computer recorded the mass of the parent ions present in Q1.

RESULTS AND DISCUSSION

Tandem mass spectrometry. Spectra of synthetic paf were obtained by FAB-MS. One can observe, in Table 1, that the *m/z* = 184 ion corresponding to the phosphocholine moiety is an intense common ion in paf and related compounds. Consequently, this ion was chosen for further identification of paf synthetized from PMN by FAB-MS-MS. In Figure 1, the parent scan obtained using the HPLC fractions purified from the PMN preparation is shown. To detect the doublets spaced by 2 AMU in paf and analogs, resolution was set below 1 AMU. This explains the abundance of peaks in the spectrum. As previously described, the two major species present were the 1-*O*-alkyl 16:0 (*m/z* = 524) and 1-*O*-alkyl 18:0 (*m/z* = 552) derivatives of paf. In addition, two other compounds with *m/z* = 538 (524 + 14) and 566 (552 + 14) were detected. These ions could be assigned to three different species of molecules: (1) 1-*O*-alkyl 17:0 (*m/z* 538) and 19:0 (*m/z* 566) analogs of paf; (2) analogs of 16:0-paf (*m/z* 538) and 18:0-paf (*m/z* 566) with a propionyl group at the *sn*-2 position (APGPC); or (3) 1-*O*-acyl 16:0 (*m/z* 538) and 18:0 (*m/z* 566) species (acyl-acetyl-GPC). All ions were accompanied by homologous ions that differed by 2 atomic mass units and these presumably corresponded to the unsaturated paf analogs (particularly 18:1-AAGPC). These species were quantitatively negligible in comparison with 16:0-paf and 18:0-paf. Further differentiation among AAGPC, APGPC and acyl-acetyl-GPC species could not be made using FAB-MS-MS, because these three species exhibited similar spectra (data not shown). If MS-MS generally offers the opportunity to study mixtures of

TABLE 1

Fragmentation of Paf and Analogs With FAB Ionization

Compounds	Phosphocholine	Glycerol matrix ions		(MH)+
16:0-paf	184 (96)	277 (100)	369 (20)	524 (50)
18:0-paf	184 (100)	277 (50)	369 (10)	552 (30)
16:0-APGPC	184 (100)	277 (80)	369 (21)	538 (21)
18:0-APGPC	184 (100)	277 (31)	369 (5)	566 (29)

FAB mass spectra of synthetic 16:0-paf, 18:0-paf, alkyl-propionyl-16:0-GPC (16:0-APGPC) and alkyl-propionyl-18:0-GPC (18:0-APGPC) obtained with a 1 μg sample (abundance %).

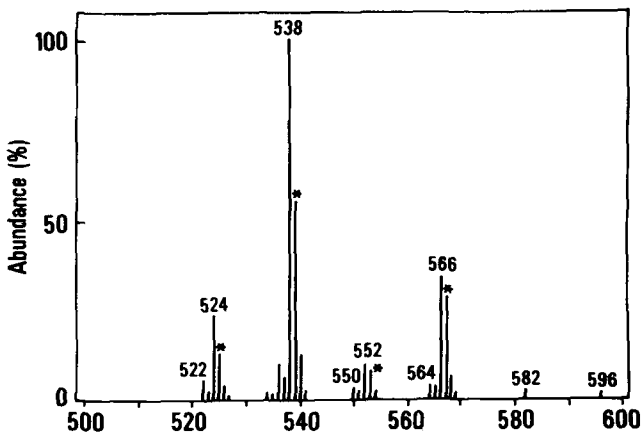

FIG. 1. FAB-MS-MS of paf formed by Io-stimulated human PMN with *m/z* = 184 for parent scans. Abundance (%). *Resolution on Q_1 < 1 AMU.

lipids, for our purpose, it does not appear a straightforward method for identifying isomers. However, this technique allowed us to characterize the molecular species undetectable when using GC-MS. In fact, the sensitivity of the latter method is too low to detect paf and derivatives generated by stimulated PMN.

Capillary GC-ECD and biological assays. Differences in the polarity of paf and derivatives allows separation by capillary GC-ECD. The analysis of synthetic standards: 17:0-AAGPC, 16:0-APGPC, 18:0-APGPC and acyl-acetyl-GPC 16:0 and 18:0 was performed on fused silica capillary column GC as HFB derivatives (25). The capacity factor k' was measured for each compound and is reported in Table 2. Good separations were obtained (Fig. 2) for those of the same molecular weight, i.e., APGPC-HFB, acyl-acetyl-GPC-HFB and AAGPC-HFB. Each peak was identified by GC-MS in the chemical ionization (isobutane) mode (25). The analysis of PMN supernatants by GC-ECD showed the presence of unknown peaks (X). These compounds were also present in extracts from unstimulated PMN, but they were biologically inactive. Thus, we assumed that the presence of these unidentified peaks would not alter the interpretation of our results. As previously described (28), we found 80% of the 16:0 and 20% of the 18:0 species. Small amounts of 17:0-paf-HFB and acyl-acetyl 16:0-HFB were observed (Fig. 2), but no 16:0-APGPC-HFB, 18:0-APGPC-HFB, 19:0paf-HFB or unsaturated paf-HFB was detected.

In the next series of experiments, we added sodium acetate or sodium propionate to Io-stimulated PMN. Five samples in each group were analyzed by GC-ECD. About the same amount of paf (16:0 + 18:0) was formed in Io-stimulated PMN in the absence (257 ± 35 pmol/1 x 10^7 cells, mean ± SEM) or in the presence of sodium acetate (298 ± 70). By contrast, paf formation was decreased by 50% when Io-stimulated PMN were supplemented with sodium propionate (134 ± 24). These results were confirmed using bioassay (data not shown), which ensured that the molecules of interest were not altered or lost during the isolation procedure. In addition, both methods of quantitation gave similar results. The lack of incorporation of the propionyl moiety into paf molecules and an inhibition of paf synthesis (50%) by sodium propionate were also demonstrated.

TABLE 2

GC/ECD Analysis of Paf and Analogs as HFB Derivatives

Compounds	k'	RT (min)
16:0-paf	5.4	26
16:0-acyl-acetyl-GPC	6.6	30
17:0-AAGPC	6.9	32
16:0-APGPC	7.3	33
18:1-AAGPC	9.2	41
18:0-paf	10.3	45

Each synthetic compound was hydrolyzed with phospholipase C followed by heptafluorobutyric derivatization for 10 min at 70°C. The GC analysis of paf and paf analogs was performed on a fused silica OV1 capillary column (0.25 mm i.d. × 25 m) using ECD detection.

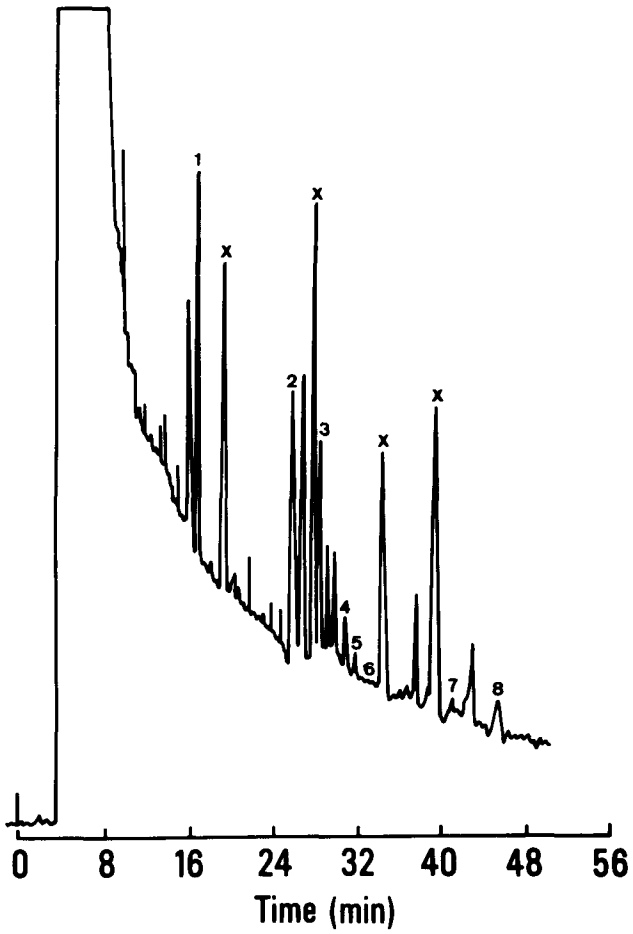

FIG. 2. GC-ECD chromatogram obtained from heptafluorobutyrate (HFB) derivatives of paf formed by Io-stimulated PMN. 1: 16:0-lyso paf, 2: 16:0-paf, 3: 18:0-lyso paf, 4: acyl-acetyl 16:0-GPC, 5: 17:0-AAGPC, 6: 16:0-APGPC, 7: 18:1-AAGPC, 8: 18:0-paf, X: unknown compounds.

The current investigations, together with those of Weintraub et al. (20), Mueller et al. (10) and Ramesha and Pickett (21), demonstrate that stimulated PMN synthesize 16:0-paf and 18:0-paf, as well as several other saturated or unsaturated paf analogs. In contrast with Weintraub et al. (20) and Mueller et al. (10), we did not detect 15:0, 20:0 or 22:0 paf analogs, but we found other molecular forms, such as 19:0- or 17:1-AAGPC, 16:0-acyl-acetyl-GPC or 18:0-acyl-acetyl-GPC with their unsaturated analogs. These apparently conflicting data can be explained by differences in both the purification phase and the sensitivity of analytical measurements utilized. In previous studies (7,20,21,28), a Bligh and Dyer extraction (29) was followed by thin layer chromatography and HPLC separation. According to Jouvin-Marche et al. (30), we used the ethanolic extraction followed by normal phase HPLC purification. This method is equivalent to Bligh and Dyer's (29) extraction, as far as paf is considered. In our studies, thin layer chromatography was not necessary. Our results are in good agreement with those of Kim and Salem (31) who, using thermospray HPLC-MS, did not find 2-propionyl analog of paf in commercial paf preparation from beef heart.

Finally, the combination of the high selectivity in the isolation procedure and the high specificity of the MS-MS

and GC-ECD analysis lead to the unequivocal conclusion that the human PMN produce principally 16:0-paf and 18:0-paf and very limited quantities of 17:0-paf. Analogs of paf, such as 16:0-acyl-acetyl are also formed, but detectable levels (limits of detection: 20 pg) of the 2-propionyl analog of paf were not observed under the conditions of stimulation that were used.

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Thiobarbituric Acid-reactive Malondialdehyde Formation During Superoxide-dependent, Iron-catalyzed Lipid Peroxidation: Influence of Peroxidation Conditions

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A systematic study of the influence of biological lipid peroxidation conditions on lipid hydroperoxide decomposition to thiobarbituric acid-reactive malondialdehyde is presented. A superoxide-dependent, iron-catalyzed peroxidation system was employed with xanthine oxidase plus hypoxanthine plus ferric iron-adenosine diphosphate complex as free radical generator. Purified cardiac membrane phospholipid (as liposomes) was the peroxidative target, and 15-hydroperoxy-eicosatetraenoic acid was used as a standard lipid hydroperoxide. Exposure of myocardial phospholipid to free radical generator at physiological pH (7.4) and temperature (37°C) was found to support not only phospholipid peroxidation, but also rapid lipid hydroperoxide breakdown and consequent malondialdehyde formation during peroxidation. Under lipid peroxidation conditions, oxidative injury to the phospholipid polyunsaturated fatty acids required superoxide radical and ferric iron-adenosine diphosphate complex, whereas 37°C temperature and trace iron were sufficient for lipid hydroperoxide decomposition to malondialdehyde. Harsh thiobarbituric acid-test conditions following peroxidation were not mandatory for either lipid hydroperoxide breakdown or thiobarbituric acid-reactive malondialdehyde formation. However, hydroperoxide decomposition that had begun in the peroxidation reaction could be completed during a subsequent thiobarbituric acid test in which no lipid autoxidation took place. Iron was more critical than heat in promoting the observed hydroperoxide decomposition to malondialdehyde during the lipid peroxidation reaction at 37°C and pH 7.4. These data demonstrate that the radical generator, at physiological pH and temperature, serves a dual role as both initiator of membrane phospholipid peroxidation and promotor of lipid peroxide breakdown and thiobarbituric acid-reactive malondialdehyde formation. Consequently, peroxidation reaction conditions can directly influence lipid hydroperoxide decomposition, malondialdehyde production and system thiobarbituric acid-reactivity. In vivo, decomposition of lipid peroxides to malondialdehyde during radical-mediated, metal-catalyzed membrane peroxidation may represent an integral component of oxidative tissue injury rather than a mere consequence of hydrolyzing the peroxidized biological sample in a thiobarbituric acid test.

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Abbreviations: ADP, adenosine diphosphate; BHT, butylated hydroxytoluene; Desferal, desferrioxamine B methanesulphonate; Fe^{3+}, ferric iron; Fe^{3+}•ADP, ferric iron-adenosine diphosphate complex; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; HPETE, hydroperoxy-eicosatetraenoic acid; HX, hypoxanthine; MDA, *malondialdehyde*; NBT, *nitro*-blue tetrazolium; $O_2^{\cdot-}$, superoxide anion radical; PUFA, polyunsaturated fatty acid; SOD, superoxide dismutase; TBA, thiobarbituric acid; TBARS, TBA-reactive substance; Tris, tris(hydroxymethyl)aminomethane; XOD, xanthine oxidase; HPLC, high performance liquid chromatography.

Free radical-mediated, metal-promoted peroxidation of membrane phospholipid polyunsaturated fatty acids (PUFA) is important to the pathogenesis of many diseases (1). Heart muscle damage during myocardial ischemia and reperfusion, for instance, is believed to involve membrane PUFA peroxidation initiated by superoxide anion radical ($O_2^{\cdot-}$) and ferric iron (Fe^{3+}) (2). During such nonenzymatic lipid peroxidation, $O_2^{\cdot-}$ establishes a suitable Fe^{3+}:Fe^{2+} ratio for the initiation of peroxidation through hydrogen atom abstraction from PUFA (3). Rearrangement of double bonds in the resulting lipid radicals yields conjugated dienes that are attacked by molecular oxygen to form peroxy radicals (4). The peroxy radicals may themselves abstract hydrogen atoms from other PUFA, forming new lipid radicals along with lipid hydroperoxides and, thereby, propagating the initiation event (5). Lipid peroxides are known to decompose at high temperature and low pH in the presence of transition metals (6). Some of the many possible decomposition products have pathological effects of their own (7).

The thiobarbituric acid (TBA) test is the most frequently used method for assessing lipid peroxidation in vitro and in vivo (8). This assay is predicated on the formation of a red adduct (absorption maximum 532 nm) between TBA and malondialdehyde (MDA), a colorless end product of lipid peroxide decomposition (9,10). Optimal color response of the TBA test to lipid hydroperoxide necessitates incubation conditions so harsh that they liberate, by sample hydrolysis, "bound" MDA (11). Antioxidant is also required to inhibit spurious PUFA autoxidation (11,12). Consequently, the TBA test is usually conducted by heating (~80–100°C) a biological sample at low (≤3) pH with TBA, one of several possible iron sources, and butylated hydroxytoluene (BHT) (4,12). These acidic hydrolysis conditions drive the breakdown of lipid peroxides to MDA and other small-molecule products (6,11,12). Any difference in the formation of TBA-reactive substance (TBARS) with or without BHT in the TBA test has been presumed to reflect the existence of lipid peroxidation products that can yield TBARS only under TBA-test conditions (3).

On a molar basis, MDA is the lipid peroxide decomposition product that reacts most efficiently with TBA to form red chromogen (10,13). Yet end products of lipid peroxide breakdown, other than MDA, are TBA-reactive and may produce orange or yellow pigments, depending, at least in part, on TBA-test reagents (14), and TBA-positive products are generated through oxidative damage to nonlipid molecules (15). The nature of MDA as a secondary indicator of PUFA peroxidation, dependent on both formation and breakdown of lipid peroxide, and the nonspecificity of TBA chemical reactivity toward MDA complicate the interrelationships among peroxide decomposition, bona fide MDA formation and TBARS generation resulting from biological lipid peroxidation. Some investigators hold that MDA is formed primarily

as a consequence of fatty-hydroperoxide decomposition in the TBA test (16). Yet, others have reported isolation of MDA from lipid peroxidation systems not reacted with TBA (17,18).

The lack of quantitative information on the course of lipid peroxide decomposition to bona fide, TBA-reactive MDA during PUFA peroxidation prompted the following investigations on the operative interrelationships among lipid peroxidation conditions, fatty-peroxide breakdown and MDA and TBARS formation. Purified, PUFA-rich (1,2) membrane phospholipid from myocardium and preformed fatty-acid hydroperoxide (15-*L*-hydroperoxy-5,8,11,13-eicosatetraenoic acid [15-HPETE]) were used as lipid study-objects. Our findings demonstrate that $O_2^{\cdot-}$-dependent, Fe-catalyzed radical reactions at physiological pH and temperature serve not only to initiate PUFA peroxidation, but also to promote the simultaneous, rapid breakdown of lipid hydroperoxide so generated to MDA. Promotion of lipid peroxide decomposition by the radical generator under physiological pH and temperature is in marked contrast to the TBA-test conditions supporting fatty-peroxide breakdown. Exposure of the peroxidized lipid sample to the harsh conditions of a TBA test does not necessarily enhance MDA production above that formed during peroxidation itself, although hydroperoxide decomposition can occur during the TBA test.

EXPERIMENTAL PROCEDURES

Materials. N-2-Hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), tris(hydroxymethyl)aminomethane (Tris), hydroxylamine hydrochloride, adenosine diphosphate (ADP), TBA, tetramethoxypropane, soybean lipoxygenase (EC 1.99.2.1) and hypoxanthine (HX) were from Sigma (St. Louis, MO). XOD (analytical preparation from bovine milk; 1.0 unit/mg) and superoxide dismutase (SOD; analytical preparation from bovine erythrocytes; 5000 units/mg) were from Boehringer-Mannheim (Indianapolis, IN). Desferal (desferrioxamine B methanesulphonate) was a gift from Ciba A.G. (Basle, Switzerland). Ferrioxamine was synthesized according to Hoe et al. (19). Chelex 100, a chelating resin with high selectivity for Fe^{3+}, was from Bio-Rad (Richmond, CA). Reagent-grade deionized water was produced with a commercial deionizer from Hydro (Research Triangle Park, NC). All organic solvents were of analytical grade and from Burdick and Jackson (Muskegon, MI).

Extraction and quantification of heart-muscle membrane phospholipid. Membranes were isolated from the perfused ventricular myocardium of the adult male Sprague-Dawley rat by differential centrifugation and salt extraction (20). The washed, purified membranes represented >70% of the total myocardial phospholipid and were some 3-fold enriched with respect to starting homogenate (18). Lipids were extracted and purified from the myocardial membranes by a modified (21) Bligh-Dyer procedure (22). The final chloroform phase containing the purified lipid represented quantitative recovery of myocardial membrane lipid by the chemical criteria detailed (18). The myocardial membrane phospholipid was isolated from the total membrane lipid extract by Sep-Pak silica column chromatography (Waters, Milford, MA) (23). All lipids were stored in chloroform under argon at −20°C (24).

The hydroxamate reaction was used to measure lipid ester (25); L-α-phosphatidylcholine dipalmitate (Avanti Polar Lipids, Birmingham, AL) was the standard.

Preparation of cardiac liposomes. A known amount of myocardial membrane phospholipid in chloroform was placed into a rotating glass flask. The solvent was evaporated under argon at room temperature (22°C), and the lipid film was resuspended in 10 mM HEPES-0.145 M KCl, pH 7.4, by indirect anaerobic sonication for 15 min at room temperature. No protein was detectable in the liposome preparation (18).

Peroxidation reaction system. Cardiac membrane phospholiposomes were subjected to $O_2^{\cdot-}$-dependent, Fe-catalyzed peroxidation as detailed (18). In brief, the complete peroxidation reaction contained 10 mM HEPES-0.145 M KCl, pH 7.4; 1.0 mM HX; 0.1 mM Fe^{3+}-1.0 mM ADP complex; 125 μg phospholipid/ml; and 10 mUnits XOD/ml (final concs.) "Free" HEPES was used, and its pH was adjusted with KOH. This concentration of HEPES was minimal for maintaining the pH at ~7.4 through the peroxidation reaction and did not influence the initial rates of peroxidation relative to an unbuffered system, which progressively decreased in pH. The reaction was started by adding the XOD, mixing and heating to 37°C in a shaking water bath. Liposome samples were also incubated in parallel to the same final lipid concentration, but without free radical generator (i.e., without XOD + HX + Fe^{3+}·ADP). Incubations of liposomes with select components of the free radical generator ("incomplete" generator) were also carried out, as specified in the Tables. Incubations were terminated by lipid extraction, acidification or SOD addition, depending on subsequent analysis. In some experiments, Desferal was included in the peroxidation reaction at a 250 μM final concentration.

Determination of conjugated diene and TBARS. For conjugated diene analysis, lipids were extracted (21) out of the peroxidation incubations and dissolved in cyclohexane. Lipid spectra were taken from 190 nm to 400 nm in a Beckman DU-7 spectrophotometer (Beckman, Palo Alto, CA). The spectra were used as detailed (18,26) to calculate net conjugated diene formation.

The TBA test was conducted as described (18). In brief, the peroxidation incubation was terminated on ice by acidification with 0.15 ml ice-cold 76% TCA-2.3 N HCl per 1.0 ml peroxidation reaction mixture. In some experiments, Desferal (250 μM, final conc.) was next added; in others, 0.1 mM Fe^{3+}-1.0 mM ADP complex was added to the specified final concentration. Finally, 0.35 ml of a mixture of H_2O/7.14 M BHT in ethanol/1.51% TBA in 0.2 M Tris, pH 7.0 (1:1:5, v/v/v) was added. After thorough mixing, the samples (pH = 2.4) were incubated in an 80°C shaking water bath for 30 min. The tubes were then plunged into an ice-water bath, and 0.5 ml ice-cold 91% TCA, followed by 2.0 ml $CHCl_3$, was added. After centrifugation for 30 min at 2000 × *g*, 4°C, the absorbance of the upper phase was read at 532 nm. MDA standard, prepared by acidification of 1,1,3,3-tetramethoxypropane with 76% TCA-2.3 N HCl, was subjected to the identical TBA-test procedure in order to construct a standard curve of TBARS as MDA-equivalents. Computer-assisted regression analysis of the standard curve was used as detailed (18) to quantify the molar amounts of MDA-equivalents in the experimental samples. Reactivity between MDA standard and TBA was not affected by

components of the radical generator, Desferal, ferrioxamine or SOD.

Chromatographic MDA isolation and quantification. When a peroxidation reaction was to be analyzed by high performance liquid chromatography (HPLC) for MDA content, peroxidation was stopped by adding SOD to a final concentration of 10^{-7} M and freezing the mixture in dry ice-acetone (18). MDA was isolated from the sample by ion-pairing HPLC with detection at 267 nm and quantified as described (17,18).

Buffer ultrapurification. Buffers were treated with Chelex resin, 100–200 mesh, to eliminate metal content (27). The Chelex column was equilibrated and optimized for divalent cation chelation as detailed (28). This protocol has been demonstrated to be particularly effective in removing divalent cations from HEPES and Tris buffers (29).

Synthesis and purification of lipid hydroperoxide (15-HPETE). Soybean lipoxygenase and 5,8,11,14-eicosatetraenoic acid (arachidonic acid; NuChek Prep, Elysian, MN) were used to synthesize 15-HPETE according to Graff (30). During extraction and purification of 15-HPETE product, sample temperature, glassware and solvents were maintained below 0°C to obviate decomposition of the lipid hydroperoxide. The conversion of arachidonate to 15-HPETE was >96%, as evaluated with [1-^{14}C]arachidonic acid (60 mCi/mmol sp. act.; New England Nuclear, Boston, MA). 15-HPETE purity was verified by thin layer chromatography (31).

Assay for $O_2^{\cdot-}$ production. The $O_2^{\cdot-}$ generated from XOD + HX was reacted with either cytochrome C or nitro-blue tetrazolium (NBT) as detector molecule. The reaction mixture, in a final volume of 3 ml, consisted of: 76 μM cytochrome C or 100 μM NBT, 1.0 mM HX, 30 mUnits XOD and 50 mM K-phosphate buffer, pH 7.4, containing 0.1 mM EDTA. The rate of SOD-inhibitable cytochrome c reduction was monitored at 550 nm (32); the rate of SOD-inhibitable NBT reduction was monitored at 560 nm (33).

RESULTS

Conjugated diene, MDA and TBARS production during myocardial membrane phospholipid peroxidation. Incubation of purified cardiac membrane phospholipid (as liposomes) under $O_2^{\cdot-}$-dependent, Fe-catalyzed peroxidation conditions led to a rapid, net formation of lipid conjugated diene (Fig. 1). Addition of more phospholipid, but not more free radical generator, to the system at 90 min could elicit a subsequent, albeit slight, increase in conjugated diene content (data not shown). The rate of $O_2^{\cdot-}$ production, assessed directly as SOD-inhibitable cytochrome c or NBT reduction, was high for ca. 25 min and was detectable, but insignificant, thereafter. Because the rate of diene formation during the period of maximal $O_2^{\cdot-}$-production was only slightly greater than the rate when $O_2^{\cdot-}$-production had virtually ceased, much of the lipid peroxide formation in Figure 1 may reflect propagatory interactions between PUFA and lipid radicals (4,5). By 90 min of reaction, the level of conjugated intermediates in the peroxidation system was maximal, suggesting that propagation had ended and/or that lipid peroxide decomposition was occurring at a rate approaching the rate of lipid peroxide formation.

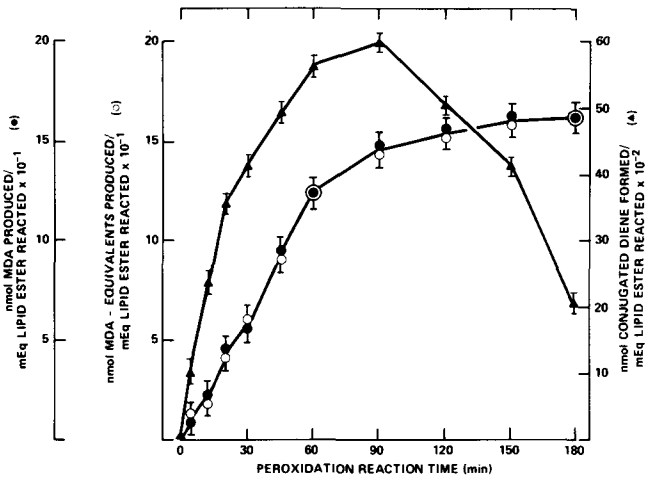

FIG. 1. Conjugated diene, MDA and TBARS formation during myocardial membrane phospholipid peroxidation. Purified rat heart-muscle membrane phospholipid was reconstituted into liposomes and subjected to peroxidative injury in a $O_2^{\cdot-}$-dependent, Fe-catalyzed system with XOD + HX + Fe^{3+}·ADP as radical generator. Liposomes were also incubated without radical generator. Three sets of samples were taken over 180 min of peroxidation reaction at the indicated times: One set of peroxidized and nonperoxidized samples was extracted for lipid, and the isolated lipids were analyzed spectrophotometrically for conjugated diene content. A second set was acidified and subjected to a colorimetric TBA test. A third set was made to 100 nM in SOD and analyzed by HPLC for MDA content. The mean differences between peroxidized and nonperoxidized samples in these three assays were taken as production of the respective lipid peroxidation product: conjugated dienes (▲), MDA enolate anion (●) and MDA-equivalents, i.e., TBARS (○). Data, normalized to the amount of phospholipid ester reacted, were graphed ± SD (n ≥ 6).

The peroxidation system developed TBA-reactivity in parallel with a net increase in MDA content (Fig. 1). No liposome-bound MDA was detected (18). A maximal linear rate of both MDA and TBARS (as MDA-equivalents) formation [2.1 ± 0.1 nmol/mEquivalent phospholipid ester reacted/min (mean ± SD, n = 7)] was maintained through the first 45 min of peroxidation and resulted in ca. 150 nmol MDA and TBARS by 2 hr. As detailed elsewhere (18), the parallelism between MDA formation and TBARS production is due to the fact that negligible nonlipid derived, nonMDA TBARS is produced. The similar yield of MDA enolate anion by HPLC and MDA-equivalents in the TBA test demonstrates that postperoxidative PUFA autoxidation did not take place during the TBA reaction. Furthermore, these kinetics indicate that the radical generator served to initiate PUFA peroxidation and to promote, although peroxidation was ongoing at physiological pH and temperature, the decomposition of lipid hydroperoxide to TBA-reactive MDA.

Influence of lipid peroxidation conditions on conjugated diene, MDA and TBARS formation. We initially studied the influence of peroxidation reaction conditions on MDA formation and TBA-reactivity. A 60-min reaction was adopted, for at that time the amplitude of peroxidation was high and the kinetics reasonably linear (Fig. 1). Parallel samples of purified cardiac membrane phospholipid were incubated for 60 min at physiological (37°C) or ambient (22°C) temperature without free radical generator, with complete generator (i.e., XOD + HX +

Fe^{3+}·ADP) or with "incomplete" generator (i.e., select generator components). The peroxidation system was then either analyzed spectrophotometrically for conjugated diene, subjected to HPLC for MDA quantification or incubated with TBA in acid at 80°C with Fe^{3+}·ADP present for TBARS determination.

Full diene, MDA and TBARS formation (cf. Fig. 1) required phospholipid exposure to complete free radical generator at 37°C (Table 1). The $O_2^{\cdot-}$-dependent, Fe-catalyzed nature of the peroxidation was apparent from the negligible diene formation when either SOD was present with complete generator or Fe^{3+}·ADP was omitted from the generator. Introduction of Fe^{3+}·ADP into the TBA test after incubating the lipid with $O_2^{\cdot-}$ (i.e., with XOD + HX) did not elicit TBARS formation. Physiological (37°C) temperature was not essential to peroxidation or to lipid peroxide decomposition, but a room-temperature (22°C) incubation of phospholipid with complete free radical generator reduced peroxidation by ~20%.

In an attempt to resolve the contributions of lipid hydroperoxide formation and lipid hydroperoxide decomposition to TBA-reactive MDA production during peroxidation, purified lipid hydroperoxide (15-HPETE) was incubated under the same peroxidation conditions as the membrane phospholipid had been. However, 100 μM SOD was included to quench the free radical generator (18). The data (Table 2) demonstrate that, during a peroxidation incubation at physiological or room temperature with complete radical generator or Fe^{3+}·ADP alone, lipid hydroperoxide readily decomposed to MDA, and the system evidenced full TBA-reactivity in a subsequent TBA test. Because 90 nmol 15-HPETE yielded 3 nmol MDA, the efficiency of lipid hydroperoxide conversion to bona fide MDA was low—ca. 3-4%. During a peroxidation incubation at 37°C in the absence of Fe^{3+}·ADP, 15-HPETE decomposition to MDA was ~80% of that when Fe^{3+}·ADP had been present; TBARS formation (with Fe^{3+}·ADP present in the TBA test) was not reduced. Similarly, during a 22°C peroxidation incubation, the absence of Fe^{3+}·ADP resulted in ~40% less MDA being produced, yet TBARS formation during a subsequent TBA test was not reduced. Therefore, lipid hydroperoxide readily decomposed to TBA-reactive MDA under PUFA peroxidation conditions. The iron component of the radical generator and heat were critical determinants of the extent of decomposition. The combined data from Tables 1 and 2 demonstrate that lipid hydroperoxide breakdown begun during peroxidation could be completed in a subsequent TBA test which did not itself elicit lipid autoxidation.

Influence of Trace Fe on MDA and TBARS formation from lipid hydroperoxide decomposition during cardiac phospholipid peroxidation. Cardiac phospholipid peroxidation was negligible without added Fe^{3+}·ADP (Table 1). Yet, without Fe^{3+}·ADP, lipid hydroperoxide decomposed to MDA and TBARS (Table 2). Aqueous, millimolar buffer solutions may contain ca. 0.2–20.0 μM divalent cation contamination (28). These data suggested that trace Fe might be present in the peroxidation reaction system which could not catalyze PUFA peroxidation itself but could promote lipid hydroperoxide decomposition. Accordingly, we investigated the influence of Fe on MDA and TBARS production from cardiac phospholipid peroxidized in the $O_2^{\cdot-}$-dependent, Fe-catalyzed system and from 15-HPETE. For these studies, Desferal was used at a concentration (250 μM) 2.5 times the Fe^{3+} concentration in the radical generator. As detailed elsewhere (18), the stoichiometric nature of Fe^{3+} chelation by Desferal required a minimal Desferal concentration of ~100 μM, the concentration of added Fe^{3+}·ADP, to inhibit peroxidation. This finding indicated that all the Fe^{3+}·ADP in the radical generator was available to peroxidation. Desferal's effects were due solely to Fe chelation; ferrioxamine (i.e., Fe-saturated Desferal [19]) was without effect (data not shown).

At either physiological (37°C) or room (22°C) temperature, excess Desferal blocked phospholipid PUFA peroxidation when complete free radical generator was present (Table 3). In peroxidation incubations containing $O_2^{\cdot-}$

TABLE 1

Influence of Peroxidation Reaction Conditions on Conjugated Diene, MDA and TBARS Formation From Myocardial Membrane Phospholipid[a]

Peroxidation conditions	Conjugated diene (% control)	MDA (% control)	TBA-test conditions	TBARS (% control)
37°C, 60 min:			80°C, 30 min:	
+ XOD; + HX; + Fe^{3+}·ADP	100	100		100
+ XOD; + HX; + Fe^{3+}·ADP + SOD	<1.0	<1.0		<1.0
+ XOD; + HX; no Fe^{3+}·ADP	<1.0	<1.0	+ Fe^{3+}·ADP	<1.0
no XOD; no HX; no Fe^{3+}·ADP	<1.0	<1.0	+ Fe^{3+}·ADP	<1.0
22°, 60 min:			80°C, 30 min:	
+ XOD; + HX; + Fe^{3+}·ADP	86 ± 5	82 ± 4		84 ± 5
+ XOD; + HX; + Fe^{3+}·ADP; + SOD	<1.0	<1.0		<1.0
+ XOD; + HX; no Fe^{3+}·ADP	<1.0	<1.0	+ Fe^{3+}·ADP	<1.0
no XOD; no HX; no Fe^{3+}·ADP	<1.0	<1.0	+ Fe^{3+}·ADP	<1.0

[a]Total myocardial membrane phospholipid (as liposomes, 125 μg/ml) was incubated for 60 min under the described peroxidation conditions. When present, the final component concentrations were: 0.1 mM Fe^{3+} − 1.0 mM ADP complex; 10 mUnits XOD/ml; 1.0 mM HX; 100 nM SOD. After 1 hr, parallel samples were assessed for conjugated diene or MDA content; other samples were subjected to the TBA test at 80°C with Fe^{3+}·ADP. Data are expressed relative to the control incubation, taken as 100%. At 60 min, 100% conjugated diene formation represents 5600 ± 300 nmol conjugated diene/mEquivalent phospholipid ester reacted, and 100% MDA or TBARS formation represents 130 ± 6 nmol MDA/mEquivalent phospholipid ester reacted. Data are means ± SD ($n \geq 6$).

(i.e., XOD + HX), but no Fe^{3+}·ADP (either with or without 250 μM Desferal), no lipid peroxidation occurred, indicating that any trace Fe present could not serve to initiate peroxidation. Fe chelation during the TBA test after lipid peroxidation had occurred did not influence formation of TBARS (Table 3), nor did it affect the TBA-reactivity of MDA standard (data not shown). The results in Table 3 are further support for the conclusion that the lipid hydroperoxide originating from cardiac phospholipid PUFA peroxidation had decomposed during the peroxidation reaction such that full TBA-reactive MDA production preceded and was not dependent on the TBA test.

A role for trace Fe, as promotor of lipid hydroperoxide decomposition and consequent MDA and TBARS production, could be demonstrated by substituting 15-HPETE for phospholipid in these experiments (Table 4). Incubation of 15-HPETE with complete free radical generator or generator without Fe^{3+}·ADP, at either 37°C or 22°C, elicited MDA formation. The peroxidation incubations, without added Fe^{3+}·ADP, produced 20–30% less MDA than did the samples containing Fe^{3+}·ADP. However, if 250 μM Desferal were included in the peroxidation incubation, either with or without Fe^{3+}·ADP present, no lipid hydroperoxide decomposition to TBA-reactive MDA took place. Similarly, buffer ultrapurification with Chelex 100 prevented 15-HPETE decomposition under the conditions of the peroxidation incubation, but had no effect on PUFA peroxidation, if $O_2^{\cdot-}$ and Fe^{3+}·ADP were present (data not shown).

In the TBA test at 80°C, partial (~50% of maximal) lipid hydroperoxide breakdown to TBARS occurred following peroxidation incubations with Desferal in which no MDA had formed (Table 4). This partial TBARS production suggested that thermal decomposition of lipid

TABLE 2

Influence of Peroxidation Reaction Conditions on MDA and TBARS Formation From Lipid Hydroperoxide (15-HPETE)[a]

Peroxidation conditions	MDA (% control)	TBA-test conditions	TBARS (% control)
37°C, 60 min, + SOD:		80°C, 30 min:	
+ XOD; + HX; + Fe^{3+}·ADP	100		100
no XOD; no HX; + Fe^{3+}·ADP	98 ± 3		94 ± 4
+ XOD; + HX; no Fe^{3+}·ADP	84 ± 5	+ Fe^{3+}·ADP	97 ± 5
no XOD; no HX; no Fe^{3+}·ADP	81 ± 4		97 ± 4
no XOD; no HX; no Fe^{3+}·ADP	82 ± 4	+ Fe^{3+}·ADP	98 ± 3
22°C, 60 min, + SOD:		80°C, 30 min:	
+ XOD; + HX; + Fe^{3+}·ADP	94 ± 5		100 ± 3
no XOD; no HX; + Fe^{3+}·ADP	96 ± 4		99 ± 5
+ XOD; + HX; no Fe^{3+}·ADP	60 ± 4	+ Fe^{3+}·ADP	98 ± 3
no XOD; no HX; no Fe^{3+}·ADP	57 ± 3		95 ± 4
no XOD; no HX; no Fe^{3+}·ADP	66 ± 5	+ Fe^{3+}·ADP	96 ± 5

[a]Lipid hydroperoxide (15-HPETE; 90 nmol/ml) was incubated for 60 min under the described peroxidation conditions. When present, the final component concentrations were: 0.1 mM Fe^{3+} − 1.0 mM ADP complex; 10 mUnits XOD/ml; 1.0 mM HX; 100 nM SOD. After 1 hr, parallel samples were either assessed for MDA content by HPLC or subjected to the TBA test under the conditions shown. Data are expressed relative to the control incubation, taken as 100%. At 60 min, 100% MDA or TBARS formation represents 3.0 ± 0.15 nmol MDA/90 nmol 15-HPETE. Data are means ± SD ($n \geq 6$).

TABLE 3

Influence of Fe and Fe Chelation on Conjugated Diene, MDA and TBARS Formation From Myocardial Membrane Phospholipid[a]

Peroxidation conditions	Conjugated diene (% control)	MDA (% control)	TBA-test conditions	TBARS (% control)
37°C, 60 min:			80°C, 30 min:	
+ XOD; + HX; + Fe^{3+}·ADP	100	100		100
+ XOD; + HX; + Fe^{3+}·ADP; + Desferal	<1.0	<1.0		<1.0
+ XOD; + HX; no Fe^{3+}·ADP	<1.0	<1.0	+ Desferal	<1.0
+ XOD; + HX; no Fe^{3+}·ADP; + Desferal	<1.0	<1.0	+ Fe^{3+}·ADP	<1.0
+ XOD; + HX; + Fe^{3+}·ADP	99 ± 3	98 ± 4	+ Desferal	99 ± 5
22°C, 60 min:			80°C, 30 min:	
+ XOD; + HX; + Fe^{3+}·ADP	83 ± 3	82 ± 4		84 ± 5
+ XOD; + HX; + Fe^{3+}·ADP; + Desferal	<1.0	<1.0		<1.0
+ XOD; + HX; no Fe^{3+}·ADP	<1.0	<1.0	+ Desferal	<1.0
+ XOD; + HX; no Fe^{3+}·ADP; + Desferal	<1.0	<1.0	+ Fe^{3+}·ADP	<1.0
+ XOD; + HX; + Fe^{3+}·ADP	78 ± 4	80 ± 4	+ Desferal	80 ± 5

[a]Total myocardial membrane phospholipid (as liposomes, 125 μg/sample) was incubated for 60 min under the described peroxidation conditions. The samples were processed and analyzed as detailed in the footnote to Table 1. Data are means ± SD ($n \geq 6$).

hydroperoxide during the TBA test was responsible for the TBA-reactivity. This TBARS appeared to be largely, if not exclusively, MDA: HPLC analysis of 15-HPETE incubated under TBA-test conditions revealed MDA formation to ~50% of maximal.

DISCUSSION

In biological systems, analysis of peroxidative PUFA injury as TBARS depends on both formation and decomposition of lipid peroxides (4,35). This study has identified and examined, in quantitative terms, at least some of the factors operative in the formation of TBA-reactive MDA from membrane-phospholipid PUFA undergoing peroxidation via $O_2^{\cdot-}$-dependent, Fe-catalyzed reactions of the type which cause tissue damage in vivo (1,2). The data highlight a complex functional interrelationship among biological lipid peroxidation and postperoxidative phenomena such as fatty-hydroperoxide decomposition and MDA formation.

Comparative analyses of MDA formation from membrane phospholipid undergoing peroxidation and from purified lipid hydroperoxide (15-HPETE) incubated under peroxidation conditions demonstrated that $O_2^{\cdot-}$-dependent, Fe-catalyzed oxyradical chemistry at physiological pH and temperature leads not only to PUFA peroxidation, but also to lipid hydroperoxide breakdown and TBA-reactive MDA formation. A subsequent TBA test at high temperature and low pH was not required for MDA formation and did not enhance MDA yield. These data define a second function of the radical generator, promoting peroxide decomposition, aside from initiating PUFA peroxidation (3,36). Accordingly, the data do not support the view that exposure of a peroxidized biological sample to the TBA test is critical to, let alone essential for, lipid peroxide breakdown to MDA (16).

Experiments in which peroxidation incubations contained Fe chelator (Desferal) or buffers treated with Fe^{3+}-chelating resin (Chelex 100) indicate that trace Fe, although unable to initiate peroxidation, can promote lipid peroxide breakdown to TBA-reactive MDA during the peroxidation incubation. Trace Fe increases TBA-test coloring (37), but the augmented signal has been taken as indication that trace Fe promotes lipid autoxidation during the TBA test (11,12,38). Our data invite an alternative interpretation, whereby the increased color could be due to enhanced lipid hydroperoxide degradation during the peroxidation reaction itself.

A subphysiological temperature in the peroxidation reaction reduced lipid peroxidation by ~20%, but did not influence lipid hydroperoxide decomposition to MDA. The 80°C heat of the TBA test incubation by itself (i.e., with no Fe^{3+} present) supported partial (~55%) lipid hydroperoxide breakdown to MDA. Thus, although both Fe and heat promote MDA formation from lipid hydroperoxide during peroxidation (Tables 3 and 4) and in the TBA test (11,12), Fe appears to be more critical for peroxide decomposition under lipid peroxidation conditions, whereas Fe and heat are about equally important for MDA formation from fatty-hydroperoxide during the TBA test.

Other studies (18), along with data herein, demonstrate the lack of PUFA autoxidation (but not lipid hydroperoxide breakdown) during the TBA test used. Yet the low (~4%) molar efficiency of lipid hydroperoxide decomposition during the TBA test to bona fide MDA noted here and elsewhere (39) should be borne in mind when using the TBA test or TBA-MDA adduct analysis by HPLC (40) as a quantitative index of lipid peroxidation. This caution is emphasized by our finding that hydroperoxide breakdown to MDA during radical-mediated lipid peroxidation at physiological pH and temperature is similarly inefficient.

Although the conversion of lipid peroxide to MDA is low, the resulting amounts of red-pigmented TBARS (determined colorimetically as MDA equivalents) and bona fide MDA (by HPLC) approximate each other. This correlation supports the view that MDA represents the major (if not sole) TBA-reactive product of biological lipid peroxide formation and decomposition detected colorimetrically at 532 nm (10,13,14). With purified lipid as oxidative-injury target, TBA-reactivity appears to be a good reflection of MDA formation and, hence, of lipid peroxide production and decomposition (Fig. 1). Yet

TABLE 4

Influence of Fe and Fe Chelation on MDA and TBARS Formation From Lipid Hydroperoxide (15-HPETE)[a]

Peroxidation conditions	MDA (% control)	TBA-test conditions	TBARS (% control)
37°C, 60 min, + SOD:		80°C, 30 min:	
+ XOD; + HX; + Fe^{3+}·ADP	100		100
+ XOD; + HX; no Fe^{3+}·ADP	82 ± 5	+ Desferal	94 ± 4
+ XOD; + HX; + Fe^{3+}·ADP; + Desferal	<1.0		60 ± 4
+ XOD; + HX; no Fe^{3+}·ADP; + Desferal	<1.0	+ Fe^{3+}·ADP	56 ± 4
+ XOD; + HX; + Fe^{3+}·ADP	98 ± 6	+ Desferal	97 ± 5
22°C, 60 min, + SOD:		80°C, 30 min:	
+ XOD; + HX; + Fe^{3+}·ADP	94 ± 5		100 ± 3
+ XOD; + HX; no Fe^{3+}·ADP	60 ± 4	+ Desferal	94 ± 5
+ XOD; + HX; + Fe^{3+}·ADP	<1.0		64 ± 5
+ XOD; + HX; no Fe^{3+}·ADP; + Desferal	₅1.0	+ Fe^{3+}·ADP	53 ± 4
+ XOD; + HX; + Fe^{3+}·ADP	97 ± 5	+ Desferal	98 ± 4

[a]Lipid hydroperoxide (15-HPETE; 90 nmol/sample) was incubated for 60 min under the described peroxidation conditions. The samples were processed and analyzed as detailed in the footnote to Table 2. Data are means ± SD ($n \geq 6$).

MDA is most certainly not the only decomposition product, for a complex variety of small molecules can be generated from lipid peroxide breakdown (10,13,35). In membrane/tissue systems where purified lipid is not the peroxidative target, bona fide MDA may represent only 20-60% of the total TBARS (18,31,39-42). Therefore, the presence of nonlipid-related, nonMDA TBARS in biological peroxidation samples represents a major limitation to the quantitative use of the TBA test in systems not having purified peroxidative target.

Formation of lipid-derived MDA under peroxidation conditions at physiological pH and temperature implies that MDA is produced locally in tissues during radical-mediated membrane damage. This proposition could help explain the association among Fe overload, oxidative stress and high tissue MDA levels (43). In vivo, local tissue effects of MDA (7,44,45) probably reflect the balance between MDA formation and catabolism (46). Because PUFA hydroperoxides are structurally similar to prostaglandin biosynthetic intermediates (16), hydroperoxide breakdown to MDA, concomitant with biological PUFA peroxidation, could obviate the pathophysiological effects of nonenzymatically produced prostaglandin-like substances. The relationships between lipid peroxide decomposition and TBA-reactive MDA formation during membrane PUFA peroxidation, as described herein, suggest a broader mechanistic outlook of radical-mediated tissue injury to encompass not only lipid peroxide formation, but also sequelae such as peroxide decomposition and the pathophysiological effects of decomposition products at the sites of tissue injury.

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Effect of Marginal Zinc Deficiency on Lipoprotein Lipase Activities in Postheparin Plasma, Skeletal Muscle and Adipose Tissues in the Rat[1]

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The activities of lipoprotein lipase in postheparin plasma, retroperitoneal adipose and gastrocnemius muscle tissues were determined in the rats fed 2.8 ppm of dietary zinc for eight weeks, as compared with pair-fed and ad libitum-fed rats given 30.8 ppm of zinc. The postheparin lipoprotein lipase activity, as determined by using a lipid emulsion labeled with [^{3}H]triolein as the substrate, was significantly lower in the first group of rats, relative to that in the second and third groups. Tissue lipoprotein lipase activities were compared using the lipid emulsion and activator serum obtained from the zinc-deficient rats and the ad libitum-fed rats. The activator sera were devoid of very low density and low density lipoproteins, but enriched in high density lipoproteins. Muscle lipoprotein lipase activities were significantly lower when assayed with the activator serum from the zinc-deficient compared with the activities determined with the activator serum from the ad libitum-fed. Similarly, muscle lipoprotein lipase activities were lower in all groups when [^{3}H]-triolein-labeled chylomicrons from the zinc-deficient were used as the substrate, compared with the activities determined using the chylomicrons from the ad libitum-fed. Lipoprotein lipase activities in the adipose tissues were not affected by the different sources of the activator sera and chylomicrons. The results strongly suggest that the decrease in postheparin lipoprotein lipase activity in zinc deficiency is not due to changes in tissue lipoprotein lipase enzyme per se, but to compositional alterations in chylomicrons and high density lipoprotein, particularly, with regard to C apolipoproteins, modulators of lipoprotein lipase activity.
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Zinc deficiency in experimental animals produces profound alterations in lipid metabolism. Evidence indicates that zinc deficiency in rats impairs the intestinal absorption of triglyceride (1) and cholesterol (2), which may be attributable to the defective formation of chylomicrons in the intestinal mucosa (3,4).

At present, however, little is known about the effect of zinc deficiency on the postabsorptive metabolism of chylomicron-carried lipids. Upon their entry into the circulation, chylomicrons are subjected to peripheral lipolysis by endothelial lipoprotein lipase (LPL), with resultant formation of chylomicron remnants, which are then rapidly taken up via the high affinity apo-E receptor (5). Our recent data (6) demonstrate that the initial plasma clearance of chylomicrons, as labeled with [^{14}C]cholesterol, is significantly delayed in marginally zinc-deficient rats. Such a delay suggests a possible impairment in the peripheral lipolysis and/or hepatic uptake of chylomicrons (remnants).

The present study was designed to specifically investigate the effect of marginal zinc deficiency on the activities of LPL in postheparin plasma, skeletal muscle and adipose tissues. In view of the important role of apo C in modulating LPL activity (7,8) and the observed decrease in chylomicron apo C in zinc deficiency (3), the LPL activities were compared between zinc-deficient (ZD) and control (CT) rats by using a natural substrate (purified chylomicrons) and an artificial substrate with the activator sera, as obtained from the ZD and CT rats.

MATERIALS AND METHODS

Animals and diets. Male Fischer rats (CDF[-344]Crl BR; Charles River Breeding Lab., Inc., Wilmington, MA) were housed individually in plastic cages with stainless-steel wire bottoms and subjected to a light cycle with the light period from 1500 to 0300 and dark period from 0300 to 1500 throughout the experiment. After acclimatization for 1 wk, the rats weighing 159 ± 8 g were divided into 3 treatment groups of 20 each.

The composition of the basal diet was the same as described previously (4). The basal diet contained 0.8 ppm of zinc. The experimental groups consisted of: 1) a zinc-deficient (ZD) group fed ad libitum the basal diet supplemented with 2.0 ppm of zinc. The total zinc content of the ZD diet was 2.8 ppm; 2) a pair-fed (PF) group fed 30.8 ppm of zinc, but with food intake restricted to the amount consumed by ZD; and 3) another control (CT) group fed the same diet ad libitum. Rats were allowed free access to deionized-distilled water delivered via an automatic watering system. All rats were housed in a windowless humidity- and temperature-controlled room in the Biomedical Research Center fully accredited by the American Association for the Accreditation of Laboratory Animal Care, and cared for in accordance with the guidelines of the National Research Council for the care and use of laboratory animals.

Determination of postheparin plasma lipoprotein lipase activity. After 8 wk of dietary treatment, 5 rats from each group were fasted for 18 hr and injected with 150 IU heparin/kg body wt (H-7005, Sigma Chemical Co., St. Louis, MO) via the jugular vein. At 10 min, blood samples were collected via the orbital sinus and plasma was obtained by centrifugation at 1700 × *g* for 1 hr and stored at −80°C in 100-μl aliquots. The substrate mixture, as prepared by the method of Nilsson-Ehle and Schotz (9), consisted of 300 mg triolein (99% purity, Sigma Chemical Co.), 0.5 mCi [^{3}H]triolein (15.4 Ci/mmole, NEN Research Products, Boston, MA), 18 mg phosphatidylcholine and 6.25 mg glycerol. The LPL assay mixture was obtained by vigorously mixing 1.1 ml of the substrate mixture in 4 volumes of 0.2 M Tris-HCl buffer, pH 8.6, containing 0.15 M NaCl and 3% bovine serum albumin (fatty acid-

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Abbreviations: HDL, high density lipoprotein; HL, hepatic lipase; LPL, lipoprotein lipase; TG, triglyceride; (V)LDL, (very) low density lipoprotein; CT, ad libitum-fed control; PF, pair-fed; ZD, zinc-deficient.

free; Sigma Chemical Co.). The mixture was then diluted by mixing with 6.38 ml of the buffer without albumin.

For LPL assay, 10 μl of postheparin plasma was preincubated for 10 min at 27°C, with 10 μl of Tris-HCl buffer (0.388 M Tris-HCl and 0.2 M NaCl), pH 8.6, with and without protamine sulfate (10). The protamine concentration of the buffer was 60 mg/ml. To the preincubated mixture, 180 μl of the assay mixture was added and incubated for 60 min at 27°C. The optimal plasma volume and assay durations were predetermined by using a series of plasma dilution and incubation intervals. LPL activity was linear with increasing concentrations of postheparin plasma from 5 to 30 μl and plateaued at 40–70 μl. With 10 μl of postheparin plasma, LPL activity was linear with time up to 90 min. At 60 min of incubation, 3.25 ml of methanol:chloroform:heptane (1.41:1.25:1.0, v/v/v), and 1.0 ml of 0.1 M boric acid and 0.1 M K_2CO_3 (pH 10.5) were added. The mixture was then partitioned by vortexing vigorously for 30 sec and centrifuged at 1500 × *g* for 30 min. One-ml aliquot of the upper phase was used to determine the ^{3}H-radioactivity of oleic acid released. All assays were performed in duplicates. LPL activity was computed by the difference between total postheparin plasma lipase and hepatic lipase (protamine-resistant) activities.

Determination of tissue LPL activities. Five rats from each group were fasted for 18 hr. Under ether anesthesia, the rats were exsanguinated and the retroperitoneal white adipose and gastrocnemius muscle of the left leg were removed. The tissues were homogenized in ice-cold 0.25 M sucrose in 1 mM EDTA buffer, pH 7.4. The soluble fractions of the tissues were prepared by centrifuging the tissue homogenates, as described previously (11).

The LPL activities were assayed by using both an artificial substrate prepared as above and a natural substrate (lymph chylomicrons) obtained from donor rats of both groups. For LPL assays using the artificial substrate, the substrate mixture was prepared as above and the activator sera were prepared by removing very low density lipoprotein (VLDL) and low density lipoprotein (LDL) fractions ultracentrifugally and heating at 62°C for 10 min. This approach was taken to enrich serum with high density lipoprotein (HDL) fraction, which was used as the source of apo Cs, modulators of LPL activity (7,8). Tissue LPL activities were determined by using the HDL-enriched serum fractions (activator sera) from ZD and CT rats, and the effects of the activator sera on the enzyme activity were compared as follows: 1.4 ml of the substrate and 5.6 ml of 0.2 M Tris-HCl buffer containing 3% albumin (pH 8.6) were mixed thoroughly and divided into 2 aliquots of 3.5 ml each. To 1 aliquot, 168 μl of activator serum from ZD rats and 532 μl of 0.15 M NaCl were added and mixed. To the other, the same amounts of activator serum from CT rats and saline were added. To 100 μl of each of the aliquots, 100 μl of the soluble fraction (enzyme source) of the adipose or muscle tissues was added and incubated for 37°C for 60 min. The assay system so prepared contained 4.0 μl of activator serum per 200 μl. The tissue LPL activity was linear with increasing the activator serum from 2 to 16 μl/200 μl of the assay system.

To prepare the chylomicron substrate, chylomicrons were obtained from the donor rats from both ZD and CT groups and purified as reported previously (2,3). The purified chylomicrons were labeled with [^{3}H]triolein by a slight modification of Fielding (12) as follows: Using a syringe with a 25-G needle, [^{3}H]triolein (500 μCi) dissolved in 1.0 ml of dimethylsulfoxide was slowly added to 4.0 ml of 10 mM Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl, 1 mM EDTA and 0.01% NaN_3 and mixed by vortexing. To this mixture, 5.0 ml of purified chylomicrons (500 mg triglyceride) was added, mixed gently and incubated at 37°C for 3 hr. After dialyzing in 4 l of 0.15 M NaCl with 0.1% EDTA (pH 7.4) at 4°C for 18 hr, the mixture was centrifuged in a Beckman 50.3 Ti rotor at 45,000 rpm for 15 min. The top chylomicron fraction was resuspended in 0.15 M NaCl (pH 7.2) and washed. The ^{3}H-radioactivity of the chylomicrons so prepared ranged from 38 to 60 μCi/100 mg triglyceride (TG). The TG concentration of the labeled chylomicrons, as obtained from both ZD and CT rats, was adjusted exactly to 68.5 mg/ml saline. The substrate was prepared by mixing the ^{3}H-chylomicrons (82.2 mg TG) from both sources in 4.8 ml of 0.2 M Tris-HCl buffer with 3% albumin (pH 8.0) and 1.2 ml saline. The LPL activity was determined by incubating 100 μl of the above substrate mixture with 100 μl of the soluble fraction of the tissues at 37°C for 2 hr. LPL activities were linear with chylomicron-TG concentrations from 0.2 to 1.5 mg/100 μl of the substrate mixture. For LPL assay using the chylomicron substrate, no activator sera were used because of the presence of apo Cs in chylomicrons, but the effects of the two chylomicron substrates, as obtained from ZD and CT rats, were compared. All assays were performed in duplicates. The ^{3}H-radioactivity of oleic acid released was partitioned and measured, as described above for plasma LPL assay.

Plasma zinc analysis. Five rats from each group were fasted for 18 hr. Blood samples were collected from the orbital sinus by using heparinized capillary tubes during the mid-dark period (0800–0900) of the light cycle. Plasma zinc was analyzed by atomic absorption spectrophotometry (Perkin-Elmer Co., Norwalk, CT). Zinc standards were prepared from a Fisher-certified reference standard (Fisher Scientific Co., Fair Lawn, NJ).

Statistics. Analysis of variance and Duncan's multiple range test were used for comparisons among three group means, and t-test was used to determine the effect of activator sera on LPL activity. The statistical analyses were performed using a computer software (PC ANOVA, Human Systems Dynamics, Northridge, CA). The level of significance was determined at $p < 0.05$.

RESULTS

General observations. The food intake, body weight, and plasma zinc status of the rats, as affected by dietary treatment, are shown in Table 1. The purpose of supplementing the basal diet containing 0.8 ppm with additional 2 ppm of zinc for ZD rats was to prevent a drastic decrease in food intake and resultant rapid weight loss in these rats and to produce a marginal level of zinc deficiency. For the 8-wk period, no significant difference in body weight was noticed between ZD and PF rats. Significant differences in food intake and body weight between ZD and CT groups were evident during wk 4 and thereafter. Plasma zinc in ZD rats, as determined at wk 8, was significantly lower when compared with PF and CT controls. However, no external symptoms of zinc deficiency such as skin lesions and alopecia were detectable, as

TABLE 1

Effects of Experimental Diets on the Food Intake, Body Weight and Plasma Zinc Status of the Rats[1]

	Food intake		Body weight		Plasma zinc
	1 wk	8 wk	Initial	8 wk	
	g/rat/day		g/rat		μg/ml
ZD	17.0 ± 0.6	14.2 ± 0.3^a	165 ± 7	277 ± 8^a	1.03 ± 0.08^a
PF	17.0 ± 0.6	14.2 ± 0.3^a	165 ± 6	281 ± 9^a	1.73 ± 0.04^b
CT	16.3 ± 0.3	16.3 ± 0.2^b	154 ± 8	335 ± 5^b	1.85 ± 0.04^b

[1]Mean ± SEM of 20 rats, except for plasma zinc from 5 rats selected from each group. Values in the same column not sharing a common superscript are significantly different ($P < 0.05$).

TABLE 2

Effect of Experimental Diets on the Activities of Postheparin Plasma, Lipoprotein (LPL) and Hepatic Lipases[1]

	Total postheparin lipase activity	LPL activity	HL activity
	μmoles of ^{3}H-oleic acid/ml plasma/hr		
ZD	10.2 ± 0.5^a	6.2 ± 0.3^a	4.0 ± 0.3
PF	12.2 ± 0.4^b	8.2 ± 0.4^b	4.0 ± 0.1
CT	12.4 ± 0.3^b	8.5 ± 0.4^b	3.9 ± 0.1

[1]Mean ± SEM of 5 rats. Values in the same column not sharing a common superscript are significantly different ($P < 0.05$).

observed in severe zinc deficiency produced with lower dietary zinc in young rats (1).

Postheparin plasma LPL activity. The effect of zinc deficiency on postheparin plasma LPL activity is presented in Table 2. Because the intravenous injection of heparin is known to release both endothelial LPL and hepatic lipase (HL) into plasma (10), LPL activity was measured by subtracting HL activity from the total activity of postheparin TG hydrolase. The total plasma TG hydrolase activity was significantly decreased in ZD, relative to PF and CT controls. The decrease was due to a significant decrease in LPL activity. The plasma LPL activity in ZD rats was 75.6 and 72.9% of that in PF and CT groups, respectively. Postheparin plasma HL activity was not affected by zinc deficiency.

Table 3 compares the effects of zinc deficiency and activator sera from ZD and CT rats on the LPL activity of retroperitoneal adipose and gastrocnemius muscle tissues. Data, as obtained by using the lipid emulsion substrate, showed that zinc deficiency per se did not affect the LPL activity of either the adipose or muscle tissue. The LPL activities of the adipose tissues from all 3 groups did not significantly differ, whether assayed with the activator serum from ZD or CT rats. However, the LPL activities of the gastrocnemius muscles were significantly decreased by the ZD activator serum, compared with the activity determined with the CT activator serum. The muscle LPL activities in ZD, PF and CT groups, as assayed with ZD activator serum, were decreased to 81.0, 80.4 and 76.5%, respectively, of the activity determined with CT activator serum.

TABLE 3

Effect of Experimental Diets and Activator Sera From ZD and CT Rats on the Tissue Activities[1] of Lipoprotein Lipase (LPL)[2]

	Source of LPL activator	
Diet group	ZD serum	CT serum
Adipose[3]	nmoles of ^{3}H-oleic acid/mg protein/min	
ZD	7.5 ± 0.4^a	7.7 ± 0.7^a
PF	8.8 ± 0.4^a	8.9 ± 0.5^a
CT	7.3 ± 0.3^a	7.9 ± 0.5^a
Muscle[4]	nmoles of ^{3}H-oleic acid/mg protein/hr	
ZD	9.4 ± 0.3^a	11.6 ± 0.5^b
PF	8.2 ± 0.3^a	10.2 ± 0.4^b
CT	9.1 ± 0.4^a	11.9 ± 0.7^b

[1]Mean ± SEM of 5 rats. Values in the same column or row not sharing a common superscript are significantly different ($p < 0.05$).

[2]As determined by using an artificial lipid emulsion labeled with [^{3}H]triolein as the substrate.

[3]Retroperitoneal white adipose tissue.

[4]Gastrocnemius muscle.

TABLE 4

Effect of Experimental Diets on the Tissue Activities[1] of Lipoprotein Lipase[2]

	Source of LPL substrate	
Diet group	ZD chylomicron	CT chylomicron
Adipose[3]	nmoles of ^{3}H-oleic acid/mg protein/min	
ZD	25.6 ± 2.8^a	21.8 ± 2.4^a
PF	26.2 ± 1.4^a	26.1 ± 1.5^a
CT	24.3 ± 2.3^a	23.6 ± 4.0^a
Muscle[4]	nmoles of ^{3}H-oleic acid/mg protein/hr	
ZD	31.0 ± 4.2^a	47.9 ± 1.0^b
PF	32.4 ± 0.9^a	46.1 ± 1.0^b
CT	33.5 ± 1.7^a	46.0 ± 2.5^b

[1]Mean ± SEM of 5 rats. Values in the same column or row not sharing a common superscript are significantly different ($p < 0.05$).

[2]As determined by using lymph chylomicrons labeled with ^{3}H-triolein as the substrate, as obtained from ZD and CT donor rats.

[3]Retroperitoneal white adipose tissue.

[4]Gastrocnemius muscle.

Similarly, the LPL activities of the adipose tissues from ZD, as well as control groups, remained unchanged, regardless of the source of the chylomicrons used as the substrates (Table 4). As with the artificial substrate, the muscle LPL activities were significantly lower in all 3 groups, when measured with the chylomicron substrate from ZD group, compared with the activities assayed with CT chylomicrons. The LPL activities in ZD, PF and CT groups, when measured with ZD chylomicrons as the substrate, were 64.7, 70.3 and 72.8%, respectively, of the activities obtained with CT chylomicrons.

DISCUSSION

Dietary TG is carried primarily by chylomicrons from intestinal mucosa to plasma and is rapidly cleared from the circulation by the action of endothelial lipoprotein lipase (LPL) of extrahepatic tissues and by the subsequent uptake by liver of the chylomicron remnants formed after lipolysis by LPL (13). In this regard, LPL plays a key role in the clearance of chylomicron TG from plasma, as well as in the peripheral utilization of its hydrolytic products (fatty acids). The present data demonstrate that zinc deficiency significantly lowers the postheparin plasma activity of LPL.

In several earlier studies (14–16), significant elevations in plasma (or serum) TG have been observed in zinc-deficient rats, suggesting a possible impairment in the plasma clearance of TG. However, some studies (17,18) have reported decreases in serum TG in zinc-deficient animals. The conflicting observations may be ascribable to the differences in the degree of zinc deficiency produced in different studies and also the well-characterized difference in feeding pattern between zinc-deficient and pair-fed control animals. In a recent study (19), in an effort to overcome such variables, Park et al. have used the method of intragastric force-feeding for both zinc-deficient and pair-fed controls and demonstrated a marked elevation in serum TG in zinc-deficient rats. This finding (19) reaffirms that the hypertriglyceridemia observed in zinc-deficient animals is not related to a difference in food intake or feeding pattern, but due to zinc deficiency.

Whether the increase in serum TG results from endogenous or dietary triglyceride is not presently known. However, the contribution of endogenous synthesis to serum TG may not be significant, considering the general catabolic state induced by zinc deficiency, as characterized by hypoinsulinemia, ketosis and reduction in fat reserves and muscle mass (14,19–21). Therefore, the elevation in serum TG, as demonstrated by intragastric feeding (19), may be due primarily to the impaired removal of TG of dietary origin. This is consistent with the observed low LPL activity and with the significant delay in the initial plasma clearance of [^{14}C]cholesterol-labeled chylomicrons (6) observed in zinc-deficient rats. Under nonforce-feeding conditions, however, the diet-induced hypertriglyceridemia may not be apparent in zinc-deficient rats despite the low LPL activity, because of the decrease in food intake and/or impaired absorption of fat (1,2).

The results obtained by using an artificial lipid emulsion and chylomicrons as the substrates (Tables 3 and 4) demonstrate that the LPL activity of the gastrocnemius muscle was consistently lower, when the assay system contained either the chylomicrons as the substrate or the HDL-enriched serum as the activator derived from ZD rats, compared with those from CT rats. Under the same assay conditions, however, the LPL activity of the retroperitoneal adipose tissue was unresponsive to the change in the substrate or the activator. This suggests that adipose LPL enzyme may be less sensitive to the concentrations of the modulator (activator) apolipoproteins (apo Cs) than muscle LPL. It has previously been shown that LPL enzymes from muscle and adipose tissues are distinct with regard to their molecular weights and kinetic properties (22,23).

The heparin-releasable LPL activity in the plasma represents the composite activity of endothelial-surface LPL from various peripheral tissues. The total endothelial LPL activity in postheparin plasma would, therefore, depend on the total tissue masses and, hence, the degree of tissue vascularization. It might be possible that the lower LPL activity in postheparin plasma is partly related to a general reduction in total tissue mass. However, in the present study, no relationships were found between postheparin LPL activities and body weights of rats. The data on tissue LPL activities from both experiments with activator sera and chylomicrons from the two different sources (Tables 3 and 4) strongly suggest that the decrease in heparin-releasable plasma LPL activity may be attributable, at least partly, to zinc deficiency-induced alterations in the concentrations of LPL-modulator (activator) apolipoproteins (apo Cs) in the chylomicrons and HDL-enriched serum (activator serum). This conclusion is also in line with the significant decreases in the total apo-C contents of lymph chylomicrons (3) and plasma HDL (24) and a reduction in the total plasma concentration of HDL apo C (25) in marginally zinc-deficient rats. Because chylomicrons, upon their release into the plasma, acquire apo C mainly from plasma HDL (26), the decrease in HDL apo C would consequently affect the peripheral lipolysis and removal of chylomicron triglyceride. In view of the evidence that apo C-II activates and apo C-III inhibits LPL activity (7,8), it is suggested that zinc deficiency may lower the circulating level of apo C-II or the ratio of apo C-II to apo C-III. Previously, it has been shown (27) that the ratio of apo C-II to apo C-III was significantly lower in zinc-deficient rats, relative to the ratio in ad libitum-fed controls, although it did not differ from that in pair-fed rats.

In summary, the present study provides evidence that zinc deficiency significantly lowers the LPL activity in postheparin plasma and in the gastrocnemius muscle. The data and earlier observations suggest that the lowering of LPL activity in marginal zinc deficiency may be due to a significant decrease in activator apo C in the plasma, rather than a change in the enzyme. However, in the present study, we examined the total tissue LPL activities. Further studies are needed to determine separately the endothelial-surface (heparin releasable) and cell-associated (nonheparin releasable) LPL activities. The effects of the low LPL activity on the utilization of dietary fat by muscle and adipose tissues, and the clinical implications of a high fat intake and ensuing possible hyperlipidemia in zinc deficiency should be further examined.

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Alpha- and Gamma-Tocopherol Levels in Lipoproteins Fractionated by Affinity Chromatography

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Alpha- and gamma-tocopherol levels of nine women were measured in whole serum and in serum lipoproteins separated by heparin affinity chromatography. Alpha-tocopherol levels (mean ± SD) in whole serum, low density plus very low density lipoproteins and high density lipoproteins were 10.8 ± 2.7, 6.4 ± 1.6 and 4.6 ± 1.4 (μg/ml), respectively. Corresponding values (μg/ml) for gamma-tocopherol were 1.2 ± 0.5, 0.7 ± 0.3 and 0.6 ± 0.2. Recoveries of serum alpha- and gamma-tocopherol from the heparin columns were 102 ± 5% and 105 ± 7%, respectively. Serum alpha-tocopherol was linearly correlated with components of high density lipoprotein (apolipoproteins, high density lipoprotein cholesterol), but not with serum total lipids or indices of low density lipoprotein, even though high density lipoprotein carried less than half of the serum alpha-tocopherol. However, serum gamma-tocopherol was highly correlated with indices of serum lipids, such as serum cholesterol ($r = 0.92$, $p = 0.005$). The coefficient for the correlation of low density lipoprotein (+ very low density lipoprotein) tocopherol with high density lipoprotein tocopherol was $r = 0.66$ ($p = 0.06$) for alpha-tocopherol and $r = 0.84$ ($p = 0.004$) for gamma-tocopherol. These differences in the relationships of the two tocopherols to lipids and lipoproteins support the view that when the two tocopherols are present at normal dietary levels, gamma-tocopherol partitions between lipoproteins based on their relative lipid content, but a portion of the alpha-tocopherol in high density lipoprotein is specifically bound.
Lipids 24, 137-140 (1989).

In plasma, tocopherols are transported primarily by high density lipoproteins (HDL) and low density lipoproteins (LDL). Fasting, normolipemic humans have relatively minor amounts of tocopherol carried by very low density lipoproteins (VLDL) (1,2). It is widely believed that the concentration of alpha-tocopherol in serum is primarily a function of serum lipid levels, and that alpha-tocopherol rides passively and nonspecifically in the lipid medium of lipoproteins (3-5). However, the exact location of tocopherol in lipoproteins is not known. Behrens and Madere (6,7) have recently suggested that alpha-tocopherol concentration in plasma may be controlled by specific carriers or transport proteins that regulate absorption, transport and tissue uptake. They also suggested that sex differences in distribution of alpha-tocopherol among lipoproteins may be related to the parallel sex differences in protein distribution among lipoproteins. Because women generally have higher ratios of HDL protein (predominantly apolipoprotein [apo] A) to LDL protein (apo B) than do men, women would tend to carry greater proportions of alpha-tocopherol in HDL relative to LDL.

Typically, studies to determine the distribution of alpha-tocopherol among lipoproteins or the regulation of alpha-tocopherol within lipoproteins have used ultracentrifugation, sometimes combined with gel filtration, to separate lipoproteins. These techniques are labor intensive and require equipment not readily available to most laboratories. More importantly, substantial losses of both alpha-tocopherol (1,2) and apoproteins (8) occur during isolation of lipoproteins by these techniques. Another approach for lipoprotein separation would be the use of precipitation techniques (9,10), which are commonly used to isolate HDL for cholesterol analysis. However, LDL tocopherol would be calculated by difference and the LDL moiety would be lost for further study.

Affinity chromatography offers an attractive alternative for lipoprotein separation not only because lipoprotein fractions remain in solution, but also because it is a rapid and simple technique. The present study was designed to evaluate tocopherol content of lipoproteins separated on heparin affinity columns. Based on this methodology, some initial findings are reported that relate alpha- and gamma-tocopherol to apoprotein and cholesterol content of lipoproteins.

MATERIALS AND METHODS

Nine women, 20-40 years of age, were randomly selected from a group participating in a controlled diet study (11). All subjects ate a low-fat (20% of calories as fat) diet, which met the Recommended Dietary Allowances. (All procedures were approved by the Human Studies Review Committees of the U.S. Department of Agriculture, Agricultural Research Service and the Georgetown University Institutional Review Board.) Estimated average daily intake of alpha-tocopherol was 15 mg per day and included dl-alpha-tocopheryl acetate from fortified breakfast cereals. Subjects did not take vitamin supplements, estrogens or other medications. Following a 12 hr fast, blood was drawn, cold clotted and serum separated by low speed centrifugation at 4°C.

Serum lipoproteins were fractionated on heparin columns (LDL Direct, Isolab Inc., Akron, OH) according to the manufacturer's instructions. Briefly, after applying whole serum (200 μl) to the column, HDL and LDL (+ VLDL) were eluted with low and high ionic strength buffers, respectively. Volumes were adjusted to exactly 1.2 ml. For comparison, a separate sample of HDL was produced from each serum sample by precipitating non-HDL lipoproteins with phosphotungstate-Mg^{2+} (9). Column eluates (100 μl) containing HDL or LDL (+VLDL) and HDL from the precipitation method (50 μl) were mixed with 1 ml of 100% methanol, and tocopherols from 75 μl of the methanolic extracts were measured by a high performance liquid chromatographic (HPLC) procedure

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Abbreviations: HDL, high density lipoprotein; (V)LDL, (very) low density lipoprotein; HPLC, high performance liquid chromatograph(ic,y).

previously described for whole plasma (12). The entire process, including tocopherol analysis, was performed in one day.

Serum triglycerides were assayed by an enzymatic method (13) using a centrifugal analyzer. An enzymatic method (14) was used to determine cholesterol in whole serum and in HDL column fractions. Cholesterol content of LDL (+VLDL) was calculated as the difference between serum total cholesterol and the HDL cholesterol measured from HDL column fractions. Total serum lipids were estimated as (2 × cholesterol) + triglycerides (15). Apo A and apo B were assayed in triplicate from whole serum by electroimmunoassay (16) using rabbit antisera to human apoproteins (Behring Diagnostics, La Jolla, CA). Antisera to apo A was raised against total apo A with apo A-I and A-II present in a ratio of 65:35, the approximate ratio of these apoproteins in human serum (17). Serum samples for apo A analysis were diluted 1:50 (v/v) in electrophoresis buffer, containing (per liter) tris, 2.9 g; sodium barbital, 4.9 g; barbital, 1.2 g; EDTA, 0.1 g and NaN_3, 0.1 g; pH 8.6. These samples were heated at 52°C for 3 hr to expose antigenic sites. For apo B analysis, serum was diluted 1:10 (v/v) in electrophoresis buffer and assayed without delipidation. (In our hands, the within-assay coefficient of variation for the electroimmunoassay was 2.3% for apo A and 2.9% for apo B; both calculated from 26 replicates of a pooled plasma sample. The between-assay coefficient of variation was typically 5% for apo A and 6% for apo B.) Samples for apoprotein analysis were stored at −20°C prior to analysis.

Data were analyzed using the SAS system (SAS Institute, Cary, NC). Correlation coefficients were determined by the Pearson product-moment method, and the paired t-test was used to compare mean values for tocopherols by method of lipoprotein separation.

RESULTS

Heparin affinity chromatography. Recoveries (mean ± SD) of serum alpha- and gamma-tocopherol from heparin affinity columns were 102 ± 5% and 105 ± 7%, respectively. LDL (+VLDL) contained 59 ± 6% of the recovered alpha-tocopherol and 54 ± 8% of the gamma-tocopherol. To assess the accuracy of the column recoveries, HDL tocopherol values from HDL column fractions were compared with tocopherol values from HDL obtained by precipitation of non-HDL lipoproteins. Values (mean ± SD) for HDL tocopherol isolated by affinity columns and by the precipitation technique were, respectively, 4.6 ± 1.4 and 4.4 ± 1.3 μg/ml for alpha-tocopherol; 0.6 ± 0.2 and 0.6 ± 0.3 μg/ml for gamma-tocopherol. These means were not significantly different by method of separation.

Tocopherol levels and relationships to lipoproteins. Table 1 shows means and ranges for tocopherol, lipid and apoprotein values of serum, HDL and LDL (+VLDL). Coefficients for linear correlations of alpha- and gamma-tocopherol in serum, HDL and LDL (+VLDL) with serum lipids, apoproteins and lipoprotein cholesterol are in Table 2. In serum, alpha-tocopherol was linearly associated ($p \leq 0.05$) with apo A and with HDL cholesterol, and gamma-tocopherol was linearly associated with total lipids, serum cholesterol, apo B and LDL (+VLDL) cholesterol. Within HDL, only alpha-tocopherol was linearly

TABLE 1

Tocopherol, Lipid and Apoprotein Levels in Serum, HDL and LDL (+VLDL) of Nine Women

Component	Mean ± SD	Range
Serum:		
Alpha-tocopherol (μg/ml)	10.8 ± 2.7	7.4–14.2
Gamma-tocopherol (μg/ml)	1.2 ± 0.5	0.6–2.1
Total lipids (mg/dl)[a]	462 ± 81	385–613
Cholesterol (mg/dl)	197 ± 36	165–263
Triglycerides (mg/dl)	68 ± 15	43–90
Apo A (mg/dl)	186 ± 42	116–270
Apo B (mg/dl)	83 ± 12	65–99
HDL:		
Alpha-tocopherol (μg/ml)	4.6 ± 1.4	2.5–6.4
Gamma-tocopherol (μg/ml)	0.6 ± 0.2	0.3–1.0
Cholesterol (mg/dl)	53 ± 15	38–76
LDL (+VLDL):		
Alpha-tocopherol (μg/ml)	6.4 ± 1.6	4.7–8.8
Gamma-tocopherol (μg/ml)	0.7 ± 0.3	0.3–1.3
Cholesterol (mg/dl)[b]	144 ± 36	122–223

[a]Calculated: total lipids = (2 × cholesterol) + triglycerides.

[b]Calculated: LDL (+VLDL) cholesterol = serum cholesterol − HDL cholesterol.

TABLE 2

Coefficients (r) for Linear Correlations of Alpha- and Gamma-Tocopherol (α-T, γ-T) in Serum, HDL and LDL (+VLDL) with Serum Lipids, Apoproteins and Lipoprotein Cholesterol

		Serum		HDL		LDL (+VLDL)	
Component		α-T	γ-T	α-T	γ-T	α-T	γ-T
Serum lipids							
Total lipids	r	0.49	0.92	0.30	0.91	0.60	0.84
	p[a]	NS	0.0004	NS	0.0006	NS	0.005
Triglycerides	r	0.003	0.51	−0.07	0.54	0.006	0.44
	p	NS	NS	NS	NS	NS	NS
Cholesterol	r	0.55	0.92	0.35	0.90	0.67	0.84
	p	NS	0.0005	NS	0.0008	0.05	0.004
Serum apoproteins							
Apo A	r	0.76	0.38	0.67	0.49	0.57	0.26
	p	0.02	NS	0.05	NS	NS	NS
Apo B	r	0.49	0.75	0.32	0.82	0.55	0.71
	p	NS	0.02	NS	0.007	NS	0.03
Lipoprotein cholesterol							
HDL cholesterol	r	0.81	0.08	0.87	0.26	0.48	−0.14
	p	0.009	NS	0.003	NS	NS	NS
LDL (+VLDL) cholesterol	r	0.23	0.91	0.002	0.82	0.49	0.92
	p	NS	0.0007	NS	0.007	NS	0.0004

[a]Probability of ≤0.05 indicates that there was a linear correlation between the indicated variables.

NS, not significant at the probability of 0.05 under the Ho: Rho = O/N = 9.

associated with apo A and with HDL cholesterol. Within LDL (+VLDL), only gamma-tocopherol was linearly associated with apo B and LDL (+VLDL) cholesterol. Also, there were linear associations between apo A and HDL cholesterol ($r = 0.85$, $p = 0.004$) and between apo B and both serum total cholesterol ($r = 0.82$, $p = 0.007$) and LDL (+VLDL) cholesterol ($r = 0.74$, $p = 0.02$). The coefficient for the correlation of LDL (+VLDL) tocopherol with HDL tocopherol was $r = 0.66$ ($p = 0.06$) for alpha-tocopherol and $r = 0.84$ ($p = 0.004$) for gamma-tocopherol.

DISCUSSION

Affinity columns. By binding apo B, heparin columns retain VLDL and LDL, whereas HDL passes through. VLDL and LDL are then eluted with a saline solution of increased ionic strength. Values for HDL tocopherols obtained by this fractionation method were comparable to tocopherol values obtained by phosphotungstate-Mg^{2+} precipitation. Although not significantly different, HDL tocopherol values from the precipitation method tended to be slightly lower. That is consistent with reports that HDL cholesterol values by the phosphotungstate-Mg^{2+} precipitation method are about 7% lower than HDL cholesterol values from ultracentrifugation or heparin-Mn^{2+} precipitation techniques (18).

In contrast to the precipitation technique, heparin affinity columns allowed direct determination of tocopherol in both LDL (+VLDL) and HDL fractions, with the added benefit of retaining lipoproteins in a soluble state suitable for subsequent chemical analysis. The lack of separation of VLDL from LDL did not present a problem in this study, because VLDL contribution to total serum alpha-tocopherol is negligible—less than 3% in fasting, normolipemic women (1,2). Similarly, the presence of apo B in both VLDL and LDL did not greatly distract from using apo B as an index of LDL concentration because ca. 90% of apo B is associated with LDL.

Alpha-tocopherol levels in whole serum were similar to those reported by Behrens and coworkers (1,2) for women of similar age (Table 3). Yet, in those studies, alpha-tocopherol content of lipoproteins, isolated by ultracentrifugation and subsequent gel filtration, was 72% (1) and 68% (2) of the original serum value. In contrast, recoveries of alpha-tocopherol from lipoproteins fractionated by the heparin affinity columns were 102%. Our alpha-tocopherol values and recoveries for LDL (+VLDL) were twofold higher than those of Behrens and coworkers, but were strikingly similar for HDL. These observations indicate that tocopherol loss associated with the ultracentrifugation and gel filtration method of isolating lipoproteins was selective for LDL. Our experience has been that once LDL is isolated from serum, tocopherols in this fraction are unstable. The improved recovery of LDL (+VLDL) tocopherol by the affinity column method may be a function of the decreased time lapse (hours rather than days) from blood collection to tocopherol analysis of lipoprotein fractions.

Relationship of tocopherols to lipoproteins. Correlation data have the limitation of only showing strengths of associations and cannot be used to prove cause and effect. However, if there is a cause and effect that has a linear association, then the correlation data must reflect that relationship. For example, if the distribution of tocopherol between lipoproteins is in equilibrium, then the correlation of tocopherol contents of HDL and LDL (+VLDL) should be very strong. Such was the case for gamma-tocopherol. On the other hand, no significant linear relationship between alpha-tocopherol contents of LDL (+VLDL) and HDL was found. These findings are consistent with the concept that alpha-tocopherol distribution among lipoproteins is not solely a function of nonspecific diffusion into a lipid medium. One explanation would be that some portion of the alpha-tocopherol in HDL is not in equilibrium with serum total lipids or serum cholesterol, but instead, is specifically bound by some component of HDL (1).

Gamma-tocopherol content of serum, HDL and LDL (+VLDL) was strongly correlated not only with serum total lipids and cholesterol, but also with LDL cholesterol, which accounts for the majority of cholesterol in serum. It should be noted that correlations of gamma-tocopherol in serum, HDL and LDL (+VLDL) with total lipids was similar to the correlation of these variables with serum cholesterol. (This was because values for total lipids were derived using a formula that largely depends on cholesterol levels and, to a lesser extent, triglyceride levels, which were consistently low in these subjects. Thus, for our subjects, the strong association of gamma-tocopherol with total lipids appears to be due to the correlation with serum cholesterol.) Although our results are consistent

TABLE 3

Distribution of Alpha-Tocopherol (α-T) in Lipoprotein Fractions of Women: Comparison of Values in Literature to Those of Present Study

	Ultracentrifugation and gel filtration				Affinity column	
	Ref. 1, n = 7		Ref. 2, n = 6		Present study, n = 9	
Component	μg/ml (mean ± SD)	% of serum α-T	μg/ml (mean ± SE)	% of serum α-T	μg/ml (mean ± SD)	% of serum α-T
Serum	8.97 ± 2.84	100	10.35 ± 0.86	100	10.8 ± 2.7	100
VLDL	0.09 ± 0.09		0.15 ± 0.03		—	
LDL	2.73 ± 0.72		2.85 ± 0.30		—	
LDL (+VLDL)[a]	2.82	31	3.00	29	6.4 ± 1.6	59
HDL	3.66 ± 1.78	41	4.00 ± 0.71	39	4.6 ± 1.4	43

[a]For Ref. 1 and 2, LDL (+VLDL) is the sum of individual measures of VLDL and LDL tocopherol. For the present study, LDL (+VLDL) was measured from column fractions that included both VLDL and LDL.

with the view that distribution of gamma-tocopherol among lipoproteins is nonspecific, increased alpha-tocopherol intake is known to result in the displacement of gamma-tocopherol by alpha-tocopherol in serum (19), as well as in red blood cells, platelets and lymphocytes (20). Therefore, results may differ when dietary intake of tocopherol is lower than in this study. Also, the fact that subjects consumed a low-fat diet may have bearing on these data.

In contrast to gamma-tocopherol, alpha-tocopherol contents of serum, HDL and LDL (+VLDL) were not generally related to indices of serum lipids. This conflicts with some previous studies that report correlations between serum alpha-tocopherol and either total cholesterol (15,21,22) or serum total lipids (22,23). However, this discrepancy may only reflect a more limited range of total lipid levels in this study.

A strong affinity of alpha-tocopherol for HDL was observed by Granot et al. (24) in their in vitro studies of [H_3] alpha-tocopherol distribution between lipoproteins. The association of alpha-tocopherol with lipoproteins was not a function of lipid mass ratios; in fact, most of the alpha-tocopherol remained associated with HDL even when LDL lipid mass was tenfold that of HDL. Transfer of alpha-tocopherol from HDL to LDL was decreased from 55 to 20%, and transfer from LDL to HDL was increased from 40 to 70%, by decreasing the mass ratio of LDL (the LDL to HDL protein ratio) from 4 to 1. Under physiological conditions, the mass ratio (estimated as the apo B to apo A ratio) would be about 0.5 for normolipemic women. Mass ratios in the physiological range would, therefore, be expected to favor retention of alpha-tocopherol by HDL and donation of alpha-tocopherol by LDL.

Behrens et al. (1,2) have speculated that HDL protein (presumably apo A, which accounts for ca. 90% of HDL protein mass) binds alpha-tocopherol, thus regulating alpha-tocopherol concentrations in HDL. Consistent with their studies, we found evidence that HDL alpha-tocopherol is linearly related to serum apo A, but no such relationship was found between LDL alpha-tocopherol and serum apo B, even though in this study, LDL carried a larger proportion (59 $\pm$ 6%) of the total serum alpha-tocopherol than did HDL. These observations support the concept that HDL contains one or more components specific for binding alpha-tocopherol. However, the HDL component responsible for this postulated alpha-tocopherol binding could not be identified in this study.

Even though some target tissues may acquire alpha-tocopherol as a component of LDL, as suggested by alpha-tocopherol entry into fibroblasts via the LDL receptor (25,26), it may be the alpha-tocopherol in HDL that ultimately reaches other target tissues. In the rat, those tissues with the highest levels of alpha-tocopherol (adrenal and ovary) (27) are also known to selectively bind HDL, the source of cholesterol for steroid synthesis in these tissues (28,29). If some target tissues take up alpha-tocopherol preferentially from HDL, then selective association of tocopherol with HDL would provide a biological advantage to those tissues by ensuring supply when plasma alpha-tocopherol levels are low.

Our data support assertions by Behrens and coworkers (1,2) that gamma-tocopherol may be carried passively in serum lipoproteins, but that alpha-tocopherol content of HDL is, to some degree, regulated. The biological advantage of regulating alpha-tocopherol content in HDL is unknown, but may be related either to the size advantage HDL would have over other lipoproteins in moving from plasma to peripheral tissues, or to the apo A component of HDL, which may allow certain tissues to selectively take up HDL-associated alpha-tocopherol.

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Fatty Acid Pattern of Tissue Phospholipids in Copper and Iron Deficiencies

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Because copper and iron have been reported to be essential cofactors in Δ9 desaturation of fatty acids, the effects of different dietary intakes of copper and iron on tissue fatty acids were studied. Male Long-Evans rats (ten per group) were fed diets containing adequate, deficient or excess copper or iron. On day 42 of the dietary regimen, the animals were killed and tissues and blood were removed for analysis of metals and fatty acids of phospholipids. Compared with the copper-adequate rats, the copper-deficient rats showed increased 18:0 in liver and decreased 16:1ω7 in liver, heart and serum. There were no differences for 16:0 or 18:1ω9. Intake of excess copper did not cause an increase in products of Δ9 desaturation. Comparisons between iron-deficient and iron-adequate rats showed that iron deficiency increased 18:2ω6 in liver and serum and decreased 20:4ω6 in serum only. Relative percentages of 16:0, 18:0, 16:1ω7 and 18:1ω9 in liver and serum phospholipids were similar for both groups. Intake of excess iron caused a decrease in 18:2ω6; and 16:0 and 18:1ω9 were higher in the liver of the iron-excess group than the iron-deficient group. This study did not support the requirement for copper or iron in the Δ9 desaturation of fatty acids as expressed in phospholipids of liver, heart and serum.

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In rats and pigs (1–3), copper was shown to be a cofactor in the Δ9 desaturation of stearic acid (18:0) to oleic acid (18:1ω9). Male rats fed low-copper had decreased oleic acid content in tissue phospholipids (PL); copper supplementation had an opposite effect (4). High-copper intake caused increased tissue 18:1ω9, and subsequently, 20:3ω9, the major polyunsaturated metabolic product of 18:1ω9 and the fatty acid elevated in essential fatty acid (EFA) deficiency. With an increase in 20:3ω9 and because of the competitive inhibition between the ω6 and ω9 metabolic pathways, the amount of arachidonic acid (20:4ω6) decreased and its precursor, linoleic acid (18:2ω6), increased (5). In several species, copper deficiency is accompanied by decreased growth rates, neurological lesions, bone lesions and defects in structure of hair (6), symptoms characteristic of EFA deficiency in humans (7).

Iron has been shown to be a structural component of the Δ9 and Δ6 desaturases (8,9). Rao et al. (10) found decreased 16:1ω7 and 18:1ω9 in liver and in other tissues of iron-deficient (FeD) rats; however, 18:2ω6 and 20:4ω6 were 2- to 3-fold greater in iron-deficient than in iron-adequate (FeA) animals. Decreased desaturation of stearic to oleic acid by Δ9 desaturase was shown in rat liver microsomes from animals fed low-iron diets. This could cause the decreases in 16:1ω7 and 18:1ω9 in tissue lipids (11).

Because copper and iron have been reported to be involved in Δ9 fatty acid desaturation (1,2,10,11) and because similar symptoms are found in deficiencies of copper, zinc and EFA (7), the current study was undertaken to elucidate the involvement of copper and iron in fatty acid metabolism.

MATERIALS AND METHODS

Animals and experimental design. Weanling (age 21 days), outbred male Long-Evans [Crl:(LE)BR] rats were purchased from Charles River Breeding Laboratories, Inc. (Wilmington, MA). Upon arrival, rats were placed in stainless steel cages and fed ad libitum a nonpurified diet (Purina Rodent Laboratory Chow No. 5001, Ralston Purina, St. Louis, MO) and tap water. At age 28 days, the rats were matched by weight (ten animals per group), placed individually in suspended stainless steel cages and given deionized water. In each study, three groups of ten animals each were fed diets containing deficient, adequate or excess copper or iron. Daily food intake and weekly weight changes of the animals were measured. The deficient, adequate and excess groups in both studies were given free access to their diets. Pair-fed (PF) rats were fed the copper-adequate (CuA) or FeA diets in amounts equal to the average amount consumed on the previous day by the corresponding deficient group.

Diets. Purified diets contained 21.3% casein protein, 5% safflower oil, and other nutrients as listed in Table 1. The Cu-deficient (CuD) FeA diet contained 0.5 μg Cu and 41.0 μg Fe per gram; CuA–FeA, 5.4 μg Cu and 38.2 μg Fe per gram; Cu-excess (CuE)–FeE, 94.1 μg Cu and 43.8 μg Fe per gram; CuA–FeD, 5.4 μg Cu and 5.6 μg Fe per gram; CuA–FeA, 5.3 μg Cu and 41.7 μg Fe per gram; and CuA–Fe-excess (FeE), 5.2 μg Cu and 731 μg Fe per gram.

Sampling and measurements. On day 42 of the dietary regimen (age 70 days), between 0800 and 0900 hr, and on each of the next 4 days, equal numbers of rats from each dietary group were anesthetized with ether, bled by cardiac puncture and killed. Two ml of blood were placed in a vacutainer (47 × 10.25 mm, #6384, Becton Dickinson, Rutherford, NJ) for hematocrit determinations and the remainder was placed in Radiac-washed, deionized water-rinsed glass tubes. Hematocrits were determined by centrifuging the blood in microhematocrit tubes (Chase Instrument, Lindenhurst, NY). The remaining blood was allowed to clot for 1 hr at room temperature, followed by 2 hr at 4°C. The serum was collected and diluted 1:5 with deionized water. Copper and iron concentrations were determined by flame atomic absorption spectrophotometry (Model 1250 Atomic Absorption Spectrophotometer, Perkin-Elmer, Norwalk, CT).

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Abbreviations: EFA, essential fatty acid; FAME, fatty acid methyl ester; PL, phospholipid; CuA, copper-adequate; CuD, copper-deficient; CuE, copper-excess; FeA, iron-adequate; FeD, iron-deficient; FeE, iron-excess; NR, ratio of experimental value to control value (normalcy ratio); PF, pair-fed.

TABLE 1

Diet Composition

Ingredient	Basal mixtures[a] (g/kg) Cu-deficient	Fe-deficient
Casein	212.766	212.766
DL-methionine	3.192	3.192
Sucrose	409.991	410.203
Cornstarch	308.511	308.511
Fiber (cellulose)	21.276	—
Microcrystalline cellulose	—	21.276
Vitamin mix, Teklad[b]	10.638	10.638
Modified AIN-76 mineral mix		
Calcium carbonate	0.798	0.798
Calcium phosphate	18.617	18.617
Potassium citrate, monohydrate	8.192	8.192
Sodium chloride	2.755	2.755
Potassium sulfate	1.936	1.936
Magnesium oxide	0.894	0.894
Ferric citrate (16.7% Fe)	0.223	—
Manganese carbonate	0.130	0.130
Zinc carbonate	0.060	0.060
Chromium potassium sulfate	0.020	0.020
Potassium iodate	0.0004	0.0004
Sodium selenite	0.0004	0.0004
Cupric carbonate	—	0.0112
Cu-mixes		
Cu-deficient mix:	0.0 g $CuSO_4 \cdot 5H_2O$ plus 1000 g cornstarch	
Cu-adequate mix:	2.0 g $CuSO_4 \cdot 5H_2O$ plus 998 g cornstarch	
Cu-excess mix:	40.0 g $CuSO_4 \cdot 5H_2O$ plus 960 g cornstarch	
Fe-mixes		
Fe-deficient mix:	0.0 g $FeSO_4 \cdot 7H_2O$ plus 1000 g cornstarch	
Fe-adequate mix:	18.0 g $FeSO_4 \cdot 7H_2O$ plus 982 g cornstarch	
Fe-excess mix:	360.0 g $FeSO_4 \cdot 7H_2O$ plus 640 g cornstarch	

	Deficient		Adequate		Excess	
Complete diets						
Diet (g/kg)	Cu	Fe	Cu	Fe	Cu	Fe
Basal mixture	940	940	940	940	940	940
Safflower oil[a]	50	50	50	50	50	50
Cu-mixes	10	—	10	—	10	—
Fe-mixes	—	10	—	10	—	10
Final diet (μg/g)						
Cu	0.5	5.4	5.4	5.3	94.1	5.2
Fe	41.0	5.6	38.2	41.7	43.8	731.0

[a]Teklad Test Diets, Madison, WI.

[b]Teklad (vitamin fortification mix) cat. no. 40060, Teklad Test Diets, Madison, WI. Composition of vitamin mix was (in grams/kilogram): p-amino-benzoic acid, 11.0132, ascorbic acid, coated (97.5%), 101.6604, biotin, 0.0441, vitamin B-12 (0.1% trituration in mannitol), 2.9736, calcium pantothenate, 6.6079, choline dihydrogen citrate, 349.6916, folic acid, 0.1982, inositol, 11.0132, menadione (vitamin K3), 4.9559, niacin, 9.9119, pyridoxine/HCl, 2.2026, riboflavin, 2.2026, thiamine/HCl,2.2026, dry vitamin A palmitate (500,000 U/g), 3.9648, dry ergocalciferol (500,000 U/g), 0.4405, dry vitamin E acetate (300 U/g), 24.2291 and cornstarch, 466.6878.

Hearts and livers were removed from individual rats, frozen at −10°C, lyophilized, transferred to crucibles (Vycor Brand, Corning Glass Works, Corning, NY) and ashed at 450°C in a muffle furnace. The residue remaining after ashing was dissolved in 2.0 ml concentrated HCl (reagent grade, Fisher Scientific, Fair Lawn, NJ) and then diluted with 0.12 N HCl, as required, to perform copper and iron analyses by flame atomic absorption spectroscopy. Copper and iron contents of the diets were also measured by flame atomic absorption spectroscopy after ashing at 450°C.

Fatty acid analyses. Lipids of liver, heart and serum were analyzed in the copper study. Only liver and serum lipids were analyzed in the iron study. The tissues and serum were frozen immediately and stored at −20°C, until shipment on dry ice to the Hormel Institute, Austin, MN, for lipid analysis.

Lipids were extracted from the samples with chloroform/methanol (2:1, v/v) and the PL separated from the neutral lipids using silicic acid plates developed in petroleum ether/diethyl ether/acetic acid (80:20:1) (12). The PL were converted to fatty acid methyl esters (FAME) and were analyzed on a Packard GC 428 gas chromatograph using a 50 m × 0.22 mm FFAP fused silica capillary column (Scientific Glass Engineering, Austin, TX) and the same operating conditions as described (12). Relative percentage composition was calculated for the FAME, and peak identifications were made by comparing with authentic FAME standards.

Data were evaluated by analysis of variance followed by Tukey's studentized range test (13).

RESULTS

The CuD and FeD rats did not consume less food than CuA and FeA rats, respectively. On day 42 of the dietary regimen, the CuD, CuA and CuE rats consumed equivalent amounts of food (21 ± 1.7, 24 ± 0.8 and 22 ± 0.6, g/day ± SE) and gained equivalent weight (274 ± 15.8, 302 ± 12.9 and 293 ± 9.8, g ± SE), respectively. Similarly, on day 42 of the dietary regimen, FeD, FeA and FeE rats consumed equivalent amounts of food (21 ± 2.5, 22 ± 1.1 and 21 ± 1.0, g/day ± SE) and gained equivalent weight (252 ± 11.8, 281 ± 12.4 and 270 ± 12.4, g ± SE), respectively. Because of equivalent food intake by deficient and adequate rats, data for the PF groups are not presented.

Copper. Copper concentrations were decreased in serum, liver and heart of CuD rats (Table 2). The copper concentration of serum was equivalent for CuA and CuE rats, but increased in liver and heart of CuE rats.

Few differences in fatty acid patterns of PL were found in tissues of CuD and CuA rats (Tables 3-5). Relative percentages of 18:2ω6 and 20:4ω6 were not different in tissues from CuD and CuA rats. In CuD and CuA rats, 16:0 and 18:0 in PL were similar for heart and serum. In liver, 16:0 was unchanged and 18:0 was increased in CuD rats. 16:1ω7, the Δ9 desaturation product formed from 16:0, was significantly lower in liver, heart and serum PL of CuD than CuA rats. 18:1ω9, the Δ9 desaturation product formed from 18:0, was unchanged in tissues of CuD rats. Total Δ9 desaturation products, which include all fatty acids formed by Δ9 desaturation, were lower in liver and serum (but not heart) of CuD than CuA rats. The final metabolic product formed from serial desaturation and elongation of 18:0 (20:3ω9) was not different in tissues from CuD and CuA rats; also, 22:6ω3 was not significantly different in heart or serum.

The total metabolites formed from a single essential precursor showed limited differences in tissues from CuD and CuA rats. Total ω6 metabolites are those formed from serial desaturation and elongation of 18:2ω6. Total ω3 metabolites are those formed from serial desaturation and

TABLE 2

Copper Content in Serum, Liver and Heart of Copper-Adequate (CuA), Copper-Deficient (CuD) and Copper-Excess (CuE) Rats (Mean ± SE)

Tissue	Units	Diet groups CuA (n = 10)	CuD (n = 10)	CuE (n = 10)
Serum	μg/dl	146.0 ± 3.8[a]	1.0 ± 0.0[a,b]	136.5 ± 4.7[b]
Liver	μg/g	13.8 ± 0.2[a]	2.5 ± 0.3[a]	16.2 ± 0.4[a]
Heart	μg/g	21.2 ± 0.2[a]	6.7 ± 0.4[a]	23.4 ± 0.4[a]

Values with similar superscripts are significantly different at $p < 0.05$.

TABLE 3

Fatty Acid Contents in Liver Phospholipids (Relative %, Mean ± SE) in Copper-Adequate (CuA), Copper-Deficient (CuD) and Copper-Excess (CuE) Rats

Fatty acid	Diet groups CuA (n = 10)	CuD (n = 10)	CuE (n = 5)
18:2ω6	10.2 ± 0.3	11.3 ± 0.6	10.1 ± 0.5
20:4ω6	32.0 ± 0.8	30.5 ± 0.9[a]	35.5 ± 1.1[a]
16:0	15.3 ± 0.2[a]	15.4 ± 0.3[b]	13.8 ± 0.5[a,b]
16:1ω7	1.5 ± 0.1[a]	0.9 ± 0.1[a]	1.2 ± 0.1
18:0	19.0 ± 0.7[a]	21.8 ± 0.7[a,b]	17.4 ± 1.1[b]
18:1ω9	2.9 ± 0.1	2.5 ± 0.2	2.5 ± 0.1
20:3ω9	0.1 ± <0.1	0.1 ± <0.1	0.1 ± <0.1
22:6ω3	1.6 ± 0.1	1.8 ± 0.1	1.8 ± 0.1
Δ9 desaturation products	4.3 ± 0.3[a]	3.4 ± 0.2[a]	3.7 ± 0.2
ω3 Metabolites	1.7 ± 0.1	1.9 ± 0.1	1.8 ± 0.1
ω6 Metabolites	40.5 ± 1.0[a]	39.1 ± 1.0[b]	45.1 ± 1.1[a,b]
ω9 Metabolites	0.4 ± 0.1	0.3 ± <0.1	0.3 ± <0.1

Pairs with same superscript are significantly different than each other at $p < 0.05$.

TABLE 4

Fatty Acid Contents in Heart Phospholipids (Relative %, Mean ± SE) in Copper-Adequate (CuA), Copper-Deficient (CuD) and Copper-Excess (CuE) Rats

Fatty acid	Diet groups CuA (n = 10)	CuD (n = 10)	CuE (n = 10)
18:2ω6	22.1 ± 0.5[a]	21.5 ± 0.5	19.7 ± 0.9[a]
20:4ω6	22.5 ± 0.4	23.2 ± 0.4	23.9 ± 0.5
16:0	8.7 ± 0.2	7.9 ± 0.2	9.1 ± 0.8
16:1ω7	0.6 ± <0.1[a]	0.4 ± <0.1[a,b]	0.6 ± <0.1[b]
18:0	22.7 ± 0.3	22.9 ± 0.4	22.6 ± 0.2
18:1ω9	3.0 ± 0.1	3.0 ± 0.2	2.6 ± 0.1
20:3ω9	0.1 ± <0.1	0.1 ± <0.1	0.1 ± <0.1
22:6ω3	1.6 ± 0.1	1.8 ± 0.1	1.7 ± 0.1
Δ9 desaturation products	3.7 ± 0.1	3.5 ± 0.2	3.3 ± 0.1
ω3 Metabolites	1.8 ± 0.1	2.0 ± 0.1	1.8 ± 0.1
ω6 Metabolites	32.6 ± 0.4	34.1 ± 0.6	34.7 ± 0.7
ω9 Metabolites	0.2 ± <0.1	0.2 ± <0.1	0.2 ± <0.1

Pairs with same superscript are significantly different than each other at $p < 0.05$.

TABLE 5

Fatty Acid Contents in Serum Phospholipids (Relative %, Mean ± SE) of Copper-Adequate (CuA), Copper-Deficient (CuD) and Copper-Excess (CuE) Rats

Fatty acid	Diet groups CuA (n = 10)	CuD (n = 8)	CuE (n = 9)
18:2ω6	16.6 ± 0.6	16.9 ± 0.6	14.7 ± 0.4
20:4ω6	23.6 ± 0.8	23.8 ± 0.7	24.6 ± 0.6
16:0	18.2 ± 0.4	16.9 ± 0.5	19.1 ± 0.7
16:1ω7	0.9 ± 0.1[a]	0.5 ± <0.1[a]	0.7 ± <0.1
18:0	22.4 ± 0.8	23.7 ± 0.6	23.0 ± 0.6
18:1ω9	3.2 ± 0.2	2.7 ± 0.2	2.7 ± 0.1
20:3ω9	0.1 ± <0.1	0.1 ± <0.1	0.1 ± <0.1
22:6ω3	0.9 ± <0.1	1.2 ± 0.2	0.9 ± 0.1
Δ9 desaturation products	4.2 ± 0.3[a]	3.2 ± 0.2[a]	3.4 ± 0.2
ω3 Metabolites	0.9 ± <0.1[a]	1.3 ± 0.2[a]	1.0 ± 0.1
ω6 Metabolites	29.6 ± 0.8	30.8 ± 0.7	31.0 ± 0.7
ω9 Metabolites	0.4 ± 0.1	0.4 ± <0.1	0.3 ± <0.1

Pairs with same superscript are significantly different than each other at $p < 0.05$.

elongation of linolenic acid (18:3ω3). Total ω9 metabolites are those formed from 18:1ω9. Total ω6 metabolites were not different for CuD and CuA rats. Total ω3 metabolites for liver and heart were not different in CuD and CuA rats, but were elevated in serum of CuD rats. Total ω9 metabolites were not different for liver, heart or serum of CuD and CuA rats.

Intake of excess copper did not cause a significant increase in Δ9 desaturation. Only 16:1ω7 in heart PL was increased. Except for reduced 16:0 in liver of CuE rats, 16:0 and 18:0 were equivalent in tissues of CuA and CuE rats. 18:2ω6 was lower in hearts of CuE than CuA rats, but not in liver or serum. 20:4ω6 did not differ in tissues of CuA and CuE rats. Only in liver, were total ω6 metabolites higher in CuE rats than CuA rats. Total ω3 and ω9 metabolites and Δ9 desaturation products were not different for tissues of CuA and CuE rats.

To illustrate the maximum possible effects of copper, Figure 1 shows the normalcy ratio (NR, ratio of experimental value to the control value) profile of relevant fatty acids in liver PL of CuD compared with CuE rats. In CuD rats, 20:4ω6 and total ω6 metabolites were lower ($p < 0.05$) than in CuE rats; but, in liver of CuD rats, 16:0 and 18:0 were higher ($p < 0.05$) than in CuE rats. The NR profile for heart and serum PL of CuD rats compared with CuE rats showed only a significant decrease ($p < 0.05$) in heart 16:1ω7 (figure not shown).

Iron. Hematocrits and iron concentrations of serum and liver were decreased in FeD rats (Table 6). Only liver iron was increased in rats fed excess iron.

Fatty acid compositions of PL in liver and serum from FeD, FeA and FeE rats are given in Tables 7 and 8. In liver and serum of FeD rats, 18:2ω6 was higher than in FeA rats. FeD rats showed lower serum 20:4ω6 than FeA rats. Liver and serum 16:0, 18:0, 16:1ω7 and 18:1ω9 were similar for FeD and FeA rats. Liver and serum 22:6ω3 were higher in FeD than in FeA. Total ω3 metabolites of liver and serum were higher for FeD rats than FeA rats.

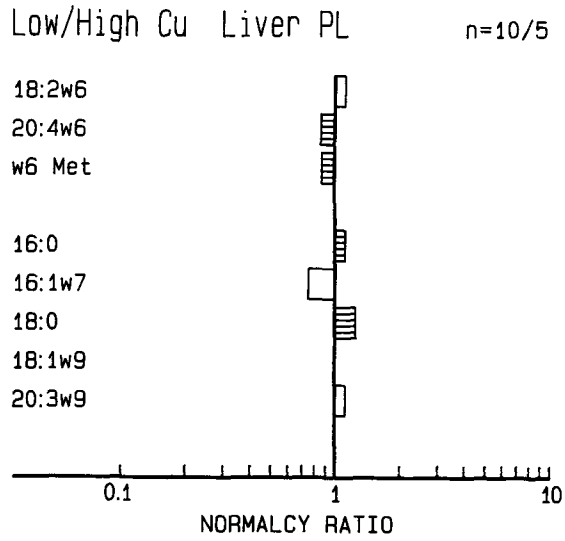

FIG. 1. Normalcy ratio profile of animals fed low-copper diet (CuD) compared with high-copper diet (CuE). Horizontal shading indicates significance at $p < 0.05$; open bars are not significant.

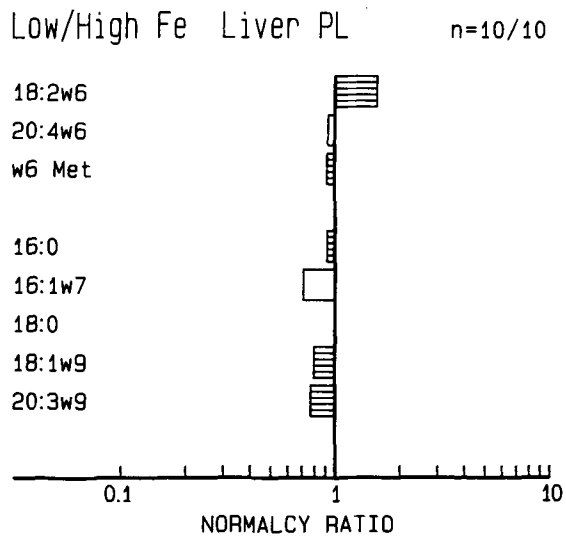

FIG. 2. Normalcy ratio profile of animals fed low-iron diet (FeD) compared with high-iron diet (FeE). Horizontal shading indicates significance at $p < 0.05$; open bars are not significant.

TABLE 6

Hematocrit and Iron Content in Serum and Liver of Iron-Adequate (FeA), Iron-Deficient (FeD) and Iron-Excess (FeE) Rats (Mean ± SE)

Tissue	Diet groups FeA (n = 10)	FeD (n = 10)	FeE (n = 10)
Hematocrit (%)	39.8 ± 0.3[a]	27.2 ± 1.7[a,b]	40.3 ± 0.9[b]
Serum (μg/dl)	254.5 ± 12.0[a]	117.0 ± 18.8[a,b]	313.0 ± 31.1[b]
Liver (μg/g)	195.5 ± 12.3[a]	66.6 ± 1.5[a]	369.1 ± 38.5[a]

Paris with same superscript are significantly different than each other at $p < 0.05$.

TABLE 7

Fatty Acid Contents in Liver Phospholipids (Relative %, Mean ± SE) of Iron-Adequate (FeA), Iron-Deficient (FeD) and Iron-Excess (FeE) Rats (Mean ± SE)

Fatty acid	Diet groups FeA (n = 10)	FeD (n = 10)	FeE (n = 10)
18:2ω6	10.5 ± 0.4[a]	14.3 ± 0.8[a,b]	9.1 ± 0.2[b]
20:4ω6	30.8 ± 1.1	30.4 ± 1.0	32.7 ± 0.8
16:0	17.6 ± 0.3	16.4 ± 0.2[a]	17.9 ± 0.3[a]
16:1ω7	1.3 ± 0.1	0.9 ± 0.1	1.3 ± 0.1
18:0	20.8 ± 1.3	20.2 ± 1.1	20.0 ± 0.8
18:1ω9	2.9 ± 0.1	2.4 ± 0.2[a]	3.1 ± 0.1[a]
20:3ω9	0.1 ± <0.1[a]	0.1 ± <0.1[b]	0.1 ± <0.1[a,b]
22:6ω3	1.5 ± 0.1[a]	2.0 ± 0.1[a,b]	1.5 ± 0.1[b]
Δ9 desaturation products	4.2 ± 0.2	3.4 ± 0.3[a]	4.4 ± 0.2[a]
ω3 Metabolites	1.5 ± 0.1[a]	2.1 ± 0.1[a,b]	1.6 ± 0.1[b]
ω6 Metabolites	38.6 ± 1.2	37.2 ± 0.9[a]	40.6 ± 0.6[a]
ω9 Metabolites	0.2 ± <0.1	0.2 ± <0.1	0.2 ± <0.1

Pairs with same superscript are significantly different than each other at $p < 0.05$.

TABLE 8

Fatty Acid Contents in Serum Phospholipids (Relative %, Mean ± SE) of Iron-Adequate (FeA), Iron-Deficient (FeD) and Iron-Excess (FeE) Rats

Fatty acid	Diet groups FeA (n = 10)	FeD (n = 10)	FeE (n = 10)
18:2ω6	16.8 ± 0.6[a]	21.5 ± 1.2[a]	12.9 ± 0.8[a]
20:4ω6	23.0 ± 0.7[a]	18.8 ± 1.3[a]	22.0 ± 0.6
16:0	20.0 ± 0.4	19.9 ± 0.5	21.2 ± 0.8
16:1ω7	0.7 ± 0.1	0.6 ± 0.1	0.7 ± 0.1
18:0	22.2 ± 1.2	21.2 ± 1.1	25.1 ± 0.9
18:1ω9	3.0 ± 0.2	2.8 ± 0.2	3.4 ± 0.2
20:3ω9	0.1 ± <0.1	0.5 ± 0.3	0.1 ± <0.1
22:6ω3	0.9 ± <0.1[a]	1.1 ± <0.1[a,b]	0.8 ± 0.1[b]
Δ9 desaturation products	3.8 ± 0.2	3.4 ± 0.2	4.2 ± 0.2
ω3 Metabolites	0.9 ± 0.1[a]	1.2 ± <0.1[a,b]	0.8 ± 0.1[b]
ω6 Metabolites	29.2 ± 0.8[a]	24.5 ± 1.3[a,b]	28.2 ± 0.6[b]
ω9 Metabolites	0.3 ± <0.1	0.4 ± 0.2	0.4 ± <0.1

Paris with same superscript are significantly different than each other at $p < 0.05$.

Serum total ω6 metabolites were lower for FeD rats than FeA rats. Liver and serum total Δ9 desaturation products and ω9 metabolites for FeD and FeA groups were not different. Intake of excess iron caused higher liver 20:3ω9 and lower serum 18:2ω6 in FeE than FeA rats (Tables 7 and 8).

To illustrate the maximum possible effects of iron, the NR profile of relevant fatty acids in liver PL of FeD compared with FeE rats is shown in Figure 2. FeD liver had higher 18:2ω6 than FeE liver. Total ω6 metabolites, 16:0, 18:1ω9 and 20:3ω9, were lower in liver of FeD than FeE rats. The NR profile for serum showed higher 18:2ω6, and decreased total ω6 metabolites in FeD compared to FeE rats, but no other differences in serum PL (figure not shown).

DISCUSSION

Wahle et al. (14) reported decreased ratios for monounsaturated to saturated fatty acids of both C_{16} and C_{18} chain lengths in subcutaneous adipose tissue from CuD rats. They also reported decreased desaturation of stearic to oleic acid by liver microsomes from CuD animals (14). The ratio of oleic to stearic acid was higher in the whole back fat of pigs given a copper supplement (15). In the current study, few consistent significant differences in fatty acid composition occurred in the tissues and serum of CuD animals. The ratios were calculated for the product/precursors of 16:1ω7/16:0 and 18:1ω9/18:0. Only a decreased 16:1ω7/16:0 ratio was found in the liver of CuD animals.

When compared with CuA rats, CuD rats showed only elevated liver 18:0 and depressed liver, heart and serum 16:1ω7. When the extremes of copper deficiency and copper excess were compared, CuD rats exhibited elevated liver 16:0 and 18:0 and depressed heart 16:1ω7. If copper was a cofactor for Δ9 desaturation, a larger difference in the precursors and products of Δ9 desaturation, that is, 16:0, 18:0, 16:1ω7 and 18:1ω9, should have occurred between CuD and CuE rats than between either CuD and CuA or CuA and CuE rats, because desaturation in CuD rats would have been inhibited by low copper and enhanced in CuE rats by high copper. However, neither of the studies showing the effect of copper on Δ9 desaturation in the adipose tissue of rats or pigs (14,15) reported the fatty acids of liver or heart. Adipose tissue lipid is largely triglyceride, and oleic acid is the principal fatty acid. On the other hand, polyunsaturated fatty acids are major components of PL which are the major lipids of liver, heart and serum. If copper is a required cofactor for desaturation from stearic to oleic acid, its effect may be more pronounced in the adipose tissue that accumulates the product. Lawrence et al. (16) analyzed the fatty acid composition of rat liver mitochondria and found only slightly less 20:4ω6 and more 22:5ω6 in CuD than CuA rats. They found no increase in 18:0 and no decrease in 18:1ω9 (16). PL synthesis in liver mitochondria was shown to be depressed by copper deficiency (17), which could help explain the minimal changes of fatty acids found in our study of the liver, heart and serum PL. The changes in fatty acids caused by diminished Δ9 desaturation may not be expressed in the membranes of liver and heart because of the depressed synthesis of PL.

Some previous studies of iron deficiency in rats have shown decreased percentages of 16:0, 16:1ω7, 18:0 and 18:1ω9 and increased 18:2ω and 20:4ω6 in tissue lipids (10). Cunnane and McAdoo showed higher linoleic acid in plasma PL and triglycerides of FeD rats and no difference in liver fatty acid composition (18). The present study revealed a significantly higher content of 18:2ω6 in liver and serum PL of FeD than in FeA rats. Excess iron caused reduction of 18:2ω6 in serum of FeE rats. Saturated and monounsaturated fatty acids were generally lower in FeD rats than in FeE rats, but were not significant (Fig. 2). If iron was a required cofactor for Δ9 desaturation, there should have been more consistent, significant differences in the levels of 16:1ω7 and 18:1ω9 between FeD and FeE groups.

The present study did not confirm the requirement for copper or iron in the Δ9 pathway of fatty acid desaturation as indicated in phospholipids. A few significant changes in the saturated and monoenoic fatty acids affected by Δ9 desaturase occurred, but these were inconsistent in the various tissues studied. Possibly, the failure of the present study to show these consistent, significant changes in Δ9 desaturase found previously (14,15,18) could be due to the differences in the tissues and lipid classes chosen for analysis in the various studies.

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Bradykinin-Increased Phospholipid Deacylation-Reacylation in Rat Renal Medulla is Inhibited by dBc AMP

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The effect of bradykinin on the mobilization of arachidonic acid was analyzed separately by acylation and degradation.

Acylating activity was determined by the incorporation of [^{14}C]arachidonic acid into the phospholipids at different times. Different concentrations of bradykinin had no effect on the phospholipid acylating activities.

The degradation of the phospholipids was performed on renal medullary slices prelabeled with [^{14}C]arachidonic acid. Treatment with bradykinin produced an initial degradation of phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol, with a concomitant increase in lysophosphatidylcholine, lysophosphatidylethanolamine and lysophosphatidylinositol within 5 min of incubation. Phosphatidylcholine-, phosphatidylethanolamine- and phosphatidylinositol-labeling increased thereafter and reached the control values after 10 min of incubation.

After 30 min, incubation of prelabeled slices with bradykinin produced a significant concentration-dependent increase in the phospholipid-labeling by reutilization of [^{14}C]arachidonic acid.

The effect of bradykinin on the phospholipid-labeling was blocked by preincubation with increasing concentrations of dBc AMP.

Mepacrine also blocked the bradykinin stimulation in phosphatidylcholine and phosphatidylethanolamine, but had no effect on bradykinin-induced changes in the phosphatidylinositol arachidonic acid moiety.
Lipids 24, 146–150 (1989).

When different cell types are activated, one of the early changes observed is the increased turnover and metabolism of unsaturated fatty acyl moieties of membrane phospholipids. Metabolism of arachidonic acid (AA) to prostaglandins via the cyclooxygenase enzyme system is one of the responses to such triggering stimuli. Because the level of free AA in the cells is generally very low, the synthesis of prostaglandins is limited by the availability of the free AA after it is released from membrane phospholipids (1,2).

The stimulatory effect on renal prostaglandin synthesis exerted by bradykinin (BK) is very important for the renal function modulation (3). It has been postulated that this stimulatory effect is produced by inducing renal phospholipase activity (4–6). Replenishment of phospholipids deacylated by these phospholipases occurs through the reacylation of lysophosphatides (7). This replenishment is essential for the integrity of membrane structure (8), and its efficiency may determine the extent to which the phospholipase mechanism can be utilized for maintaining high levels of prostaglandins. Because arachidonate is an essential fatty acid, the continuous supply for phospholipid replenishment must come from an exogenous source (9).

The precursor of prostaglandins is a part of a cyclic biochemical process that involves three different steps: a) an acylation reaction for the incorporation of plasma AA into phospholipids, b) an active deacylating process that uses these phospholipids as substrate and c) a coupled and efficient reacylating system that maintains membrane integrity.

In the present study, we present evidence of the bradykinin effect on all these processes in rat renal medullary slices.

MATERIALS AND METHODS

[^{14}C]AA (specific activity 52.8 mCi/mmol) was obtained from New England Nuclear (Boston, MA); and phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE) and lysophosphatidylinositol (LPI) were obtained from Supelco (Bellefonte, PA). Silica Gel H plates (0.25 mm thick) were purchased from Applied Science (State College, PA) and Silica Gel G plates (0.25 mm thick) from Brinkmann Instruments, Inc. (Westbury, CT). Bradykinin, mepacrine (MP) and dibutyril cyclic adenosine monophosphate (dBc AMP) were provided by Sigma Chemical Co. (St. Louis, MO), and X-ray films for autoradiography were from Eastman Kodak Company (Rochester, NY).

Bradykinin effect on rat renal medullary phospholipid labeling. After decapitation of male Sprague-Dawley rats (250–280 g), both kidneys were removed and maintained on ice-cold Krebs solution. Each kidney was cut in half through its longitudinal axis and the renal medulla was separated by scissor and scalpel dissection (10). The renal medullary tissue was sliced (ca. 0.5 mm thick) using a Stadie-Riggs microtome. For each experiment, 100 mg of tissue slices were collected in 1.25 ml of Krebs Ringer bicarbonate buffer containing 5.5 mM of glucose. The medium was gassed with carbogen (95% O_2, 5% CO_2). Samples were incubated at 37°C with 0.25 μCi of [^{14}C]AA in a metabolic shaking bath under an atmosphere of carbogen for different lengths of time. In some experiments, the influence of BK on this acylation reaction was investigated. In these cases, the slices were preincubated with various concentrations of BK at 37°C for 10 min before the addition of AA. The reactions were stopped by the addition of 4.0 ml of chloroform/methanol (1:2, v/v) (11). The samples were homogenized in glass tubes with Teflon pestles at 3,000–3,500 rpm. Phases were split by adding 1.25 ml of water (12). In the

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Abbreviations: AA, arachidonic acid; BK, bradykinin; dBc AMP, dibutyril cyclic adenosine monophosphate; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPI, lysophosphatidylinositol; MP, mepacrine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; TLC, thin layer chromatography.

experiments measuring LPI, the extraction was performed using chloroform/methanol/concentrated HCl (50:100:1, v/v), and the phases were separated by adding 0.6 ml of chloroform and 0.6 ml of 2 M KCl (13). The lower chloroform phases were removed. The inorganic phases were washed twice with chloroform and the organic phases were pooled and dried under a stream of nitrogen at 25°C. This extraction procedure ensures 80–85% recovery of the lipids. The extracts were submitted to thin layer chromatography (TLC) and quantitated as will be described.

Effect of BK on deacylation and reacylation of prelabeled slices. The slices prelabeled for 60 min were removed from the tubes and washed three times with ice-cold fresh Krebs' solution containing 10% albumin. The labeled slices were incubated with BK for different lengths of time. However, when the influence of dBc AMP or MP on the degradation of the phospholipids was investigated, the labeled slices were preincubated with these compounds for 10 min. Appropriate control incubations were done in the presence of diluents instead of drug solutions. The reactions were stopped as previously described. Then, the tissues were homogenized and the lipids extracted as described for the acylation. The extracts were separated by TLC.

Chromatographic lipid separation. The labeled phospholipid extracts were redissolved in chloroform, applied onto activated Silica Gel H TLC plates (0.25 mm thick) and developed in a solvent system consisting of chloroform/methanol/acetic acid/water (75:45:12:3, v/v/v/v) (14). In this solvent system, PI and PS migrated as a single spot, whereas PC, PE, LPC, LPE and sphingomyelin were well separated.

Separation of PI from PS was achieved by two-dimensional chromatography on 1% ammonium oxalate-impregnated Silica Gel H plates using the previous solvent system in the first dimension and chloroform/methanol/13.5 N ammonia/water (70:30:0.5:4, v/v/v/v) in the second dimension (15).

The LPI was separated on thin layer Silica Gel G plates impregnated with 1% potassium oxalate containing 2 mM ethylenediaminetetraacetic acid using chloroform/methanol/4 N NH_4OH (45:35:10, v/v/v) (16).

For the separation of neutral lipids and free fatty acids, the upper phase of a mixture of ethyl acetate/2,2,4-trimethylpentane/acetic acid/water (9:5:2:10, v/v/v/v) as a solvent system was used. The migration of the lipids was detected by I_2 vapors or autoradiography, as appropriate.

The positional distribution of the incorporated, labeled fatty acid was analyzed by the degradation of PC, PE or PI with phospholipase A_2. The enzyme activity was evaluated as previously described (14). The amount of radioactivity recovered from the phospholipids and the free fatty acid fraction indicated that the fatty acid was incorporated in position 2 of the phospholipid molecule. The degradation rate exceeded 96%. No spontaneous hydrolysis of the phospholipids was observed in controls under identical conditions without addition of phospholipase A_2.

Quantitation. Zones of the TLC plates corresponding to the various phospholipids fractions were scraped off and added to 5.0 ml of a toluene-omnifluor 0.4% mixture. Quantitation was performed in a liquid scintillation counter. Counting efficiency for ^{14}C was 75%.

For the quantitation of the individual lysophospholipids, specific areas of the plates were digested with 70% of perchloric acid and the resulting inorganic phosphate was assayed (17).

Areas of silica gel were used as blanks for cpm or phosphorus assay and the results were corrected by subtraction of blank values from all samples.

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

RESULTS

[^{14}C]AA incorporation. Effect of BK. Fatty acid incorporation into phospholipids appears to be a rapid phenomenon (Fig. 1), because 35–45% of the maximal radioactivity incorporated into the phospholipids is present within 5 min of incubation. The equilibrium is reached at 60 min of incubation, when the maximal incorporation of radioactivity is achieved. Of the phospholipids studied, PC accounts for most of the radioactivity (60%). A smaller amount of AA is incorporated into PE (19%). PI incorporates 21%, and no radioactivity is associated to PS.

The addition of BK for 10 min prior to labeling does not affect the time course of AA incorporation into the renal medullary phospholipids at any concentration studied 0.5–2.0 μg/ml).

Bradykinin-induced changes in radioactive phospholipids. Figure 2 shows the time course degradation of PC,

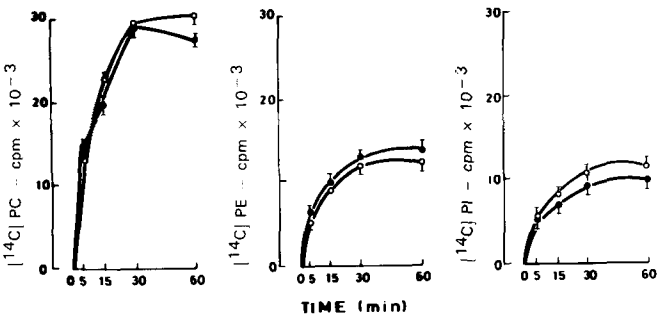

FIG. 1. Time course incorporation of [^{14}C]AA on rat renal medullary phospholipids—the BK effect. Tissue slices were incubated with [^{14}C]AA in the absence (○) and the presence (●) of BK (1 μM) for different lengths of time. Each point represents the mean of 5 experiments ± SE.

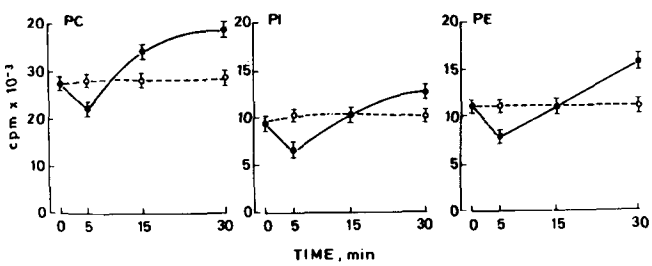

FIG. 2. Effect of BK on prelabeled rat renal medullary phospholipids. The slices prelabeled with [^{14}C]AA during 60 min were incubated in fresh Krebs' solution in the absence (○) and presence (●) of BK for different lengths of time. Each point represents the mean of 5 experiments ± SE.

PI and PE prelabeled with [^{14}C]AA. In the experiments achieved with BK (1.0 μg/ml), a transient decrease of radioactivity was observed within 5 min. This degradation was followed by a considerable increase in the labeling of the phospholipids. The BK action was observed in PC, PE and PI. Phosphatidylserine does not participate in this phenomenon. No change of radioactivity was observed in the absence of BK.

Table 1 shows the increase in the net amount of lysophospholipids as a consequence of the BK-effect. This increase, together with the decrease of radioactivity in the phospholipids, reflects the degradation of phospholipids. No radiolabeled lysophospholipids were detected. Because AA is incorporated into position 2 of the phospholipids, the lack of lysophospholipid radioactivity indicates that no phospholipase A_1 activity was present.

The reacylation of the phospholipids, shown by the increase of the labeling at 30 min of incubation, was analyzed at different concentrations of BK. In Figure 3, a concentration-dependent increase in PC-, PI- and PE-labeling can be observed ($p < 0.05$), with a maximal stimulation up to 39.8%, and 32.7% of the control values in PC, PI and PE, respectively.

Table 2 shows that arachidonate is very efficiently incorporated into triglycerides and, after 60 min of incubation, a considerable amount of radioactive fatty acid remains in a free form in the system. This free fatty acid is not extractable by washing with albumin. After treatment with bradykinin, a decrease of radioactivity in the free fatty acid pool and in the triglycerides was observed.

TABLE 1

Rat Renal Medullary Slices Were Incubated at 37°C for 5 min Without (Control) or with BK[a]

	Control	BK (1 μg/ml)
PC	139.1 ± 22.1	101.9 ± 19.0
LPC	ND	33.3 ± 6.6
PE	127.5 ± 24.0	50.6 ± 7.6[b]
LPE	ND	85.4 ± 16.1
PI	27.0 ± 4.5	18.5 ± 3.0[b]
LPI	ND	4.0 ± 0.7

[a]Data represent average of 5 experiments ± SE, expressed as ng P/mg (wet weight tissue).

[b]$p < 0.005$.

ND, not determined.

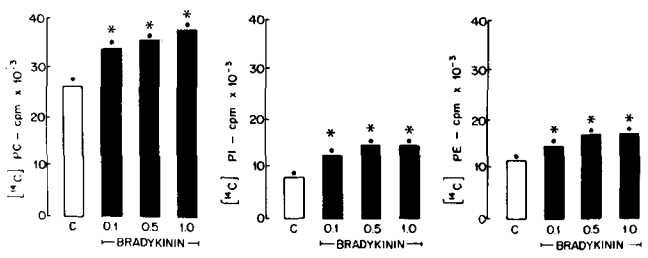

FIG. 3. Effect of BK on prelabeled rat renal medullary phospholipids. The slices prelabeled with [^{14}C]AA were incubated for 30 min in the presence of different concentrations of BK. Each bar represents the mean of 5 experiments ± SE.

MP inhibits the BK effect on PC and PE, but not the effect on PI. MP at 0.1-mM concentration blocks the effect of BK on medullary PC by 72%. At 0.5 mM, a complete inhibition is observed; but at a higher concentration of MP (1.0 mM), a decrease of radioactivity is shown, reaching values even lower than those obtained in the absence of BK. A similar pattern is observed with PE, but the value reached at 1 mM MP is not lower than the one obtained in the absence of BK (Fig. 4). On the contrary, MP (0.1 mM) failed to block the BK increase of radioactivity on PI. Moreover, at higher concentrations, it produced a significant ($p < 0.05$) stimulation of labeling.

dBc AMP blocks the BK effect on medullary phospholipids. Different concentrations of dBc AMP (0.05–0.1 M) prevented the BK-labeling increase of PC, PI and PE.

The dBc AMP lower concentration (0.05 M) exerted a partial blockade, but, at higher concentrations, a total inhibition was observed (Fig. 5). A single concentration of

TABLE 2

Radiolabeled Rat Renal Medullary Slices Were Incubated at 37°C for 30 min in the Presence of BK[a]

		BK (1 μg/ml)
Diglyceride	ND	ND
Triglyceride	(16.43 ± 3.1)10^3	(9.8 ± 2.5)10^{3b}
FFA	(18.03 ± 4.1)10^3	(12.05 ± 2.9)10^{3b}

[a]Data is the average of 5 experiments ± SE expressed as cpm.

[b]$p < 0.05$.

ND, not determined.

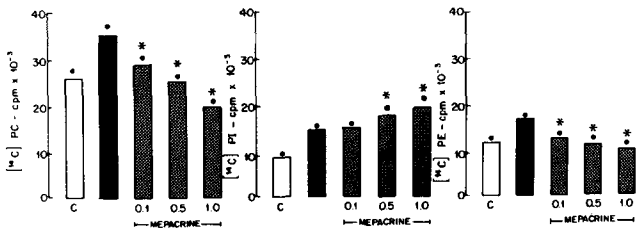

FIG. 4. Effect of MP on BK-stimulation of rat renal medullary phospholipids. The slices prelabeled with [^{14}C]AA were preincubated with different concentrations of MP and then incubated for 30 min in the presence of 1 μg/ml of BK. Each bar represents the mean of 5 experiments ± SE.

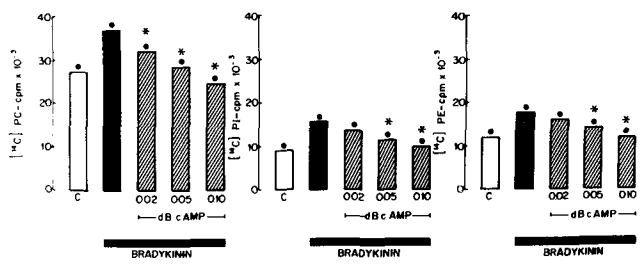

FIG. 5. Effect of dBc AMP on BK-stimulation of rat renal medullary prelabeled slices. The slices prelabeled with [^{14}C]AA were preincubated with different concentrations of dBc AMP and then incubated for 30 min in the presence of 1 μg/ml of BK. Each bar represents the mean of 5 experiments ± SE.

0.1 M dBc AMP also prevented the breakdown of the various phospholipids observed after 5 min of incubation (data not shown).

DISCUSSION

Bradykinin is an important physiological stimulator of the renal prostaglandin synthesis (3), and it has been generally accepted that this hormone exerts its stimulatory effect through phospholipase A_2 activation (4-6,18).

The present paper was aimed at obtaining direct evidence on the ability of BK to influence the mobilization of the prostaglandin precursor, AA. Arachidonic acid is introduced into phospholipids by acylation of the lysophospholipids, rather than by de novo synthesis (18-21).

Our studies of incorporation of AA showed that, under the conditions used, the acylation step is active in rat renal medullary phospholipids, and BK does not influence this enzymatic step (Fig. 1).

To determine the effect of BK on the activation of the deacylation-reacylation cycle, slices prelabeled with [^{14}C]AA were prepared and reincubated in physiological medium. This study demonstrates that BK perturbs the deacylation-reacylation cycle.

We have previously shown (4) that AA is incorporated at position 2 of the phospholipids. Therefore, the transient degradation of the radiolabeled phospholipids promoted by BK (Fig. 2), with the concomitant increase of the corresponding nonradioactive lysophospholipids, can be considered a sign of phospholipase A_2 activation. This result may represent a direct evidence of the generally accepted concept that BK exerts its prostaglandin synthesis stimulatory effect through phospholipase A_2 activation (4-6,18). On the other hand, the breakdown of the phospholipids promoted by BK is followed by what appears to be a compensating resynthesis (Figs. 2 and 3). The most reasonable interpretation of these data encompasses the concept that both components of the deacylation-reacylation cycle, a membrane bound phospholipase A_2 and an acyl-CoA transferase, increase their activities in the presence of BK.

To determine the source of the fatty acid used during the bradykinin stimulation, we explored the mobilization of the arachidonate from the triglyceride and the free fatty acid pool. Under these conditions, a decrease of radioactivity in both of them is observed, perhaps indicating that the free arachidonate and/or the triglyceride pool could act as the source of the fatty acid.

We used MP to ascertain the relative implication of phospholipase A_2 in the reacylation evoked by BK. Although MP is considered to be an inhibitor of phospholipase A_2, and it has been used to differentiate phospholipase A_2 from phospholipase C activities (22), it is not possible to analyze the effect of MP from this exclusive point of view.

The data indicate (Fig. 3) that BK exerts its effects on PI even in the presence of 1 mM of MP and completely blocks the action on PC and PE. One interpretation of these results is the possibility that the BK increase of reacylation on PI occurs through a mechanism distinct from the one that operates on PC and PE. Therefore, MP would produce a selective inactivation of such processes, maybe as the consequence of different phospholipase A_2 activities.

However, this interpretation is only speculative, because in our experience, MP alone exerts mobilization of AA from the kidney phospholipids by a mechanism independent of phospholipases (manuscript in preparation). Moreover, Dise et al. reported a direct interaction of MP with erythrocyte and platelet phospholipids (23).

On the other hand, dBc AMP exerts a concentration-dependent blockade in both effects of bradykinin: the deacylation (a reflection of the phospholipase A_2 activation) and the recomposition of the renal phospholipids. This inhibition is effective on PC, PE and PI. The mechanism by which dBc AMP produces this inhibition could be related to our previous observations that bradykinin induces PI-specific phospholipase C activity in rat renal medulla. The stimulation of this phospholipase C occurs at 2 min and is prevented by dBc AMP (24). From these two observations, we suggest that the bradykinin stimulation of the breakdown and recomposition of the kidney phospholipids, as presented here, could be secondary to the stimulation of phospholipase C. Therefore, in rat renal medulla, BK could stimulate a sequence of reactions that involves both phospholipase C and A_2 activation. Such a sequence of reactions has been described for thrombin in platelets (25).

Of interest, is the absence of BK stimulation in the acylation process.

One adequate explanation could be that the AA incorporated into phospholipid during deacylation-reacylation (Fig. 2) arises from an intermediate pool (such as triglycerides) which has not become sufficiently labeled to show transfer to phospholipids under the conditions of acylation (Fig. 1).

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Dietary Induction of Cholesterol Gallstones in Hamsters From Three Different Sources

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Cholesterol gallstones were produced in young male, golden Syrian hamsters, obtained from three different suppliers, by administering a nutritionally adequate, semipurified diet for periods of either 5 or 10 weeks. The major components of the lithogenic diet were casein, cornstarch, butterfat, corn oil and 0.3% cholesterol. The hamsters were obtained from Sesco, Harlan Sprague-Dawley (Engle hamster) and Charles River (Lakeview hamster). There were profound differences among the three groups with respect to gallstone formation and cholesterol metabolism: The highest incidence of gallstones occurred in Sesco hamsters, 44.4% and 63.6% after 5 and 10 weeks on the lithogenic diet, respectively. In the Engle hamster, after a 5-week feeding, cholesterol crystals and gallstones were absent. When the feeding period was extended to 10 weeks, cholesterol gallstones were present in 45.5% of the animals. In the Lakeview hamsters, neither gallstones nor cholesterol crystals were found in the gallbladder after a 5-week period. After 10 weeks, cholesterol gallstones were found in only a single hamster. In all groups, the lithogenic diet produced large increases of liver, serum and biliary cholesterol concentrations and increased liver weights. When the animals were fed for 5 weeks, only the bile of Sesco hamsters became supersaturated. Supersaturated bile was induced in all groups after a 10-week feeding of the lithogenic diet with cholesterol saturation ranging from 1.47 to 1.97. These data indicate that it is possible to induce cholesterol gallstones in hamsters by means of a nutritionally adequate, semipurified diet of moderate cholesterol content. The source of the animals appears to be an important variable, because there were significant differences among the hamsters of differing origins, in cholesterol metabolism and rates of gallstone formation.
Lipids 24, 151-156 (1989).

Cholesterol cholelithiasis has been studied for many years, yet the mechanism of gallstone formation is still not fully understood. In order to elucidate its cause and to produce and evaluate new cholelitholytic agents, several animal models of cholesterol gallstones have been studied, for example, the squirrel monkey (1), prairie dog (2,3), hamster (4,5) and mouse (6). Hamsters are small, easy to handle and have a bile acid composition similar to that of man (7), which makes the hamster a useful model. Two hamster models of cholesterol gallstone disease have been described by Dam and Christensen (4) and by Pearlman et al. (5). The former model used a fat-free, nutritionally inadequate diet that inhibited normal growth of the animals and frequently resulted in premature deaths, whereas the latter was not readily reproducible. It is known, however, that hamsters from different commercial suppliers differ in their susceptibility to gallstone disease and exhibit differences in the metabolism of cholesterol and the other biliary lipids (8,9). The present study was designed to determine whether significant differences in cholesterol gallstone formation and cholesterol metabolism existed among male, golden Syrian hamsters from three suppliers. We now report that a nutritionally adequate semipurified diet of moderate cholesterol content induces cholesterol gallstones at widely divergent rates in these groups of hamsters. This model should prove useful for investigating mechanisms of gallstone formation and for developing and testing new bile acids and bile acid analogs designed to prevent or dissolve gallstones.

MATERIALS AND METHODS

Male, golden Syrian hamsters *(Mesocricetus auratus),* 4 wk of age, were obtained from three different suppliers, namely Sesco Inc., Omaha, NE (Sesco hamster, golden Syrian outbred hamster derived from closed colony from the University of Nebraska Medical Center, Eppley Cancer Institute); Harlan Sprague-Dawley, Indianapolis, IN (Engle hamster, golden Syrian brown and white outbred hamster derived from a nucleus colony obtained from the National Institutes of Health); and Charles River Breeding Labs, Wilmington, MA (Lakeview hamster, golden Syrian outbred hamster descended from two original colonies acquired in 1949 and 1951). During a 1-wk quarantine period the animals were maintained with Purina Rodent Chow and water ad libitum. The animals from each supplier were divided into two groups and maintained with a semipurified diet (SPD) (control diet: groups I, III and V) or SPD containing 0.3% cholesterol (cholesterol diet: groups II, IV and VI) (Dyets Inc., Bethlehem, PA). One kg of diet contained 200 g casein, 20 g corn oil, 40 g butterfat, 434 g cornstarch, 146 g Dyetrose (soluble starch), 100 g fiber (cellulose), 50 g salt mix (modified USP XIV salt mix, #200951), 5 g vitamin mix (#300000), 2 g choline chloride, with or without 3 g cholesterol. Cholesterol was added at the expense of cornstarch. Food and water were given ad libitum, and the animals were kept under an alternating 12-hr light/dark cycle.

After a 5-wk feeding (short-term feeding), a portion of the animals of each group were killed according to the procedure described below. The remainder was continued on the same diets for 10 wk (long-term feeding).

At the end of the experiment, the animals were anesthetized with 20 mg ketamine hydrochloride (Bristol Labs, Syracuse, NY). Blood was withdrawn by cardiac puncture to determine the serum cholesterol concentration, and the animals were killed by exsanguination. The gallbladder was exposed and examined visually. When cholesterol gallstones were present, they could usually be seen through the gallbladder wall as yellowish-white

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Abbreviations: GLC, gas liquid chromatography; LD, lithogenic diet; mol %, molar percentage; SPD, semipurified diet.

solids. Gallbladder bile was removed using a 50 μl Hamilton syringe. The gallbladder was excised and stones, if present, were removed and stored in a desiccator until analysis. In a few cases, the cystic duct was obstructed by a concrement and the gallbladder contained little bile. One drop of fresh bile and gallbladder contents were immediately examined under a polarizing microscope (Olympus MCHAP microscope, Olympus Corp., Lake Success, NY) to determine the presence of cholesterol crystals and gallstones (10). Bile samples were centrifuged at 2000 × *g* for 10 min, and aliquots were used for determination of biliary lipids. The liver was excised and weighed, and aliquots were removed for cholesterol determination.

A portion of bile was hydrolyzed with alkali (2 N NaOH), and biliary bile acids were determined as the methyl ester acetates using an SPB-5, 15 m capillary column (Supelco, Bellefonte, PA) attached to a Hewlett-Packard 5830A gas chromatograph (9). Biliary phospholipid concentration was determined by an enzymatic colorimetric procedure using the Wako phospholipid B kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Cholesterol concentrations of bile, serum and liver were analyzed by gas liquid chromatography (GLC), using the trimethylsilyl ether derivatives (11). The gallstones were washed with distilled water, desiccated at 100°C in vacuum at least 3 days, then used for the determination of cholesterol content. The stones were placed in 2.0 ml 10% KOH in ethanol using 5α-cholestane as an internal standard and heated at 70°C for 1 hr. After the stones were completely dissolved, cholesterol was extracted with hexane and analyzed by GLC, using the procedure described above for biliary cholesterol.

Cholesterol saturation indices of bile were calculated using published methods (12,13). The data are reported as the mean ±SEM. Statistical differences among the groups were calculated using one-way analysis of variance followed by Student's t-test (14). The significance of differences in gallstone incidence was determined by chi-square (14).

RESULTS

Animals of the 12 experimental groups were fed the diets for 5 wk (short term) or 10 wk (long term). Food intake for each animal averaged 9 g/day. All animals were healthy throughout the experimental period and gained similar amounts in weight: 46.8 ± 1.9 g for the short-term animals and 49.3 ± 4.9 g for the long-term hamsters.

The incidence of gallstones and cholesterol crystals was determined at sacrifice by polarizing light microscopy (Table 1). Neither cholesterol gallstones nor biliary cholesterol crystals were found in any animals fed the cholesterol-free semipurified control diet (groups Ia and b; IIIa and b; and Va and b), irrespective of the duration of the feeding period. The addition of 0.3% cholesterol to the semipurified diet (lithogenic diet, LD) led to the production of cholesterol crystals and stones, but the incidence and course of development was not the same for the three groups. With the short feeding period, cholesterol gallstones were found only in Sesco hamsters (44.4%). In the Engle hamster, gallstones and cholesterol crystals were not detected. Similarly, the Lakeview hamsters had neither gallstones nor cholesterol crystals after a 5-wk feeding of the lithogenic diet.

The longer feeding period produced a higher incidence of gallstones in groups IIb and IVb receiving the lithogenic diet. In the Sesco hamster, there was a higher incidence of cholesterol crystals and stones (63.6%), significantly different in comparison with animals fed no cholesterol (group Ib). In the Engle group, 45% of the hamsters now had cholesterol gallstones. Attempts to

TABLE 1

Incidence of Gallstones and Cholesterol Crystals in Three Groups of Hamsters

Source	Group[a]	Diet[a]	No. of wk on diet	No. of animals	Incidence of biliary CH gallstones	Incidence of biliary CH crystals
Sesco	Ia	SPD	5	5	0/5	0/5
	Ib	SPD	10	18	0/18	0/18
	IIa	SPD + 0.3% CH	5	9	4/9[b]	4/9[b]
	IIb	SPD + 0.3% CH	10	11	7/11[c,d]	7/11[c,d]
Engle	IIIa	SPD	5	5	0/5	0/5
	IIIb	SPD	10	5	0/5	0/5
	IVa	SPD + 0.3% CH	5	10	0/10	0/11
	IVb	SPD + 0.3% CH	10	11	5/11[c,e]	5/10[c,e]
Lakeview	Va	SPD	5	5	0/5	0/5
	Vb	SPD	10	4	0/4	0/4
	VIa	SPD + 0.3% CH	5	10	0/10	0/10
	VIb	SPD + 0.3% CH	10	11	1/11	1/11

[a]Animals were fed a semipurified diet (SPD) or SPD plus cholesterol (CH) for 5 wk (short-term feeding; groups Ia–VIa) or 10 wk (long-term feeding; groups Ib–VIb). See Materials and Methods.

[b]Differs from groups IVa and VIa; $p < 0.01$, by chi-square test.

[c]Differs from corresponding control groups; $p < 0.005$, by chi-square test.

[d]Differs from group VIb; $p < 0.01$, by chi-square test.

[e]Differs from group VIb; $p < 0.01$, by chi-square test.

induce cholesterol stones in the Lakeview hamsters failed: only one animal of this group had cholelithiasis.

The size of stones varied from tiny sand-like solids to 2 mm in diameter. All the stones had a yellowish-white color and were hard to crush. To determine the cholesterol content of the stones, several samples were analyzed by GLC. These analyses revealed that the stones contained at least 70% cholesterol by weight.

The effect of the feeding period and dietary cholesterol content on liver weight and tissue cholesterol concentrations are shown in Table 2. In animals fed the lithogenic diet, liver weight, liver cholesterol, serum cholesterol and biliary cholesterol were increased, compared with those of the corresponding control groups; and all of these differences were statistically significant. In all groups, the liver cholesterol concentrations were markedly increased by cholesterol feeding: 8.5 to 27.7 times above that of the corresponding controls. The values for liver cholesterol appear to increase sharply for the first 5 wk (groups IIa, IVa and VIa) and then decline or increase slightly after 10 wk (IIb and IVb vs VIb, respectively). Average food intake did not change during the entire experiment. In comparison with Engle and Lakeview hamsters, the liver cholesterol levels of the Sesco hamster were the highest, both in the groups fed the semipurified diet alone (groups Ia and Ib) and in those fed the lithogenic diet (groups IIa and IIb). Biliary cholesterol levels exhibited changes paralleling those of liver cholesterol. Serum cholesterol levels were increased in animals maintained with the lithogenic diet. In addition, in the Engle hamsters, serum cholesterol levels were increased significantly by the longer feeding of the semipurified diet; there was a significant difference between groups IIIa and IIIb. A similar change was observed when comparing groups IVa and IVb. Lakeview hamsters also showed increasing values of serum cholesterol as the duration of the feeding period was increased.

Biliary lipid compositions and lithogenic indices are shown in Table 3. The total biliary lipid concentrations were, in most instances, not affected by the addition of 0.3% cholesterol to the semipurified diet. Sesco hamsters had greater total lipid concentrations than the other two groups. The molar percentage (mol %) of phospholipid in the Sesco groups seemed low in comparison with the other hamsters. In all groups fed the high cholesterol lithogenic diet, the mol % of biliary cholesterol was increased significantly compared with that of each corresponding control group.

The lithogenic indices were calculated from the total lipid concentration using a computer program and data depicted in Table 3. The cholesterol saturation of bile was below 1.00 in all six control groups (Ia and b; IIIa and b; and Va and b), although in the Engle and Lakeview hamsters, the lithogenic indices on the SPD alone showed a definite dependence on the length of the feeding period. (The values for groups IIIb and Vb were significantly higher than those of groups IIIa and Va, respectively.) After a 5-wk feeding of the lithogenic diet, all the Sesco hamsters had supersaturated bile (mean LI = 1.24). Long-term feeding of the lithogenic diet (10 wk) caused supersaturation of bile in all three groups of hamsters. All animals in groups IIb and IVb had lithogenic indices exceeding unity; 8 out of 10 animals in group VIb had bile with a lithogenic index above 1.0.

Table 4 shows the composition of the biliary bile acids. Total bile acid concentration was highest in the Sesco hamster. The percentages of four bile acids normally found in the hamster (cholic acid, chenodeoxycholic acid, deoxycholic acid and lithocholic acid) were very similar in all groups fed the semipurified diet. Feeding the lithogenic diet caused a decrease in cholic acid and a corresponding increase in chenodeoxycholic acid, although in some groups these differences were not significant.

TABLE 2

Liver Weight and Tissue Cholesterol Levels in Three Groups of Hamsters

Source	Group	Diet	No. of animals	Liver weight (g)	Cholesterol concentration		
					Liver (mg/g)	Serum (mg/dl)	Bile (mg/ml)
Sesco	Ia	SPD	5	3.29 ± 0.48	5.03 ± 0.23	172 ± 18	1.63 ± 0.20
	Ib	SPD	18	3.89 ± 0.17	6.51 ± 0.59	144 ± 7	1.71 ± 0.13
	IIa	SPD + 0.3% CH	9	5.08 ± 0.32[a]	62.50 ± 3.08[a]	316 ± 15[a]	5.32 ± 0.42[a]
	IIb	SPD + 0.3% CH	11	5.57 ± 0.29[a]	55.09 ± 3.61[a]	294 ± 10[a]	4.61 ± 0.69[a]
Engle	IIIa	SPD	5	4.33 ± 0.30	2.35 ± 0.28	87 ± 19	1.02 ± 0.32
	IIIb	SPD	5	4.48 ± 0.33	3.11 ± 0.32	317 ± 34[b]	1.11 ± 0.33
	IVa	SPD + 0.3% CH	10	5.94 ± 0.37[c]	50.08 ± 3.94[a]	284 ± 16[a]	3.38 ± 0.48[a]
	IVb	SPD + 0.3% CH	11	7.26 ± 0.36[a]	36.10 ± 2.30[a]	510 ± 46[a]	5.55 ± 1.02[a]
Lakeview	Va	SPD	5	5.34 ± 0.27	1.76 ± 0.07	136 ± 16	0.88 ± 0.14
	Vb	SPD	4	4.28 ± 0.71	1.46 ± 0.58	181 ± 13	0.70 ± 0.18
	VIa	SPD + 0.3% CH	10	6.49 ± 0.27[c]	33.21 ± 1.95[a]	261 ± 19[a]	2.53 ± 0.64[d]
	VIb	SPD + 0.3% CH	11	6.92 ± 0.55[d]	40.37 ± 4.45[a]	347 ± 34[a]	3.04 ± 0.60[a]

[a]Differs significantly from value in corresponding control group (IIa vs Ia; IIb vs Ib; IVa vs IIIa; IVb vs IIIb; VIa vs Va; VIb vs Vb); $p < 0.01$, respectively, by t-test.

[b]Differs significantly from group IIIa; $p < 0.01$.

[c]Differs significantly from corresponding control group (IVa vs IIIa; VIa vs Va); $p < 0.02$.

[d]Differs significantly from corresponding control group (VIa vs Va; VIb vs Vb); $p < 0.03$.

DISCUSSION

Models of cholesterol cholelithiasis in the hamster have been described by Dam and Christensen (4) and Pearlman et al. (5). Hamster gallstone models are important. The biliary lipid and bile acid composition of the hamster is similar to that of man and, because of their small size, many animals can be studied simultaneously without using excessive amounts of synthetic bile acids or bile acid analogs that are being tested for their cholelitholytic properties. On the other hand, this model presents a disadvantage in that only small quantities of bile and gallstones are available per animal. Dam and Christensen (4) described the induction of cholesterol gallstones in young, growing golden Syrian hamsters by administering a fat-free, semipurified diet for about 6 wk. However, this model had two serious disadvantages: First, because of the lack of essential fatty acids in the diet, the animals did not gain weight and frequently a considerable proportion of the hamsters died from intestinal infections before completion of the experiment. Second, dihydroxy bile acids, chenodeoxycholic acid (15,16) and ursodeoxy-

TABLE 3

Biliary Lipid Composition and Saturation Index in Three Groups of Hamsters

Source	Group	Diet	No. of animals	Total lipid (g/dl)	Biliary lipid composition mol %			Lithogenic index
					Bile acid	Phospholipid	Cholesterol	
Sesco	Ia	SPD	5	10.0 ± 1.3	85.0 ± 0.6	12.7 ± 0.3	2.3 ± 0.3	0.48 ± 0.07
	Ib	SPD	18	11.1 ± 0.8	84.7 ± 0.9	13.2 ± 0.8	2.1 ± 0.1[d]	0.43 ± 0.02
	IIa	SPD + 0.3% CH	8	11.3 ± 1.0	80.2 ± 0.6[a]	13.4 ± 0.5	6.4 ± 0.4[a,e]	1.24 ± 0.09[a]
	IIb	SPD + 0.3% CH	11	8.9 ± 1.2	80.5 ± 1.4[a]	12.6 ± 1.1	6.9 ± 0.4[a,f]	1.47 ± 0.05[a]
Engle	IIIa	SPD	5	7.5 ± 2.1	77.7 ± 1.7	20.4 ± 1.6	1.9 ± 0.1	0.31 ± 0.01
	IIIb	SPD	5	5.4 ± 1.0	89.6 ± 1.0	7.8 ± 1.2	2.6 ± 0.4	0.83 ± 0.14
	IVa	SPD + 0.3% CH	10	9.4 ± 0.7	76.2 ± 1.2	18.9 ± 1.0	4.9 ± 0.5[a]	0.78 ± 0.07[a]
	IVb	SPD + 0.3% CH	11	7.3 ± 0.8	76.4 ± 1.4[a]	14.0 ± 1.3[a]	9.6 ± 1.0[a]	1.97 ± 0.21[a]
Lakeview	Va	SPD	5	5.6 ± 0.6	80.0 ± 2.6	17.8 ± 2.5	2.2 ± 0.3	0.40 ± 0.05
	Vb	SPD	4	3.1 ± 0.8	84.3 ± 6.5	12.6 ± 6.4	3.1 ± 0.2	0.89 ± 0.17
	VIa	SPD + 0.3% CH	10	7.5 ± 1.2	77.4 ± 1.3	18.3 ± 1.0	4.3 ± 0.5[a]	0.71 ± 0.05[a]
	VIb	SPD + 0.3% CH	10	5.5 ± 0.5[b]	79.3 ± 2.2	13.6 ± 1.5	7.1 ± 1.0[a]	1.51 ± 0.15[c]

[a]Differs significantly from corresponding control group; $p < 0.01$ (see Table 2 for details on control grouping).

[b]Differs significantly from corresponding controls; $p < 0.03$.

[c]Differs significantly from corresponding control group; $p < 0.05$.

[d]Differs from group Vb; $p < 0.01$ by t-test.

[e]Differs from groups IVa and VIa; $p < 0.03$ and $p < 0.01$ by t-test.

[f]Differs from group IVb; $p < 0.03$ by t-test.

TABLE 4

Composition of Biliary Bile Acid in Three Groups of Hamsters

Source	Group	Diet	No. of animals	Total free bile acid (mg/ml)	Composition of bile acid%				
					CA	CDCA	DCA	LCA	Others[a]
Sesco	Ia	SPD	5	65.3 ± 8.7	42.9 ± 3.4	30.2 ± 5.7	9.7 ± 3.0	1.6 ± 0.2	15.6 ± 2.2
	Ib	SPD	18	71.8 ± 5.2	47.5 ± 1.0	25.0 ± 1.0	10.8 ± 0.7	1.1 ± 0.2	15.5 ± 0.9
	IIa	SPD + 0.3% CH	8	69.7 ± 6.5	34.0 ± 1.5[c]	40.3 ± 2.4	8.7 ± 1.2	3.3 ± 0.2[b]	13.8 ± 1.0
	IIb	SPD + 0.3% CH	11	55.2 ± 8.2	31.9 ± 1.0[b]	39.3 ± 1.5[b]	9.5 ± 0.9	2.8 ± 0.4[b]	16.5 ± 1.9
Engle	IIIa	SPD	5	42.9 ± 12.4	38.3 ± 7.7	26.9 ± 2.7	16.3 ± 3.2	3.2 ± 0.8	15.4 ± 8.1
	IIIb	SPD	5	38.1 ± 7.4	43.1 ± 1.6	28.2 ± 1.3	12.8 ± 1.7	2.7 ± 0.3	13.2 ± 1.5
	IVa	SPD + 0.3% CH	10	53.0 ± 3.8	35.9 ± 2.3	38.5 ± 1.6[b]	9.5 ± 0.6	3.6 ± 0.4	12.6 ± 2.8
	IVb	SPD + 0.3% CH	11	43.2 ± 5.1	32.5 ± 1.6[b]	43.7 ± 1.6[b]	7.4 ± 0.7[b]	3.7 ± 0.5	12.7 ± 1.1
Lakeview	Va	SPD	5	33.7 ± 4.6	40.8 ± 2.0	33.9 ± 1.8	11.4 ± 2.8	1.2 ± 0.6	12.6 ± 1.6
	Vb	SPD	4	28.8 ± 10.7	36.9 ± 5.8	32.9 ± 2.9	8.8 ± 3.8	1.6 ± 0.6	19.8 ± 5.0
	VIa	SPD + 0.3% CH	10	42.6 ± 6.1	35.8 ± 2.4	43.2 ± 1.9[b]	8.0 ± 0.8	1.4 ± 0.3	11.5 ± 1.4
	VIb	SPD + 0.3% CH	11	36.9 ± 4.9	34.4 ± 3.5	44.0 ± 3.8	7.6 ± 1.2	2.0 ± 0.3	12.0 ± 1.4

See footnotes to Tables 1 and 2.

[a]Others include hyodeoxycholic, ursodeoxycholic and keto bile acids.

[b]Differs from corresponding control groups; $p < 0.01$ by t-test.

[c]Differs from corresponding control groups; $p < 0.02$ by t-test.

cholic acid (17), shown to be cholelitholytic in man are ineffective in preventing or dissolving cholesterol gallstones in this model.

The model of Pearlman et al. (5) employed a chow diet supplemented with 0.24% cholesterol and small levels of ethinyl estradiol to obtain a 90% incidence of cholesterol gallstones during a period of 12 wk. (When the estrogen was omitted, the incidence was 50%.) The cholelitholytic dihydroxy bile acids (ursodeoxycholic acid and chenodeoxycholic acid) were effective in preventing or dissolving cholesterol gallstones. Unfortunately, probably because of a genetic change in these hamsters (the source of the hamsters was not reported), this model was not reproducible (18). Our laboratory had similar experiences with a hamster model of pigment lithiasis (9).

During studies designed to elucidate the dietary factors to establish a stable, reproducible model of pigment cholelithiasis in the hamster (9), we used semipurified diets containing various components including casein, cornstarch and butterfat, as exemplified by the cholesterol-free control diet used in the present study. This basal diet produced a 10–20% incidence of pigment stones in the Engle hamster; no pigment stones were found in the other two strains studied (9). However, when 0.3% cholesterol was added to this diet and fed to the hamsters for 5–10 wk, cholesterol gallstones developed in two of the three groups used, namely in the Sesco hamster and, to a lesser extent, in the Engle hamster (Table 1). This new model of cholesterol cholelithiasis has proved to be reproducible, uses a nutritionally adequate diet and does not require the administration of estrogens or other hormones. All of the 104 experimental animals gained weight at a normal rate and did not develop symptoms of disease or die during the feeding period used.

We reported previously that there were apparent strain differences between Lakeview hamsters and the BIO strain (Bio-Research Institute, Cambridge, MA) with respect to cholesterol metabolism (8). In that study, too, the BIO hamster model of prostatic hypertrophy (19) became nonreproducible, although differences in cholesterol metabolism persisted. In the present study, hamsters from three suppliers were used to investigate whether they differed with respect to cholesterol gallstone formation and cholesterol metabolism when a lithogenic diet was administered. Only Sesco hamsters had lithogenic bile at the end of 5 wk and exhibited a higher incidence of gallstones than the other two groups (Table 1). All hamsters had lithogenic bile at the end of 10 wk (Table 3). The lithogenic index, indicating supersaturation of bile with cholesterol, may explain in part the greater incidence of gallstones in the Sesco animals. In the other two strains, it took much longer for the bile to become supersaturated with cholesterol (Table 3). It cannot be concluded that more prolonged feeding of the lithogenic diet alone, particularly in the Lakeview hamsters, would lead to an increased incidence of cholesterol cholelithiasis. Factors other than cholesterol, such as nucleating/antinucleating proteins, gallbladder motility and mucus secretion in the gallbladder, may be involved in gallstone formation.

Sesco hamsters in the control groups (groups Ia and Ib) had higher concentrations of liver and biliary cholesterol than those of the other two strains. When the total weight of cholesterol per liver was calculated for all three groups (liver weight times liver cholesterol concentration), Sesco hamsters clearly accumulated higher amounts of cholesterol than the other hamsters. However, this finding alone does not enable us to conclude that there is a quantitative difference in hepatic cholesterol secretion among the three groups. It will be necessary to measure cholesterol secretion into the bile, before and after cholesterol feeding, to obtain definitive information on this point.

The parameters of cholesterol metabolism examined in the present experiment do not provide clear-cut clues to the differences in gallstone incidence observed in the three groups of hamsters. Tissue cholesterol concentrations and liver weights were all increased in the groups fed the lithogenic diet (Table 2), but the Sesco hamsters that had the highest degree of cholelithiasis did not differ greatly from the other two groups. Other investigators, including our group, have previously reported similar responses to high cholesterol diets in the hamster (20–22), although the intensity of the response appeared to be lower when a basal diet of rodent chow was used. It is noteworthy that in previous studies with the Engle and Lakeview hamsters, marked increases of serum and liver cholesterol were reported with diets similar but not identical to that used in the present experiment (9). The earlier experiments used diets containing cholesterol and ethinyl estradiol, but not butterfat and cornstarch; the former diet also led to high cholesterol saturation of bile, but only pigment stones were produced (9). The control diet used in the present experiment, which contained no cholesterol, nevertheless produced a significant increase of serum cholesterol in the Engle hamster (Table 2). There can be little doubt that the change from sucrose to cornstarch and partial substitution of butterfat for corn oil, plus the added cholesterol, played an important role in producing cholesterol cholelithiasis. Butterfat was reported to produce cholesterol gallstones in hamsters (23); complex carbohydrate (cornstarch) rather than simple carbohydrate was similar to the type of carbohydrate that produced cholesterol gallstones in another hamster model (5). The role of these same dietary components in gallstone formation has been described by Hashimoto (24), but the exact role of individual dietary components in stimulating the production of nucleating/antinucleating factors (mucin, protein) remains to be established.

In summary, cholesterol gallstones can be produced in the hamster, but the incidence seems to depend on the source from which the animals are obtained. This suggests that inherent genetic differences that alter the incidence of cholesterol cholelithiasis may exist in hamsters.

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METHODS

Determination of Ethane and Pentane in Free Oxygen Radical-induced Lipid Peroxidation

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It has been proposed that ethane and pentane reflect free oxygen radical-induced lipid peroxidation. However, methodological difficulties limit the use of these gases for assessment of free oxygen radical activity. In the present report we describe an improved method for the accurate analysis of picomole quantities (≥1 pmol) of ethane and pentane. They are first quantitatively trapped into an adsorbent and then heat-desorbed directly into a capillary column for gas chromatographic quantitation. During oxidation of linolenic (n-3) and linoleic (n-6) acid, ethane and pentane were formed, respectively. Nonstimulated granulocytes formed pentane. Upon addition of phorbol 13-myristate 12-acetate, the generation of pentane was increased by 540%. Addition of superoxide dismutase plus catalase inhibited lipid peroxidation in both a cell-free system and in isolated cells.

The present method is useful in the evaluation of free oxygen radical induced damage.

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Free oxygen radicals are produced during normal metabolism and in pathological states (1). Sources include redox-reactions of electron transport chains and certain reactions catalyzed by soluble enzymes like xanthine oxidase (1-3). A burst of free oxygen radical formation takes place during phagocytosis (4). Free oxygen radicals damage lipids, DNA and proteins (1,2). There are several defense systems against free oxygen radicals. Of these, superoxide dismutase catalyzes the autoxidation of superoxide radical, and catalase promotes hydrolysis of the resulting hydrogen peroxide that may form highly reactive hydroxyl radicals in the presence of a transition metal (1).

Because of the transient nature of the free oxygen radicals, direct measurement of these molecules is difficult (1,2). They have been determined by chemiluminescence or by following the reduction of cytochrome c added in vitro (5,6). In vivo assessment of free oxygen radicals is based mainly on analysis of products of lipid peroxidation in extracellular fluid, but the specificity of these methods is low (2,7-9). However, peroxidation of n-3 and n-6 fatty acid families results in the formation of ethane and pentane, respectively (10). In animals, these volatile alkanes are generated only by free oxygen radical-mediated lipid peroxidation that may take place as a result of free oxygen radical activity (11).

Previously published methods for determination of ethane and pentane require large injection volumes or separation of ethane and pentane prior to quantitation by gas chromatography with packed columns (12-15). In the present study, we describe an accurate method for the analysis of ethane and pentane as indicators of lipid peroxidation.

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Abbreviations: CAT, catalase; PMA, phorbol 13-myristate 12-acetate; SOD, superoxide dismutase; FID, flame ionizing detector.

MATERIALS AND METHODS

Standards and reagents. Ethane and propene standard gases were purchased from AGA SpecialGas, Lidingö, Sweden. Pentane was from Rathburn Chemical Ltd. (Walkerburn, Scotland, U.K.). Gas standard bag, gas bulbs for standard dispension and the capillary column were from Chrompack (Middelburg, The Netherlands). Linolenic acid, linoleic acid, phorbol 13-myristate 12-acetate (PMA), superoxide dismutase (SOD) and catalase (CAT) were obtained from Sigma Chemical Company (St. Louis, MO). Porasil-C was purchased from Alltech Associates (Deerfield, IL), and Ficoll-Paque from Pharmacia Chemicals (Uppsala, Sweden). All chemicals were of the highest grade of purity available.

Collection of ethane and pentane. The gaseous samples were collected using acid washed stainless steel columns (5 mm i.d.) packed with 80/100 mesh of Porasil-C packing material for gas chromatography. The headspace of the air-tight reaction vial was flushed into the gas trap with an excess of analytical grade of N_2 containing 0.5 pmol/ml propene as an internal standard. A precolumn of powdered NaOH, for elimination of water, and CO_2 was connected to the gas trap containing from 2.5 to 5 ml of Porasil-C adsorbent. Because of the low boiling point of ethane, the trap was immersed in liquid N_2/ethanol slurry.

Gas chromatography of ethane and pentane. The gases were analyzed using a Hewlett-Packard 5890 gas chromatograph with a Chrompack Al_2O_3/KCl (50 m, 0.4 mm i.d.) capillary column and high purity He as carrier gas. The hydrocarbons were detected with a flame ionizing detector (FID) and quantitated with a Hewlett-Packard 3390A integrator. A shunt with a high-pressure valve on the carrier gas line was connected to the trap. The other end of the trap was connected through a needle to the injector. The sample was eluted into the capillary column by heating the trap to 120°C for 10 min. During the elution, the proximal 30 cm loop of the capillary column was immersed in a liquid N_2 bath. The gas chromatography was started by rapid transfer of the 30 cm loop into the oven. The temperature was programmed from 50 to 200°C at 15°C/min.

Lipid peroxidation in a cell-free system. In the studies of alkane liberation from linolenic and linoleic acid, the fatty acids (10 μmol each) were incubated for 48 hr at room temperature (22°C) in the dark. The headspace of the air-tight 20-ml reaction vessel initially contained clean air.

In another experiment, 1.0 μmol of linoleic acid with 5 μmol palmitic acid was sonicated in 5 ml 200 mM phosphate buffer, pH 7.8, in an ice-cold water bath for 45 min. Lipid peroxidation was induced with 10 mM H_2O_2 and 0.2 mM $FeSO_4$ in 37°C for 18 hr. The headspace (15 ml) initially consisted of N_2.

Isolation and incubation of the granulocytes. Peripheral blood granulocytes were isolated from buffy-coats obtained from the Finnish Red Cross Blood Transfusion Service, by density gradient sedimentation and subsequent Ficoll-Paque centrifugation (16). The viability of the cells was more than 90% by the Trypan-Blue exclusion method (17). Altogether, from 280 to 580 × 10^6 cells were incubated in air-tight silicone-coated vials (100 ml) for 2 hr at 37°C in 50 ml preoxygenated phosphate-buffered saline containing 5 mM glucose, and 0.5 mM $CaCl_2$. (N-2-Hydroxyethylpiperazine-N-2-ethanesulfonic acid [HEPES] should not be used as buffer because it produces ethane in the presence of activated granulocytes.) Constant shaking of the incubation vessels (60 strokes/min) prevented aggregation of the cells.

Before incubation, the headspace was flushed with 100% O_2. During incubation pO_2, pCO_2 and pH were measured in a 0.5 ml specimen taken at the beginning and the end of the experiment. A maximal decrease in pH was from 7.4 to 7.2 and pO_2 decreased from 600 to 200 mm Hg.

Fatty acids were analyzed by gas chromatography as described previously (18).

Statistical method. The results are presented as mean ±SE. Unpaired Student's t-test was used to examine statistical significance.

RESULTS

Method for quantitation of ethane and pentane. The capillary column sharply separated the hydrocarbons under the conditions described. The retention time of ethane was 5.5 ± 0.1 min and that of pentane 10.9 ± 0.2 min (n = 15) (Fig. 1). The detector response to the standard gases compared with the constant amounts of propene as an internal standard proved to be linear (Fig. 2). The detection limit for ethane and pentane was 1 pmol. The recoveries of ethane and pentane were over 90% in the range of 15–140 pmol. With larger concentrations, the saturation of the adsorptive capacity of Porasil-C became evident. This concerned ethane and propene in particular, their boiling points being −89°C and −48°C, respectively.

Experiment with the cell free system. Linolenic and linoleic acid were incubated for 48 hr in clean air. In the headspace of the vial containing 10 μmol linolenic acid, 2.0 nmol of ethane was formed, whereas 2.4 nmol of pentane was detected in the vial containing 10 μmol linoleic acid (Fig. 1). In order to demonstate the free oxygen radical dependence on the lipid peroxidation, linoleic acid in slightly basic phosphate buffer was sonicated in a basin sonicator for 45 min at +4°C. Thereafter, H_2O_2 and ferrous-ion were added. As shown in Table 1, addition of SOD (50 U/ml) and CAT (50 U/ml) inhibited the formation of pentane by 94%.

Experiment with the granulocytes. The pooled granulocytes were divided into three different groups and incubated in pure oxygen. One group was kept as control.

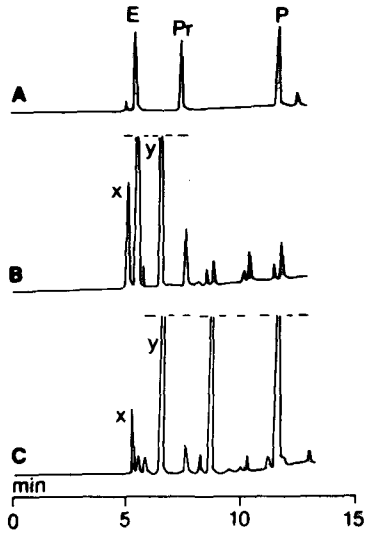

FIG. 1. Chromatograms of hydrocarbons trapped on Porasil-C; A, ethane (E), propene (Pr) and pentane (P) standards; B, hydrocarbons formed from linolenic acid; C, hydrocarbons from linoleic acid; X, air peak; and Y, contaminant from rubber stopper.

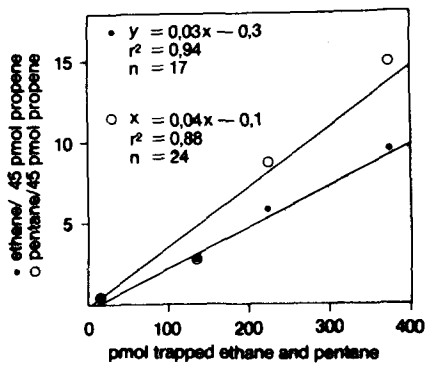

FIG. 2. Relationship between constant amount of propene (45 pmol) and varying concentrations of ethane and pentane.

TABLE 1

Formation of Ethane and Pentane from Linolenic and Linoleic Acid

Addition	Form	Gas formed
Linolenic acid (n-3), 10 μmol	Dry[a]	2.0 nmol ethane/48 hr
Linoleic acid (n-6), 10 μmol	Dry[a]	2.4 nmol pentane/48 hr
Linoleic acid, 1 μmol + 10 mM H_2O_2 + 0.2 mM Fe^{2+}	Liposomes[b,c]	1.0 nmol pentane/18 hr
Linoleic acid, 1 μmol + 10 mM H_2O_2 + 50 U/ml SOD + 50 U/ml CAT	Liposomes[b,c]	0.1 nmol pentane/18 hr

[a]Temperature: 22°C.

[b]Temperature: 37°C.

[c]Liposomes were prepared by sonicating the fatty acid for 45 min on ice bath on 200 mM KH_2PO_4-K_2HPO_4, pH 7.8.

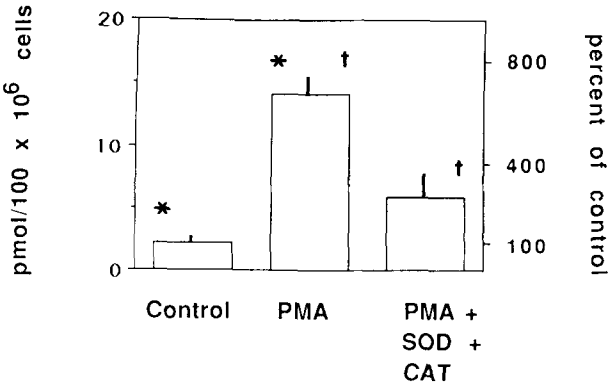

FIG. 3. Production of pentane by granulocytes. The cells were treated with PMA alone and with PMA in the presence of SOD + CAT. Pentane formation in control cells represents 100%. Each group consisted of six separate experiments. Pentane production increased after activation of granulocytes with PMA (*, $p < 0.0005$), and pentane production in PMA-activated cells was decreased by SOD + CAT (†, $p < 0.025$).

The second group of cells was treated with 50 ng/ml PMA to induce oxygen radical formation (19). The third group was incubated with 50 ng/ml PMA, after addition of 200 U/ml SOD and 200 U/ml CAT. The control cells remained in homogenous suspension during the incubation, whereas PMA-induced aggregation and attachment of the cells onto the walls of the reaction vial. However, the cells treated with PMA, SOD and CAT remained homogenously suspended in the incubation medium in a similar way to the controls.

Nonactivated granulocytes produced 2.2 ± 0.3 pmol pentane per 100 × 10^6 cells. Stimulation of granulocytes by PMA increased pentane production to 14.1 ± 1.4 pmol/100 × 10^6 cells (540%, $p < 0.0005$). In the presence of SOD and CAT, the effect of PMA on pentane production was significantly inhibited ($p < 0.025$). Ethane production was close to the detection limit (0.3 ± 0.3 pmol/100 × 10^6 cells), and it was affected by neither PMA nor PMA + SOD + CAT (Fig. 3).

Analysis of nonactivated granulocytes revealed no detectable linolenic acid (n-3).

DISCUSSION

In the present study, we describe a quantitative method for the determination of ethane and pentane with a sensitivity exceeding that of previous methods by at least one order of magnitude (12-15). Because there is also a high recovery, the present method enables measurement of the volatile products of lipid peroxidation both in a chemically well-defined system and in isolated cells.

The present system allowed quantitative measurement of the hydrocarbons ranging from 1 to 150 pmoles. The capillary column enhanced the sensitivity because of distinct resolution of the peaks. The use of an internal standard enabled exact quantitation and monitoring of the recovery of the sample. In this method, special interest was focused on the injection of the enriched specimen into the gas chromatograph. The Porasil-C trap was connected to a shunt built on the carrier gas line of the chromatograph. The sample was desorbed with heat, led directly into the column and concentrated in the proximal loop of the column immersed in liquid N_2. The present technique reduces the sample loss compared with the conventional syringe injection. The problem of saturation of the adsorption capacity at higher than 400 pmole of trapped hydrocarbon can be avoided by adjusting the gas volume.

The nature of the alkanes formed in lipid peroxidation is dependent on the fatty acid composition of the cell membrane. Nonactivation of granulocytes produced pentane, and PMA increased only pentane in the headspace. Therefore, the phagocytic stimulation of granulocytes involving activation of the respiratory burst was associated with selective peroxidation of n-6 fatty acids.

Although it has been proposed that free oxygen radicals are important in pathogenesis of many diseases, direct evidence of their role is often based on unreliable techniques or is missing altogether (2,20). As demonstrated by Dillard et al. (13), ethane and pentane are specific markers of free oxygen radicals that can be measured in expired air. However, measurement of these alkanes has been hampered by low sensitivity of previous gas chromatographic methods. The present method conceivably improves the accuracy of measurement of expired ethane and pentane. It may further enable detection of these peroxidation products in specimens recovered from transcellular space such as cerebrospinal and synovial fluid.

ACKNOWLEDGMENTS

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ERRATUM

"Location of Methyl Branchings in Fatty Acids: Fatty Acids in Uropygial Secretion of Shanghai Duck by GC-MS of 4,4-Dimethyloxazoline Derivatives," by Q. T. Yu, B. N. Liu, J. Y. Zhang, and Z. H. Huang, *Lipids 23*, 804-810, 1988. Abstract, lines 8-12, should read:

Continuing our previous study, 66 out of a total of 68 fatty acids obtained from the preen gland wax of Shanghai duck now have been identified by gas chromatography-mass spectrometry (GC-MS) of their oxazoline derivatives.

Table 3, footnote a, should read:

[a]Peaks #3, 64 and 65 represent minor unsaturated acids, which have been identified according to the empirical rule of "12 mass interval" as described (17). These acids were probably originated from contaminations in gland sampling and handling.

Figure 1 should be replaced by the following gas chromatogram:

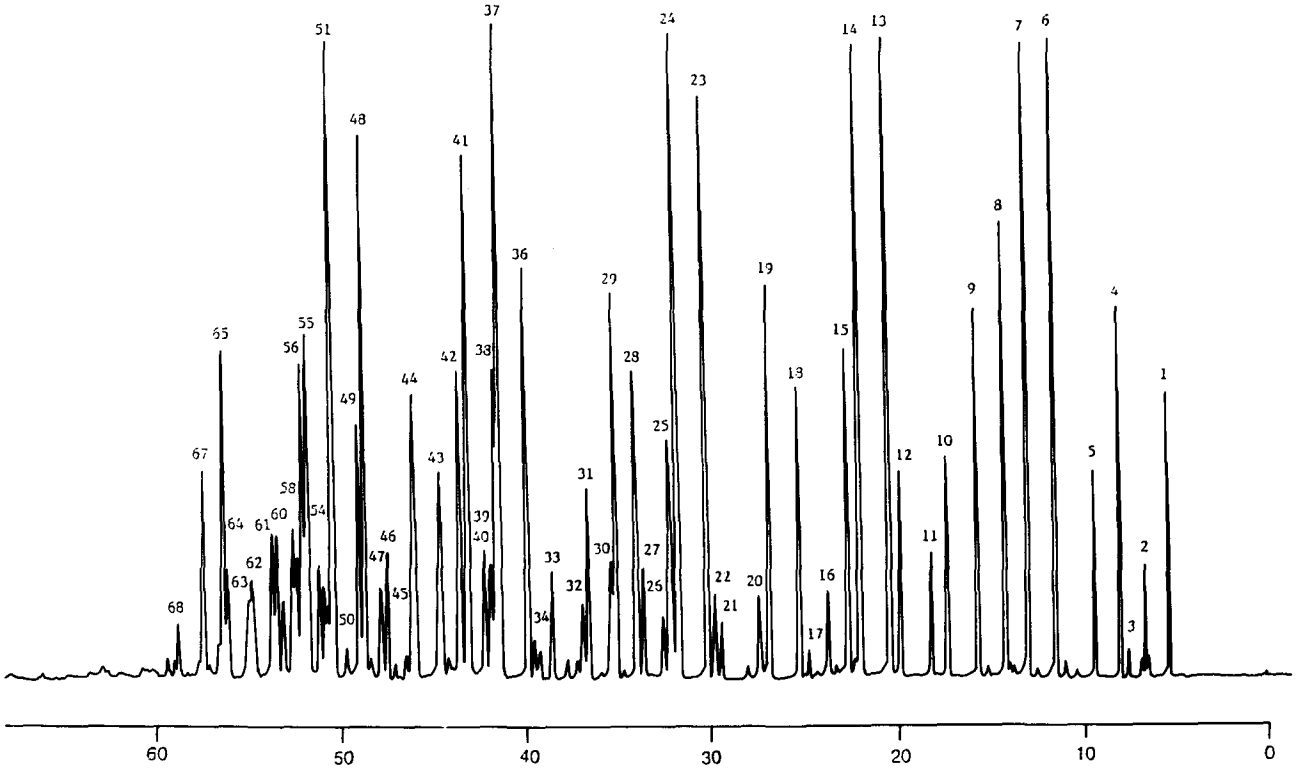

FIG. 1. Gas chromatogram of DMOX of fatty acid mixture obtained from the preen gland secretion of Shanghai white duck *(A. platyrhynchos)*.

Effects of Dietary Casein and Soy Protein on Metabolism of Radiolabelled Low Density Apolipoprotein B in Rabbits

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Rabbits fed semipurified diets containing casein have elevated plasma cholesterol levels compared to those fed soy protein. As part of continuing studies on the mechanism of casein-induced hypercholesterolemia, two groups of six rabbits were fed these diets for 14 to 16 weeks. Animals fed the casein diet were found to have significantly higher plasma concentrations of protein, cholesterol, triacylglycerol, phospholipid and apolipoprotein B (apo B) associated with low density lipoprotein (LDL) than those fed the soy protein diet. Kinetic studies showed that the fractional catabolic rate of LDL-apo B was significantly lower in animals fed casein than in those fed soy protein regardless of whether the tracer LDL was obtained from donors fed casein or soy protein. The production rate of LDL-apo B was higher in casein-fed animals but this was not statistically significant. These results show that the efficiency of removal of LDL is significantly reduced in animals fed casein compared to those fed soy protein, and that the source of LDL did not affect the efficiency of its subsequent removal. The accumulation of LDL in casein-fed animals is consistent with down-regulation of the LDL receptor.
Lipids 24, 169–172 (1989).

The differential effects of dietary casein and soy protein on plasma cholesterol levels in rabbits are well established (1,2). Casein, fed as part of a cholesterol-free semipurified diet, raises plasma cholesterol, while soy protein maintains it at a low level (3,4). Although the overall lipoprotein pattern is similar in both dietary groups, the rise in plasma cholesterol level in casein-fed animals is characterized by a much higher level of low density lipoprotein (LDL) (5).

The rise in LDL in casein-fed animals is accompanied by changes in composition of the lipoproteins. Recent studies in our laboratory have shown that the LDL is enriched in cholesterol at early stages of dietary treatment (6). As part of ongoing studies of the mechanism of casein-induced hypercholesterolemia, LDL apolipoprotein B (apo B) turnover was investigated in rabbits fed diets containing either casein or soy protein.

MATERIALS AND METHODS

Animals and diets. Young, male New Zealand White rabbits (1.5–2.0 kg) were obtained from Riemen's Fur Ranches (Guelph, Ontario), and housed individually in a controlled environment as described previously (5). The animals were maintained on rabbit Chow (Ralston Purina Co., St. Louis, MO) for up to two weeks before being transferred to semipurified diets containing either casein or soy protein, the composition of which has been described (7). The animals, six per group, were fed these diets for 14 to 16 weeks.

Isolation and iodination of LDL. Prior to the metabolic studies, blood was obtained from the marginal ear vein of unanesthetized animals in the fasted state. Plasma was separated and LDL ($1.019 < d < 1.063$ g/ml) isolated from the pooled plasma of six animals from each dietary group by sequential ultracentrifugation (8). The isolated LDL was ultracentrifuged a second time to wash and concentrate it. LDL obtained from animals fed casein and soy protein diets was radiolabeled with ^{131}I and ^{125}I (Amersham, Oakville, Ontario), respectively, as described by Huff and Telford (9), followed by dialysis to remove unbound radioiodine. To determine the intramolecular distribution of the radioiodine, 10 μl aliquots of LDL were added to carrier bovine serum albumin (5% solution) and then precipitated by trichloroacetic acid (TCA, 5% final concentration). In addition, aliquots of the labelled lipoprotein were added to 200 μg of carrier LDL and then precipitated using isopropanol (50% final concentration). The isopropanol pellet yielded the amount of label associated with apo B (10), while the TCA supernatant yielded the free iodine (11). The distribution of label was similar for both groups, with 92% of the label in apo B and less than 2% as free iodine.

In vivo studies. Animals were fasted for 12–16 hr prior to the kinetic studies. Each animal was injected with approximately 3.7 μCi ^{125}I-LDL (obtained from soy protein-fed donors) and 1.9 μCi ^{131}I-LDL (obtained from casein-fed donors). The amount of protein injected amounted to less than 0.01% of the circulating pool of LDL protein. Both tracers were injected simultaneously into the marginal vein of the left ear followed by 1–2 ml of phosphate-buffered saline to flush the cannula. Blood samples were then taken from the marginal vein of the right ear at 3 min, 45 min, 1.5, 3, 4.5, 6, 12, 24, 36, 48, 60 and 72 hr postinjection. Plasma was separated and the radioactivity in apo B determined following precipitation by isopropanol (12). A radioactivity disappearance curve was then constructed for both tracers by taking the radioactivity in apo B at three min as 100% of the injected dose. The curves were analyzed according to the method described by Matthews (13). The fractional catabolic rate (FCR), as defined by Kushwaha and Hazzard (14), was determined for both tracers.

Plasma total cholesterol was assayed enzymatically (CHOD PAP Monotest, Boehringer Mannhein Canada, Dorval, P.Q.) on samples obtained at 3 min and 24, 48 and 72 hr. Aliquots of plasma (0.5 ml) from all time points from each animal were then pooled and ultracentrifuged as described above to obtain the LDL fraction. The cholesterol, triacylglycerol and phospholipid concentrations of LDL were analyzed using enzymatic assay kits (Boehringer Mannheim Canada, Dorval, P.Q.). Total protein concentration was determined using the modification of Markwell et al. (15) of the Lowry method (16). To determine the concentration of apo B, 100 μg of protein was precipitated by isopropanol (10). The supernatant was

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Abbreviations: apo B, Apolipoprotein B; FCR, fractional catabolic rate; LDL, low density lipoprotein; VLDL, very low density lipoprotein.

assayed for protein and apo B was calculated as the difference between this value and the total protein concentration. Production rates (or turnover rates) for homologous LDL were calculated as the product of the FCR and the plasma concentration of apo B and expressed as mg/hr/dl.

Statistical analyses. Statistical differences between dietary groups were determined using Student's t test. To determine statistical differences within a group, the paired Student's t test was used.

RESULTS

Both groups of animals maintained equal weight gains throughout the experimental period such that the final weight was 3.4 ± 0.1 kg (SEM) for each dietary group. However, the two dietary proteins had divergent effects on plasma cholesterol concentrations, with casein-fed animals exhibiting a marked hypercholesterolemia (399 ± 62 mg/dl) compared to those fed soy protein (66 ± 16). These concentrations of total cholesterol were maintained throughout the 72 hr of the kinetic experiments consistent with the animals being in a steady state.

All components of LDL were significantly higher in casein-fed animals than in those fed soy protein, with the cholesterol and phospholipid concentrations showing a seven-fold difference (Table 1). In addition, LDL-apo B metabolism in casein-fed animals was slower than in those fed soy protein, with the FCR, or the efficiency of removal, being significantly lower (Fig. 1, and Table 2). The FCR in casein-fed rabbits was low regardless of the source of tracer LDL, that is, whether it came from casein- or soy protein-fed donors had no significant influence on its clearance rate, which was determined by the diet fed the recipient animal. There was a significant relationship between the FCR and plasma LDL-apo B concentrations, the FCR diminishing with increasing concentrations of LDL-apo B in soy protein-fed animals. However, for animals fed casein, there was little or no change in FCR

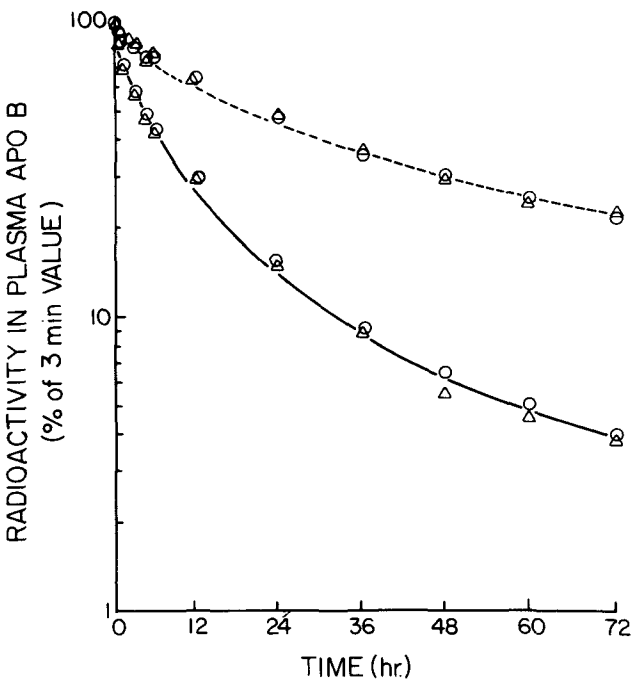

FIG. 1. Decay of radioactivity of plasma apo B obtained using LDL from (△) casein-fed or (○) soy protein-fed donor rabbits injected into (– – –) casein-fed or (—) soy protein-fed recipients. Each data point represents the mean value for 6 animals; SEM did not exceed 8% for any data point.

TABLE 1

The Effect of Dietary Casein and Soy Protein Diets on the Composition of Plasma LDL

Diet	Protein (mg/dl)	Cholesterol (mg/dl)	Triacylglycerol (mg/dl)	Phospholipid (mg/dl)
Casein	74.2 ± 12.7**	144.7 ± 23.1**	10.2 ± 2.7*	69.2 ± 10.2**
Soy protein	15.6 ± 3.1	19.1 ± 2.9	3.2 ± 0.5	10.3 ± 1.6

All values expressed as Mean ± SEM, n = 6.

Protein, cholesterol, triacylglycerol and phospholipid concentrations in the group fed casein diet were significantly higher than their soy protein-fed counterparts, as determined using Student's t test (**P < 0.001, *P < 0.05).

TABLE 2

Effects of Dietary Casein and Soy Protein Diets on the Metabolism of LDL-apo B in Rabbits

Diet	Plasma LDL-apo B (mg/dl)	FCR (per hr)		Production rate (mg/hr/dl)
		Casein-fed donor	Soy protein-fed donor	
Casein	65.4 ± 10.5**	0.023 ± 0.002*	0.023 ± 0.002	1.41 ± 0.38
Soy protein	12.5 ± 2.9**	0.089 ± 0.018	0.079 ± 0.013*	0.82 ± 0.11

All values expressed as mean ± SEM, n = 6.

Mean values sharing a common symbol are statistically different from each other as determined using Student's t test (**P < 0.001, *P < 0.05).

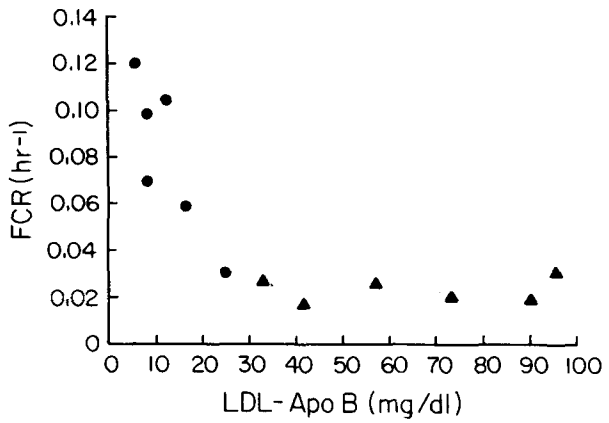

FIG. 2. The relationship between FCR (/hr) and LDL-apo B (mg/dl) in rabbits fed (▲) casein or (●) soy protein diets for 14 to 16 weeks.

over a three-fold increase in LDL-apo B concentration (Fig. 2).

Casein-fed animals had a larger plasma pool and a higher production rate of LDL-apo B than those fed soy protein, but the latter did not reach statistical significance.

DISCUSSION

The results of these experiments show, for the first time, that the hypercholesterolemic effect of dietary casein compared to soy protein is due primarily to the reduced efficiency of removal of LDL. This effect is consistent with the down-regulation of the LDL receptor which determines the metabolic fate of LDL (17).

The LDL particles in casein-fed animals are enriched in cholesterol compared to those fed soy protein diets. From time-course studies of the effect of dietary protein on plasma lipoproteins, these changes occur within 3-7 days of dietary treatment (6,18). It is thus plausible that the cholesterol-enriched LDL particles in casein-fed animals are removed less efficiently than those from animals fed soy protein, which could in turn result in raised levels of LDL. However, results of these experiments showed that the source of LDL, whether it came from a normocholesterolemic soy protein-fed donor animal or a hypercholesterolemic casein-fed donor animal, was unimportant in determining the efficiency of its removal. These results are in contrast to those obtained for the metabolism of very low density lipoproteins (VLDL) and intermediate density lipoproteins which are influenced by diets of both the donor and recipient animals (7).

Thus, our results suggest that the changes seen in the LDL particle do not contribute to any functional abnormality and that the effect is consistent with the down-regulation of the LDL receptor. The in vitro experiments of Chao et al. (19) showing a down-regulation of the receptor in animals fed wheat starch-casein diets lend support to this suggestion.

Another possible explanation of the raised levels of LDL is an increase in its production rate. Our data show a trend for a higher production rate in animals fed casein than in those fed soy protein diet, although large variations and small numbers of animals prevented this from reaching statistical significance. In the rabbit, most of the LDL is derived from a VLDL-independent pathway (20,21). Current work in our laboratory suggests that casein-fed animals produce more LDL by such a pathway than do animals fed soy protein diets (Khosla, P., Samman, S., and Carroll, K.K., unpublished data). This, together with a reduced efficiency of removal of LDL (Fig. 2), contributes toward an increased pool size of LDL in casein-fed animals.

The relationship between FCR and LDL-apo B concentration shown in Figure 2 is of interest because of the role apo B plays in targetting the LDL particle to its receptor in both hepatic and nonhepatic cells (22). The decreased FCR with increasing LDL-apo B concentration reflects the saturation of the removal mechanism, possibly due to the expanded LDL pool.

Beynen et al. (23) have suggested that the casein-induced rise in LDL is a consequence of "primary events" which take place in the intestinal lumen. They suggest that the first step involves stimulation of cholesterol and/or bile acid absorption as demonstrated in our laboratory (24). The subsequent increase in hepatic cholesterol concentrations may elicit down-regulation of the apo B/E receptor and increase the output of cholesterol associated with lipoproteins. However, it remains to be established what component of casein induces increased cholesterol absorption. Thus, further research on the digestibility of the proteins (25), interactions with micronutrients (26) or interference with the enterohepatic circulation (27) may help to determine the primary causes of casein-induced hypercholesterolemia.

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Incorporation of Arachidonic, Dihomogamma Linolenic and Eicosapentaenoic Acids Into Cultured V79 Cells

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The uptake and distribution of three common dietary polyunsaturated fatty acids was studied using Chinese hamster lung fibroblasts (V79 cells). Treatment of V79 cells with arachidonic (20:4), eicosapentaenoic (20:5) and dihomogammalinolenic (20.3) acids for 24 hr produced a marked uptake of 20:3 and 20:4, both of which were assimilated to a considerably greater degree than 20:5. All polyunsaturated fatty acids were incorporated primarily into phospholipids; however, there were considerable differences in their distribution into individual phospholipid species. Although 20:4 was incorporated primarily into phosphatidylcholine, 20:3 entered largely into phosphatidylethanolamine and phosphatidylglycerol, and 20:5 was distributed about equally between phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol. A marked conversion of 20:3 to 20:4 was found after 24 hr and, in several phospholipids, there was as much derived-radiolabeled 20:4 as there was radiolabeled 20:3. There was little evidence of 20:4 and 20:5 metabolism. V79 cells undergo substantial changes in phospholipid fatty acid composition following supplementation with these polyunsaturated fatty acids; however, these fatty acids are assimilated to different degrees and their distribution among cellular phospholipids is distinct, suggesting incorporation via independent mechanisms. ***Lipids 24,*** **173–178 (1989).**

Polyunsaturated fatty acids (PUFA) are most frequently found associated with complex lipids such as phospholipids and triacylglycerols, whereas low cellular levels of these fatty acids in free form are usually maintained by active reacylation reactions. Indeed, there have been numerous reports on the uptake and distribution of fatty acids (1–3) and, more specifically, arachidonic acid (20:4) (1,4) in cell culture systems. It is generally concluded from these and other studies (5) that it is possible to experimentally modify the lipids of cultured cells by addition of fatty acids to the growth media. Release of these PUFA from complex lipids is largely influenced by the action of phospholipases, where, for example, phospholipase A2 hydrolysis represents the first step in the conversion of these PUFA to eicosanoids (6). Phospholipases appear to influence the uptake, distribution and release of 20:4 and related PUFA. Specific phospholipase activities towards different phospholipid species have been reported under in vitro conditions (7). Nakagawa et al. (8) demonstrated that stimulated rabbit alveolar macrophages utilize 20:4 for eicosanoid synthesis mostly from choline glycerophospholipids, leaving the other glycerophospholipids, as well as triacylglycerols, practically unaffected. Similar substrate preferences were shown in other cell systems such as platelets (9), lymphocytes (10) and fibroblasts (11). It has been proposed that different classes of phospholipids serve as sources of arachidonic acid for eicosanoid production (12). Moreover, the release of arachidonic acid can be influenced by its distribution in specific phospholipid pools.

Eicosanoids are synthesized from several 20-carbon PUFA in many cell types where they participate in numerous physiological and pathophysiological reactions (13). Although the most prominent of these fatty acids is 20:4, prostaglandins and leukotrienes are also produced from other 20-carbon n-3 and n-6 PUFA. Replacement of (20:4) by dihomogammalinolenic acid (20:3) or eicosapentaenoic acid (20:5) is known to generate prostaglandins and leukotrienes with an accordingly altered number of double bonds and, in general, these eicosanoids exert weaker or, in some cases, distinct, biological effects compared with 20:4. This suggests that the potential for adverse effects produced by 20:4 metabolism may be circumvented by appropriate substitution with the other eicosanoid precursors (14).

Chinese hamster embryonic lung fibroblasts (V79 cells) were used to investigate the uptake of added 20:4, 20:3 and 20:5 into the different lipid pools. We were specifically interested in determining whether these fatty acids were assimilated into similar or different phospholipid species because this could have a bearing on their subsequent availability for eicosanoid synthesis. V79 cells were selected due to their ease of culture and rapid growth combined with an apparent low level of eicosanoid synthesis.

METHODS

Cell culture. Chinese hamster embryonic lung fibroblasts (V79 cells) were grown in 100-mm petri dishes (Corning Glass Works, Corning, NY, NY) as described previously (15,16). The culture medium in all cases consisted of 5 ml Dulbecco's Minimum Eagles Medium, containing 10% heat-inactivated dialyzed fetal calf serum (Gibco, NY).

Logarithmically growing cell cultures (ca. 5×10^5 cells per dish) were incubated for 24 hr with one of the following radioactively labeled PUFA: [1-^{14}C] 20:4 (n-6) (1.0 μCi/μmol) or [2-^{14}C] 20:3 (n-6) (4.0 μCi/μmol), which were >98% pure, or [1-^{14}C] 20:5 (n-3) (0.55 μCi/μmol), which was 96% pure, as determined by high pressure liquid chromatography (HPLC) according to methods herein described. Radiolabeled lipids were obtained from Amersham (Arlington, IL) and nonlabeled fatty acids were purchased from NuChek Prep (Elysian, MN). Unlabeled 20:5 was found to be only 65% pure and was, therefore, purified further by Prep-HPLC using a 5-μ (9 mm × 25 cm i.d.) RP-18 column (Alltech, Inc., Los Altos, CA) eluted with 20% H_2O in acetonitrile at a flow rate of 6 ml/min.

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Abbreviations: CL, cardiolipin; FAME, fatty acid methyl ester; LPC, lysophosphatidylcholine; PA, phosphatidic acid; PBS, phosphate buffered saline; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; PUFA, polyunsaturated fatty acids; SM, sphingomyelin; GC, gas chromatography; HPLC, high performance liquid chromatography; TLC, thin layer chromatography.

The eluant was monitored by refractive index. The concentrations of fatty acids used varied depending on the experiments performed and are, thus, indicated in the Results section. All fatty acids were dissolved in ethanol and added to the culture medium prior to culture. The final concentration of ethanol was 0.5% (v/v). The cells were then harvested by washing the monolayers with cold PBS (phosphate buffered saline, pH 7.2) three times and then scraping off the plastic into 50-ml PBS. After centrifuging for 15 min at 1000 rpm, the cell pellet was recovered and total lipids extracted.

Extraction of cells. The cells (ca. 10^8 cells in 0.7-ml buffer) were extracted using a modified Bligh & Dyer method (17) by means of freeze-thawing in liquid nitrogen 3 times. The cell homogenate was subsequently mixed with 2 ml of chloroform/methanol/water (2:2:1.8, v/v) and the mixture kept for 14 hr under argon in a refrigerator. After centrifugation, the supernatant was saved and the pellet subjected to brief extraction using the same solvents. Extraction of the pellet was repeated twice and the supernatants pooled. The combined chloroform phases, containing 98–100% of the total radioactivity, were further separated by two chromatographic procedures. In the first system the neutral lipids were separated by thin layer chromatography (TLC) on 0.2-mm thick silica gel G-60 plates (EM Science, Cherry Hill, NJ) using a solvent system containing petroleum ether/diethylether/acetic acid (80:20:1, v/v) (18). Under these conditions the phospholipid fraction remained at the origin. Areas corresponding to triacylglycerols and free fatty acids were identified by comparison with authentic standards as visualized by iodine vapor. The plates were scanned for radioactivity (radio-TLC) (Berthold Instruments, Wildbad, FRG), regions of interest scraped from the plates and isolated lipids recovered by elution with chloroform/methanol (1:1, v/v). After drying, the samples were stored under argon in a −20°C freezer until further analysis. The phospholipid fraction was separated using a second TLC system (19) containing chloroform, methanol, triethylamine, isopropanol and an aqueous solution of 0.25% (w/v) potassium chloride (30:9:18:25:6, v/v). Individual phospholipid species were identified using a standard mixture containing lysophosphatidylcholine (LPC) (0.07), phosphatidylcholine (PC) (0.17), phosphatidylglycerol (PG) (0.47), cardiolipin (CL) (0.56), phosphatidylserine (PS) (0.29), phosphatidylinositol (PI) (0.40), phosphatidylethanolamine (PE) (0.43), phosphatidic acid (PA) (0.77) and sphingomyelin (SM) (0.12) (Rf-values in parenthesis), which were applied to each plate.

The authentic standards, which were developed along the margins of the plates were charred (19,20) in order to identify the location of each phospholipid. The margins were broken away prior to charring and then rejoined to locate the isolated phospholipid. The recovered phospholipid and triacylglycerol fractions were then subjected to transesterification by heating in 1 ml 4 N HCl/methanol at 90°C for 1 hr under argon. The resulting fatty acid methyl esters (FAME) were extracted with 4 ml hexane. The methanol phases were analyzed for phosphorous content according to Bartlett (21), whereas the hexane phases were evaporated and the residues prepared for gas chromatography (GC) by dissolving them in 25 μl acetonitrile, containing 0.01% BHT and 25 μg methylheptadecanoate (GC-standard). This solvent also was used to dissolve the free fatty acid fractions that were methylated using ethereal diazomethane (22).

The FAME composition was determined by GC using a 30 m × 0.53 mm i.d.DB-225 capillary column. Chromatographic conditions were as follows: helium carrier gas at 5.0 ml/min, initial column temperature at 185°C (using on-column injection), temperature gradient of 2°C/min beginning at 8 min postinjection, 220°C final temperature held for 12 min, detector temperature of 250°C. Authentic fatty acid methyl ester standards were obtained from NuChek Prep (Elysian, MN) and used to identify the fatty acids in samples. Chromatography was performed with a Hewlett-Packard 5730A gas chromatograph interfaced to a Perkin-Elmer 3600 chromatographics data station. The FAME were quantified according to their peak areas and individual FAME were calculated as the percentage of total FAME identified in a given sample.

FAME derived from all samples were also analyzed by HPLC using a Perkin-Elmer Liquid Series 4 Chromatograph, connected in series to a liquid scintillation flow-detector (radio-HPLC) (Ramona-LS (IN/US, Fairfield, NY). The radiochromatogram was displayed and analyzed by computer utilizing integrated chromatographics software. UV absorbance at 205 nm was simultaneously recorded. A spherisorb ODS-2, 3-μ (150 mm × 4.6 mm i.d.) column (Chromanetics Corp., Jessup, MD) was eluted with acetonitrile: water (65:35, v/v) at a flow rate of 1.0 ml/min. After 12 min, the composition of the mobile phase was raised to 90% acetonitrile linearly over a 4-min interval and then maintained as such for 15 min. Thereafter, the mobile phase was returned to its original composition over a 4-min interval, thus ending the analytical run.

Data are reported as the mean and standard deviation calculated from 3–5 independent analyses. Statistical analysis, where applicable, was performed using the Students t-test.

RESULTS AND DISCUSSION

The analysis of the various lipid fractions isolated from V79 cells after 24 hr of culture revealed that most of the fatty acids (70%) were present in phospholipids, whereas the free fatty acid and triacylglycerol fractions each contained ca. 15% of the total fatty acids. The high level of free fatty acids, along with high levels of LPC, appear to be due to freeze thawing of samples during the extraction of lipids from cells. When cells were extracted without freeze-thawing, the levels of free fatty acids were reduced to <7%, whereas LPC levels were lowered to 3%. This suggests that the amounts of radiolabel present in these lipid fractions are anomalously high and, as presented in Tables 1 and Figures 1 and 2, likely do not represent incorporation into these lipid pools accurately. The determination of individual FAME derived from phospholipids (Table 1) showed that palmitate (16:0), stearate (18:0) and oleate (18:1) were the predominant fatty acids. 18:1 was the major fatty acid in the free fatty acid fraction (48.8%), whereas 16:0 was predominant in the triacylglycerol fraction (30.6%). PC amounted to 40% of the total isolated phospholipids; PE made up 23%, whereas the contribution of all other phospholipids was <10%. The major fatty acid of the phospholipids was 18:1 (43.9% of total FAME). Considerable amounts of 16:0 (10.6%), 18:0 (13%) and 20:4 (5.5%) were present, whereas other identified fatty acids were <2% each (Table 1).

TABLE 1

Fatty Acid Composition of Cell Phospholipids

	% of total FAME[a]	% composition[b]							
		PC	PE	PG	PI	PS	LPC	CL	SM
14:0	0.2	0.1	0.1	0	0	0	0	0.1	0
16:0	10.6	1.0	2.2	1.3	1.4	0	1.7	1.0	2.1
16:1	2.8	0.7	0.6	0.3	0.4	0.2	0.3	0.2	0.2
18:0	13.0	6.6	0.1	1.9	1.3	1.6	0.7	0.6	0.3
18:1	43.9	29.2	8.5	2.2	1.0	0.7	0.8	1.0	0.4
18:2	1.7	0.3	0.8	0	0.5	0	0	0.1	0
18:3	0.8	0	0.1	0.2	0.2	0.2	0.1	0	0
20:0	3.0	0.2	0.3	0.5	0.2	1.6	0.2	0.1	0.1
20:1	0.8	0.5	0.2	0	0	0	0	0	0.1
20:2	0.9	0.2	0.1	0	0.7	0	0	0	0
20:3 ω-6	0.3	0.2	0.1	0	0	0	0	0	0
20:3 ω-3	0.9	0	0	0	0.9	0	0	0	0
20:4	5.5	1.5	1.4	1.3	0.5	0.2	0.4	0.1	0.2
20:5	0	0	0	0	0	0	0	0	0
22:0	4.4	0.4	1.3	0.2	0.3	0.3	1.5	0.2	0.3
22:1	1.1	0.1	0.2	0.2	0.3	0	0.2	0	0
22:6	3.3	0.6	1.9	0.5	0.2	0	0	0	0
24:0	2.8	0.5	0.7	0.4	0.1	0.2	0.1	0.2	0.5
Total		41.8	18.5	9.0	8.0	5.0	5.3	3.6	4.2

[a] Percentage composition of fatty acids in total phospholipids as determined by GC.

[b] The values shown are the percentage of total fatty acids found in all phospholipid species. Percentages less than 0.1 are shown as 0. Values are the mean of 3 determinations with variations not greater than 10% of the mean for any component shown.

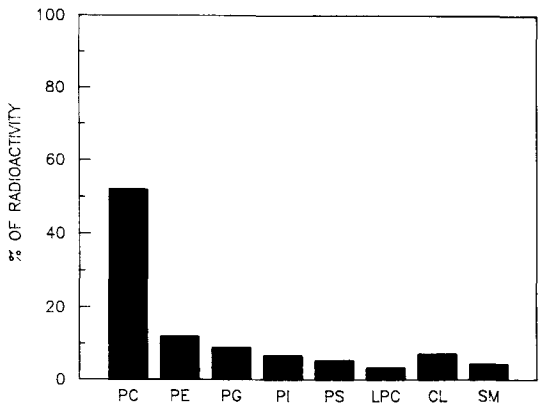

FIG. 1. The uptake and distribution of 10 μM [^{14}C]-20:4 after 24 hr incubation with V79 cells is shown. The incorporation of labeled fatty acid into individual phospholipids is presented as percentage of radioactivity incorporated into total phospholipids.

After 24 hr of culture in presence of 10 μM additions of [2-^{14}C] 20:3, [1-^{14}C] 20:4 or [1-^{14}C] 20:5, most of the radioactivity added to the medium was incorporated into the cells. Uptake of the three labeled fatty acids by V79 cells is shown in Figures 3, 4 and 5. Each fatty acid was incorporated in a biphasic manner over the 24-hr incubation period. A rapid initial phase of uptake occurred during the first 3-5 hr, followed by a more gradual uptake for the remaining incubation period. The initial uptake of fatty acids appeared to be independent of cell division,

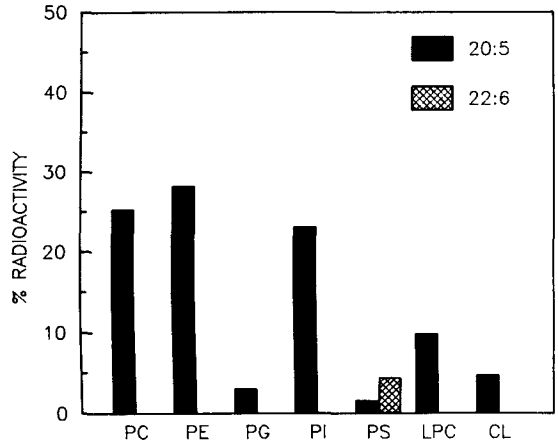

FIG. 2. The uptake and distribution of 10 μM [^{14}C]-20:5 after 24 hr incubation with V79 cells. Data are presented in a manner identical to Figures 1 and 6. Also shown is the extent of radioactivity associated with 22:6, determined by radio-HPLC analysis of the fatty acid methyl esters derived from each phospholipid species as described in the text.

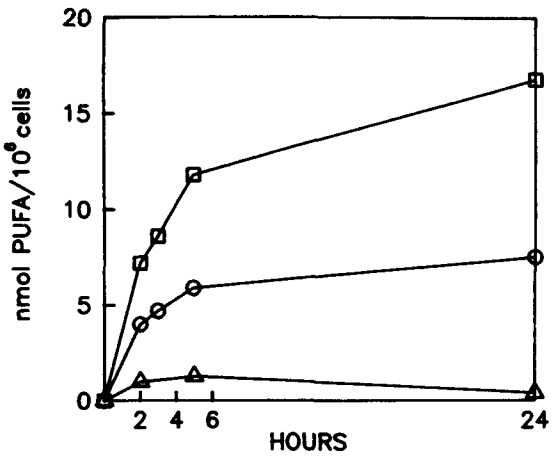

FIG. 3. The uptake of [^{14}C]-20:3 over 24 hr of incubation. The values shown at each time point present the total amount of radioactive fatty acid associated with isolated cells. The extent of incorporation was determined in the presence of 1 μM (△), 5 μM (○) and 10 μ (□) additions of each fatty acid to the culture medium.

as cell numbers were not significantly different during the first 6 hr of incubation. The second phase of uptake was associated with cell division and cell numbers had more than doubled by 24 hr. The uptake of each fatty acid was concentration dependent, where doubling the concentration in the medium approximately doubled the amount of fatty acids assimilated. Both 20:3 and 20:4 were incorporated to nearly equal extents, whereas about half as much 20:5 was incorporated under similar incubation conditions. Radio-TLC measurements showed that only minor amounts (~5%) of the total cellular radioactivity was present in the free fatty acid fraction, whereas negligible amounts of radioactivity (<1%) were detected in the triacylglycerol fraction at 24 hr. The major portion of the extracted radioactivity (~95%) was present in the phospholipid fraction.

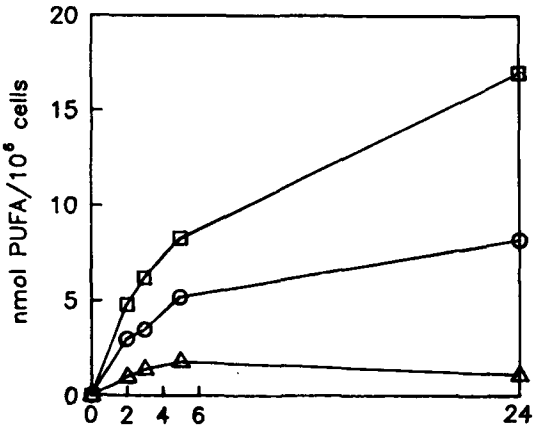

FIG. 4. The uptake of [^{14}C]-20:4 over 24 hr of incubation. See Figure 3 for details.

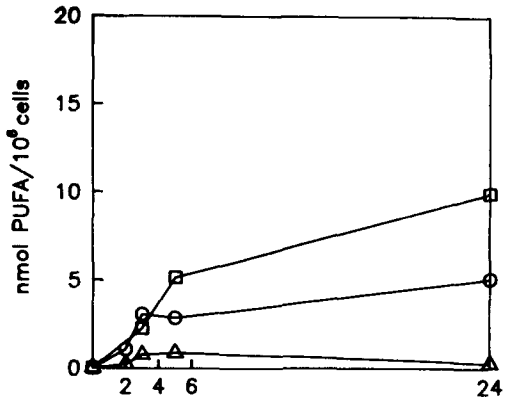

FIG. 5. The uptake of [^{14}C]-20:5 over 24 hr of incubation. See Figure 3 for details.

TABLE 2

Major Fatty Acid Composition in Cells Grown in the Presence of the Indicated Polyunsaturated Fatty Acids

	% composition[a]			
	20:3 ω-6	20:5	20:4	None[b]
16:0	19.3	7.1	17.5	10.6
18:0	18.8	21.9	19.1	13.0
18:1	25.3	33.3	33.8	43.9
20:3 ω-6	3.9	0.7	0.9	0.3
20:4	5.1	5.2	6.9	5.5
20:5	0	3.7	0	0

[a]Fatty acid methyl ester composition of total lipid extracts from V79 cells incubated for 24 hr in the presence of 10 μM of the fatty acids noted. Values are the mean of 3 independent analyses with maximum variation being no greater than 10% of the mean.

[b]Fatty acids methyl ester composition of cells grown under standard conditions in complete medium only. Values shown are the mean of 3 independent analyses with maximum variation being no greater than 10% of the mean.

Table 2 summarizes the data for incorporated radioactive fatty acids after 24 hr. The distribution and proportions of the major fatty acids 16:0, 18:0 and 18:1 were altered after addition of 20:3, 20:4 and 20:5. The most pronounced changes occurred with 18:1 where addition of 20:3 lowered the proportion of 18:1 by ~40%. The changes in saturated fatty acids and 18:1 were least evident after incubation with 20:5. It is conceivable that these responses represent adjustments of lipid composition because PUFA are known to affect membrane fluidity (23). One possible stage of regulation may occur through elongation and desaturation reactions that can be influenced by incorporation of PUFA into cultured cells (24). Increases in saturated fatty acids on addition of PUFA may compensate for the elevated assimilation of unsaturated fatty acids and result from decreased desaturase activity (25).

After cultivation of cells for 24 hr in the presence of 10 μM [^{14}C]-20:3, the levels of this fatty acid increased from 0.3 to 3.9% of total FAME. Cultivation in the presence of 10 μM [^{14}C] 20:5 increased 20:5 content from 0 to 3.7%. The percentage of 20:4 was not significantly altered after addition of 10 μM [^{14}C] 20:4, i.e., it remained in the range of 5-7% of total FAME. The small increase in the proportion of 20:4 upon supplementing V79 cells with this fatty acid may be misleading when considered only in terms of percentage of total FAME composition rather than in terms of absolute amounts of lipid. Considerable amounts of [^{14}C]-20:4 were incorporated into the various phospholipids—with marked uptake into PC. However, these data do not indicate whether preexisting pools of 20:4 were redistributed during assimilation of added 20:4 and preferential uptake into PC (which would serve as a large phospholipid pool). This effect, combined with redistribution of 20:4 from other minor phospholipids, could permit substantial amount of 20:4 to be assimilated with little effect on the overall proportions of 20:4.

It should be noted that the lipid composition of these cells is probably determined in large measure by the serum lipid composition of the culture medium. Therefore, the fatty acid composition of the culture medium was analyzed in order to establish the inherent levels of the PUFA that were under study. GC measurements of derived FAME indicated that 20:4 concentrations were 2.5 μM. Accordingly, the final concentration of 20:4 in supplemental cultures was 12.5 μM. 20:5 and 20:3 concentrations were less than 0.05 μM, suggesting that supplementing the medium with 10 μM concentrations of these PUFA represented marked elevations over standard culture conditions.

Radio-HPLC analysis of the FAME isolated from cellular phospholipids indicated that most of the incorporated [^{14}C]-20:4 was found in the PC fraction, whereas all of the other phospholipids showed markedly less uptake (Fig. 1). Figure 6 describes the distribution of [^{14}C]-20:3 among cell phospholipids. It can be seen that [^{14}C]-20:3 was present mainly in PE and PG. Substantial amounts of 20:3 were converted to 20:4. Conversion of 20:3 to 20:4 was most extensive in PG and least in PS. No further metabolic products of 20:3 were detected (Fig. 7A). Figure 2 shows that 20:5 was incorporated mainly into PC, PE and PI. Conversion of this fatty acid to 22:6 occurred to a some extent where considerable

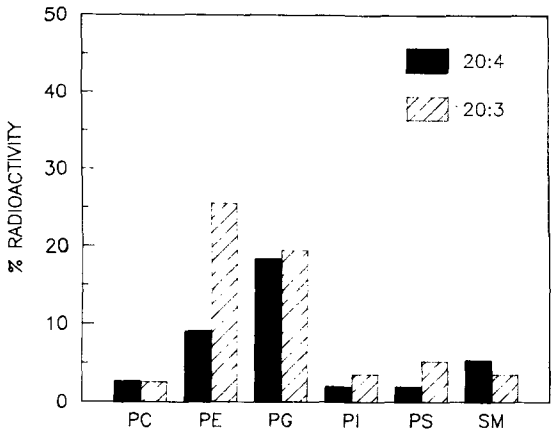

FIG. 6. The uptake and distribution of 10 μM [^{14}C]-20:3 after 24 hr incubation with V79 cells is shown. Data are presented in a manner identical to that described for Figure 1. Also shown is the extent of radioactivity associated with 20:4, determined by radio-HPLC analysis of the fatty acid methyl esters derived from each phospholipid species as described in the text (also, see Fig. 7 for an example).

amounts of [1-^{14}C]-22:6 were associated with PS. The amount of 22:6 formed represented ~2.5% of the total 20:5 incorporated into phospholipids. Included in Figure 7 are sample chromatograms for 20:3 uptake into PE (Fig. 7A), 20:4 uptake into PC (Fig. 7B) and 20:5 uptake into PS (Fig. 7C).

These findings show that V79 cells contain low levels of PUFA under standard culture conditions, however, there is a marked capacity to assimilate these fatty acids on supplementation of the culture medium. After 24 hr, most of these fatty acids were incorporated into phospholipids, but because specific pools were not examined in detail at time intervals less than 24 hr, we cannot ascertain into which lipid pools the fatty acids may have entered during earlier periods of incorporation. The uptake of 20:3 and 20:4 into cell phospholipids was quite similar although the pattern of uptake into specific phospholipid species differed in several respects. This might suggest that 20:3 and 20:4 may use similar binding sites and uptake mechanisms, whereas a distinct pathway may exist for 20:5. However, PC was the major site for 20:4 incorporation, whereas 20:3 was incorporated primarily into PE and PG, and 20:5 accumulated largely into PC, PE and PI. Consequently, the assimilation of different PUFA may be occurring by separate uptake mechanisms in these cells. This suggests that the uptakes of these fatty acids are independent of each other and that each has a preferred incorporation into specific phospholipid pools. The differential uptake of these PUFA into phospholipids may also depend on the degree of unsaturation of the phospholipid pool into which the fatty acids are incorporated. PC, for example, is the most saturated phospholipid (i.e., saturated and monoenoic fatty acids make up >90% of all fatty acids) and 20:4 is incorporated mostly into PC. 20:3 is incorporated largely into PE and PG and 20:5 into PE and PI, all of which are considerably more unsaturated than PC.

It is also interesting to note that these fatty acids are subject to different degrees of metabolic conversion. There was little evidence of 20:4 metabolism, where more than 90% of the added 20:4 was recovered as 20:4 (Fig. 7B). As shown in Figure 7B, there was a small radioactive peak eluting after 20:4, which indicates that some metabolic conversion had taken place. This was seen

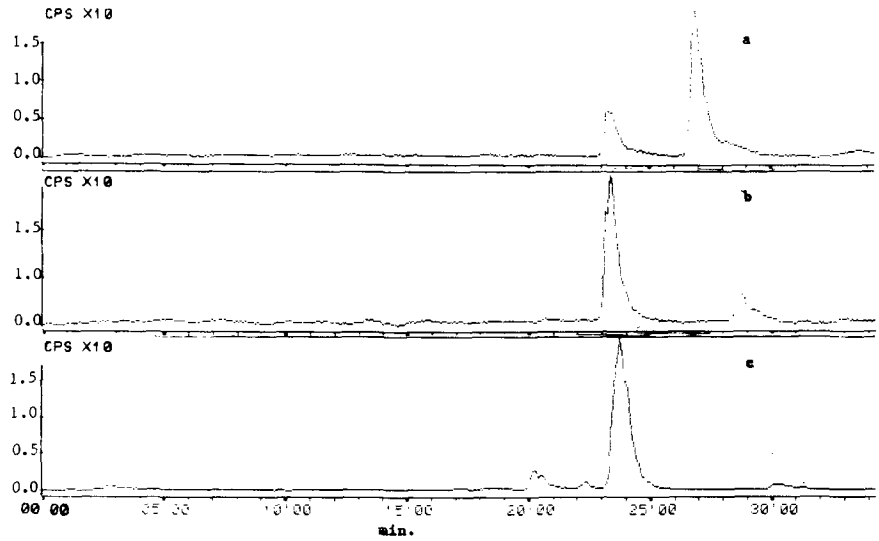

FIG. 7. Radio-HPLC chromatograms characterizing labeled fatty acids isolated from cell phospholipids after 24 hr incubation with 10 μM additions of [^{14}C]-20:3 (A), [^{14}C]-20:4 (B) and [^{14}C]-20:5 (C). A representative chromatogram of radioactivity associated with FAME derived from PE is shown in A. Peaks correspond to authentic standards of 20:4 (23.1 min) and 20:3 (26.5 min). A representative chromatogram of radioactivity associated with FAME derived from PC is shown in B. Peaks correspond to authentic standard of 20:4 (23.1 min) and an unknown component (28.7 min). A representative chromatogram of radioactivity associated with FAME recovered from PS is shown in C. Peaks correspond to authentic standards of 20:5 (20.2 min) and 22:6 (23.8 min).

only in PC and the identity of this product is presently unknown. 20:5 was metabolized to a minor extent where the product was identified as 22:6. On the other hand, 20:3 was extensively converted to 20:4, indicating that these cells possess Δ5-desaturase activity. The metabolic conversions of PUFA have been described in a similar cell line (CHO cells), which also possessed Δ5-desaturase, but lacked Δ-6 desaturase (24). The proportions of 20:3 and derived 20:4 were similar in virtually all the phospholipid species after 24 hr (with the exception of PE), despite the finding that the fatty acids were incorporated differently into the various phospholipid species (Fig. 6). The rather facile conversion of 20:3 to 20:4 may, in part, account for the comparable uptake of these fatty acids into cell lipids and, accordingly, it should be possible to enrich different phospholipid pools with 20:4 by incubating cells with 20:3 instead of 20:4.

These studies indicate that interaction of ω-3 and ω-6 may not be direct because these fatty acids appear to be incorporated into different phospholipid pools. The noted inhibitory effects of 20:5 on 20:4 metabolism and eicosanoid synthesis in some cases, as well as conversion of the former to less active eicosanoid products (2,26,27), suggests that these fatty acids are interacting via common pools. This may also be the case in V79 cells, in so far as PC represents a common depot for 20:4 and 20:5. It may thereby be possible to influence the release and availability of 20:4 by additions of 20:5, thus altering the production of eicosanoids. These findings can be viewed in the light of other reports comparing the uptake of these three fatty acids. For example, Punnonen et al. (28) described the uptake of 20:3, 20:4 and 20:5 into human keratinocytes. In these cells, incorporation into phospholipids was predominant and the extent of uptake was of the order: 20:3 ∼ 20:4 > 20:5. Similar findings have been described by others (29). Nevertheless, there are some clear differences in terms of which phospholipid species incorporated these fatty acids. The most notable difference occurred with 20:4, where Punnonen et al. (28) described uptake primarily into PE along with considerable incorporation into triacylglycerols. Because PE is the major phospholipid in keratinocytes (30) and PC is the major phospholipid in V79 cells, the extent of 20:4 uptake may reflect the pool size of these phospholipids. Other differences in the uptake and distribution of PUFA can be found in the literature, particularly with respect to studies with platelets, fibroblasts and endothelial cells (9,10,29,31). These discrepancies may be due to differences in phospholipid composition or to the utilization of PUFA in events such as eicosanoid synthesis. Differences in uptake and distribution may also be accounted for by the concentrations of fatty acids in the medium. Others have reported that supplementations with high concentrations of fatty acids (>10 μM) increase the proportionate uptake into triacylglycerols and change the distribution of fatty acids among phospholipid species (10-12). This was also found in V79 cells, where concentrations of >10 μM 20:4 resulted in a greater proportion of the fatty acid being incorporated into triacylglycerols (data not shown). These findings suggest that care must be taken during treatment of cells with specific fatty acids, when attempts are made to enrich specific lipid species. Another parameter influencing uptake, subsequent conversion and utilization of PUFA is the phospholipid species into which the fatty acids are incorporated. Accordingly, release of specific fatty acids (e.g., for the purpose of prostaglandin synthesis) could involve different phospholipid pools and, thus, be separately influenced.

ACKNOWLEDGMENTS

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Effects of Fish Oil, Corn Oil and Lard Diets on Lipid Peroxidation Status and Glutathione Peroxidase Activities in Rat Heart[1]

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In this study, we investigated the effect of various types of fats on heart lipid peroxidation status and on blood lipid parameters. Rats were fed either a low-fat diet (2.2% lard plus 2.2% corn oil), a corn oil diet (17%), a salmon oil diet (12.5%) supplemented with 4.5% corn oil, or a lard diet (15%) supplemented with 2% corn oil. All diets were supplemented with 1% cholesterol. Rats were fed for eight weeks. When compared with the low-fat diet, the salmon oil-diet intake resulted in a lower blood cholesterol, triglyceride and phospholipid concentrations (−50, −56 and −30%, respectively). Corn oil only tended to lower blood lipids; this decrease was significant for triglycerides only (−40%). The hypocholesterolemic effect of salmon oil diet is even more pronounced, if blood cholesterol values are compared with those of rats fed the lard diet. Heart lipid composition was not affected by dietary manipulations. Fatty acid composition of cardiac phosphatidylcholines and phosphatidylethanolamines, however, were altered by high-fat diets. In phosphatidylcholine, salmon oil induced a twelvefold decrease in the n-6/n-3 ratio and a 26% increase in the unsaturation index. For phosphatidylethanolamine, the n-6/n-3 ratio decreased 7.7-fold and the unsaturation index increased by 13%. A 50% decrease of the n-6/n-3 ratio was observed in animals fed the lard diet. Ultramicroscopic examination of ventricles revealed that those of the salmon oil group significantly accumulated lipofuscin-like or ceroid material, whereas this accumulation was barely detectable in hearts of the other groups. Selenium-dependent glutathione peroxidase activity tended to be the highest in hearts of rats fed the salmon oil diet; this increase is significant (+36% and +54% for total and specific activities, respectively), if values are compared with those of the rats fed the lard diet. Liver glutathione peroxidase and heart glutathione S-transferases activities remained unchanged. These results indicate that fish oil did not lower the selenium involved in glutathione peroxidase activity. This rules out that a deficiency in this enzyme was at the origin of heart lipofuscinosis. Also, it is concluded that the n-6/n-3 ratio of the diet is likely more determinant in the alteration of heart lipid peroxidation status than is the polyunsaturated/saturated ratio.
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Fish consumption is considered as a promising nutritional approach to preventing cardiovascular diseases (1,2). Although fish oils are also expected to give the same effects (3–5), they, however, may induce an increase in sensitivity to membrane peroxidation because of their very high unsaturation degree. Indeed, n-3 polyunsaturated fatty acids (PUFA) comprising fish oils are efficiently incorporated into membrane phospholipids of various organs (6). Among these, the heart has been shown to respond to the dietary n-3 PUFA supplied by fish oils (7–12). This appears largely attributable to cardiomyocytes (13,14). As these n-3 PUFA (20:5 n-3, 22:5 n-3, 22:6 n-3) are oxidatively unstable, a higher sensitivity to membrane peroxidation can be expected in animals fed highly polyunsaturated oils (15–17). Consequently, a higher requirement of vitamin E may be needed (16–18). This could explain why the serum concentration of vitamin E in animals fed fish oils was always less than that in animals fed standard diets or polyunsaturated fats other than fish oils (12,19), although the dietary supply of the vitamin was largely adequate. This subnormal circulating vitamin E level was supposedly responsible for the mild, but significant, accumulation of lipofuscin-like or ceroid material observed in hearts of rats fed fish oil (12,20). This was not the case in hearts of pigs fed mackerel oils (21,22), although lipofuscin accumulated in their livers (21). It is possible that the lower amount of fish oil used or the species of animals tested may account for the differences observed in hearts.

Another component of the defense system against lipid peroxidation is the selenium-dependent glutathione peroxidase (GSH-PX; E.C. 1.11.1.9) (23) that has been reported in various tissues (20). This enzyme reduces hydroperoxides of PUFA, provided they are first released by intracellular phospholipase A_2 (24).

The increase in membrane unsaturation induced by fish-oil intake may lead to a higher requirement of GSH-PX and, consequently, of selenium. If the selenium status is subnormal or near the limit recommended, enzyme activity may be altered, thus resulting in an increase in the peroxidation level. The selenium metabolism is the object of intense studies (25). It is well established that a decrease in GSH-PX activity reflects a selenium deficiency (26,27) and that repletion of the element increases GSH-PX activity (27–29). However, the mechanism of enzyme regulation during repletion appears complex. A recent study (29) has shown that the chemical form of the element, as fed, did not affect enzyme activity, although in tissue cytosol the estimated percentage of selenium associated with the enzyme changed. Also, it has recently been demonstrated (28) that during selenium repletion the rate of increase in rat liver GSH-PX protein was significantly higher than that of its activity. From these results the authors (28) deduced that an active and an inactive form of GSH-PX coexisted. As the increase in activity is hyperbolic, the same authors concluded that the level

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Abbreviations: ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase; FAME, fatty acid methyl ester; GSH-PX, selenium-dependent glutathione peroxidase; GST, glutathione S-transferase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PL, phospholipid; PUFA, polyunsaturated fatty acid; TG, triglyceride; ANOVA, analysis of variance; GLC, gas liquid chromatography; HPLC, high performance liquid chromatography; P/S, polyunsaturated/saturated.

of total selenium is not the primary factor regulating enzyme activity in selenium-adequate animals.

Thus, our purpose was to study the effect of fish oil on the heart selenium status, considering this element as an active component of GSH-PX. To this end, we compared the effects of a salmon oil diet with those of a corn oil, a saturated fat and a low-fat diet. Heart lipid status was studied by investigating lipid composition, GSH-PX and glutathione S-transferase (GST, E.C. 2.5.1.18) activities and ultramicroscopic aspects of myocardium. Some plasma parameters were also analyzed to assess the effectiveness of fish oil in lowering a diet-induced rise in plasma cholesterol and to evaluate cell injury indicated by enzyme release.

MATERIALS AND METHODS

Animals and feeding procedures. Four groups of 6 male Wistar rats (IFFA-CREDO, L'Arbresle, France), weighing 190–210 g, were used. One group was fed a low-fat diet (4.4%, w/w) consisting of an equal amount of lard and corn oil with a polyunsaturated/saturated (P/S) ratio of 1.2. A second group received a corn oil diet (17%, w/w) with a P/S ratio of 5. The third group received a salmon oil diet (12.5%, w/w), supplemented with 4.5% corn oil and a P/S ratio of 1.9, and the last group was fed a 15% lard diet supplemented with 2% corn oil and with a P/S ratio of 0.4. Table 1 presents the composition of the diets. All the diets were supplemented with 1% cholesterol (w/w) and contained ca. 60 μg/kg of selenium. Corn oil contained 45 mg of α-tocopherol per 100 g of oil. Salmon oil was supplemented with 20 mg of α-tocopherol and 20 mg of α-tocopheryl acetate per 100 g of oil. Lard contained 3 mg/100 g of α-tocopherol. Thus, the control diet supplied a total of 171 mg/kg of tocopherol and the lard, corn oil and salmon oil diets supplied 175, 246 and 232 mg/kg, respectively. Peroxide values of corn oil and salmon oil were zero and 3.8, respectively. In the middle of the experimentation period, the daily intake of 20:5n-3 was estimated at 1 g/kg body weight. The low-fat, corn oil and lard diets were prepared to last one month and stored at −20°C in plastic bags. The salmon oil diet was prepared every two weeks and stored at −20°C in sealed containers flushed with nitrogen. The rats were fed for 8 weeks ad libitum, and uneaten food was discarded in the morning. They had free access to tap water.

TABLE 1

Composition of the Diets (g/100 g)

	Low-fat	Corn oil	Salmon oil	Lard
Casein[a]	27.4	27.4	27.4	27.4
Lard[a]	2.2	—	—	15.0
Corn oil[b]	2.2	17.0	4.5	2.0
Salmon oil[c]	—	—	12.5	—
Starch[a]	33.5	26.4	26.4	26.4
Glucose[a]	23.2	17.7	17.7	17.7
Minerals[a]	5.0	5.0	5.0	5.0
Vitamins[a]	1.0	1.0	1.0	1.0
Cellulose[a]	4.5	4.5	4.5	4.5
Cholesterol[a]	1.0	1.0	1.0	1.0
n-6/n-3	46.0	71.0	0.8	22.0
UI	107	150	184	77

[a]From Unite Alimentation Rationelle (U.A.R.), Villemoisson (France).

[b]From CPC Europe Consumer Products, Heilbronn (FRG).

[c]From Unit Specific Aliment, St Waast-La-Vallée (France).

Detailed information on mineral, vitamin and fatty acid composition of the diets are given elsewhere (12,30). On the basis of the information given by U.A.R., the selenium content was estimated at 60 μg/kg.

UI, Unsaturation Index.

Tissue treatment and blood sampling. At the end of the feeding period, the rats were weighed and guillotined, in the morning and at the same time for each group. Blood was collected in lithium heparin-containing tubes. The heart was quickly excised and a biopsy of ventricular tissue was taken immediately and immersed in a 2.5% solution of glutaraldehyde, with further treatment by ultramicroscopy performed as previously described (12). Hearts were thoroughly rinsed at 4°C in a Tris-HCl buffer 20 mM (pH 7.5) containing 0.22 M mannitol and 70 mM sucrose. Tissue homogenization and lipid extraction were done as previously described (30). All lipid extracts were kept at −20°C under nitrogen until analysis. Livers were excised and homogenized with a Potter-Elvehjem in a buffer consisting of 50 mM KH_2PO_4, pH 7.4, 30 mM EDTA, 70 mM KCl, 1 mM dithiothreitol and 0.1 M sucrose.

Blood was centrifuged at 2,000 *g* for 20 min at 4°C and the plasma was collected for immediate analysis. The biopsies were observed with a Philips EM 300 microscope (Endoven, The Netherlands) at 80 kV. For each heart (2 per group), the frequency of lipid droplets or lipofuscin material was expressed as the ratio of the number of structures/number of unoverlapped fields examined (30 μm^2 each). The total number of fields examined (four grids issued from two hearts) was 214, 197, 184, 233 for low-fat, corn oil, salmon oil and lard diets, respectively. The person who did the observations was not aware of the nature of the samples.

Lipid analysis, plasma parameters and enzyme assays. Choline- and ethanolamine-containing phospholipids (PL) were separated by high performance liquid chromatography (HPLC) using a Waters-Associates liquid chromatograph system (Milford, MA) as previously described (31). Separated phospholipids were quantified by phosphorus determination (32). Fatty acid methyl esters (FAME) of PC and PE were prepared according to a rapid and convenient method used for vegetable oil triglycerides (TG) (33). FAME were separated as previously described (12, 30) using gas liquid chromatography (GLC) (Girdel 3000, Paris, France) equipped with a peak integrator (Girdel Co., Enica 10, Delsi, Suresne, France) and a 50-m capillary column (Spirawax FS 1493, Spiral, Dijon, France).

The TG concentrations of plasma and heart lipid extracts were determined by using the Technicon-Smac analyzer (Technicon, Tarry-Town, NY) according to the method of Bucolo and David (34). For heart-TG measurement, a sample of the heart lipid extract was evaporated to dryness under nitrogen and then dispersed by sonication in 20 mM Tris-HCl buffer, pH 8.0. Cholesterol concentration in the heart was evaluated from the same aqueous lipid dispersion with a cholesterol esterase–cholesterol oxidase kit (Boerhinger Mannheim, Mannheim, FRG) using a two-point kinetic method. Measurements were made with an automatic Multistat III (Instrumentation

Laboratory, Lexington, MA) at 30°C. Plasma cholesterol was determined with the Technicon-Smac analyzer according to the method of Lie et al. (35). Plasma PL were assayed with the Biolyon Kit (Ref. 44711, Wako Chemicals, Neuss, FRG) using the Technicon RA 1000.

Plasma alanine aminotransferase (ALAT) (E.C. 2.6.1.2) and aspartate aminotransferase (ASAT) (E.C. 2.6.1.1) were assayed according to the method of Kessler et al. (36), using the Technicon SMAC analyzer system. Alkaline phosphatase (E.C. 3.1.3.1) was assayed according to the method of Morgenstern et al. (37). Heart (100 μg protein) and liver (20 μg protein) GSH-PX activity (E.C. 1.11.1.9) was assayed, as described by Levander et al. (38), by using t-butylhydroperoxide to initiate the reaction. Heart glutathione S-transferase (E.C. 2.5.1.18) activity (100 μg protein) was assayed according to Habig et al. (39) by using 1-chloro-2,4-dinitrobenzene as substrate. Kinetics were recorded with an automatic DU 40 spectrophotometer (Beckman Instruments, Palo Alto, CA).

Chemicals and statistical analysis. FAME standards were from Interchim (Paris, France). Solvents were of HPLC grade and were filtered through a 0.2-μm Millipore filter before use. Results presented in the tables are means ± SD of 6 animals. Statistical significance of mean differences between dietary groups was investigated by analysis of variance (ANOVA) and by the Sheffé multiple comparison method at $p < 0.05$ or $p < 0.01$.

RESULTS

Over the 8-week feeding period, no significant difference ($p > 0.05$) was observed in body weight gains (in g): 162.0 ± 32.7, 185.0 ± 6.8, 178.0 ± 34.8 and 207.0 ± 34.8 for rats fed low-fat, corn oil, salmon oil and lard diets, respectively. Heart weights were not significantly different (in g): 0.80 ± 0.04, 0.84 ± 0.05, 0.91 ± 0.11 and 0.87 ± 0.07 in rats fed low fat, corn oil, salmon oil and lard diets respectively. Table 2 shows some of the relevant blood parameters. Total protein concentration was not affected by the diets ($p > 0.05$). Alkaline phosphatase, ALAT and ASAT activities remained unchanged ($p > 0.05$). The most striking effect of diets on plasma concerned lipid composition. Compared with the low-fat diet, salmon oil-diet intake resulted in a reduction by a factor of about 2 ($p < 0.01$) of both esterified and free cholesterol; with corn oil, this effect was much less pronounced and not significant. Salmon oil- and corn oil-diet intake led to a significant lower serum-TG concentration (−55% and −38%, respectively). The hypocholesterolemic property of salmon oil is even more pronounced, when comparing plasma cholesterol values of this group with those of the lard-diet group. The same observation was made for plasma-PL concentration.

Heart composition is shown in Table 3. Protein, total PL, cholesterol and TG concentrations were not

TABLE 2

Blood Parameters of Rats Fed Various Diets

	Low-fat	Corn oil	Salmon oil	Lard
Proteins (g/L)	73.2 ± 2.5	72.6 ± 3.8	76.3 ± 6.7	77.6 ± 8.3
Alk. Phos. (UI/L)	141.0 ± 47.0	137.0 ± 22.1	168.0 ± 25.2	145.0 ± 38.3
ALAT (UI/L)	47.3 ± 6.5	74.0 ± 26.0	60.5 ± 9.0	62.0 ± 22.1
ASAT (UI/L)	164.0 ± 25.3	190.0 ± 31.2	183.0 ± 16.5	161.0 ± 43.2
Cholesterol (total) mM	2.8 ± 0.4^{a,c,*}	2.3 ± 0.5^{c,b,*}	1.4 ± 0.3^{b,**}	3.6 ± 1.2^{a,**}
Cholesteryl ester mM	2.4 ± 0.4^{a,c,*}	1.9 ± 0.5^{a,b,*}	1.2 ± 0.3^{b,*}	3.1 ± 1.1^{a,**}
Cholesterol (free) mM	0.4 ± 0.1^{a,c*}	0.4 ± 0.1^{a,c,*}	0.2 ± 0.03^{a,*}	0.5 ± 0.2^{b,c,*}
Phospholipids mM	1.3 ± 0.5^{a,c,*}	1.4 ± 0.4^{a,c,*}	0.9 ± 0.1^{c,*}	1.8 ± 0.4^{a,*}
Triglycerides mM	1.6 ± 0.3^{c,*}	1.0 ± 0.3^{a,b,*}	0.7 ± 0.2^{a,*}	1.4 ± 0.4^{b,c,*}

Values not bearing the same superscript letter are different at $p < 0.05$ (*) or $p < 0.01$ (**). Values are means ± SD ($n = 6$).

Alk. Phos., alkaline phosphatase; ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase.

TABLE 3

Composition of Hearts of Rats Fed Various Diets

	Low-fat	Corn oil	Salmon oil	Lard
Protein mg/g	123.5 ± 7.9	122.8 ± 4.8	128.4 ± 23.7	135.1 ± 12.7
Phospholipids μmol/g	32.8 ± 1.7	33.8 ± 1.7	34.9 ± 2.0	33.0 ± 1.8
Cholesterol μmol/g	3.5 ± 0.3	3.7 ± 0.3	3.3 ± 0.3	3.4 ± 0.2
Triglycerides μmol/g	3.2 ± 0.4	4.3 ± 1.3	4.3 ± 0.9	4.2 ± 0.9
PC %	50.7 ± 0.7	50.6 ± 0.9	53.5 ± 4.6	50.4 ± 2.4
PE %	42.3 ± 1.1	42.3 ± 0.9	38.6 ± 4.1	42.7 ± 3.1
SPH %	3.2 ± 0.4	3.6 ± 0.3	3.8 ± 1.8	3.0 ± 0.5
LysoPC %	0.9 ± 0.3	1.0 ± 0.3	0.8 ± 0.3	1.2 ± 0.5
LysoPE %	3.0 ± 1.6	2.4 ± 1.0	3.2 ± 1.4	2.8 ± 1.0

Protein, phospholipid, cholesterol and triglyceride concentrations are expressed per g of wet tissue. Phospholipid composition is in molar percentage. Values are means ± SD ($n = 6$).

PC, phosphatidylcholine; PE, phosphatidylethanolamine; SPH, Sphingomyelin.

significantly altered ($p > 0.05$) by the dietary manipulations. Only TG tended to increase in the hyperlipidic groups. The composition of choline- and ethanolamine-containing PL was not modified except for a slight, but insignificant, increase in PC and decrease in PE in the hearts of rats fed salmon oil. The amount of lyso derivatives of PC and PE remained unchanged ($p > 0.05$) in the four groups.

Marked variations were observed in the fatty acid composition of PC and PE (Table 4). In PC and PE, palmitic acid had the lowest levels in the corn oil group, whereas it had the highest level in PE of rats given the salmon oil diet. Monounsaturated fatty acids (18:1, essentially) were affected differently, depending on the nature of the diet and the class of phospholipid. When compared with the low-fat diet, the salmon oil diet lowered 18:1n-9 and n-7 in the two classes of PL, whereas corn oil and lard diets lowered 18:1n-7 in both classes. A drastic drop in arachidonic acid (20:4n-6) was induced by salmon oil in both PC and PE. In the latter, the value was 3.5-fold lower than that in the low-fat diet. In PE, corn oil provoked a slight, but significant, decrease in 20:4n-6. It induced a rise in the 22:4n-6 and 22:5n-6, mainly in PE. The primary feature arising from salmon oil intake was the appearance of eicosapentaenoic acid (20:5n-3) in cardiac PC and PE of rats fed salmon oil. In addition, 22:5n-3 and mainly 22:6n-3 were efficiently incorporated in the cardiac PL. The 22:6n-3 was increased by a factor of 7.9 and 2.8 in PC and PE, respectively. The lard diet also provoked a significant rise in 22:5n-3 and 22:6n-3, especially in PE. As shown in Table 4, the n-6/n-3 ratio of cardiac PC and PE of rats given the salmon oil diet dropped sharply as a consequence of n-3 PUFA incorporation. A decrease in this ratio was also observed in cardiac PL of rats fed the lard diet, but was less pronounced than that observed with the salmon oil diet. The highest increase in the unsaturation index of cardiac PL was provoked by the salmon oil diet (+26% in PC and +13% in PE). Compared with the low-fat diet, the lard diet induced a higher level of cardiac-PL unsaturation (+8% for both classes). This index was slightly increased in corn oil-fed animals: +8 and +3% in PC and PE, respectively.

Ultramicroscopic examination of ventricles revealed that a few lipid droplets were occasionally present in cardiomyocytes of rats fed low-fat as well high-fat diets (Table 5). The most striking feature of the hearts of rats given the salmon oil diet was the significant accumulation (Fig. 1 and Table 5) of dense granules structurally identified as lipofuscin or ceroid material (40).

As shown in Table 6, heart GSH-PX total and specific activities tended to be higher in the salmon oil group than in the other groups. These increases were significant ($p < 0.05$), when compared with those of the saturated lard diet. They were +36% and +54% for total and specific activities, respectively. Heart-GST activity, as well as liver GSH-PX activity, was not significantly modified among the four groups.

DISCUSSION

The high-fat diets were well accepted by the rats, none of which showed any significant difference in body weight at the end of the experiment. The previously mentioned hypocholesterolemic effect of fish oil (3,4,12) is confirmed. It even tended to be more pronounced than that of vegetable oil, if comparison is made with the saturated lard diet. The fact that most of the plasma lipid components were lowered, suggests that a drop in circulating lipoproteins occurred, as has been demonstrated for very low density, as well as low density lipoproteins in man (41). Heart lipid composition was not affected by the dietary manipulations. This is in agreement with previous studies performed in rats fed fish oils (7,8,12,30), but not with another report showing a global increase in heart TG, PL and cholesterol in rats fed higher amount of fish oil (42). However, we observed an average of 2.8%

TABLE 4

Fatty Acid Composition of Phosphatidylcholine and Phosphatidylethanolamine of Heart From Rats Fed Various Diets

	Phosphatidylcholine				Phosphatidylethanolamine			
Fatty acid	Low-fat	Corn oil	Salmon oil	Lard	Low-fat	Corn oil	Salmon oil	Lard
16:0	$17.7^{a,*}$	$15.0^{b,*}$	$17.9^{a,*}$	$15.6^{a,b,*}$	$7.0^{a,*}$	$5.3^{b,*}$	$9.8^{c,*}$	6.7
16:1n-7	0.70	—	0.54	—	0.18	—	—	—
18:0	$25.5^{a,*}$	$27.6^{a,b,*}$	$26.3^{a,b,*}$	$29.5^{b,*}$	24.8	24.3	26.9	26.2
18:1n-9	$5.3^{a,*}$	$3.6^{b,*}$	$3.5^{b,*}$	$5.1^{a,*}$	$5.6^{a,*}$	$4.7^{a,b,*}$	$4.0^{b,*}$	$4.5^{a,b,*}$
18:1n-7	$6.5^{a,*}$	$3.0^{b,*}$	$3.9^{c,*}$	$4.3^{c,*}$	$3.2^{a,**}$	$1.6^{b,**}$	$2.0^{b,**}$	$1.6^{b,**}$
18:2n-6	$13.8^{a,b,*}$	$16.0^{a,*}$	$12.3^{a,b,*}$	$9.4^{b,*}$	$7.0^{a,*}$	$11.0^{b,*}$	$6.0^{a,*}$	$4.5^{a,*}$
20:0	—	$0.10^{a,**}$	$0.33^{b,**}$	—	—	$0.2^{a,**}$	$0.4^{b,**}$	—
20:1n-9	—	$0.10^{a,**}$	$0.20^{b,**}$	—	0.10	0.11	tr	—
20:2n-6	—	$0.44^{a,**}$	$0.14^{b,**}$	—	$0.11^{a,**}$	$0.42^{b,**}$	tr	—
20:4n-6	$28.0^{a,*}$	$30.7^{a,*}$	$20.3^{b,*}$	$31.3^{a,*}$	$32.1^{a,**}$	$28.0^{b,**}$	$9.2^{c,*}$	$27.6^{a,b,*}$
20:5n-3	—	—	4.02	—	—	—	3.0	—
22:4n-6	$0.45^{a,*}$	$0.85^{b,*}$	—	$0.49^{a,*}$	$2.1^{a,*}$	$3.9^{b,*}$	tr	$1.7^{c,*}$
22:5n-6	$0.33^{a,b,*}$	$0.59^{a,*}$	$0.12^{b,*}$	$0.27^{b,*}$	$3.7^{a,c,**}$	$5.9^{a,**}$	$0.35^{b,**}$	$2.6^{c,b,**}$
22:5n-3	$0.43^{a,**}$	$0.51^{a,**}$	$1.8^{b,**}$	$1.2^{c,**}$	$1.8^{a,*}$	$2.0^{a,*}$	$2.4^{b,*}$	$3.2^{c,*}$
22:6n-3	$1.4^{a,**}$	$1.2^{a,**}$	$10.7^{b,**}$	$2.7^{c,**}$	$12.5^{a,**}$	$12.3^{a,**}$	$35.5^{b,**}$	$20.8^{c,**}$
n-6/n-3	23.8	27.6	2.0	10.7	3.1	3.4	0.4	1.5
UI	165	178	208	179	263	271	297	286

Values not bearing the same superscript letter are significantly different at $p < 0.05$ (*) or $p < 0.01$ (**) ($n = 6$).
Tr, traces (<0.1%); UI, Unsaturation Index.

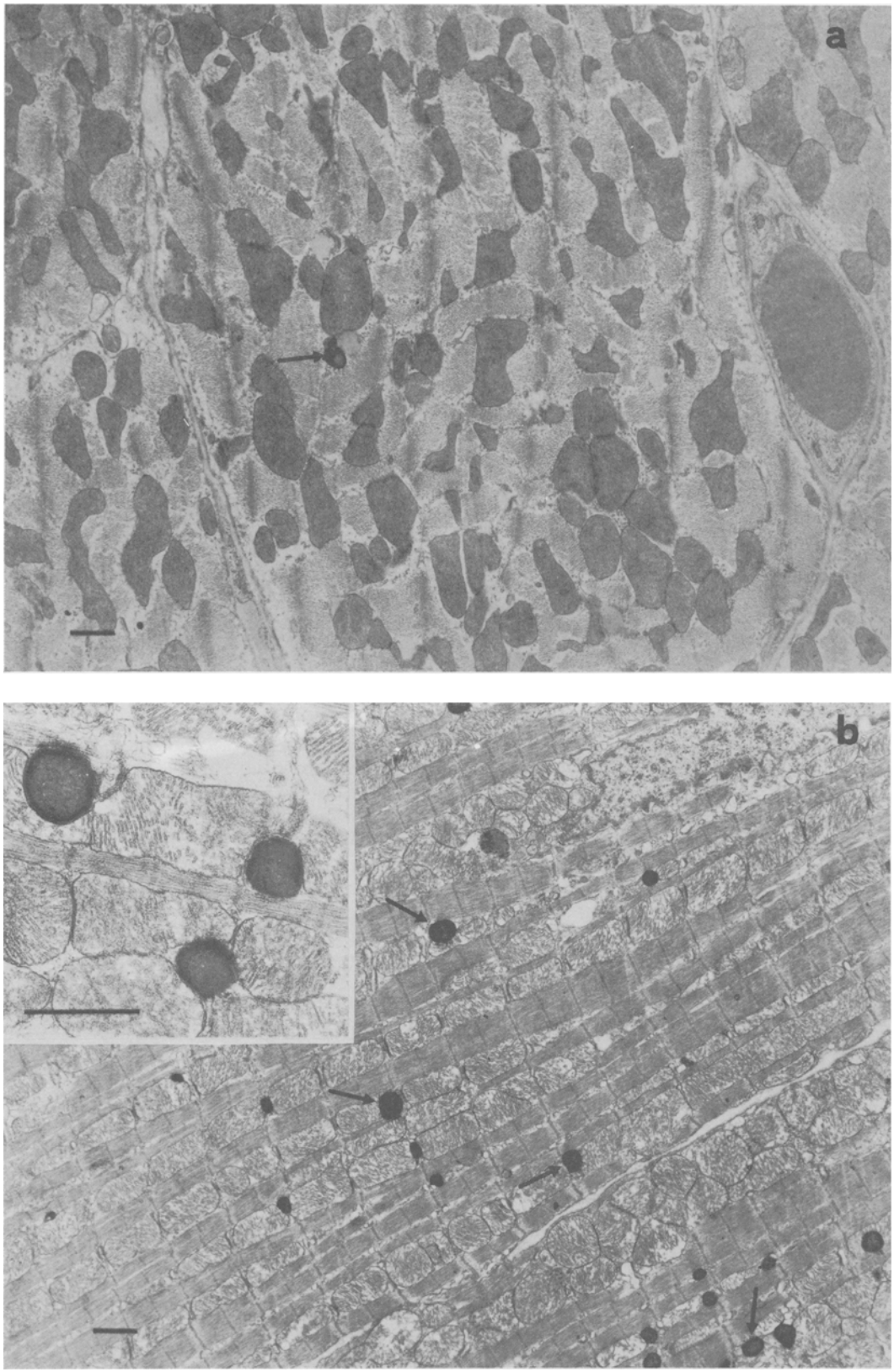

FIG. 1. Ultramicroscopic examination of ventricle of heart of rats fed low-fat (a) or salmon oil (b) diet. Arrows show some examples of lipofuscin or ceroid-like material. The bars indicates 1 μm.

TABLE 5

Estimation of Lipidosis and Lipofuscinosis in Heart Ventricle Myocytes of Rats Fed Various Diets

	Low-fat	Corn oil	Salmon oil	Lard
Intracytoplasmic lipid droplets	0.39 ± 0.10	0.34 ± 0.06	0.16 ± 0.03	0.35 ± 0.00
Lipofuscin-like material	0.11 ± 0.01^{a,**}	0.16 ± 0.09^{a,**}	1.63 ± 0.19^{b,**}	0.11 ± 0.04^{a,**}

Values that represent the frequency of structures (number per 30 μm^2) are means ± SEM; (n = 2). Procedures for counting are detailed in the Materials and Methods section. Values not bearing the same superscript letter are significantly different at $p < 0.01$ (**). Values without superscript letter are not statistically different ($p > 0.05$).

TABLE 6

Activities of Selenium-Dependent Glutathione Peroxidase and Glutathione S-Transferases in Heart and Liver of Rats Fed Various Diets

	Low-fat	Corn oil	Salmon oil	Lard
Heart				
GSH-PX Units/mg	0.40 ± 0.03^{a,b}	0.38 ± 0.04^{a,b}	0.51 ± 0.14^{a}	0.33 ± 0.05^{b}
GSH-PX Units/g	48.9 ± 6.6^{a,b}	47.0 ± 4.6^{a,b}	60.5 ± 10.9^{a}	44.1 ± 3.1^{b}
GSH S-Tr. Units/g	2.8 ± 0.4	2.6 ± 0.3	2.3 ± 0.5	2.2 ± 0.3
Liver				
GSH-PX Units/mg	1.3 ± 0.3	1.2 ± 0.1	1.5 ± 0.2	1.4 ± 0.3

One Unit corresponds to a micromol of substrate catalyzed/min. Activities are expressed either per g of wet tissue (total activity) or per mg of protein (specific activity). Values not bearing the same superscript letter are significantly different at $p < 0.05$; values are means ± SD (n = 6).

of cardiac lysoPE, whereas in our previous reports (12,30) this figure was ca. 1%. Because the percentages of lyso PC were similar, the increase in cardiac lysoPE might be specific and reflect a metabolic change induced by the cholesterol supplementation in the diets.

A more pronounced effect on cardiac fatty acid composition appeared with 12.5% salmon oil than with 17% corn oil. This may reflect different metabolic properties of n-3 and n-6 PUFA (43). It has been shown that n-3 PUFA competitively inhibit n-6 desaturation. Also, n-3 PUFA may be preferred as substrate by the acylCoA: lysoPCacyltransferase for acylation into lysophosphoglycerides. A cardiac cytosolic acyltransferase has been described as being selective towards some n-3 PUFA (44), although data with 22:5n-3 and 22:6n-3 were lacking. So, all these events may converge to enhance incorporation of n-3 PUFA in cardiac-membrane PL, especially the 22:6n-3, which became the predominant fatty acid in PE. A higher level of the 22:6n-3 was also induced by the lard diet in both PC and PE. This is the result of the preferential metabolism of n-3 by desaturases, especially when dietary supply of 18:n-6 is low. Thus, the n-6/n-3 ratio of the diet appears to be a more determinant factor in the modification of cardiac-PL fatty acid composition than is the P/S ratio or the amount of fat. This is in agreement with studies showing that lower (9,22), as well as higher, amounts (7,8,10,12) of dietary fish oils induced significant incorporation of n-3 PUFA in cardiac PL. Comparison of our results with previous studies (12,30) indicates that addition of cholesterol to the diets does not modify the rate of incorporation of either n-6 or n-3 PUFA in hearts. This was also found by others in rat heart and kidney, but not in liver (10).

As a result of n-3 incorporation, the unsaturation index of heart-membrane PL was increased in the salmon oil group. This may increase membrane-PL sensitivity to peroxidation. Indeed, ultramicroscopic examination of the ventricles revealed significant accumulation of lipofuscin-like or ceroid material in the salmon oil group, indicating a higher level of lipid peroxidation. Selenium deficiency is known to generate lipid peroxidation, as measured by pentane production (45). A minimal value of 40 μg of selenium per kg of diet had been recommended for optimal growth of rats (20), but this minimal value should now be around 70 μg/kg. Some authors (22) have supplemented their fish oil diets with selenium to prevent any deficiency induced by a higher demand to neutralize hydroperoxides. As our purpose was to study the effect of dietary PUFA on the GSH-PX activity in selenium basal conditions, no selenium was added. Our basal diet contained ca. 60 μg of selenium/kg, which is near the recommended limit. The specific activities of the GSH-PX measured in heart and liver of rats fed low-fat diet gave values in the range of those reported by Levander et al. (38). This suggests that our basal diet (low-fat) induced no deficiency in the active GSH-PX. However, it may be thought that the fish oil diet induced some deficiency in the selenium available for GSH-PX. This was not the case, because heart GSH-PX activity was not lowered in the salmon oil group and, significantly, was 50% higher than that measured in hearts of rats fed the saturated lard diet. Moreover, liver GSH-PX activity, which is used as an index of selenium status in animals (46), remained unchanged in all the experimental groups. This indicates that selenium availability for GSH-PX was not altered in the salmon oil group. It can be argued that if diets had

been supplemented with selenium, we might have observed a more pronounced activation of heart GSH-PX in rats fed salmon oil. But, on the other hand, if GSH-PX activity had been insufficient in the salmon oil group, we should have observed some activation of the GST, because these enzymes are known to compensate for inadequate activity of the selenium-dependent enzyme (38,47). In fact, GST activities remained unchanged, which again argues against a subnormal level of active GSH-PX in the salmon oil group.

It is interesting to compare the fish oil group with the lard group. In the latter, no significant lipofuscin accumulation was observed in heart, although 22:5n-3 and 22:6n-3 levels were higher than those of the low-fat group. GSH-PX, in this group, was even lower than that of the fish oil group. This would indicate that selenium-dependent enzyme activity is not the most important factor protecting lipids against peroxidation and that it was not involved in heart lipofuscin or ceroid accumulation in the fish oil group.

Deficiency or subnormal level of vitamin E, on the other hand, is well known to increase the level of peroxidation and ceroid accumulation in tissues (12,18,20,21,48–50). An increased P/S ratio in the diet has been proposed as a major factor leading to a higher susceptibility to peroxidation in rats (16). Our previous study (12) suggested that the n-6/n-3 ratio and the unsaturation index of the diets might be a more accurate parameter to investigate their effects on vitamin E status. Indeed, we showed that a salmon oil-enriched diet, more significantly lowered the plasma vitamin E concentration of rats than did a corn oil diet having a higher P/S ratio, but a lower unsaturation index and n-3/n-6 ratio than the salmon oil diet. In the present study, rats were given diets similar to those previously used (12), except they were supplemented with 1% cholesterol. Herein, we can reasonably infer a similar lowering effect of salmon oil on serum vitamin E. Nevertheless, two biological parameters allow us to suggest that vitamin E deficiency was not attained in the salmon oil group. First, blood transaminases were not elevated in this group, which is in contrast to the results in vitamin E and selenium-deficient pigs (50). Second, Cao et al. (51) have recently reported a higher level of lysoPC in the hearts of vitamin E-depleted rats. In our present experimental conditions, no significant increase in heart-lysoPC concentration could be detected in the salmon oil group. This lets us suggest that, although a subnormal vitamin E level was probably induced in this group, a serious deficiency level was not attained.

In conclusion, high intake of fish oil given for 2 months did not affect the enzymatic defense system against fatty acid hydroperoxides. Other factors like vitamin E or phospholipid hydroperoxide glutathione peroxidase (53) have to be considered when explaining heart lipofuscinosis.

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Isolation and Identification of Acetyl-CoA Carboxylase From Rainbow Trout (*Salmo gairdneri*) Liver

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Acetyl-CoA carboxylase is the pivotal enzyme in the de novo synthesis of fatty acids and is the only carboxylase with a biotin-containing subunit greater than 200,000 daltons. The biotin moiety is covalently linked to the active site and has a high affinity ($K_d = 10^{-15}$ M) for the protein avidin. This relationship has been used in previous studies to identify acetyl-CoA carboxylase isolated from mammalian species. However, acetyl-CoA carboxylase has not been isolated and characterized in a poikilothermic species such as the rainbow trout. The present study describes the isolation and identification of acetyl-CoA carboxylase in the cytosol of rainbow trout *(Salmo gairdneri)* liver. The enzyme was isolated using two distinct procedures—polyethylene glycol precipitation and avidin-Sepharose affinity chromatography. Identification of the isolated protein as acetyl-CoA carboxylase was made by the following: (1) sodium dodecyl sulfate-polyacrylamide gel electrophoresis; (2) avidin binding; (3) in vivo labeling with [^{14}C]biotin; and (4) acetyl-CoA carboxylase-specific activity. The subunit molecular weight of the major protein was 230,000 daltons ± 3.3%. This protein was shown to bind avidin (M_r = 16,600) prior to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, indicating the presence of biotin. In addition, protein isolated from fish that had previously received intraperitoneal injections of [^{14}C]biotin, showed the majority of radioactivity associated with the 230,000 dalton protein. The polyethylene glycol precipitation yielded 200 μg protein (4.4 μg/g liver), with a specific activity of 5 nmol malonyl-CoA/min/mg protein, whereas avidin affinity chromatography yielded 1.75 ± 1.1 mg protein (9.0 μg/g liver), with a specific activity of 1.37 ± 0.18 μmol malonyl-CoA/min/mg protein. The enzyme was citrate dependent showing maximum activity between 10 and 20 mM. Acetyl-CoA carboxylase-specific activity decreased by 50% in the presence of 0.2 M NaCl. These findings suggest that the major protein (M_r = 230,000) purified from rainbow trout liver is acetyl-CoA carboxylase with enzyme characteristics comparable to mammalian acetyl-CoA carboxylase.
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Acetyl CoA-carboxylase (ACC) [EC 6.4.1.2] is a biotin-containing enzyme responsible for the first committed step in the de novo synthesis of fatty acids (1-3). The enzyme has been isolated and characterized from several mammalian tissues, including rat liver (4-7), rat mammary gland (8) and rabbit mammary gland (9,10), as well as from chicken liver (11) and goose uropygial gland (12). These studies reported a subunit (M_r) for ACC that ranged from 225,000 to 260,000 daltons. However, this enzyme has not been characterized or purified to homogeneity in a poikilothermic species.

The rainbow trout *(Salmo gairdneri)* is currently being used as an alternative vertebrate model (13-15) for testing the carcinogenic effects of environmental and dietary toxicants, such as nitrosamines (16,17), aflatoxin B_1 and cyclopropenoid fatty acids (CPFA) (18-20). These studies have shown that the liver is the primary site of toxicity for these compounds. Because the liver is also the principle organ for lipogenesis in fish (21), it is possible that these compounds, as well as others, may adversely affect the primary lipogenic enzymes ACC and/or fatty acid synthetase.

In this laboratory, preliminary studies using rainbow trout identified ACC (M_r = 220,000) from the microsomal fraction of liver homogenates and revealed that the enzyme was closely associated with, but not bound to, liver endoplasmic reticulum. It was also observed that fish exposed to dietary CPFA showed a significant reduction in liver microsomal ACC (22). The presence of this enzyme in the microsomal fraction is of interest, because it is well known that the biochemical pathway involved with lipogenesis occurs in the cytosol of cells. Previous studies (4-12) have, therefore, concentrated on isolating the enzyme from the soluble fraction of tissue preparations. Recently, other workers have reported mammalian ACC to be associated with the mitochondira (23) and endoplasmic reticulum (24). These investigations not only support our earlier findings in trout, but also suggest that fatty acid synthesis and, hence, ACC are not confined to the cytosol.

Before toxicity data obtained from a poikilotherm model can be used to predict similar effects in a homoiotherm model, it is necessary to fully understand the similarities and differences of the enzyme affected in both systems. We report here the first purification of ACC from the cytosol of rainbow trout liver.

MATERIALS AND METHODS

Chemicals. [^{14}C]Biotin and [^{14}C]sodium bicarbonate were purchased from Amersham Inc. (Arlington Heights, IL). Polyacrylamide (99%), protein standards and all other electrophoresis supplies were obtained from Bio Rad (Richmond, CA). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO) and were of the purest grade available.

Animals and diets. Mt. Shasta strain rainbow trout *(Salmo gairdneri)* were used for all analyses. These were spawned and reared at the Oregon State University Food Toxicology and Nutrition Laboratory. The control diet (diet A) consisted of a dextrose and casein-gelatin mixture containing 10% lipid. The test diet (diet B) contained 20% carbohydrate with no lipid or biotin. All animals were ca. 1 yr of age and weighed 300-700 g.

Isolation of acetyl-CoA carboxylase. Fish were killed by a sharp blow to the head and the livers were excised immediately and placed in ice-cold homogenization buffer

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Abbreviations: ACC, Acetyl-CoA carboxylase; CPFA, cyclopropenoid fatty acid; PEG, polyethylene glycol precipitation; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

(Tris-HCl 100 mM pH 7.4, sucrose 0.25 M, EDTA 2 mM, β-mercaptoethanol 15 mM and phenylmethylsulfonyl fluoride 0.4 mM) as a rinse. After rinsing, the livers were placed in an ice-cold glass blender pitcher that contained a small volume of the homogenization buffer. When all livers had been removed and placed in the pitcher, three volumes of ice-cold homogenization buffer were added and the livers homogenized by pulsating (5-sec intervals) at high speed for 20 sec. The homogenate was allowed to settle and the foam was removed by aspiration. The homogenate was filtered through cheese cloth to remove fibrous materials and then centrifuged at 100,000 × *g* for 1 hr at 4°C. The supernatant was subjected to ammonium sulfate precipitation (35% of saturation) and two 3% polyethylene glycol (PEG) precipitations, as described previously (9). Protein concentrations were determined either by absorbance at 280 nm or the Bradford assay (25).

Acetyl-CoA carboxylase activity. ACC activity was assayed using the [^{14}C]bicarbonate fixation procedure (26,27). The reaction buffer (Tris-Acetate, 70 mM, pH 7.5; potassium acetate, 120 mM; reduced glutathione, 4 mM; potassium citrate, 10 mM; ATP, 1 mM; bovine serum albumin, 0.6 mg/ml; acetyl-CoA, 0.35 mM; and magnesium acetate, 10 mM) was essentially as described by Allred and Roehrig (26), with the following modifications: nonlabeled bicarbonate was omitted to increase sensitivity, and samples were incubated with 7 mM (0.25 μCi) of sodium [^{14}C]bicarbonate (0.1 μCi/μmole) at 37°C for 2–3 min. Final reaction volumes were 0.5 ml and reactions were started by addition of enzyme. Controls were performed in the absence of enzyme or acetyl-CoA.

Binding avidin to PEG-isolated acetyl-CoA-carboxylase prior to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Ten fish (45 g liver tissue) were killed and prepared according to the method previously described. Avidin was bound to ACC essentially as described by Goodson et al. (5). ACC (3 μg) was added to 0.15 ml of sample buffer (Tris-HCl, 0.0625 M, pH 6.8; 4% sodium dodecyl sulfate (SDS), w/v; 2% dithiothreitol; 5% glycerol and 0.003% bromophenol blue) and the mixture was heated 2–3 min at 95°C. After cooling, 50 μg of N-acetylated monomeric avidin was added and the mixture was allowed to stand for 5 min. The sample was loaded onto an acrylamide gel (18.0 cm × 16.5 cm × 0.2 cm) consisting of a 3.5% stacking gel and a 5% resolving gel (28). The sample was electrophoresed at 21 mA constant current for 7 hr, stained overnight with Coomassie Blue and destained with a 50% methanol, 10% acetic acid solution for several hours.

In vivo [^{14}C]biotin incorporation into acetyl-CoA carboxylase. Ten fish (450–600 g) were fasted for 1 month, then fed diet B for 5 days. During this feeding period, each fish received daily i.p. injections of 2 μCi [^{14}C]biotin in 0.2 ml distilled water. After sacrifice, ACC was isolated by the PEG procedure run on SDS-PAGE and stained Coomassie Blue, as previously described. The gel was sliced into 2 mm segments, each of which was placed into a 1.5 ml Eppendorf micro centrifuge tube and dried in an oven at 60°C. Following the addition of 0.1 ml 30% hydrogen peroxide, each segment was incubated again at 60°C to solubilize the gel. Once solubilized, each tube received 0.4 ml of water and 5.0 ml ACS scintillation fluid and the radioactivity in each segment was determined using a Beckman model LS 3801 scintillation counter, as previously described (7).

Avidin affinity chromatography. Avidin was cross-linked to the Sepharose according to Thampy and Wakil (4), as modified from Beaty and Lane (11). The biotin binding capacity of the column was 13 nmoles/ml of packed column. Ca. 200 g of liver tissue was removed and prepared as described above, except that the PEG precipitations were omitted. The 100,000 × *g* supernatant was subjected to ammonium sulfate precipitation (35% or 26% of saturation) and the pellet collected by centrifugation at 20,000 × *g* for 20 min. The $(NH_4)_2SO_4$ pellet was resuspended in a minimum volume of column buffer (Tris-HCl, 100 mM, pH 7.5; EDTA 1.0 mM; dithiothreitol 0.1 mM; NaCl 0.5 M; glycerol 5.0% (v/v) and phenylmethylsulfonyl flouride 0.4 mM) and added to the avidin-Sepharose gel, forming a slurry. To prevent settling and ensure uniform mixing, the preparation was gently shaken at 4°C for 4–6 hr. The suspension was then gently poured into a 150-ml glass fritted funnel mounted on a vacuum flask and washed with 4–6 liters of column buffer (precooled to 4°C) to remove unbound protein. The cleaned avidin-Sepharose gel, now containing bound ACC, was transferred to a Bio Rad econo column, 10 cm in length by 2.5 cm in diameter with a 50-ml capacity. ACC was eluted with 0.2 mM biotin in column buffer without the 0.4 mM phenyl-methylsulfonyl flouride. The column was attached to a peristaltic pump and the flow rate set at 3–5 ml/hr. Fractions were collected in 1.0-ml aliquots and analyzed for protein content on a Beckman spectrophotometer at 280 nm. The fractions containing protein were pooled and dialyzed 4–5 hr at 4°C against column buffer without NaCl. This dialysis is important, because NaCl will greatly decrease ACC activity. ACC-specific activity and protein content were then determined. The final preparation was aliquoted, frozen rapidly in liquid nitrogen and stored at −70°C.

RESULTS

ACC was initially isolated and purified using the PEG procedure first described by Hardie and Cohen (9). The protein concentration in the final preparation was 200 g (45 g liver tissue, 4.4 μg ACC/g liver). A portion of the final preparation was subjected to SDS-PAGE and the protein visualized by Coomassie Blue staining. As shown in Figure 1, lanes 2 and 3, the major band detected had an (M_r) of 230,000 daltons ± 3.3%, as determined by its electrophoretic mobility (Fig. 2). Two minor protein components (Fig. 1, lanes 2 and 3, [a,b]) were closely associated with the putative ACC.

Biotin is covalently bound to all known carboxylases. Therefore, to show the presence of biotin in the 230,000-dalton protein, another portion of the PEG-isolated protein was incubated with N-acetylated monomeric avidin. Because the binding affinity between avidin and biotin is high, (Kd = 10^{-13}, for monomeric avidin), the avidin (M_r = 16,600) bound to the putative ACC forms a stable ACC-avidin complex. The molecular weight of this complex was found to be approximately equal to the sum of the two subunits—248,000 daltons (Fig. 1, lane 1; and Fig. 2). In Figure 1, lane 1, both the major 230,000-dalton band and minor component (b) bind to avidin. This results in an increased molecular weight and a decreased

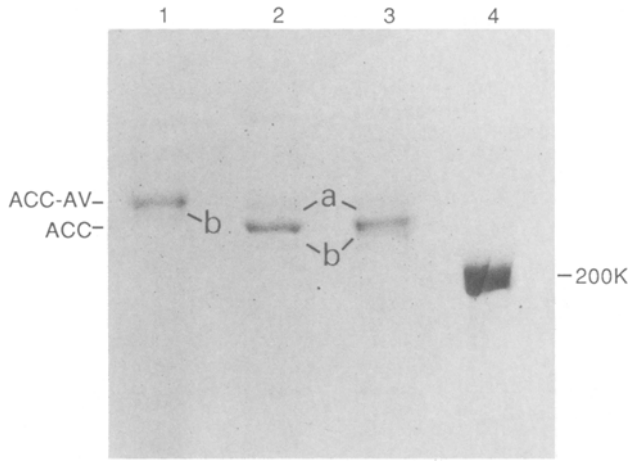

FIG. 1. SDS-PAGE analysis (5% polyacrylamide) and subsequent staining with Coomassie Blue of the putative acetyl-CoA carboxylase (ACC) at 230,000 daltons obtained from the PEG-precipitation procedure. Lane 1 shows the formation of a carboxylase-avidin complex (ACC-AV) and the subsequent increase in mol wt from 230,000 to 248,000. Lane 2 represents 3 μg ACC after 4 hr dialysis. Lane 3 is 3 μg ACC without dialysis. Lane 4 is a myosin standard marker. The bands identified as (a) and (b) are minor components (see Results).

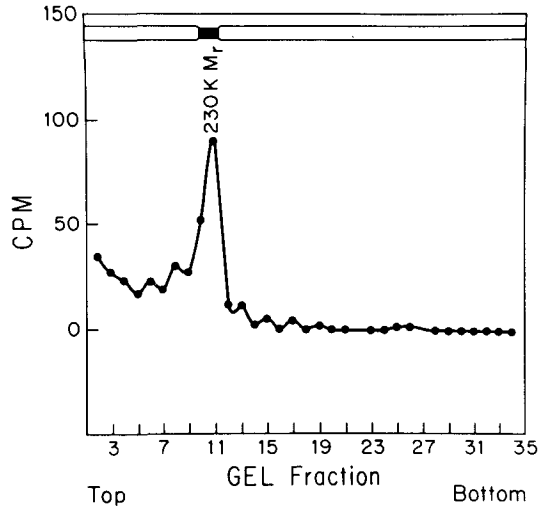

FIG. 3. SDS-PAGE analysis (5% polyacrylamide) of [^{14}C]biotin labeled acetyl-CoA carboxylase, isolated by the PEG procedure. The amount of protein used was 25 μg (12.0 CPM/μg protein). After electrophoresis at 21 mA constant current for 7 hr, the gel was stained with Coomassie Blue (gel not shown) and sliced into 2-mm segments. The radioactivity content of each slice was determined as described in Materials and Methods and the distribution of [^{14}C]biotin plotted. The majority of the radioactivity was observed in the 230,000-dalton region of the gel.

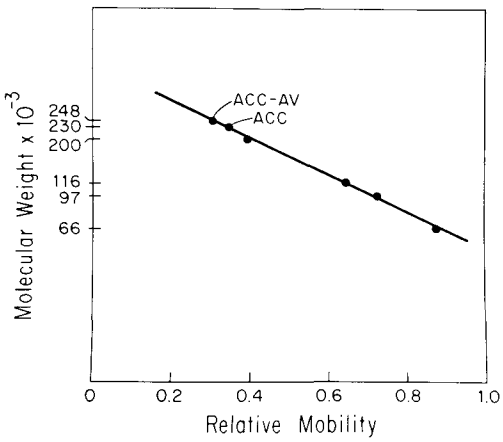

FIG. 2. Mol wt determination for rainbow trout acetyl-CoA carboxylase (ACC). Subunit mol wt (MW) of the Bio Rad protein standards, ($\times 10^{-3}$) were as follows: Myosin (200), β-galactosidase (116), phosphorylase b (97) and bovine serum albumin (66). The putative ACC was (230), and the acetyl-CoA carboxylase-avidin complex (ACC-AV) was (248). The graph was derived by plotting the $\log_{10}$ values of the subunit (MWs) against their relative mobility. Actual MW ($\times 10^{-3}$) are shown for easier evaluation.

electrophoretic mobility. Subsequently, both bands appear above their counterparts in lanes 2 and 3. This evidence suggests that protein (b) also possesses biotin and is most likely a proteolytic fragment of the major 230,000-dalton band. Protein (a) on the other hand did not bind avidin and did not change its position relative to lanes 2 and 3. Therefore, component (a) is not visible in lane 1 (the ACC-AV complex is coeluting with protein [a]), suggesting that it did not react with avidin and, therefore, could not be an active subunit of ACC.

Additional evidence showing the presence of biotin in the 230,000-dalton protein is shown in Figure 3. These data represent the in vivo incorporation of [^{14}C]biotin into the 230,000-dalton protein. The protein was isolated by the PEG procedure and analyzed by SDS-PAGE. The graph depicts the distribution of radioactivity in the gel, showing the majority of activity present in the 230,000-dalton region.

The PEG isolation was adequate for making a preliminary identification of the 230,000-dalton band as ACC. However, the final preparation was not homogeneous and proteolysis was apparent. Several attempts to improve the quality of the isolation were unsuccessful. In addition, the specific activity of citrate-activated (10 mM) ACC (5.0 nmoles/min/mg protein) was extremely low in this procedure.

Therefore, to improve the purification procedure and verify the data obtained from the PEG isolation, fresh liver tissue was extracted, homogenized and centrifuged at 100,000 × *g* and subjected to 26% $(NH_4)_2SO_4$ precipitation, as described previously for the PEG procedure. However, instead of continuing with the PEG purification, the $(NH_4)_2SO_4$ fraction was further purified using avidin-affinity chromatography. Figure 4 shows the elution of ACC from the column, as determined by its absorbance at 280 nm. Total ACC activity (μmol malonyl-CoA/min/ml) in selected fractions showed a single peak that coincided with the protein peak. The amount of protein recovered was 1.75 mg ± 1.1 (9.0 μg ACC/g liver), and the activity in the combined fractions was 1.37 ± 0.18 μmol malonyl-CoA/min/mg ACC. A sample of the column-purified ACC was analyzed by SDS-PAGE (Fig. 5). The Coomassie Blue stained gel revealed a single protein band with a subunit (M_r) of 230,000 daltons. The two minor components observed in the PEG isolation procedure (Fig. 1) were not present.

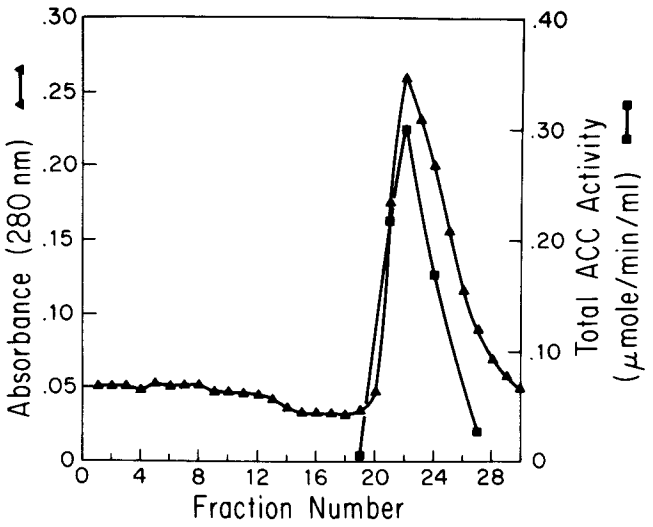

FIG. 4. Elution of ACC from the avidin-Sepharose column using 0.2 mM biotin in column buffer. Fractions were collected in 1.0 ml aliquots at a flow rate of 3–5 ml/hr. ACC total activity coincided with the protein peak. The biotin binding capacity was 13 nmoles/ml packed gel.

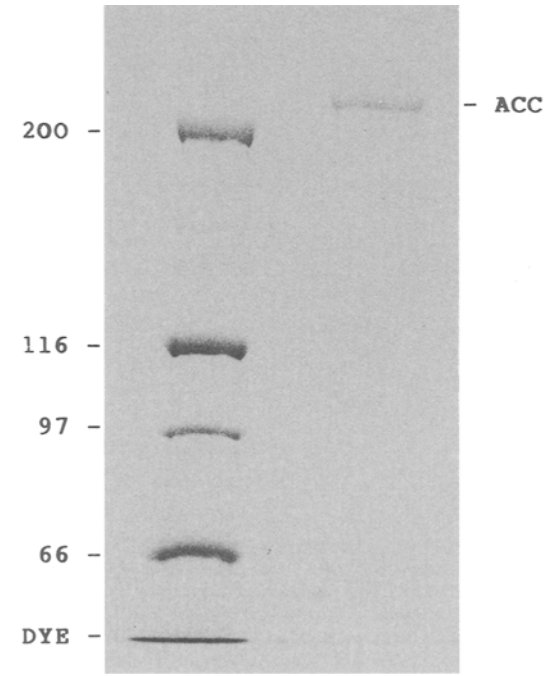

FIG. 5. SDS-PAGE analysis (5% polyacrylamide) and subsequent staining with Coomassie Blue of protein recovered from the avidin-Sepharose column. Lane 1 contains Bio Rad high mol wt standards ($\times 10^{-3}$). Lane 2 received 5 μg protein and shows a single protein (M_r = 230,000) component.

A typical purification using avidin affinity chromatography is shown in Table 1. Data obtained from mammalian and avian species using essentially the same protocol are included for comparison. It is difficult to accurately access ACC activity in crude cell fractions due to interference by malonyl-CoA decarboxylase and contamination by mitochondrial pyruvate carboxylase (29). However, the values are useful as a relative comparison between species and to monitor the success of the purification procedure. Data presented in Table 1 can be summarized as follows: (1) Throughout the purification,

TABLE 1

Purification of Acetyl-CoA Carboxylase (ACC) From Rat, Chicken, Goose and Rainbow Trout Using Avidin-Sepharose Affinity Chromatography

Fraction	Species	Tissue	Total protein (mg)	Total[a] Act. (U)	Specific Act. (U/mg)	Purification (-fold)
100,000 × *g*						
	Rat[b]	Liver	11,169	40.1	.004	1.0
	Chicken[c]	Liver	4,000	400.0	.010	1.0
	Goose[d]	Uropy	2,240	44.0	.020	1.0
	Trout[e]	Liver	11,800	21.0	.002	1.0
$(NH_4)_2SO_4$						
35%	Rat	Liver	1,248	31.2	.025	6.25
25%	Chicken	Liver	360	162.0	.450	45.00
30%	Goose	Uropy	60	35.0	.590	30.00
26%	Trout	Liver	172	3.0	.020	10.00
Avidin-Sepharose column						
	Rat	Liver	20.0	37.10	1.830	458.00
	Chicken	Liver	29.4	153.00	5.200	520.00
	Goose	Uropy	3.0	15.00	5.000	250.00
	Trout	Liver	1.0	1.24	1.240	620.00

[a]Activity in all studies was determined using the [^{14}C]bicarbonate fixation assay at 37°C. One unit (U) equals μmol malonyl-CoA produced per min at 37°C.

[b]From 200 g liver; ACC production was induced by diet (Ref. [6]).

[c]From 500 g liver; citrate was included throughout the isolation (Ref. [11]).

[d]From five uropygial glands; gel filtration preceded the 30% $(NH_4)_2SO_4$ (Ref. [12]).

[e]From 200 g liver; fish received standard diet.

ACC total activity in trout was significantly lower than in the other species; (2) each step in the procedure resulted in a several-fold increase in purity for all species; (3) the specific activity of trout ACC in the final fraction, although lower, was comparable with values obtained in rat, chicken and goose; and (4) the overall recovery of trout ACC per gram of liver was less than in the other species.

Activity assays performed at various citrate concentrations (0, 2, 5, 10, 20 and 40 mM) revealed the enzyme was citrate dependent with optimal citrate levels between 10 and 20 mM. At 40 mM and at higher citrate concentrations, ACC-specific activity decreased. In addition, sodium chloride (0.2M) in the reaction mixture decreased ACC-specific activity by 50%. These findings are all characteristic of ACC isolated from homoiotherms and are consistent with data reported in previous studies (12).

DISCUSSION

ACC has been identified and characterized in several mammalian species. The activity of ACC in various fish species has been studied only in crude cell fractions (30–32). The present investigation provides the first description and characterization of ACC purified from rainbow trout liver cytosol. To accomplish this, advantage was taken of the fact that ACC is predominantly a cytosolic enzyme with a subunit (M_r) greater than 200,000 daltons. The remaining carboxylases are confined to the mitochondria and possess a subunit (M_r) less than 200,000 daltons (3,5). In addition, ACC (as well as the other carboxylases) contains a covalently linked biotin moiety associated with each subunit. Thus, a protein isolated from the soluble fraction that possesses biotin and has a subunit (M_r) greater than 200,000 daltons is consistent with the characteristic properties of ACC.

The subunit (M_r) of the major protein isolated from rainbow trout using two different techniques was determined to be 230,000 daltons. This is in agreement with the (M_r) range of 225,000–260,000 daltons reported for mammals. To establish that this protein also possessed a biotin moiety essential for carboxylase activity, it was incubated with monomeric avidin. The resulting protein-avidin complex showed an increase in the (M_r), as judged by a reduction in its electrophoretic mobility. These data suggest that the entire 230,000-dalton band in Figure 1, lane 1, was composed of the biotinyl protein. The fact that protein (b) also bound avidin, strongly suggests that it is a proteolytic fragment of the larger protein. This proteolytic fragment was also observed by Hardie and Cohen (10). Protein (a) (Fig. 1, lane 1) did not move up relative to its position in lanes 2 and 3. This suggests that (a) did not possess biotin and therefore must be a contaminant. The electrophoretic properties and close association of band (a) with ACC suggests that protein (a) is fatty acid synthetase, which has been shown to electrophorese slightly above ACC (10). To be certain that the observed ACC-avidin complex (Fig. 1, lane 1) was the result of a specific biotin-avidin interaction and not random association of avidin with the 230,000-dalton protein, [^{14}C]biotin was incorporated into the carboxylase in vivo. The results of this experiment confirmed the presence of biotin in the 230,000-dalton band and showed that the observed interaction between avidin and the suspected ACC (M_r = 230,000) was associated with the presence of this moiety.

The PEG-isolation procedure was useful in establishing the presence of biotin in the 230,000-dalton protein. However, ACC-specific activity, as well as the amount of protein obtained (4.4 μg/g liver), was extremely low. Additionally, small amounts of contaminant proteins were apparent. Therefore, avidin-Sepharose affinity chromatography was used to purify trout ACC to homogeneity.

This procedure yielded a homogeneous ACC preparation without the proteolysis observed using the PEG procedure. In addition, ACC-specific activity in the final fraction was substantially higher than the PEG isolation and comparable with ACC activity reported in homoiothermic species. The average amount of ACC recovered per gram of liver increased from 4.4 μg to 9.0 μg.

The data presented here provides good evidence that the 230,000-dalton protein isolated from rainbow trout liver cytosol is ACC, with a subunit (M_r) and enzyme characteristics comparable with mammalian ACC. These similarities are important, because the rainbow trout is being used to study the effects of various toxicants on lipogenesis. The fact that the fish ACC possesses many of the same biochemical properties as rat ACC suggests that toxicants directly affecting this important lipogenic enzyme in trout may also cause the same effects in mammals. Finally, these data provide an important impetus to studies currently underway in our laboratory and aimed at understanding the mechanisms by which xenobiotics affect ACC and the processes involved with lipogenesis in the rainbow trout model.

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In vivo Incorporation of Lauric Acid Into Rat Adipose Tissue Triacylglycerols

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An in vivo approach was taken to examine fatty acid esterification in adipose tissue using a coconut oil-enriched diet. Rats were fed a diet containing coconut oil (50% lauric acid) for six weeks. Triacylglycerols from perirenal adipose tissue were fractionated by silver nitrate–thin layer chromatography and, then, preparative gas chromatography. The distribution of 169 triacylglycerol types accounting for 97% of total triacylglycerols was determined. There was evidence for a very high content of mixed triacylglycerols composed of intermediate (12:0 and 14:0) and long acyl moieties. No significant differences were observed between the experimental distribution of triacylglycerol types and the random distribution, calculated from the total fatty acid composition. This indicated that most long chain triacylglycerols stored before coconut oil feeding would have been rearranged after the six weeks of coconut oil feeding. The experimental proportion of trilauroylglycerol reached 2%, as expected from its random proportion, and the proportions of dilauroylacylglycerols were slightly higher than the random values. Present results were compared with those previously obtained from triacylglycerols of adipose tissue of rats fed a low-fat standard diet (1,2). From our results and those of other authors, it is suggested that lauric acid is a good substrate for *sn*-glycero-3-phosphate acyltransferase and diacylglycerol acyltransferase in rat adipose tissue.
***Lipids 24,* 193–203 (1989).**

Adipose tissue, as most tissues, is able to synthesize only fatty acids longer than C14 (3), but may incorporate shorter chains (4), if present in the plasma. Lauric acid, probably by passive permeation (5), is transported through the plasma membrane of rat adipocytes; and although this fatty acid is transported less efficiently than longer chain acids (6), it is easily stored in the triacylglycerols of body fat of coconut oil (50% lauric acid)-fed animals, whereas shorter chain acids are not deposited (7). The mechanism of triacylglycerol biosynthesis in adipose tissue has been the subject of some interest (8), but much remains to be learned of the factors (including the chain length of fatty acids) that control intracellular esterification.

In the present study, rats were given CO for 6 weeks, and the distribution of triacylglycerol types in the triacylglycerols of the perirenal adipose tissue (TCO) was determined. The results were compared with those previously obtained from the triacylglycerols of the adipose tissue of rats fed a low-fat standard diet (TC), analyzed under similar conditions (1,2). The present results provide useful information about controlling the chain length of the fatty acids that acylate the glycerol moiety during triacylglycerol synthesis in rat adipose tissue. The results, however, cannot be used to define the precise mechanism of biosynthesis.

Abbreviations: $AgNO_3$, silver nitrate; CO, coconut oil; TC, triacylglycerols from rats fed a standard diet; TCO, triacylglycerols from rats fed a standard diet with 20% CO; VLDL, very low density lipoproteins.

MATERIALS AND METHODS

Source of triacylglycerols. Male Wistar rats of the same age (Lessieux, Sèvres, France) were supplied a low-fat standard diet (UAR, Villemoisson, France) ad libitum for 4 weeks before any experiment. A preliminary study was undertaken to determine the pattern of deposition of intermediate chain fatty acids (12:0 + 14:0) into adipose tissue. Rats weighing 150 g were fed the standard diet supplemented with 20% (w/w) CO for 7 weeks. Three rats were killed each week and perirenal adipose tissue was excised from both sides. Total lipids were extracted according to Delsal (9). Aliquots of total lipids obtained from 3 rats killed at a fixed time were pooled and, then, analyzed by gas chromatography for their fatty acid profiles.

In a second step, 2 groups of 6 rats weighing ca. 285 g each were maintained on either the standard diet or the 20%-CO diet for 6 weeks. All rats were weighed each week and sacrificed after 6 weeks. Perirenal adipose tissue was excised and weighed, and total lipids were extracted. The 6 rats fed the standard diet were only used as control animals to assess body growth, and weight and lipid content of the adipose tissue. Aliquots of total lipids from the 6 CO-fed rats were pooled and fractionated on a column of silicic acid according to Fillerup and Mead's procedure (10) for preparing pure triacylglycerols (TCO). The purity of the triacylglycerols was controlled by thin layer chromatography using a mixture of hexane/diethyl ether/acetic acid (90:30:1, v/v/v) as the solvent system. TCO were then fractionated by $AgNO_3$-thin layer chromatography and preparative gas chromatography to determine the distribution of triacylglycerol types.

The standard diet contained only 3.7% (w/w) lipids made up of 67% triacylglycerols, 15% free fatty acids, 9% phospholipids, 6% diacylglycerols, 2% monoacylglycerols and 0.8% sterol esters (as the mol % of fatty acids for all the lipids). CO (Astra Calvé, Asnières, France) consisted of 96% triacylglycerols and 4% diacylglycerols. The main constituents (wt %) of the standard diet enriched with 20% CO were carbohydrates (46.4%), lipids (23.0%), proteins (13.6%), water (9.0%), cellulose (4.0%), minerals (4.0%) and vitamins. Lipids in the 20%-CO diet, therefore, consisted of CO (87.1%) and long chain lipids (12.9%) originating from the standard diet.

TC (triacylglycerols from perirenal adipose tissue of rats fed a standard diet) have previously been studied (1,2), and some results are used here for comparison. The rats had been fed the same low-fat standard diet as that described above.

$AgNO_3$-thin layer chromatography. Total TCO were fractionated according to the degree of unsaturation on Silica Gel G (Merck, Darmstadt, FRG) impregnated with 10% silver nitrate in three steps using chloroform

(stabilized with 0.4% ethanol) made more or less polar by adding methanol (0–1.5%). The bands were scraped and the triacylglycerols were extracted as previously described (1). The proportions of the bands were determined using two internal standards—tritridecanoylglycerol added at the beginning of the extraction of triacylglycerols from $AgNO_3$-silica powder and pentadecanoic acid added before preparing the butyl esters. The proportions of the bands were eventually corrected of contaminations due to overlapping or tailing. The extent of these contaminations was calculated from the fatty acid composition of the bands.

Gas chromatography. Although triacylglycerols can be resolved by high performance liquid chromatography into numerous peaks, depending on both the degree of unsaturation and chain length, gas chromatography was chosen for several reasons—separation of triacylglycerols according to only one precise characteristic (total carbon number), elution of long chain triacylglycerols in a relatively short time and possible fractionation of minute amounts of triacylglycerols. Resolutions of triacylglycerols were performed, therefore, by a Packard 7400 gas chromatograph equipped with an on-column injector heater. The glass column (0.40 m × 2 mm I.D.) was packed with 3% JXR on 100–120 mesh Gas Chrom Q (Applied Science Laboratories, State College, PA) and operated with a gas (helium) flow of 100 ml/min. Analyses were made by linear-temperature programming from 220 to 350°C at 4°C/min. Weight calibration factors were determined from known composition mixtures of saturated triacylglycerols (Applied Science Laboratories). The factors for a well-conditioned column and for a 1–10 μg/peak range of injected triacylglycerols were: 1.17 for trioctanoylglycerol (C24), 1.07 for tridecanoylglycerol (C30), 1.00 for trimyristoylglycerol (C42), 1.03 for tripalmitoylglycerol (C48), 1.10 for tristearoylglycerol (C54) and 1.35 for triarachidoylglycerol (C60), when the factor for trilauroylglycerol (C36) was equal to 1. The correction factors for intermediate molecular weight triacylglycerols were interpolated from the curve obtained with the standard triacylglycerols.

The micropreparative fractionation of triacylglycerols also was carried out by gas chromatography under similar conditions. However, quantities of triacylglycerols as large as 150–300 μg (about 50 μg for a major peak) dissolved in $CHCl_3$ or CS_2 (100 μg/μl) could be injected into a wider glass column (0.30 m × 4.4 mm I.D.). Helium flow was increased to 200 ml/min, 88% of the column effluent was directed with a stream-splitter to a Packard 852 gas collector modified to maintain its temperature at 335°C. Each peak of the triacylglycerols was trapped at room temperature in a glass cartridge partially filled with glass wool and eluted with pentane. Purity of the collected peaks was checked by gas chromatography under the analytical conditions described above. When the purity was less than 98%, the peaks of the triacylglycerols were purified under the micropreparative conditions.

Fatty acid compositions were determined by gas chromatography of the butyl esters, as previously described (11). When microquantities (0.5–50 μg) of triacylglycerols collected by micropreparative gas chromatography were analyzed, fatty acid butyl esters were prepared in sealed glass tubes (70–100 μl of inner volume), as reported elsewhere (1).

Nomenclature and calculations. When triacylglycerols are fractionated by $AgNO_3$-thin layer chromatography, the bands consist of one or several classes of triacylglycerols. A class is symbolized by a triad of digits, each digit representing the number of double bonds in a constituent fatty acid, without positional reference. Following the separation of triacylglycerols by gas chromatography on apolar phase, each chromatographic peak is equivalent to a group of triacylglycerols characterized by the same total number of carbon atoms of the three acyl moieties. Triacylglycerol types are combinations of three fatty acids, without positional reference.

The experimental distribution of triacylglycerol types, as proposed by Bezard et al. (12), was first calculated in each group as follows: the fatty acids found in one group were combined three by three so that the carbon number of each combination (triacylglycerol type) corresponded to the group. Equations were established from the experimental percentages of the fatty acids in the group and from the unknowns (mol % of the triacylglycerol types, which had to be determined). The solution of the equations was such that the fatty acid composition of the group, calculated from the triacylglycerol type distribution, had to be as close to the experimental fatty acid composition as possible. The distribution of the triacylglycerol types in the total triacylglycerols was calculated by using the experimental proportion of the group in the corresponding class and that of the class in the total triacylglycerols.

The random distribution of triacylglycerol types was calculated according to the method described by Bailey (13) and Baltes (14) and as used by Kuksis et al. (15,16). If a, b and c are the experimental percentages of the fatty acids A, B and C in the total triacylglycerols, the random percentages of the triacylglycerol types AAA, AAB and ABC are equal to $a \times a \times a \times 10^{-4}$, $a \times a \times b \times 3 \times 10^{-4}$ and $a \times b \times c \times 6 \times 10^{-4}$, respectively.

RESULTS

Time course of CO fatty acid incorporation into adipose tissue. Rats were fed a 20%-CO diet for 7 weeks, and total lipids from perirenal adipose tissue were analyzed. Changes in the fatty acid composition are shown in Figure 1. The fatty acid distribution was nearly stabilized within 3 weeks. The intermediate chain fatty acids (12:0 + 14:0), accounting for 2.5% before CO feeding, reached 24% after 1 week and stayed at a plateau (about 37%) during the last 4 weeks.

Total fatty acids and triacylglycerol groups. Rats were fed either a standard diet or a 20%-CO diet for 6 weeks. The growth of CO-fed animals was comparable with that of control animals, as shown in Figure 2. The perirenal adipose tissue of CO-fed rats was 1.3 times as heavy as that of control rats. Total lipids accounted for 96.1% ± 0.3 and 92.5% ± 0.5 ($p < 0.001$) of the fresh perirenal adipose tissue mass in the CO-fed and control rats, respectively. In Table 1, the fatty acid and triacylglycerol group compositions of triacylglycerols from adipose tissue of the CO-fed rats (TCO) are compared with those of triacylglycerols from the standard diet, CO and adipose tissue of rats fed a low-fat standard diet (TC) (1). In CO-fed rats, saturates represented two-thirds of fatty acids and lauric acid was the major fatty acid. The shortest

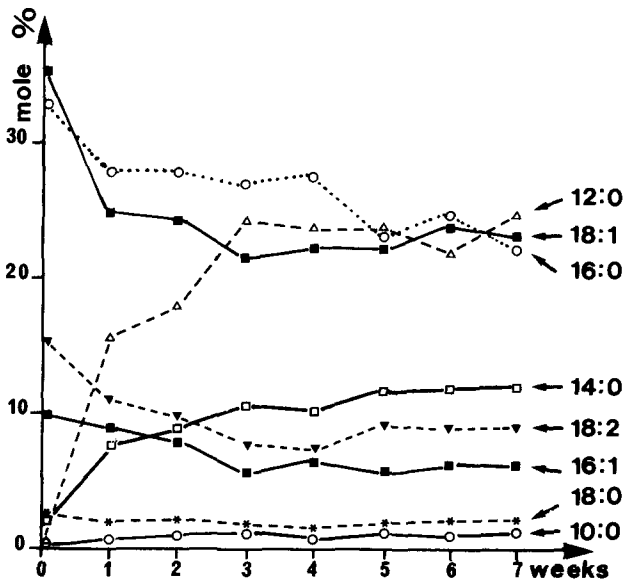

FIG. 1. Time course of CO fatty acid incorporation into total lipids of adipose tissue of rats fed a 20%-CO diet.

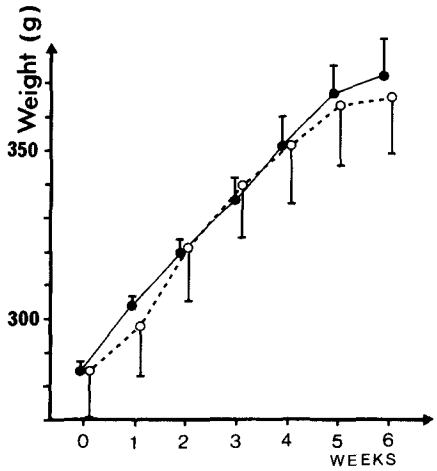

FIG. 2. Growth of rats fed a standard diet supplemented with 20% CO (continuous line) versus rats on a standard diet (dotted line). Means ± SE (n = 6).

chains (6:0 + 8:0) present in CO were not incorporated into TCO. Linoleic acid, an essential fatty acid that is known to have a regulatory action on adipose tissue (17), was still present in TCO, because its content (w/w) in the CO diet was about 1.5%—as much as in the standard diet. In contrast to TCO, three main classes of long chain fatty acids—saturates (35%), monoenes (37%) and diene (27%)—had been observed in adipose tissue triacylglycerols from rats on the low-fat standard diet (TC) (1), like rats on a fat-free diet supplemented with 2% linoleate (17). The distribution of triacylglycerol groups in TCO reflected neither that in the standard diet and in TC (1), nor that in CO. The triacylglycerol groups C42-C48, which represented only 7% in TC (1) and 14% in CO, reached 64% in TCO. This provides evidence for mixed triacylglycerols made up of long and intermediate acyl moieties.

Triacylglycerol classes. Although TC had been fractionated into 13 bands by only one chromatographic run (1), the separation of TCO was made more difficult because of the widening of the bands due to differences

TABLE 1

Fatty Acid and Triacylglycerol Group Distributions (mol %) in Total Triacylglycerols From Standard Diet, Coconut Oil (CO) and Perirenal Adipose Tissue of Rats[a]

Fatty acids[b]	Standard diet[c]	TC	TCO	CO	Triacylglycerol groups[d]	Standard diet	TC	TCO	CO
6:0				1.4	C26				0.2
8:0			0.1	12.6	C28				0.8
10:0			1.5	7.9	C30				3.7
12:0	0.3	0.1	27.5	48.4	C32			0.1	14.9
14:0	1.5	2.4	14.1	15.6	C34			0.6	17.9
16:0	20.9	28.9	19.5	6.9	C36			2.3	20.4
16:1	1.3	7.4	3.1		C38			3.9	16.8
18:0	4.7	3.9	2.3	1.8	C40			6.8	9.9
18:1	22.0	29.4	17.9	4.6	C42			14.7	6.7
18:2	42.5	26.8	12.5	0.8	C44			14.7	3.7
20:0	0.5			t[e]	C46	0.4	0.9	18.8	2.0
18:3	3.7	1.1	0.8	t	C48	0.7	5.7	16.1	1.5
20:1	1.2		0.6		C50	6.3	24.8	10.0	0.8
20:4	0.5	t	0.1		C52	34.9	44.0	8.4	0.5
22:1	0.3				C54	54.5	23.4	3.4	0.2
24:1	0.1				C56	2.7	1.2	0.2	
22:6	0.5	t			C58	0.5	t		

[a]Rats were fed the standard diet alone (TC) (Ref. 1) or supplemented with 20% CO (TCO).

[b]Fatty acids identified by total number of carbon atoms and double bonds.

[c]The fatty acid composition of the triacylglycerol fraction was similar to that of the total lipids.

[d]Groups of triacylglycerols identified by the total number of carbon atoms of the three constituent fatty acids.

[e]t, trace amounts (<0.1%).

in the R_f values according to triacylglycerol chain length, because R_f increases along with molecular weight of triacylglycerol (18–20). Figure 3 shows the 10 bands obtained by using 3 different developing solvent systems.

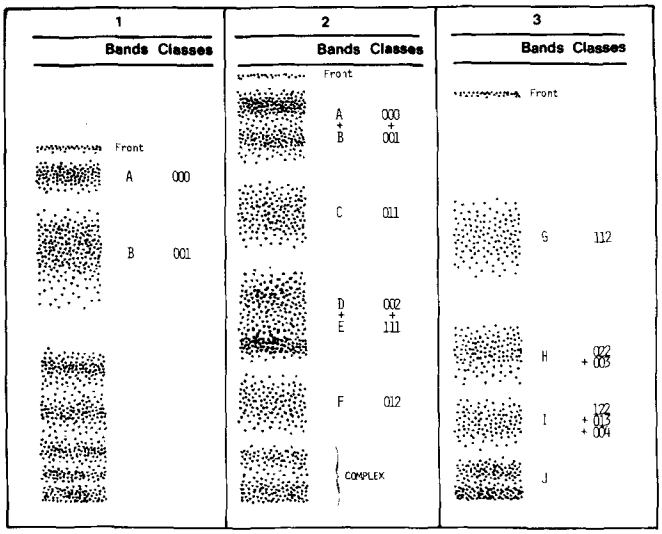

FIG. 3. Separation of triacylglycerols from perirenal adipose tissue of CO-fed rats (TCO) by ascending thin layer chromatography on $AgNO_3$-impregnated Silica Gel G. Plate 1: The classes 000 and 001 were separated using chloroform alone. Plate 2: Total triacylglycerols were fractionated using chloroform/methanol (99.3:0.7, v/v). Plate 3: The complex band isolated on the plate 2 was rechromatographed in chloroform/methanol (98.5:1.5, v/v). The bands were located under UV light after spraying with 0.05% ethanolic dichlorofluorescein.

Bands D and E were collected together, because they were incompletely separated as already observed for TC (1) and other fats (21,22). The bands contained one or several classes, which were identified by gas chromatography of fatty acids as shown in Table 2. The order of elution of the triacylglycerol classes followed that determined experimentally by Gunstone and Padley (23), but some classes were not separated (classes 022 + 003 in band H and classes 122 + 013 + 004 in band I). The triacylglycerol structure of band I was not studied. Triacylglycerol compositions of bands were obtained by gas chromatography under analytical conditions (Table 3). The calculation of average chain length of triacylglycerols shows a good correlation between fatty acid and triacylglycerol compositions for all bands except the complex, polyunsaturated band (J). The experimental triacylglycerol composition of TCO, or that calculated from the triacylglycerol compositions of the bands, too, is very close to the random distribution.

The proportion of a class is the mean of the determinations based on three internal standards, namely tridecanoic and pentadecanoic acids used for fatty acid analyses and tritridecanoylglycerol used for triacylglycerol gas chromatography. Table 4 shows the experimental and random distributions of classes in TCO. The triacylglycerols of the three major classes (000, 001 and 002) accounted for 73.5% and contained at least two saturated acyl moieties as the consequence of the high content of saturates (65%). In contrast with TCO, there was only one major class (012) in TC (1), as the reflection of the three main fatty acids (16:0, 18:1 and 18:2) (see Table 1). The experimental and random proportions of

TABLE 2

Distribution (mol %) of Fatty Acids in Bands Isolated by Silver Nitrate-Thin Layer Chromatography From TCO

Fatty acids	Bands									Total triacylglycerols (TCO)	
	A	B	C	D+E	F	G	H	I	J	Experimental	Calculated[a]
8:0	0.2	0.2					0.2	t[b]		0.1	0.1
10:0	2.6	1.3	0.6	1.4	0.6	t	0.8	0.5	0.6	1.5	1.5
12:0	43.5	29.0	14.0	26.2	13.9	2.2	18.2	8.7	10.2	27.5	27.8
14:0	23.1	13.5	6.6	11.6	6.3	1.1	8.8	4.0	5.2	14.1	13.4
16:0	27.3	20.2	12.6	19.4	12.0	2.8	17.2	7.6	10.8	19.5	19.5
16:1		4.8	10.3	1.8	6.2	11.2	t	4.7	4.2	3.1	3.8
18:0	2.9	2.8	1.4	2.6	1.6	t	2.4	1.8	2.4	2.3	2.4
18:1	0.4	27.5	52.6	5.8	27.0	45.9	1.4	23.9	17.0	17.9	18.7
18:2			0.1	31.1	31.1	35.7	42.4	35.9	29.4	12.5	11.7
18:3							8.6	11.1	15.3	0.8	0.6
20:1		0.7	1.8	0.1	1.3	1.1		0.9	0.3	0.6	0.4
20:4								0.9	4.6	0.1	0.1
Saturates[c]	99.6	67.0	35.2	61.2	34.4	6.1	47.6	22.6	29.2	65.0	64.7
Monoenes	0.4	33.0	64.7	7.7	34.5	58.2	1.4	29.5	21.5	21.6	22.9
Diene			0.1	31.1	31.1	35.7	42.4	35.9	29.4	12.5	11.7
Triene							8.6	11.1	15.3	0.8	0.6
Tetraene								0.9	4.6	0.1	0.1
Classes[d]	000	001	011	002 + 111	012	112	022 + 003	122 + 013 + 004	Others		

[a] Calculated from fatty acid compositions of bands and proportions of bands (see Table 4).

[b] t, trace amounts (<0.1%).

[c] Saturates, monoenes, diene, triene, and tetraene are fatty acids with 0, 1, 2, 3 and 4 double bonds per molecule.

[d] Classes are triacylglycerols identified by number of double bonds of acyl moieties without positional reference.

TABLE 3

Distribution (mol %) of Triacylglycerol Groups in Bands Isolated by Silver Nitrate–Thin Layer Chromatography From TCO

Triacylglycerol groups[a]	Bands									Total triacylglycerols (TCO)			
	A	B	C	D+E	F	G	H	I	J	Experimental	Calculated[b]	Random[c]	Difference %[d]
C32	0.2									0.1	t[e]	0.04	
C34	2.8	t	0.2							0.6	0.6	0.4	+50
C36	10.1	0.4	0.4							2.3	2.7	2.5	− 8
C38	15.8	0.8	0.4	t			t	t		3.9	3.9	3.9	0
C40	23.6	4.4	1.0	2.8		t	0.6	t		6.8	7.5	7.9	−14
C42	20.1	18.7	1.1	17.5	0.7	2.2	3.9	0.9		14.7	14.5	13.7	+ 7
C44	16.0	21.0	2.6	17.6	1.2	2.1	4.8	0.9	0.6	14.7	14.6	14.5	+ 1
C46	7.1	29.3	12.2	30.7	9.0	2.8	9.1	3.5	2.4	18.8	19.0	17.3	+ 9
C48	3.5	14.1	30.7	14.2	34.8	3.0	30.8	18.3	16.2	16.1	15.5	17.1	− 6
C50	0.7	9.2	23.1	9.9	21.0	5.1	16.4	9.6	11.7	10.0	9.8	10.4	− 4
C52	0.1	1.9	24.2	4.0	28.9	24.8	28.4	20.0	20.7	8.4	8.2	7.9	+ 6
C54		0.2	3.9	3.1	4.4	55.9	5.6	44.5	41.7	3.4	3.5	4.1	−17
C56			0.2	0.2	t	4.1	0.4	2.1	6.7	0.2	0.2	0.2	0
C58						t		0.2	t			0.01	
Average carbon numbers If	40.9	45.2	49.1	46.0	49.6	52.5	49.2	51.7	52.0	45.6	45.5		
IIg	41.1	45.3	49.3	46.3	49.6	52.7	48.4	51.2	50.8	45.7	45.6		

[a] Groups are triacylglycerols identified by total number of carbon atoms of constituent acyl moieties.

[b] Calculated from triacylglycerol compositions of bands and proportions of bands (see Table 4).

[c] Theoretical distribution of triacylglycerol groups calculated from total fatty acid composition (shown in Table 1) according to Bailey's method (13).

[d] Difference % = (Experimental % − Random %)/(Random %) × 100.

[e] t, trace amounts (<0.1%).

[f] Average number of carbon atoms of triacylglycerols in bands and TCO, calculated from triacylglycerol compositions.

[g] Average number of carbon atoms of triacylglycerols in bands and TCO, calculated from fatty acid compositions (×3) given in Table 2.

TABLE 4

Experimental and Random Distributions of Triacylglycerol Classes in TCO

Classes	Experimental (mol %)	Random (mol %)	Difference %[a]
000[b]	24.7	27.5	−11
001	30.3	27.2	+12
011	9.1	9.1	0
002	18.5	15.8	+17
111	1.1	1.0	+13
012	9.7	10.8	−10
112	1.7	1.8	− 5
022	2.0	3.1	−36
003	0.7	1.0	−31
122	0.8	1.0	−19
013	0.5	0.6	−23
004	0.04	0.1	−69
Others	0.7	1.0	−27

[a] Difference % = (Experimental % − Random %)/(Random %) × 100.

[b] Classes were underlined when the experimental and random proportions were estimated to be significantly different.

this class in TC reached 24.0% and 20.7%, respectively (1). The experimental and random distributions of classes were fairly close in both fats. However, it can be noticed that the experimental proportion (24.7%) of the saturated class (000) in TCO was lower than the random proportion (27.5%). Similar results had been obtained in TC (2.0% and 4.3%, respectively) (1). On the other hand, the mixed classes 001 and 002 in TCO, and 012 in TC (1), showed experimental values higher than the random proportions expected.

Triacylglycerol types. Bands of triacylglycerols were fractionated into groups by micropreparative gas chromatography and fatty acid compositions of groups were determined. Figure 4 shows triacylglycerol chromatograms obtained for the analysis and fractionation of band F (class 012), which accounted for 9.7% in TCO. Under analytical conditions, all the chromatograms of the bands isolated from TCO displayed traces of triacylglycerols having an odd carbon number (Fig. 4A). The separation of peaks under analytical conditions was better than under micropreparative conditions (Figs. 4A and B), because peaks were overloaded for collecting enough triacylglycerol for fatty acid determinations. However, this separation was sufficient enough to obtain a purity greater than 98% for the peaks C46-C52 (Fig. 4C). Peak C54 (4.4%) was purified under the same chromatographic conditions (Fig. 4D). Peaks C42 (0.7%) and C44 (1.2%) were not collected. Peak tailing in chromatograms (Fig. 4) was mainly caused by the void volume of the flame ionization detector. The fatty acid composition of the triacylglycerol groups C46-C54 (collected peaks) of the class 012 (band F) was then determined (data not shown). It was observed that the proportions of fatty acid classes (saturates, monoenes and diene) were close to 33%, showing absence of selective losses during fractionation and purification of triacylglycerols.

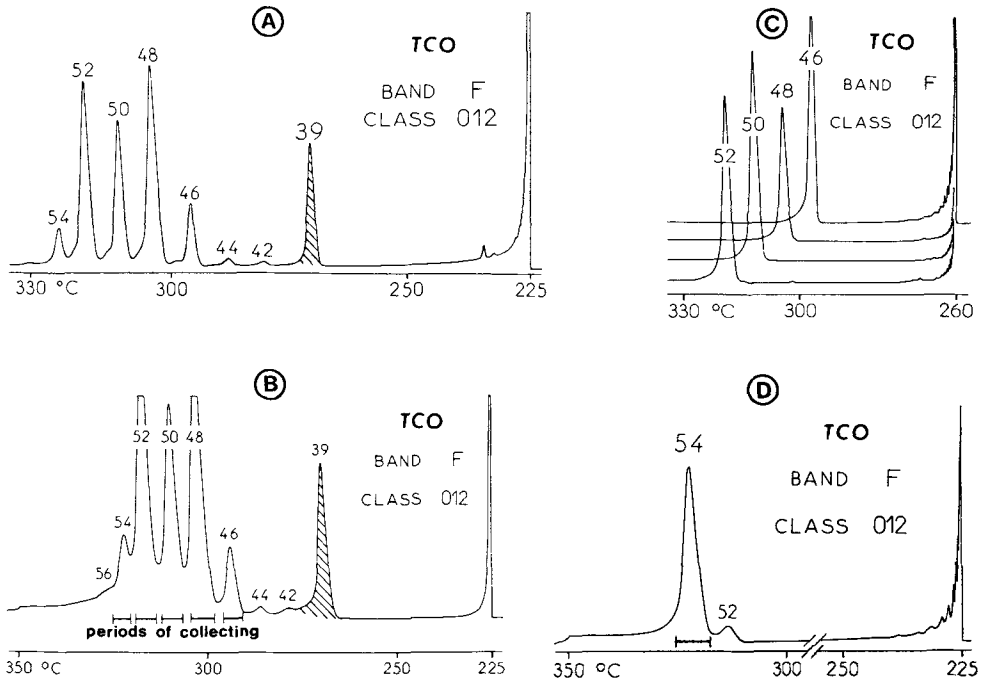

FIG. 4. Gas chromatography of triacylglycerols of band F (class 012) isolated beforehand by $AgNO_3$-thin layer chromatography from triacylglycerols of perirenal adipose tissue of CO-fed rats (TCO). Peaks identified by total number of acyl carbon atoms. Peak C39 corresponds to tritridecanoylglycerol added as an internal standard. Conditions of analytical or micropreparative gas chromatography as given in the Experimental Section. A) Chromatogram recorded under analytical conditions. B) Chromatogram recorded under micropreparative conditions and time of peak collection. C) Chromatogram recorded under analytical conditions for checking purity of peaks C46–C52 first collected by micropreparative gas chromatography. D) Chromatogram recorded during purification of peak C54.

Distributions of triacylglycerol types in groups were calculated from fatty acid compositions of collected peaks. The proportions of types in TCO were calculated from the distributions of types in groups, the distributions of groups in classes (Table 3) and the distribution of classes in TCO (Table 4). The triacylglycerols of bands A–G, accounting for 93.6% in TCO, were fractionated and analyzed. They contained 181 potential triacylglycerol types, and the proportions of most of them were assessed. Only some minor peaks accounting for 0.4% (in total) in TCO were not collected, resulting in 21 types not determined. The bands H and I were more complex and only 9 types accounting for 3.4% in TCO could be estimated. Table 5 shows the proportions of the 94 triacylglycerol types representing at least 0.1% in TCO and accounting for 95.8% in total. The proportions of 75 minor types, each one representing less than 0.1% in TCO, were also assessed, but were not shown in Table 5, because they accounted for only 1.2% in total. The first 10 main types in TCO were mixed triacylglycerols containing one or two lauroyl moieties and accounting for 42.2%. The type 16:0 18:1 18:2, which was the major one in TC (16.7%) and contained the three main fatty acids of TC (1,2), accounted for only 2.6% in TCO.

In Table 5, the experimental values were compared with the random percentages calculated from the fatty acid composition in Table 1. The differences between the two kinds of distribution were generally limited in TCO, as well as in TC (1,2), and often reflected the differences already noticeable in classes. For example, tripalmitoylglycerol accounted for 0.53% in TCO and 1.04% in TC (1,2), whereas the random proportions reached 0.74% and 2.4%, respectively. The experimental proportion of lauroylpalmitoyloleoylglycerol reached 7.0%, but its random proportion did not exceed 5.8%. The experimental and random proportions of palmitoyloleoyllinoleoylglycerol were similar (2.6%) in TCO, but they were different in TC (16.7% and 13.6%, respectively) (1,2). All these differences reflected those of classes 000, 001 and 012 in TCO (Table 4) and TC (1,2). However, sometimes differences were not homogeneous for all the types in a class. Although most of the main types of class 001 in TCO had an experimental proportion higher than the random proportion, two types—14:0 16:0 18:1 and 14:0 14:0 18:1—showed the reverse situation. The comparison of the experimental and random distributions of types in class 000 of TCO, according to the chain length of the component fatty acids, is given in Table 6. It is evident that, when the mean chain length of the saturated TCO increased, the experimental proportions of types became lower than the random proportions. This was not observed in the other classes of TCO, nor in TC (1,2) or CO (Bugaut, M., unpublished data).

DISCUSSION

Effect of CO on growth and perirenal adipose tissue size. Similar weight gain was observed in rats fed the 20%-CO

TABLE 5

Experimental and Random Distributions of Triacylglycerol Types in TCO

Triacylglycerol types[a]	Experimental (mol %)	Random (mol %)	Triacylglycerol types[a]	Experimental (mol %)	Random (mol %)
12:0 16:0 18:1	6.96	5.75	16:1 18:1 18:2	0.49	0.42
12:0 16:0 18:2	5.34	4.02	10:0 12:0 18:1	0.45	0.45
12:0 14:0 18:1	4.62	4.16	10:0 12:0 14:0	0.45	0.35
12:0 12:0 18:1	4.40	4.06	14:0 18:2 18:2	≤0.45	0.66
12:0 14:0 16:0	4.26	4.54	14:0 16:1 18:1	0.41	0.47
12:0 12:0 16:0	3.67	4.43	16:0 18:0 18:2	0.41	0.34
12:0 12:0 14:0	3.47	3.20	12:0 12:0 18:0	0.40	0.52
12:0 12:0 18:2	3.24	2.84	12:0 14:0 18:0	0.39	0.54
12:0 14:0 18:2	3.14	2.91	16:1 18:1 18:1	0.38	0.30
12:0 18:1 18:2	3.10	3.69	10:0 16:0 18:1	0.38	0.31
14:0 16:0 18:1	2.74	2.96	10:0 12:0 16:0	0.35	0.48
16:0 18:1 18:2	2.63	2.62	18:0 18:1 18:2	0.32	0.31
12:0 16:0 16:0	2.45	3.14	14:0 16:1 18:2	0.31	0.33
16:0 16:0 18:1	2.31	2.05	10:0 16:0 18:2	0.31	0.22
12:0 18:1 18:1	2.30	2.64	14:0 18:0 18:2	0.27	0.24
16:0 18:1 18:1	2.10	1.87	14:0 16:0 18:0	0.26	0.38
14:0 16:0 18:2	2.05	2.06	18:0 18:1 18:1	0.25	0.22
12:0 12:0 12:0	2.02	2.08	12:0 18:1 18:3	≤0.25	0.24
12:0 14:0 14:0	1.86	1.64	10:0 14:0 18:1	0.23	0.23
16:0 16:0 18:2	1.54	1.43	14:0 18:0 18:1	0.23	0.35
12:0 16:0 16:1	1.28	1.00	10:0 12:0 18:0	0.18	0.06
14:0 18:1 18:2	1.26	1.90	12:0 18:0 16:1	0.18	0.12
18:1 18:1 18:2	≤1.18	1.20	12:0 14:0 20:1	0.18	0.14
14:0 16:0 16:0	1.13	1.61	12:0 16:0 20:1	0.17	0.19
14:0 18:1 18:1	1.07	1.35	10:0 14:0 18:2	0.17	0.16
14:0 14:0 16:0	1.07	1.16	10:0 18:1 18:2	0.17	0.20
16:0 16:1 18:1	0.94	0.65	14:0 14:0 14:0	0.15	0.28
12:0 16:1 18:1	0.93	0.92	16:0 16:0 18:0	0.15	0.26
14:0 14:0 18:1	0.90	1.07	14:0 16:0 20:1	0.14	0.10
12:0 14:0 16:1	0.84	0.72	16:0 16:1 16:1	0.14	0.06
12:0 18:2 18:2	≤0.84	1.29	12:0 20:1 18:2	0.14	0.12
12:0 18:0 18:1	0.78	0.68	16:1 16:1 16:1	0.13	0.05
16:0 18:2 18:2	≤0.76	0.91	12:0 18:1 20:1	0.13	0.18
12:0 18:0 18:2	0.72	0.47	14:0 18:1 18:3	≤0.13	0.12
14:0 16:0 16:1	0.72	0.51	12:0 14:0 18:3	<0.13	0.19
12:0 12:0 16:1	0.69	0.70	10:0 14:0 16:0	0.12	0.25
14:0 14:0 18:2	0.66	0.74	10:0 18:1 18:1	0.12	0.14
12:0 16:1 18:2	0.66	0.64	18:0 16:1 18:1	0.12	0.08
16:0 16:1 18:2	0.66	0.45	16:0 20:1 18:2	0.11	0.09
10:0 12:0 12:0	0.62	0.34	18:0 16:1 18:2	0.11	0.05
18:1 18:2 18:2	≤0.61	0.84	14:0 20:1 18:2	0.11	0.06
10:0 12:0 18:2	≤0.56	0.31	16:0 18:0 16:1	0.11	0.08
16:0 18:0 18:1	0.54	0.48	18:0 16:0 18:1	0.11	0.02
12:0 16:0 18:0	0.53	0.74	18:0 18:2 18:2	≤0.11	0.11
16:0 16:0 16:0	0.53	0.74	10:0 16:0 16:0	0.10	0.17
18:1 18:1 18:1	0.53	0.57	12:0 16:1 16:1	0.10	0.08
16:0 16:0 16:1	0.52	0.35	16:0 18:1 20:1	0.10	0.13
			12:0 12:0 18:3	≤0.10	0.18

[a]Triacylglycerol types are triacylglycerols identified by their three component fatty acids, without positional reference.

diet and the standard diet, as reported by other authors (24,25). Nevertheless, it has been observed earlier that inclusion of hydrogenated CO (26), or saturated triacylglycerols containing C12–C18 acyl moities prepared from CO (17,27–29), depressed growth more than a fat-free diet, but there was no difference in growth when linoleate was added to the diets. On the other hand, addition of CO to the diet enlarged perirenal fatty depot, as described by Kaunitz et al. (17,27,29) in the epididymal fat of rats fed a CO fraction rich in intermediate chain fatty acids (12:0 + 14:0), when compared with rats on a fat-free diet. The enlargement of fat bodies in CO-fed rats, when compared with rats on a standard diet, would be due mainly to an increase in fat cell number in the perirenal site and an increase in adipocyte volume in the epididymal site (25). Moreover, the lipid content of perirenal adipose tissue was significantly higher in CO-fed rats than in rats fed the standard diet. Similar results were obtained by Awad (30) studying the epididymal fat pads. As far as perirenal adipose tissue is concerned, the increase of lipid content in CO-fed rats is difficult to explain, because an expected increase in adipocyte volume has not been observed (25).

Fatty acids. Rats were fed a diet enriched with medium

TABLE 6

Experimental and Random Distributions (mol %) of Triacylglycerol Types in the Saturated Class (000) of TCO[a]

Triacylglycerol groups	Triacylglycerol families[b]	TC			TCO			CO		
		Experimental	Random	Difference %[c]	Experimental	Random	Difference %	Experimental	Random	Difference %
18-30	MMM							0.1	0.4	−60
24-34	MMI				0.1	0.04	+200	5.8	10.5	−45
28-38	MML				0.3	0.04	+700	0.7	1.4	−49
30-38	MII				4.6	2.9	+ 58	41.1	32.7	+26
36-42	III				30.4	26.3	+16	30.8	31.8	− 3
34-42	MIL				2.8	3.0	− 5	8.5	8.8	− 4
40-46	IIL	2.0	1.4	+43	40.1	41.4	− 4	12.1	13.0	− 7
38-46	MLL				0.7	0.8	−16	0.3	0.6	−45
44-50	ILL	16.7	17.8	− 6	18.1	21.7	−17	0.6	0.8	−35
48-54	LLL	81.3	80.8	+ 1	2.9	3.8	−24			

[a] as a function of the chain length of component fatty acids. saturated TCO are compared with saturated triacylglycerols from adipose tissue of rats fed a standard diet (TC) and from coconut oil (CO) (Bugaut, M., unpublished data). Experimental values for TC were calculated from the experimental percentages of individual triacylglycerol types taken in Ref. 2. Random values were calculated from the fatty acid composition of TC in Table 1 according to Bailey's method (13).

[b] Families of triacylglycerols identified by the chain length of component fatty acids. M, medium chain fatty acids (6:0 + 8:0 + 10:0). I, intermediate chain fatty acids (12:0 + 14:0). L, long chain fatty acids (16:0 + 18:0).

[c] Difference % = (Experimental % − Random %)/(Random %) × 100.

(6:0 + 8:0 + 10:0) and intermediate chain triacylglycerols for 6 weeks, and perirenal adipose tissue was analyzed. The fatty acid composition of adipose tissue triacylglycerols partially reflected that of CO. However, among medium chain fatty acids, only a small amount of decanoate was incorporated in vivo into adipose tissue triacylglycerols, as already observed by other authors (3,7,31-33), in spite of linoleate in the diets. Kaunitz et al. (17,34) found that linoleate supplementation (2%) to a fat-free diet increased the incorporation of medium chain fatty acids into adipose tissue. The low level of medium chain fatty acids in the adipose tissue of the CO-fed rats did not result from the inability of adipocytes to utilize medium chain fatty acids, because octanoate can be activated to its acyl-CoA derivative (35). Octanoate is known to be incorporated in an intact form into triacylglycerols of rat perirenal fat, and its catabolism rate in this tissue is not different from palmitate (4,36,37). In fact, medium chains are absorbed via the hepatic portal vein (38) and quickly taken up by the liver (39). *Sn*-3-glycerophosphate and *sn*-1-acylglycerol-3-phosphate acyltransferases in rat liver have been reported to exclude fatty acids of chain lengths shorter than 12:0 (40). However, *sn*-1,2-diacylglycerol acyltransferase accepts octanoate as a substrate (41). As a result, medium chain fatty acids are incorporated into hepatic triacylglycerols only in a low proportion (42-44) and predominantly in position *sn*-3 (45). Therefore, in contrast to laurate (46), the medium chain fatty acids cannot be secreted at a significant level into the blood as very low density lipoprotein (VLDL). The largest portion of medium chain fatty acids is catabolized to CO_2 and ketone bodies in the liver (47). This catabolism occurs at a high rate in other organs (36,48) including the intestine (49,50). Medium chain fatty acids are cleared from the blood so rapidly that their plasma content remains at a very low level (44,51,52).

Laurate and myristate reached 28% and 14%, respectively, of the adipose tissue triacylglycerol fatty acids after 6 weeks of CO feeding. The high level of the intermediate chain fatty acids observed in the adipose tissue could be the result of the presence of linoleate (1.5% in the CO diet), which "facilitates the laying down of a depot fat more representative of that in the diet" (17). The rate of deposition of the two fatty acids (Fig. 1) was similar to that of undecanoate, which follows an experimental curve with the $t_{1/2}$ = 9 days in young rats (53) and, with increasing age, remains rapid in rats (54,55). The incorporation rate of myristate was higher than that of laurate, because the ratio of laurate to myristate was 3 in CO and only 2 in fat tissue. This could be the result of three factors. First, the proportion of dietary 14:0 drained via the lymph duct is 1.4 times higher than that of 12:0 (56). Therefore, less 14:0 than 12:0 is directly cleared by the liver. Second, intermediate chain fatty acid catabolism in the liver (39) or total organism (57) decreases when chain length increases. Finally, laurate as undecanoate (58-59), is elongated to myristate and to other longer fatty acids in the rat liver (60-62), but not in the adipose tissue (4,51). Myristate deriving from the hepatic elongation is supplied to the adipose tissue via VLDL triacylglycerol.

Because the proportions of long chain fatty acids (≥16 C), except 18:0 and 18:3, were much higher in TCO than in the CO diet, an endogenous origin for the long chain fatty acids has to be considered. Intestinal chylomicrons carry not only exogenous lipids, but also endogenous long chain fatty acids (63). These fatty acids can be synthesized de novo either by the hepatocyte before delivering to VLDL or in situ by the adipocyte (64). Finally, long chain fatty acids such as linoleate could be preferentially recycled inside the fat cell, because the store of essential fatty acid in body lipids is known to be preserved at the expense of other fatty acids (65-68). However, increased linoleate requirement has been observed in rats fed saturated intermediate chain triacylglycerols, when compared with animals fed a fat-free diet

or medium chain triacylglycerols (17,28,29). Therefore, it appears that the animals receiving intermediate chain triacylglycerols preserve their linoleate store less efficiently than the others. It is difficult to transfer these findings to rats receiving CO, which contains both medium (22%) and intermediate (64%) chain triacylglycerols.

Triacylglycerols. The decrease of long chain triacylglycerols observed in the perirenal adipose tissue of CO-fed rats paralleled the changes in the fatty acid composition as described above. The triacylglycerol groups C50-C56, which accounted for 93% in TC (1), decreased to 22% after 6 weeks. This decrease cannot be explained only by an accumulation of triacylglycerols newly synthesized from endogenous long chain and exogenous medium chain fatty acids, even though the relative content of previously stored long chain triacylglycerols is reduced because the perirenal adipose tissue increases in size during aging (69). The analyses of triacylglycerols in groups, classes and types indicated that the experimental and random distributions in TCO were very close on average. The 10 major types of triacylglycerols in TC (1,2), which were mixed combinations of 16:0, 16:1, 18:1 and 18:2 and accounted for 66.1% in total, did not exceed 13.4% in TCO, and these experimental percentages were very close to the random percentages in both fats—65.1% in TC (1,2) and 12.4% in TCO. There appears to be no evidence for a significant remainder of the primitive long chain triacylglycerols, which would not have been rearranged after 6 weeks of CO feeding. Our data suggest that intermolecular rearrangement of triacylglycerol fatty acids in adipose tissue occurs rapidly. This is probably a result of hydrolysis of stored triacylglycerols and reesterification of part of released fatty acids, along with fatty acids synthesized de novo by adipocytes or newly taken up from blood, although the rate of reesterification of lipolyzed free fatty acids (70) is not known in rats fed intermediate chain triacylglycerols. Triacylglycerol fatty acid turnover in adipose tissue through the lipolysis/reesterification cycle seems considerably faster than previous estimates (71-75).

Several authors (24,30,76-84) have shown that administration of a diet containing saturated fat, including CO, which modifies cellular lipid contents and fatty acid compositions, induces alterations in a variety of cellular functions such as enzyme activity (lipolysis and lipogenesis) and nutrient uptake. Furthermore, it has been suggested that exogenous fatty acids could control the relative proportions of the fatty acids entering each position of the triacylglycerols by factors that probably include the acyltransferases of the glycerophosphate pathway and other enzymes (85,86). Indeed, it is well-established that, with rare exceptions, linoleate is predominantly esterified to the position *sn*-2 of animal fats (87). Therefore, TC contained a higher experimental proportion of the triacylglycerol types in class 012 and a lower proportion in classes 022, 122 and 222, than was expected from their random proportions (1,2). However, the differences between the experimental and random distributions of long chain triacylglycerol types in TCO were sometimes less than in TC or disappeared, as in the case of 16:0 18:1 18:2, and even were reversed as for 16:0 16:0 18:1, 16:0 16:1 18:2 and 16:1 18:1 18:2. These results are in agreement with those of Guesnet et al. (54) who have observed a great modification of the distribution of long chain fatty acids among the three positions of rat fat triacylglycerols after CO feeding. These authors pointed out that the long chain fatty acids, except linoleate, tend to be distributed much more equally among each of the three positions than in rats on a stock diet. Though this paralleled the appearance of laurate preferentially esterified in external position, the decrease in linoleate could contribute to these modifications as well. The supply of exogenous intermediate chain fatty acids may control the relative proportions of the fatty acids entering triacylglycerols, as already suggested for exogenous long chain fatty acids (85,88-90).

It is noteworthy that, considered as a whole, the differences between the experimental and random proportions of groups, classes, and types are weak in TCO. This suggests that intermediate and long chain fatty acids could belong to a single homogeneous compartment for triacylglycerol synthesis. Apparently, this conclusion does not agree with those of other authors. It has been suggested that free fatty acids and acylglycerols in the adipocyte would be shared between several semi-independent pools according to their origin, extracellular or intracellular (fatty acids issued from either de novo synthesis or lipolysis) (88,91,92), as observed in liver (93) and intestine (94). Interestingly, Awad and Chattopadhyay (83) reported that the fatty acid composition of intracellular free fatty acids did not reflect that of triacylglycerols of adipose tissue or dietary fat when the rats were fed CO or other fats. This composition with a high level of palmitate, stearate and arachidonate and a very low level of linoleate could correspond instead to that of membrane phospholipids. Therefore, the major part of intracellular free fatty acids would not belong to the pool of triacylglycerol synthesis, which would be small and rapid.

The absence of very significant differences between the experimental and random proportions of triacylglycerol types in TCO could also suggest that there is no obvious evidence for any specificity of acyltransferases towards chain length. Although lauroyl-CoA has been found to be a much less effective substrate than long chain acyl-CoA for *sn*-1-acylglycerol-3-phosphate acyltransferase when assessed in vitro in rat liver (95) and cow mammary gland (96), the esterifying enzymes of adipose tissue would be able to transfer saturated intermediate chain fatty acids into any position of the glycerol skeleton—even into position *sn*-2—as was proven by the presence of trilauroylglycerol in TCO. Myristate has also been found to be distributed equally among the three positions of the adipose tissue triacylglycerols of CO-fed rats (54). However, under the same conditions, laurate was incorporated preferentially into the external position (97-99), especially the position *sn*-3 (54), as observed for medium chain fatty acids as well (34). This is in agreement with the results of Coleman and Bell (100) who found the highest activity of the microsomal 1,2-diacylglycerol acyltransferase from isolated rat fat cells towards decanoyl- and lauroyl-CoA. We observed that the experimental proportions of dilauroyllinoleoylglycerol (3.2%) and dilauroylglycerols (18.6%), in general, in TCO were a little higher than the calculated values of random distribution (2.8% and 15.6%, respectively). Laurate, in the presence of long

chain fatty acids, has been found to be preferentially esterified to the position *sn*-1 (54) probably for reasons of physico-chemical stability of the triacylglycerol, as compared with the position *sn*-2, to which unsaturated fatty acids are predominantly attached (101). Therefore, laurate is probably a good substrate for *sn*-glycerol-3-phosphate acyltransferase.

We also observed that the shorter the chain length of the triacylglycerol types of the class 000 in TCO, the more readily the triacylglycerols were synthesized (Table 6). Furthermore, trilauroylglycerol was largely present (2%) in TCO, as expected from its calculated proportion, whereas the experimental percentage of tripalmitoylglycerol in TC was 2.3 times lower than its random percentage (1,2). These observations agree with the fact that 1,2-dilauroyl- and 1,2-dimyristoylglycerol are better substrates than 1,2-dipalmitoylglycerol for the 1,2-diacylglycerol acyltransferase when assessed in rat (100) and chicken (102) adipose tissue. It can, therefore, be concluded that intermediate chain fatty acids are, in general, slightly better utilized by acyltransferases than saturated long chain fatty acids.

The final pattern of stored triacylglycerols may also depend on the specificities of the two lipolytic enzymes, hormone-sensitive lipase and monoacylglycerol lipase (103), through the lipolysis-reesterification cycle. Whereas, during lipolysis, some authors (73,104) found no preferential release of adipose tissue triacylglycerol fatty acids, others (98,105-107) observed a trend to a preferential release of saturated long chains. This could be the result of the positional specificity of hormone-sensitive lipase that splits external bonds (108). Reesterification by the 2-monoacylglycerol pathway (109) of part of 2-monolinoleoylglycerol, which would escape monoacylglycerol lipase, could contribute to the retention of linoleate in the body fat of animals fed a diet with low levels of essential fatty acid (65-68). Although triacylglycerol lipase activity is higher when using a CO emulsion as substrate, instead of triolein (110), intermediate chain fatty acids from adipose tissue of rats fed a CO diet have been found to be mobilized at the same rate (107) or at a lesser rate (98) than longer chain fatty acids.

In conclusion, our results support the view that lauric acid is a good substrate for adipose tissue acyltransferases in vivo. However, even if the laurate content in adipose tissue triacylglycerols from CO-fed rats is quickly stabilized (ca. 3 weeks), further studies would be necessary to gain more information on the time course of triacylglycerol reshuffle.

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Reaction Products of α-Tocopherol With A Free Radical Initiator, 2,2′-Azo*bis*(2,4-dimethylvaleronitrile)

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α-Tocopherol was reacted with an alkylperoxyl radical at 37°C in ethanol. 2,2′-Azo*bis*(2,4-dimethylvaleronitrile) was used to generate the alkylperoxyl radicals. The reaction products of α-tocopherol were isolated by reverse-phase and normal-phase high performance liquid chromatography, and their structures were characterized by infrared, ultraviolet, ^1H and ^{13}C nuclear magnetic resonances and mass spectrometry. They were 8a*S*-hydroperoxy-α-tocopherone, 8a*R*-hydroperoxy-α-tocopherone, a mixure of 7,8-epoxy-8a*S*-ethoxy-α-tocopherone and 7,8-epoxy-8a*R*-ethoxy-α-tocopherone, 8a*S*-(1*R*-cyano-1,3-dimethyl)butylperoxy-α-tocopherone, 8a*S*-(1*S*-cyano-1,3-dimethyl)butylperoxy-α-tocopherone, a mixture of 8a*R*-(1*R*-cyano-1,3-dimethyl)butylperoxy-α-tocopherone and 8a*R*-(1*S*-cyano-1,3-dimethyl)butylperoxy-α-tocopherone, 4a*S*,5*R*-epoxy-8a*R*-ethoxy-α-tocopherone, 4a*R*,5*S*-epoxy-8a*S*-ethoxy-α-tocopherone, 4a*S*,5*R*-epoxy-8a*S*-ethoxy-α-tocopherone and 4a*R*,5*S*-epoxy-8a*R*-ethoxy-α-tocopherone.
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α-Tocopherol is an important natural antioxidant in food and living cells. The fundamental chemistry of the inhibition of lipid peroxidation by α-tocopherol, both in homogeneous solution (1-3) and in aqueous dispersion (4-7), has been studied extensively. One molecule of α-tocopherol can scavenge two lipid-peroxyl radicals (1). However, the detailed reaction products of α-tocopherol with peroxyl radicals have not yet been satisfactorily elucidated, even in vitro.

α-Tocopherol is very reactive toward a variety of free radicals and active oxygen species and forms various products. For example, α-tocopherol traps radicals generated from certain radical initiators such as benzoyl peroxide (8), 2,2′-azo*bis*(isobutylnitrile) (9) and *t*-butyl hydroperoxide (10-12), and it reacts with alkyl and alkoxyl radicals to give alkylated derivatives (13-16). Free radical oxidation of α-tocopherol takes place via the tocopheroxyl radical as an intermediate (17,18). If a suitable lipid-peroxyl radical is present, a product may be formed from the coupling of the lipid-peroxyl radical with the tocopheroxyl radical. Winterle et al. (19) reported that α-tocopherol formed 8a-alkylperoxy-α-tocopherones via reaction of alkylperoxyl radical with α-tocopheroxyl radical. Matsumoto et al. (20) also reported that the reaction products of an α-tocopherol model compound, 2,2,5,7,8-pentamethylchroman-6-ol, with *t*-butylperoxyl radical were 8a-peroxychroman epoxides.

We have studied the reaction products of α-tocopherol with an alkylperoxyl radical in ethanol. 2,2′-Azo*bis*(2,4-dimethylvaleronitrile) (AMVN) was used as the alkylperoxyl radical-generating source, in which the alkyl radical from the thermal decomposition of AMVN reacts with molecular oxygen to generate the alkylperoxyl radical (2).

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Abbreviations: AMVN, 2,2′-azo*bis*(2,4-dimethylvaleronitrile); HPLC, high performance liquid chromatography; TLC, thin layer chromatography; NMR, nuclear magnetic resonance; MS, mass spec(tra, trometry); 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 purified by Sephadex LH-20 column chromatography (21). A free radical initiator, AMVN, was purchased from Wako Pure Chemical Ind. (Osaka, Japan) and used without further purification. 8a-Hydroperoxy-α-tocopherone was produced from α-tocopherol by photosensitized oxidation (22). All other chemicals were obtained from common laboratory suppliers. 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 875 UV detector. Reverse-phase HPLC was done either with a μBondasphere 5μ C18 100Å column (3.9 × 150 mm, Nihon Waters Ltd., Tokyo, Japan) or a Wakogel LC ODS-10H column (7.6 × 250 mm, Wako Pure Chemical Ind., Osaka, Japan) developed with methanol at a flow rate of 0.7 or 3.0 ml/min. Normal-phase HPLC was done with a LiChrosorb Si 60 column (4.6 × 250 mm, Merck, Darmstadt, FRG) developed with hexane/isopropanol (98:2 or 95:5, v/v) at a flow rate of 1.5 ml/min. The eluent was monitored by an absorbance at 250 nm.

Reaction procedure. α-Tocopherol (1.29 g, 3 mmol) and AMVN (3.72 g, 15 mmol) were dissolved in ethanol (500 ml) and incubated at 37°C for 34 hr under air. After the reaction was complete, hexane and distilled water (500 ml each) were added to the reaction mixture and the upper organic phase was washed twice with 100 ml of distilled water to remove ethanol, and the solvent was removed in vacuo. The oily residue obtained was then dissolved in 50 ml of ethanol and allowed to stand at −20°C for 2 hr. The resulting crystals of AMVN were removed by filtration. The filtrate was analyzed by reverse-phase HPLC. The compounds responsible for the peaks that appeared to be the reaction products of α-tocopherol were isolated by reverse-phase and normal-phase HPLC. Both the disappearance of α-tocopherol in the reaction mixture and the purities of isolated compounds were checked by thin layer chromatography (TLC) on precoated silica gel 60 plates (Merck, Darmstadt, FRG) using hexane/ethyl ether (80:20, v/v). The isolated compounds were dissolved in ethanol and stored at −20°C until analysis. The peroxide values were determined by the iodometric method (23). Benzoyl peroxide was used as the standard peroxide.

Spectroscopy. Proton (^1H) and carbon-13 (^{13}C) nu-

clear magnetic resonance (NMR) spectra were recorded on a JEOL JNM-GX-270 FT NMR spectrometer with $CDCl_3$ as the solvent and with tetramethylsilane as the internal standard. NMR spectra were measured at ca. 27°C; ^{13}C NMR at 67.8 MHz with proton decoupling, and ^1H NMR at 270.05 MHz. 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 with an ionizing energy of 70 eV. 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 Yanaco OR-50 automatic polarimeter.

RESULTS

AMVN decomposes at 37°C to form alkyl radicals which, in turn, react with molecular oxygen to form alkylperoxyl radicals. The resulting alkylperoxyl radical reacts with α-tocopherol. The reaction was monitored by TLC; the α-tocopherol (R_f 0.45) disappeared after 34 hr incubation. The reaction products of α-tocopherol with AMVN were analyzed by reverse-phase HPLC (Fig. 1). Eight major peaks, **1**, **2**, **3**, **4**, **5**, **6**, **7** and **8**, in addition to some minor peak, appeared on the chromatogram. Each peak was collected by preparative reverse-phase HPLC. Peaks **1** and **4** could each be separated into two peaks (**1a** and **1b** from **1** and **4a** and **4b** from **4**, respectively) by the normal-phase HPLC using hexane/isopropanol (95:5, v/v for **1** and 98:2, v/v for **4**, respectively) as eluents. The structures of **1a**, **1b**, **2**, **3**, **4a**, **4b**, **5**, **6**, **7** and **8** were determined as described in Scheme 1.

1a and **1b** were obtained as pale yellow oils (**1a**: R_f 0.20, yield 23.5 mg; **1b**: R_f 0.16, yield 23.3 mg). Their spectral data were essentially identical with authentic 8a-hydroperoxy-α-tocopherone (22,24,25). **1a**, 8a*S*-Hydroperoxy-α-tocopherone: MS *m/z* 462 (M^+, 1%); IR (film) ν 3350 cm^{-1} (OOH), 1640; UV (ethanol) λ 240 nm (ε 12000); ^1H NMR ($CDCl_3$) δ 0.86-0.92 ppm (*m*, 12H), 1.05-2.30 (broad *m*, 23H), 1.08 (*s*, 3H), 1.86 (*s*, 6H), 1.97 (*s*, 3H), 2.64 (broad *t*, 2H), 8.16 ppm (*s*, 1H); ^{13}C NMR ($CDCl_3$) δ 10.8 ppm, 11.3, 13.2, 19.7, 19.8, 21.1, 21.9, 22.6, 22.7, 24.5, 24.8, 25.2, 28.0, 31.8, 32.7, 32.8, 37.3 (2 atoms), 37.5 (2 atoms), 39.4, 43.4, 77.2, 98.3, 132.2, 132.6, 145.2, 148.3, 185.4; $[\alpha]_D^{25}$ +23 (*c* 0.47, ethanol). **1b**, 8a*R*-Hydroperoxy-α-tocopherone: MS *m/z* 462 (M^+, 1%); IR (film) ν 3350 cm^{-1} (OOH), 1640; UV (ethanol) λ 240 nm (ε 10200); ^1H NMR ($CDCl_3$) δ 0.83-0.88 ppm (*m*, 12H), 1.05-2.30 (broad *m*, 23H), 1.36 (*s*, 3H), 1.88 (*s*, 3H), 1.89 (*s*, 3H), 1.98 (*s*, 3H), 2.63 (broad *t*, 2H), 7.71 (*s*, 1H); ^{13}C NMR ($CDCl_3$) δ 10.8 ppm, 11.3, 13.4, 19.6, 19.8 (2 atoms), 21.4, 22.6, 22.7, 24.4, 24.8, 26.8, 28.0, 32.6, 32.8 (2 atoms), 37.3 (2 atoms), 37.5 (2 atoms), 39.4, 41.7, 77.2, 98.6, 132.3, 132.4, 145.0, 148.1, 185.4; $[\alpha]_D^{25}$ −2 (*c* 0.09, ethanol).

Compound **2** was obtained as a colorless oil (yield 39.2 mg) that gave two spots on a TLC plate (R_f 0.68 and 0.64). Compound **2** was identified as a mixture of 7,8-epoxy-8a*S*-ethoxy-α-tocopherone and 7,8-epoxy-8a*R*-ethoxy-α-tocopherone: MS *m/z* 490 (M^+, 1%), 447 (36), 445 (37), 430 (14), 417 (58), 265 (45), 225 (100), 182 (62), 165 (39), IR (film) ν 1680 cm^{-1}; UV (ethanol) λ 248 nm (ε 7880); ^1H NMR ($CDCl_3$) δ 0.84-0.88 ppm (*m*, 12H), 1.00-2.60 (broad *m*, 28H), 1.15 (*s*, 3/2H, shielded 2-CH_3), 1.37 (*s*, 3/2H, deshielded 2-CH_3), 1.46 (*s*, 3H), 1.50 (*s*, 3H), 1.80 (*s*, 3H), 2.91 (*q*, 2H); ^{13}C NMR ($CDCl_3$) δ 11.2 ppm, 12.0, 15.5 (2 atoms), 19.7, 19.8, 21.1 and 21.4, 22.3 and 22.5, 22.6, 22.7, 24.5, 24.8, 25.3 and 27.0, 28.0, 31.3 and 31.5, 32.7, 32.8, 37.3, 37.5 (3 atoms), 39.4, 41.6 and 43.4, 58.2 and 58.3, 62.4, 65.0 and 65.1, 75.6 and 75.7, 96.9 and 97.0, 128.6 and 128.7, 143.9 and 144.0, 195.8 and 195.9. The stereochemistry of the epoxy group was not investigated. Compound **2**, in the presence of 0.1 M HCl, decomposed rapidly to α-tocopherylquinone 5,6-oxide: MS *m/z* 462 (M^+, 2%), 444 (4), 419 (11), 402 (16), 237 (47), 195 (100); IR (film) ν 3550 cm^{-1}, 1685; UV (ethanol) λ 274 nm (ε 4500); ^1H NMR ($CDCl_3$) δ 0.83-0.88 ppm (*m*, 12H), 1.02-2.10

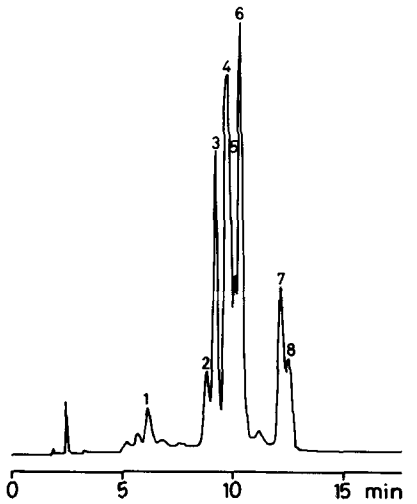
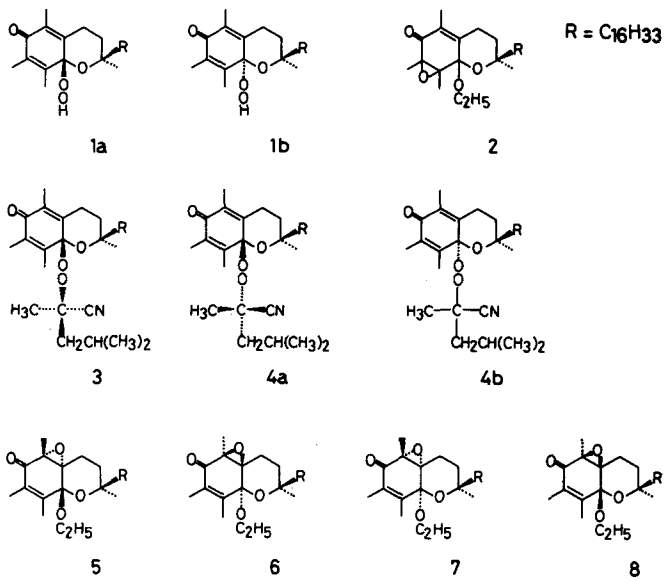


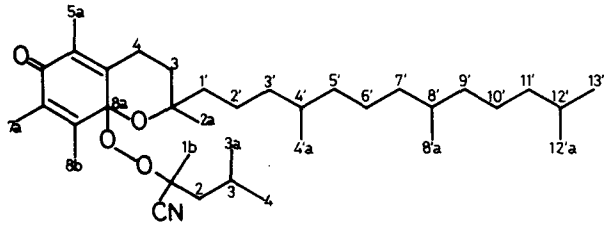
FIG. 1. Reverse-phase HPLC of the products of the reaction of α-tocopherol with AMVN in ethanol for 34 hr. HPLC was done with a μBondasphere 5μ C18 100Å column developed with methanol at a flow rate of 0.7 ml/min. The eluent was monitored by an absorbance at 250 nm.



SCHEME 1

TABLE 1

^{1}H NMR Chemical Shifts of 3, 4a and 4b



3	4a	4b	Proton assignment
0.81–0.88[a] (m[b], 18H)	0.77–0.88 (m, 18H)	0.82–0.88 (m 18H)	4'a, 8'a, 12'a, 13' 8a-(3a), 8a-(4)
1.00–2.32 (broad m, 26H)	1.05–2.70 (broad m, 26H)	1.05–2.07 (broad m, 26H)	3, 1', 2', 3', 4', 5', 6', 7', 8', 9', 10', 11', 12', 8a-(2), 8a-(3)
1.11 (s, 3H)	1.11 (s, 3H)	1.39 (s, 3H)	2a
1.55 (s, 3H)	1.60 (s, 3H)	1.56 (s, 3H)	8a-(1b)
1.86 (s, 3H)	1.84 (s, 3H)	1.858 and 1.862[c] (s, 3H)	7a
1.86 (s, 3H)	1.87 (s, 3H)	1.87 (s, 3H)	5a
2.02 (s, 3H)	1.92 (s, 3H)	2.01 and 2.02[c] (s, 3H)	8b
2.62 (t, J= 6.11 Hz, 2H)	2.82 (broad t, 2H)	2.61 (t, J = 6.40 Hz, 2H)	4

[a]Shifts in parts per million downfield relative to tetramethylsilane.
[b]Multiplicity: s, singlet; d, doublet; t, triplet; m, multiplet.
[c]A double peak due to epimeric isomers.

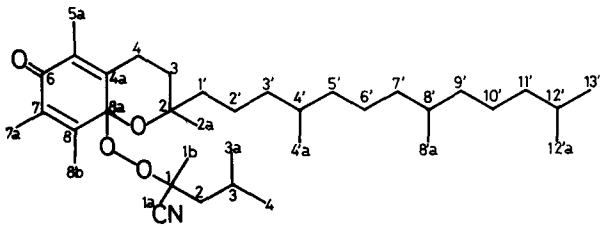
(broad m, 24H), 1.21 (s, 3H), 1.60 (s, 6H), 2.00 (s, 3H), 2.38 (m, 1H), 2.60 (m, 1H).

Compounds **3**, **4a** and **4b** were obtained as colorless oils (**3**: R_f 0.54, yield 98.5 mg; **4a**: R_f 0.62, yield 116.3 mg; **4b**: R_f 0.57, yield 82.2 mg). UV spectra of these compounds showed the same absorption maximum at 240 nm in ethanol (**3**: ε 12600, **4a**: ε 12800, **4b**: ε 11600). These spectra are very similar to those of **1a** and **1b** or to the 8a-alkylperoxy-α-tocopherone reported by Winterle et al. (19), which is formed by reaction of alkylperoxyl radicals and α-tocopherol. The ^{1}H NMR spectra of **3**, **4a** and **4b** were consistent with those expected for 8a-(1-cyano-1,3-dimethyl)butylperoxy-α-tocopherones (Table 1). In particular, the geometrical relationship between the compounds can be established by interpretation of the spectra. When the 8a-peroxy group is *trans* to the 2-methyl group (**3** and **4a**), the 2-methyl group (1.11 ppm) is localized in a shielding environment above the plane of the enone system. When the 8a-peroxy group is *cis* to the 2-methyl group (**4b**), deshielding of the 2-methyl group (1.39 ppm) is observed. Furthermore, the stereochemistry of the cyano group in the 8a-(1-cyano-1,3-dimethyl)butylperoxy group was determined. When the cyano group is *S* (**4a**), deshieldings of the 4-methylene (2.82 ppm), 5-methyl (1.87 ppm) and 8a-(1-methyl) (1.60 ppm) groups are observed. When the cyano group is *R* (**3**), deshieldings of the 7-methyl (1.86 ppm) and 8-methyl (2.02 ppm) groups are observed. When the cyano group is a mixture of *R* and *S* (**4b**), the two methyl groups resonate as close double peaks (7-methyl: 1.858 and 1.862 ppm, 8-methyl: 2.01 and 2.02 ppm). The ^{13}C NMR spectra of **3**, **4a** and **4b** show two tertiary ethers (**3**: 77.3 and 77.7 ppm, **4a**: 77.5 and 77.9 ppm, **4b**: 77.2 and 77.7 ppm), a carbon atom bearing two oxy substituents (**3**: 97.9 ppm, **4a**: 97.7 ppm, **4b**: 98.1 ppm), a nitrile carbon (**3**: 120.2 ppm, **4a**: 120.4 ppm, **4b**: 120.2 ppm) and a single carbonyl (**3**: 185.2 ppm, **4a**: 185.2 ppm, **4b**: 185.4 ppm) (Table 2). The peroxide values of **3**, **4a** and **4b** were determined to be 1.10, 0.95 and 1.19 mol/mol of the respective compounds. Thus, each compound contains one peroxy group in the molecule. From these results and other spectral data, the structures of **3**, **4a** and **4b** were identified as follows: Compound **3**, 8a*S*-(1*R*-cyano-1,3-dimethyl)butylperoxy-α-tocopherone: MS *m/z* 471 ([M-100]$^+$, 1%), 445 (9), 430 (62), 417 (14), 221 (18), 178 (16), 165 (100); IR (film) ν 1645 cm^{-1}; $[\alpha]_D^{25}$ +20 (*c* 0.38, ethanol). Compound **4a**, 8a*S*-(1*S*-cyano-1,3-dimethyl)butylperoxy-α-tocopherone; MS *m/z* 471 ([M-100]$^+$, 1%), 445 (7), 430 (65), 417 (15), 221 (18), 178 (17), 165 (100); IR (film) ν 1645 cm^{-1}; $[\alpha]_D^{25}$ −15 (*c* 0.41, ethanol). Compound **4b**, a mixture of 8a*R*-(1*R*-cyano-1,3-dimethyl)butylperoxy-α-tocopherone and 8a*R*-(1*S*-cyano-1,3-dimethyl)butylperoxy-α-tocopherone: MS *m/z* 471 ([M-100]$^+$, 1%), 445 (20), 430 (60), 417 (21), 221 (14), 178 (16), 165 (100); IR (film) ν 1650 cm^{-1}; $[\alpha]_D^{25}$ −22 (*c* 0.29, ethanol).

Compounds **5**, **6**, **7** and **8** were obtained as colorless oils (**5**: R_f 0.71, yield 60.9 mg; **6**: R_f 0.72, yield 132.9 mg; **7**: R_f 0.83, yield 88.3 mg; **8**: R_f 0.76, yield 32.1 mg). Their structures were identified to be geometric isomers of 4a,5-epoxy-8a-ethoxy-α-tocopherone. Compound **5**, 4a*S*,5*R*-epoxy-8a*R*-ethoxy-α-tocopherone: MS *m/z* 475 ([M-CH_3]$^+$, 1%), 445 (40), 430 (6), 417 (100), 265 (13),

TABLE 2

^{13}C NMR Chemical Shifts of 3, 4a and 4b



3	4a	4b	Carbon assignment
10.7[a]	10.7	10.7	5a
11.2	11.2	11.2	7a
13.6	13.1	13.7	8b
19.7, 19.8	19.8(2)	19.6, 19.8	4'a, 8'a
21.1	21.2	21.3	2'
21.9	22.0	21.8	4
22.6, 22.7	22.7(2)	22.7, 22.8	12'a, 13'
23.3	23.2	23.3	8a-(1a)
23.5	23.5	23.5	8a-(3)
24.5	24.5	24.5	6'
24.8	24.8	24.8	10'
24.8(2)[b]	25.0(2)	24.4(2)	8a-(3a), 8a-(4)
25.8	25.8	25.8	2a
28.0	28.0	28.0	12'
32.1	32.0	33.6	3
32.8(2)	32.9(2)	32.6, 32.8	4', 8'
37.3, 37.5(2), 37.6	37.3(2), 37.5(2)	37.3(2), 37.5(2)	3', 5', 7', 9'
39.4	39.4	39.4	11'
43.2	43.2	42.2	1'
46.8	46.9	46.8	8a-(2)
77.3	77.5	77.2	2
77.7	77.9	77.7	8a-(1)
97.9	97.7	98.1	8a
120.2	120.4	120.2	8a-(1a)
131.9, 132.0	131.9, 132.1	131.6, 132.0	5, 7
144.4	145.0	144.7	4a
148.1	147.5	148.3	8
185.2	185.2	185.4	6

[a]Shifts in parts per million downfield relative to tetramethylsilane.
[b]Number of carbon in parentheses.

219 (23), 167 (61), 165 (22); IR (film) ν 1680 cm^{-1}; UV (ethanol) λ 248 nm (ε 8400); ^{1}H NMR ($CDCl_3$) δ 0.84–0.88 ppm (*m*, 12H), 1.00–2.01 (broad *m*, 23H), 1.08 (*t*, J = 6.96 Hz, 3H), 1.27 (*s*, 3H), 1.47 (*s*, 3H), 1.80 (*s*, 3H), 1.90 (*s*, 3H), 2.03 (*m*, 1H), 2.51 (*m*, 1H), 3.04 (*dq*, 1H), 3.28 (*dq*, 1H); ^{13}C NMR ($CDCl_3$) δ 10.3 ppm, 12.2, 14.4, 15.5, 19.8 (2 atoms), 20.8, 21.7, 22.6 (2 atoms), 24.6, 24.8, 27.5, 28.0, 32.8 (2 atoms), 37.3, 37.5 (2 atoms), 37.6 (2 atoms), 39.4, 41.1, 58.3, 63.1, 63.4, 77.0, 96.4, 128.9, 147.2, 196.3; $[\alpha]_D^{25}$ +35 (*c* 0.38, ethanol). Compound **6**, 4a*R*,5*S*-epoxy-8a*S*-ethoxy-α-tocopherone: MS *m/z* 475 ([M-CH_3]$^+$, 1%), 445 (51), 430 (6), 417 (100), 265 (13), 219 (20), 167 (58), 165 (21); IR (film) ν 1685 cm^{-1}; UV (ethanol) λ 251 nm (ε 7110); ^{1}H NMR ($CDCl_3$) δ 0.84–0.88 ppm (*m*, 12H), 1.00–1.90 (broad, *m*, 23H), 1.07 (*t*, J = 6.96 Hz, 3H), 1.43 (*s*, 3H), 1.48 (*s*, 3H), 1.80 (*s*, 3H), 1.90 (*s*, 3H), 1.95 (*m*, 1H), 2.65 (*dt*, J = 4.40, 13.19 Hz, 1H), 3.09 (*dq*, 1H), 3.45 (*dq*, 1H); ^{13}C NMR ($CDCl_3$) δ 10.2 ppm, 12.5, 15.1, 15.3, 19.7, 19.8, 20.4, 20.7, 22.7, 22.8, 24.5, 24.7, 24.8, 28.0, 32.7, 32.9, 33.3, 37.3 (2 atoms), 37.5 (2 atoms), 39.4, 44.9, 58.4, 63.1, 63.4, 76.0, 96.3, 128.8, 147.7, 196.9; $[\alpha]_D^{25}$ −52 (*c* 0.47, ethanol). Compound **7**, 4a*S*,5*R*-epoxy-8a*S*-ethoxy-α-tocopherone: MS *m/z* 475 ([M-CH_3]$^+$, 1%), 445 (48), 417 (100), 265 (8), 219 (25), 167 (69), 165 (20); IR (film) ν 1675 cm^{-1}; UV (ethanol) λ 247 nm (ε 7560); ^{1}H NMR ($CDCl_3$) δ 0.80–0.88 ppm (*m*, 12H), 1.00–1.90 (broad *m*, 24H), 1.21 (*t*, J = 6.96 Hz, 3H), 1.44 (*s*, 3H), 1.51 (*s*, 3H), 1.787 and 1.791 (*s*, 3H), 1.854 and 1.858 (*s*, 3H), 2.56 (*dt*, J = 4.40, 13.18 Hz, 1H), 3.18 (*dq*, J = 6.96, 9.53 Hz, 1H), 3.68 (*dq*, J = 6.96, 9.52 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 11.0 ppm, 12.2, 13.9, 16.0, 19.6, 19.8, 20.7, 20.9, 22.7, 22.8, 24.5, 24.8, 25.2, 28.0, 32.6, 32.8, 35.6, 37.3, 37.4, 37.5 (2 atoms), 39.4, 44.9, 59.1, 60.4, 66.2, 76.5, 96.4, 130.6, 149.1, 193.8; $[\alpha]_D^{25}$ + 101 (*c* 0.58, ethanol). Compound **8**, 4a*R*,5*S*-epoxy-8a*R*-ethoxy-α-tocopherone: MS *m/z* 490 (M$^+$, 2%), 475 (1), 445 (31), 417 (61), 265 (14), 219 (41), 167 (98), 165 (20), 99 (100); IR (film) ν 1675 cm^{-1}; UV (ethanol) λ 247 nm (ε 7340); ^{1}H NMR ($CDCl_3$) δ 0.82–0.90 ppm (*m*, 12H), 1.00–2.00 (broad *m*, 23H), 1.10 (*s*, 3H), 1.20 (*t*, J = 6.96 Hz, 3H), 1.51 (*s*, 3H), 1.790 and 1.794 (*s*, 3H), 1.880 and 1.884 (*s*, 3H), 2.24 (broad *t*, 1H), 2.52 (*dt*, J = 4.03, 13.19 Hz, 1H), 3.15 (*dq*, J = 7.33, 9.52 Hz, 1H), 3.68 (*dq*, J = 6.96, 9.52 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 11.0 ppm, 12.3, 13.9, 16.0, 19.7, 19.8, 20.6, 21.3, 22.7, 22.8, 24.6, 24.8,

28.0, 28.6, 32.9, 33.0, 36.7, 37.4, 37.5, 37.6, 37.9, 39.4, 39.9, 59.1, 60.3, 65.9, 76.6, 96.4, 130.8, 148.8, 193.9; $[\alpha]_D^{25}$ −82 (*c* 0.23, ethanol). Compounds **5, 6, 7** and **8**, in the presence of 0.1 M HCl, decomposed rapidly to α-tocopherylquinone 2,3-oxide (26): MS *m/z* 462 (M^+, 1%), 444 (6), 419 (54), 402 (26), 237 (54), 167 (100); IR (film) ν 3500 cm^{-1}, 1680; UV (ethanol) λ 272 nm (ε 4200); ^1H NMR ($CDCl_3$) δ 0.83–0.88 ppm (*m*, 12H), 1.05–2.05 (broad *m*, 24H), 1.21 (*s*, 3H), 1.63 (*s*, 3H), 1.81 (*m*, 1H), 1.97 (*s*, 6H), 2.29 (*m*, 1H).

DISCUSSION

α-Tocopherol is a chain-breaking antioxidant and reacts with peroxyl radicals to stop autoxidation of polyunsaturated fatty acids. The resulting tocopheroxyl radicals can react with other peroxyl radicals or with each other to form some reaction products. Although Winterle et al. (19) reported that the tocopheroxyl radical reacted with alkylperoxyl radicals to form 8a-alkylperoxy-α-tocopherones and some unknown products, they could not characterize their structures. In the present study, we have succeeded in isolating and characterizing the reaction products of α-tocopherol with the alkylperoxyl radical from AMVN in ethanol. The products are 8a-hydroperoxy-α-tocopherones (**1a** and **1b**), 7,8-epoxy-8a-ethoxy-α-tocopherone (**2**), 8a-alkylperoxy-α-tocopherones (**3, 4a** and **4b**) and 4a,5-epoxy-8a-ethoxy-α-tocopherone (**5, 6, 7** and **8**) (Scheme 1). Compounds **3, 4a** and **4b** correspond to the peroxy ketals reported by Winterle et al. (19). Compounds **1a** and **1b** have been reported to be primary products of the reaction of α-tocopherol, with singlet molecular oxygen (22,24,25) and key intermediates in the superoxide-catalyzed oxygenation of α-tocopherol (27). 4a,5-Epoxy-8a-methoxy-α-tocopherones, with structures that resemble compounds **5, 6, 7** and **8**, were obtained as the reaction products of α-tocopherol with singlet molecular oxygen (26).

The reaction of α-tocopherol (TOH) with AMVN in ethanol can be accomplished by several processes:

$$\text{AMVN} \rightarrow 2\text{R}\cdot \quad (1)$$
$$\text{R}\cdot + \text{O}_2 \rightarrow \text{ROO}\cdot \quad (2)$$
$$\text{ROO}\cdot + \text{TOH} \rightarrow \text{ROOH} + \text{TO}\cdot \quad (3)$$
$$\text{TO}\cdot + \text{ROO}\cdot \rightarrow \text{molecular products} \quad (4)$$

Because the tocopheroxyl radicals produced are resonance stabilized, they do not continue the chain reaction, but are eventually destroyed by reaction with a second peroxyl radical (reaction 4) (28). Our results indicate that the tocopheroxyl radical reacts with the alkylperoxyl radical to form **3, 4a** and **4b** as the products. If the tocopheroxyl radical reacts with a hydroperoxyl radical, **1a** and **1b** might be formed. The hydroperoxyl radical (HOO·) can be produced from the autoxidation of ethanol (29). The essential steps in the process are:

$$\text{ROO}\cdot + \text{CH}_3\text{CH}_2\text{OH} \rightarrow \text{ROOH} + \text{CH}_3\dot{\text{C}}\text{HOH} \quad (5)$$
$$\text{CH}_3\dot{\text{C}}\text{HOH} + \text{O}_2 \rightarrow \text{CH}_3\text{CHO} + \text{HOO}\cdot \quad (6)$$

The ROO· in reaction 5 may be the alkylperoxyl radical (see reaction 2). Presumably, the formation of **1a** and **1b** proceeds by this mechanism.

The formation of 8a-ethoxy compounds **2** and **5–8** must occur via the carbonium ion of α-tocopherol (TO^+) followed by reaction with the ethoxide anion (EtO^-) 30,31):

$$\text{TO}\cdot + \text{ROO}\cdot \rightarrow \text{TO}^+ + \text{ROO}^- \quad (7)$$
$$\text{TO}^+ + \text{ROO}^- \rightarrow \textbf{3, 4a and 4b} \quad (8)$$
$$\text{TO}^+ + \text{EtO}^- \rightarrow \text{8a-ethoxy-α-tocopherone} \quad (9)$$

Electron transfer might be the first step in the tocopheroxyl radical reaction with the alkylperoxyl radical (reaction 7). Collapse of this ion pair would yield **3, 4a** and **4b** (reaction 8), but the ethoxide anion would presumably be perfectly able to compete with ROO^- and it would yield 8a-ethoxy-α-tocopherone (reaction 9). Epoxides can be formed by peroxyl radical addition to double bonds followed by an intramolecular homolytic substitution process. Thus, the observed products **2** and **5–8** require peroxyl radical addition to 8a-ethoxy-α-tocopherone at the 7 and 5 positions, respectively. Matsumoto et al. (20) obtained 4a,5-epoxy-8a-alkylperoxychroman as the reaction product of the α-tocopherol model compound with *t*-butylperoxyl radical. More extensive oxidations of **3, 4a** and **4b** may produce the corresponding epoxy compounds.

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The Distribution of Lipids and Sterols in Cell Types From the Marine Sponge *Pseudaxinyssa* sp.[1]

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The sponge *Pseudaxinyssa* sp., unique in sterol and fatty acid composition, was cellularly dissected into fractions enriched in each of the major cell types present in the sponge: microbial symbionts (cyanobacteria), small sponge cells (pinacocytes and choanocytes), and large sponge cells (archeocytes and cyanophytes). Three phototrophic microbial symbionts were also isolated from the cell fractions and grown in culture. An unsymmetrical distribution of fatty acids and sterols was observed for the sponge cells: small cells contained larger quantities of long chain fatty acids (> C24) and smaller quantities of sterols than were present in the larger sponge cells. Moreover, the rare sterols 24-isopropylcholesterol predominated in the smaller sponge cells, whereas its 22-dehydro analog predominated in the larger sponge cells. Long chain fatty acids and sterols were not detected in the cultured microbial symbionts. This constitutes the first report of lipid variability according to cell type for this most primitive group of Metazoa.

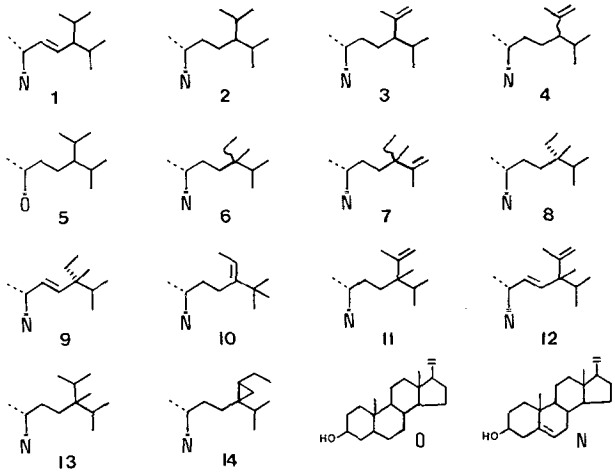
Lipids 24, 210-216 (1989).

The logical next subject for the study of lipid biosynthesis beyond the unicellular level (1) is the sponge—an organism limited to only a few cell types which are loosely cohesive without nervous cell control, but with the capacity for developing specialization and derived from the totipotent archeocyte cell. In higher organisms, the ratio of different kinds of polar lipids in membranes is characteristic of the type of membrane system, the organ and the species. Cholesterol, the most prevalent animal sterol, is not found in all membranes, and fatty acid composition varies markedly from one tissue to another (2,3). In contrast, the phylum Porifera with no real tissue development exhibits the widest diversity of sterols (4-10). Further, the major class Demospongiae has been especially noted for unusually long chain (C_{26}-C_{36}) fatty acids (11-22).

It is the common practice in this laboratory (23) and the practice of others to use whole sponges for biosynthetic studies of sterols and fatty acids. Generally included in the whole sponge are inter- and intracellular microbial symbionts. To date, few attempts have been made to assign sterol and lipid chemistry to particular sponge cell types (19,24), and only crude partial cell separations have been performed to investigate the biosynthetic roles of the symbionts (25).

It has long been known (26-28) that sponge cells can be dissociated in a calcium-magnesium-free medium to give a suspension of live cells. DeSutter and Van de Vyer (29) separated a choanocyte-enriched fraction from one enriched in archeocytes of the fresh water sponge *Ephydatia fluviatilis* by using a Ficoll gradient and noted that choanocytes aggregated very rapidly, but never formed functioning sponges. Johns et al. (30) added EDTA to further sequester the calcium and magnesium ions (to slow reaggregation), but noted that cell viability decreased. Lowenstein (31) had already shown that cells exhibited a "leakiness" at low-calcium concentrations and that hypotonically shocked cells failed to aggregate. Finally, DeSutter and Tulp (32) used a Ficoll gradient to purify gram quantities of specific sponge cell types. Hence, sponge cells can be effectively sorted according to size and density; certainly, the less small and dense pinacocytes and choanocytes can be effectively separated from the more large and dense archaeocytes and spiculocytes. In the present work, tandem Ficoll gradients were used to purify microbial symbionts and sponge cells from the demosponge *Pseudaxinyssa* sp.

Separation of sponge cells from microorganism symbionts can be more difficult than that of sponge cells from one another. Whereas eubacteria would be expected to be easily separable due to their small size and low density, cyanobacteria may not have densities much different than sponge cells. Moreover, in some sponges, bacteria are found intracellularly in the vacuoles and even within the nucleus (33). In some species, young sponges begin life with an established bacterial flora (34)—matrix bacteria are transferred into the larvae (35) or into the gemmules (36). Some chemical basis for this association already has been suggested (34,37), but any explicit role of membrane lipids in symbiosis is not known.



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Abbreviations: CMF-ASW, calcium-magnesium-free artificial sea water; TMTD, 4,8,12-trimethyltridecanoic acid; GC, gas chromatography.

The Great Barrier Reef sponge *Pseudaxinyssa* sp. is a long-lived species that contains a considerable population of cyanobacteria (blue-green algae) (25,38). Its sterol composition is quantitatively simple, but structurally unique—99% of the sterols are represented by 22-dehydro-24-isopropylcholesterol (1) and 24-isopropylcholesterol (2) (39). In addition, there are trace sterols with intriguing side chains: triply (1-10) and quadruply (11-14) methylated, including cyclopropyl (14), tert-butyl (10) and 24-dialkylated (6-9, 11-14) sterols (40,31). Conventional sterols, such as cholesterol, are absent. In addition, this sponge is known to contain unusual long chain fatty acids that Lawson (24) identified as cellular membrane constituents. *Pseudaxinyssa* sp. is, therefore, an excellent candidate for the evaluation of cell-specific lipid content.

EXPERIMENTAL

Sponge sample. Pseudaxinyssa sp. (Australian Museum specimen #Z4988) was collected by hand from the John Brewer Reef, Australian Great Barrier Reef (15 M), December 18, 1986.

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 462 mM NaCl, 8.4 mM Na_2SO_4, 11 mM KCl and 2.1 mM $NaHCO_3$.

Electron microscopy. For electron microscopy, sponge samples were prefixed in 3% glutaraldehyde/0.1 M sodium cacodylate buffer in Australia and then shipped to the USA where they were postfixed in 1% osmium tetroxide in the same buffer. After alcohol dehydration, they were embedded in epoxy resin according to Spurr (42). Silver sections were stained with 1% uranyl acetate and Reynold's (43) lead citrate and were examined with a Philips 410 electron microscope at 60 or 80 KV. The cultured cyanobacteria were fixed as above except that the prefix (in USA) was 2.5% glutaraldehyde, 4% paraformaldehyde, 2% acrolein in a 0.2 M phosphate buffer at 6.9 pH (prefix time = 18 hr).

Construction of ficoll gradients. Two types of Ficoll gradients were constructed (Fig. 1) by layering the below-designated w/w concentrations prepared from CMF-ASW. For the initial gross separation (I) and the refinement of the lighter cell fractions, Type I and II gradients were prepared by layering 5 mL aliquots of 23, 21.7, 16, 13, 9, 5, 0% and 21.7, 16, 13, 9, 5 and 0% Ficoll solutions, respectively. For the further separation and enrichment of the heavier cell fractions, Type III gradient was prepared by layering 10 mL aliquots of 35, 30, 26, 23, 21.7% Ficoll solutions. The Ficoll gradients were prepared just prior to use and kept at 3-10°C.

Dissociation of sponge cells was according to Thompson et al. (44) with the following modifications: The wet sponge (500 g) was washed 4 times in CMF-ASW, cut into cubes 0.5 cm on a side, soaked in a bath of 500 mL CMF-ASW to which 126 mg of protease enzyme had been added, and left at room temperature (23°C) for 2 hr. The resulting cloudy mixture was then filtered through cheesecloth and centrifuged at 600 *g* (MSE Mistral 41 Rotor GRMS [swing bucket]) for 5 min to produce a dark red, very dense sponge pellet. About 15% of the pellet was reserved for electron micrography (EM) analysis (Fig. 2B) and membrane analysis. The rest was suspended in 75 mL CMF-ASW and applied to the Ficoll gradients as described.

Separation of cells on ficoll gradients. To the top of a type I Ficoll gradient (Fig. 1) was added 10 mL of the resuspended pellet and the system was spun 5 min at 3°C and 600 *g* in the Mistral swing bucket centrifuge. The cells were concentrated between Ficoll layers and at the top of the column and were collected from these locations. The lightest four fractions were combined and washed free of Ficoll in CMF-ASW (250 mL × 2) via centrifugation (10,000 *g*/15 min). These cells were placed on the top of a Ficoll type II gradient and centrifuged as before. The cellular material between the different Ficoll concentrations was collected to yield the cell fractions I, II and III shown in Figure 1. Fractions IV and V were obtained from the original gradient (I) by collecting cells between the Ficoll concentrations 16-21.7 and 21.7-23%, respectively. The remaining pellet, which contained a major fraction of the original cells, was reapplied to the top of a Type III Ficoll gradient and centrifuged as before. Collection of cells between the Ficoll concentrations 22 and 26, 26 and 30, and 30 and 35% produced fractions VIa, VIb and VIc, respectively. Lipid analysis of fraction VIc and the adjacent pellet cells gave the same results, indicating that all cell types had finally been suspended. After this procedure was repeated six times, the following combined fraction weights were realized: I (42.7 mg), II (26.5 mg), III (27.2 mg), IV (19.5 mg), V (14.7 mg), VIa (2.5 mg), VIb (11.0 mg), VIc (9.9 mg), pellet

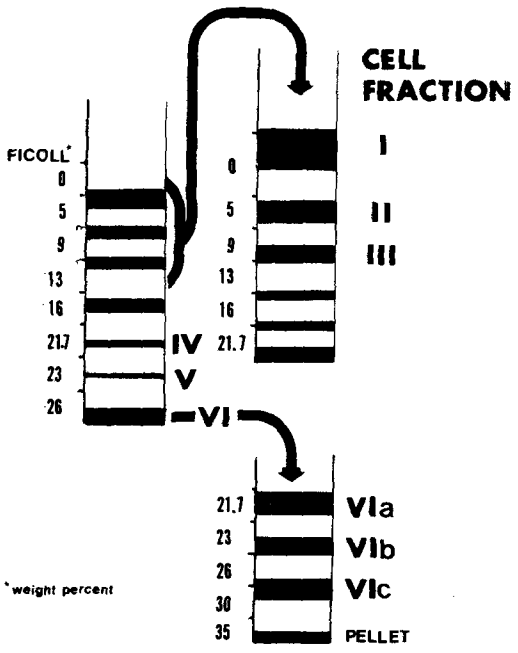

FIG. 1. Cell separation scheme using tandem Ficoll gradients. Roman numerals refer to cell fractions cited in the text. Ficoll concentrations are weight percentage in CMF-ASW.

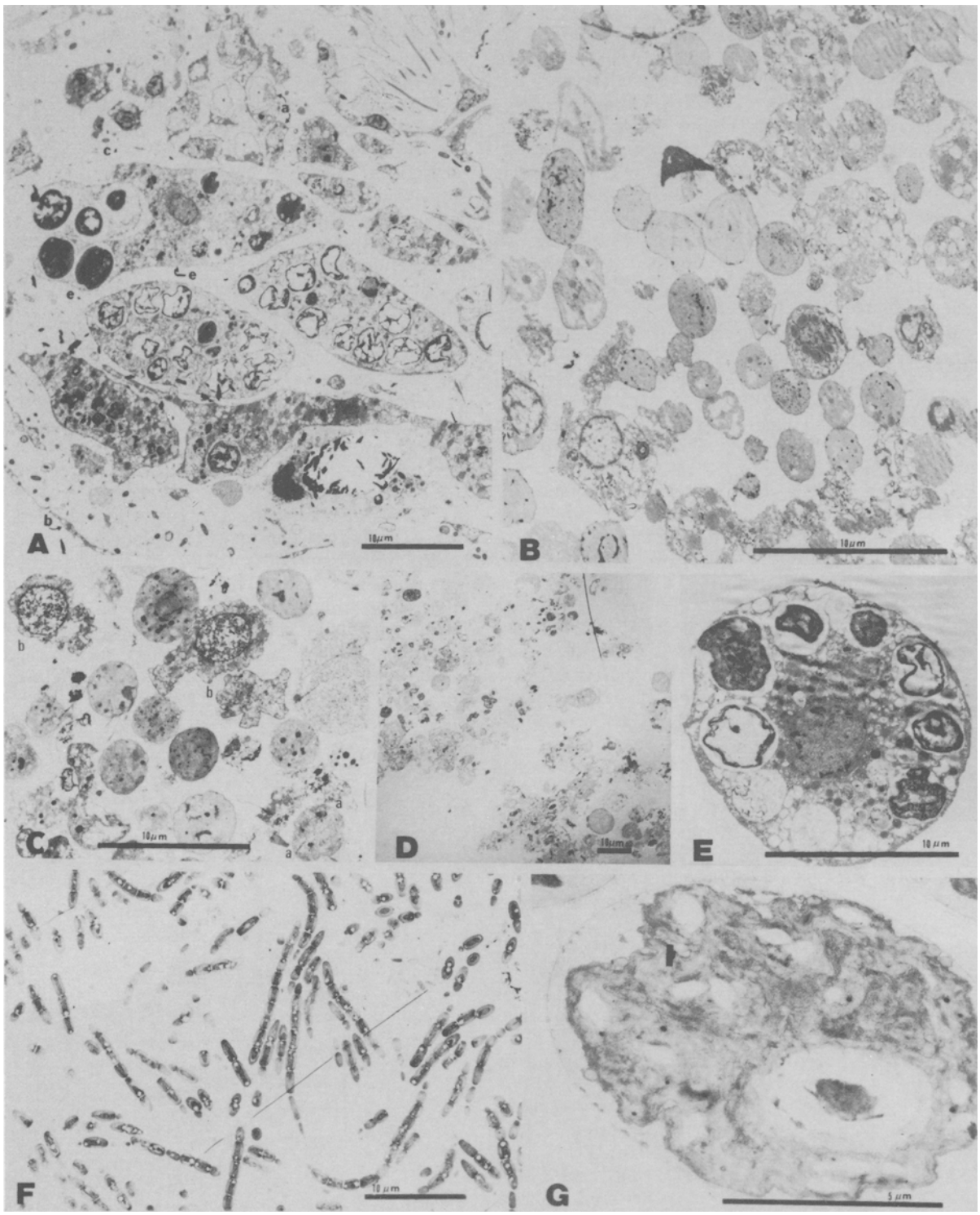

FIG. 2. ***Pseudaxinyssa*** **sp. A. Whole sponge tissue: a. choanocyte, b. pinacocyte, c. bacterium, d. archaeocyte (cyanophyte), e. intracellular cyanobacterium. B. Dissociated sponge cells prior to separation. C. Fraction II cells from Ficoll gradient cell separation: a. microbial symbiont, b. sponge cell (? choanocyte). D. Fraction IV sponge cells from Ficoll gradient cell separation. E. Fraction VIc sponge cell containing numerous microbial symbionts (a). F. Cultured cyanobacteria (CBI) from Fraction II. G. Cultured microbial symbiont (SYMIII) from Fraction II.**

(bottom cells, 43.3 mg). The cells were then identified by size and morphology.

Chemical analysis of cell fractions. A sample cell fraction was freeze-dried and extracted with 3 × 1 mL of $CHCl_3/CH_3OH$ (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 under a stream of nitrogen; then 1 mL of 1.15 N HCl (CH_3OH) was added and the reaction was heated at reflux for 1 hr. The solvents and reagents were then azeotropically removed with toluene under a stream of N_2. The resulting material was dissolved in 0.25 mL toluene and passed through a 0.5 ml florosil (toluene) column with toluene washes (1 mL × 5). After evaporation of the solvent (N_2), the resulting fatty acid methyl esters and sterols were collectively analyzed by capillary gas chromatography (GC) on a Hewlett-Packard 5790A series gas chromatograph equipped with a 25 m (0.3 mm i.d.) SE-54 coated fused silica column programmed at 170-320°C, 5°C min^{-1}; injector 250°C; detector temperature 300°C; automatic injector system (sampler model 7672A), injector (Model 3392A), sampler/event control module (model 19405A) and a computerized fatty acid system developed in our laboratory and previously used for the fatty acid analysis of *Pseudaxinyssa* sp. (45,46).

Sterol analysis. Under the above conditions of gas chromatography, the two sterols that constitute 99% of the nonsaponifiable lipid of *Pseudaxinyssa* sp., 24-isopropylcholesterol (2) and its 22-dehydro analog (1), were detected after all long chain fatty acids had come off the column. Sterol retention times were confirmed by injections of these known sterols.

Culture of symbionts. Multiple cultures were made from Ficoll fractions I-VI. Plates were inoculated directly from the Ficoll gradient on MN medium made with Difco agar (DIFCO Laboratories, Detroit, MI) as described by Rippka et al. (47). After substantial growth on solid medium, the culture was placed in liquid medium which, after several weeks, gave ~ 100 mg wet material for both chemical and EM analysis

RESULTS

Electron micrographs of the whole sponge *Pseudaxinyssa* sp. (Fig. 2A) show distinct cell types that we would expect to separate (or enrich) via a Ficoll gradient: small cells or choanocytes (a in Fig. 2A) with cell dimensions ~4.25 × 2 μm and a nuclear diameter of 0.87 μm; pinacocytes (b in Fig. 2A) with cell dimensions of 3 × 4 μm; and large cells or cyanophytes (e in Fig. 2A), which are cyanobacteria-containing arachaeocytes, with dimensions of 28.7 × 9.25 μm and nuclear diameters of 3.2 μm. Also found were other archeocytes and spiculocytes.

Dissociation of the whole sponge yielded intact cells independent of the mesophyl matrix; a small number of cells were broken by the dissociation process resulting in the presence of cell fragments (Fig. 2B). The dissociated cells took on a more rounded form, making morphological assignments difficult. The fatty acid and sterol analyses of this cell mixture (Figs. 3-4) agrees qualitatively and quantitatively with that reported from the whole sponge (45). Therefore, the dissociated cell mixture was a valid representation of the

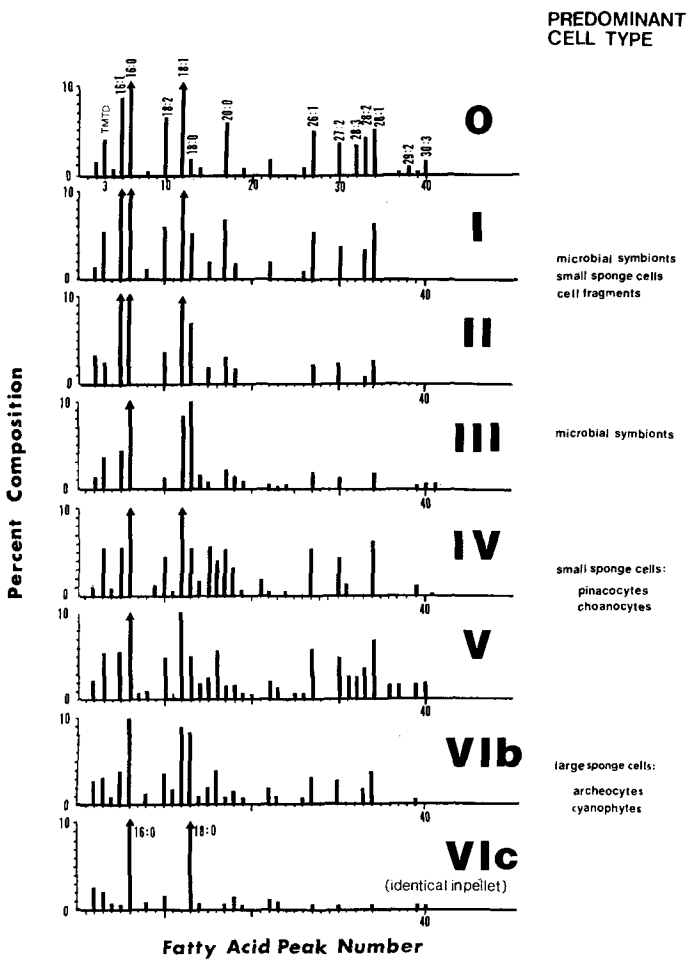

FIG. 3. Fatty acid analysis of Ficoll cell fractions. Fraction 0 represents analysis of dissociated cells from whole *Pseudaxinyssa* sp. prior to fractionation. Arrows indicate fatty acid contributions greater than 10% of total. These values are: 0 (peak 6, 18%; peak 12, 17%); I (5,13; 6,32; 12,30); II (5,12; 6,29; 12,26); III (6,39); IV (6,19; 12,11); V (6,20); IVb (6,23); VIc (6,38; 13,24). The chemical nature of the major fatty acids is indicated in Ficoll cell fraction 0 and in Table 1. (Note the percentage composition is of the fatty acids only.)

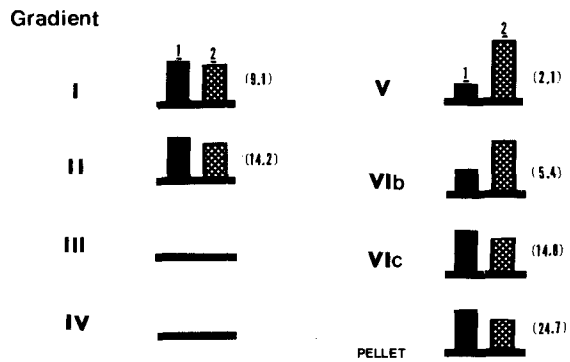

FIG. 4. Distribution of major sterols in Ficoll density gradient fractions. Graphed are the relative amount of each sterol in each cell fraction: 22-dehydro-24-isopropylcholesterol (1) vs 24-isopropylcholesterol (2). In parentheses is the percentage contribution to lipid (sterol + fatty acid) composition of the sterols in each fraction.

TABLE 1.

Fatty Acids Present in *Pseudaxinyssa* sp. According to Equivalent Chain Length (ECL), as Determined by GC

Peak no. (Fig. 3)	Abundance[a]	ECL	Identity	Peak no. (Fig. 3)	Abundance[a]	ECL	Identity
1	t	13.393	C14:1 br	22	2.2	23.788	C24:1
2	1.5	14.001	C14:0	23	t	23.861	C24:1
3	4.2	14.488	C16:0 br (TMTD)[b]	24	t	24.661	unknown
4	0.6	15.000	C15:0	25	t	24.996	C25:0
5	9.8	15.818	C16:1 *cis*9	26	0.7	25.651	C26.2 *cis*-5,9
6	18.1	15.999	C16:0	27	5.3	25.998	C26:1
7	t	16.421	C17:1 br	28	t	26.395	C26:0
8	0.4	16.999	C17:0	29	t	26.395	unknown
9	0.5	17.190	unknown	30	4.1	26.651	C27:2 *cis*-5,9
10	6.8	17.611	C18:2 *cis*-5,9	31	t	26.997	C27:0
11	t	17.719	C18:2 *cis*-9,12	32	3.8	27.489	C28:3 *cis*-5,9,21
12	16.6	17.770	C18:1 *cis*-9	33	4.5	27.561	C28:2 *cis*-5,9
13	1.9	18.000	C18:0	34	5.8	27.639	C28:1
14	0.8	19.000	C19:0	35	t	27.641	unknown
15	t	19.608	C20:2 *cis*-11	36	t	27.899	unknown
16	t	19.705	C20:1	37	0.6	27.999	C28:0
17	6.2	19.773	C20:1 *cis*-11	38	1.0	28.620	C29:2
18	t	20.000	C20:0	39	0.2	29.000	C29:0
19	1.1	21.399	C22:4	40	1.8	29.510	C30:3 *cis*-5,9,23
20	t	21.557	C22:4	41	t	30.000	C30:0
21	t	21.693	unknown				

[a]Percentage fatty acid composition free cells, fraction 0 (Fig. 3); t = < 0.2%.
[b]TMTD, 4,8,12-trimethyltridecanoic acid.

whole sponge and our cell separation was focused on obtaining cells of each cell type.

Cell separation. The first observation made from the cell separation was that the lighter fractions (I-III) were green, whereas the heavier fractions were dark red. This suggests a gross separation of phototrophic symbionts (green) from sponge cells. However, the Ficoll separation was refined enough to enrich fractions by cell type. Each fraction, therefore, was used as an inoculum for the culture of cyanobacterial symbionts, and subjected to electrom microscopy and fatty acid analysis.

The lightest four fractions (Fig. 1) were collected, combined and resubmitted to another Ficoll gradient (II). The resulting top three interfaces (between Ficoll concentrations 0-5, 5-10 and 10-14%) were collected to give fractions I, II and III. EM of these fractions (Fig. 2C) showed intercellular microbial symbionts (a), small sponge cells (b) and cell fragments. Both symbiont cells and sponge cells were of relatively small diameters (3-8 μm) compared with those cells found in the last Ficoll gradient (VIc, Fig. 2E), where cells of 15 μm diameter were common. The cells isolated with low density Ficoll appeared to suffer some osmotic shock, which likely explains the minimal observed reaggregation (31). Nonetheless, some of the small cells (b in Fig. 2C) have the gross morphology of a choanocyte (48), although a strict assignment cannot be made. The gross lipid analysis for these light fractions (I-III) is shown in Figure 3. In order to know the fatty acid contribution of the sponge cells, the contribution from the microbial symbionts had to be ascertained.

The major phototrophic symbionts of Pseudaxinyssa sp. Because the Ficoll gradient is a cell enrichment technique, each Ficoll fraction was used as a culture inoculum in order to give the broadest spectrum of phototrophic microbial symbionts. The medium chosen (MN) (47) favored growth of cyanobacteria because chlorophyll-a analysis had indicated large populations of these bacteria in the sponge (25). From this survey, three microbial symbionts were repeatedly isolated and grown in bulk. Their fatty acid content apparently makes a major contribution to the fatty acid analysis of the whole sponge (Table 1), although other microbial symbionts cannot be strictly excluded.

A green cyanobacterium (CBI) was found predominantly in the lighter Ficoll layers I-III; a red cyanobacterium (CBII) was found in all the Ficoll layers; and a green unicellular symbiont (SYMIII) was found predominantly in the lighter layers. The cyanobacteria CBI and CBII are seemingly identical in EM analysis with the red color of the latter lost during fixation. The EM (Fig. 2F) shows cylindrical cyanobacterial cells growing in straight trichomes, many more than 10 cells in length, although all cyanobacteria appear to be unicellular in the sponge, in agreement with previous studies (49). Each cyanobacterium has a dense peripheral thylakoid system surrounding a central nucleoplasm. In culture, the cells grew to 2 μm in length and 0.9 μm in width. The cells are separated from each other by deep constrictions and each cell has one and sometimes more gas vacuoles. Although seemingly identical in morphology, the cyanobacteria CBI and CBII do differ.

The two cyanobacteria CBI and CBII differ both in color and fatty acid composition. The cyanobacterium CBI which was isolated from the lighter Ficoll fractions I-III was green, whereas cyanobacterium CBII, which was present in all fractions, but dominant in the heavier ones (i.e., IVc), was red. The red bacterium CBII likely was present intracellularly, imparting the distinct red color of the cyanophytes. Both cultures contained fatty acids (Table 2) typical of cyanobacteria—primarily, C_{16} and C_{18} acids (50). In addition, both

TABLE 2

Fatty Acid Content of Cultured Microbial Symbionts Isolated from Ficoll Fraction II

	CBI	CBII	SYMIII
14:0	0.4	0.6	1.
15:0	1.	2.4	—
15:1	—	0.2	—
16:0	32.	40.	28.
16:1	21.	5.4	5.
17:0	1.	2.5	—
17:1	1.7	1.1	—
18:0	2.2	2.3	—
18:1	16.1	33.6	41.3
18:2	8.	6.3	15.
20:1	—	0.3	—
Cyclopropyl	*d*	*e*	—
Other	*b*		*c*
C16/C18	1.25	1.07	0.59

[a]Cyanobacteria (CBI and CBII) and Unknown Phototrophic Microorganism (SYMIII)
[b]Unknown % (ECL): 2.3% (15.53), 2% (15.585), 3.6% (17.42), 0.8% (17.68).
[c]Unknown % (ECL): 2% (15.586), 10% (15.654), 2% (19:1).
[d]0.6%, 19:0 cyclo C11-12.
[e]0.3%, 17:0 cyclo; 0.2%, 19:0 cyclo C11-12.

cyanobacteria have C16/C18 fatty acid ratios greater than one, another common characteristic of cyanobacteria (51). Moreover, Gillan et al. (46) has identified C16:1 *cis*-9 as a marker acid for cyanobacteria, and this is present in both the obvious cyanobacteria (CBI, II) and in the third symbiont (SYMIII) described below.

The third symbiont (SYMIII) is not an obvious cyanobacterium. It is apple green in color with an average cell diameter of 10 μm and with no obvious nucleus (Fig. 2G). Its size and morphology agree closely with another Great Barrier Reef symbiont recently reported (52) which has an ultrastructure resembling that of the chlorophyll-b-containing prokaryote *Prochloron* sp. Moreover, the fatty acid analysis of SYMIII was not typical of a cyanobacterium. The C16/C18 ratio for SYMIII (Table 2) is less than 1, suggesting that SYMIII may be more closely related to the green algae. In general, the unsaturated C18 fatty acids are predominant in all species of green algae, whereas, with some exceptions, C16 fatty acids are predominant in blue-green algae (53).

Nonsaponifiable lipids in symbionts. Sterols are generally considered to be present only in eukaryotic organisms, whereas prokaryotes seem to use carotenoids and other polyterpenoids; however, some crossover is known i.e., one class of prokaryote, Mycoplasma, requires environmental sterols (54). The present analysis ascertained by capillary GC that the major sterols in *Pseudaxinyssa* sp. were not present (to 0.03% of lipid content) in any of the three cultured symbionts.

The lipid analysis (Figs. 3-4) of the less dense Ficoll fractions (I-III) is largely dominated by the symbiont fatty acid contribution (Table 1, Fig. 3). The minor sterol and long chain fatty acid contribution is presumably from small sponge cells and cell fragments. Fraction III exemplifies heavy symbiont population, being composed largely of C16:0, C18:1, and C18:0, with no significant amount of sterols (Fig. 4). Small sponge cells largely comprise fraction IV with very little contamination from symbionts and fragments.

The ficoll gradient fractions mainly containing small sponge cells (IV, V). Progressing in descending order from fraction IV, the Ficoll density gradient contains mainly sponge cells. This is most clearly shown from the EM (Fig. 2D) which shows sponge cells of ~ 4 μm in length and very few cyanocytes or larger cell types. Some choanocytes are visible, but mostly the morphology is obscure. By an extrapolation of DeSutter's Ficoll gradient (32), the cells in fraction IV are likely pinacocytes and choanocytes. According to fatty acid analysis (Fig. 3), these fraction IV cells contain the highest concentration of long chain fatty acids—twice as many as fraction VIb—whereas fraction VIc has almost none. Coupled with this, fractions IV and V have a very low sterol content (Fig. 4). Moreover, the cells in fraction V (Fig. 4) contain a small amount of 24-isopropylcholesterol (2), but far less 22-dehydroisopropyl cholesterol (1). This discrimination favoring the saturated side chain persists into fraction VIb and thereafter is reversed (Fig. 4). (It is known that our sterol isolation procedure would not have separated these sterols.) The ratio of these sterols in the whole sponge is 1:1. Different cell types appear to be using these two sterols selectively, implying a functional dependence on whether the sterol has a double bond at position 22 in the side chain. Fractions IV and V differ from those that follow by containing the smaller sponge cells (pinacocytes and choanocytes), the cells in direct contact with the aqueous environment and those that insulate the interior of the sponge from the environment. Thus, it is not surprising that they differ in lipid constitution from the interior cells.

The ficoll gradient fractions containing mainly large sponge cells (VIb, VIc). Fraction VIc and the pellet (Fig. 1), which together constitute 33% of the dissociated cell mass, were identical in lipid content. These fractions contained large (14.7 μm diam.) cyanobacteria-containing archaeocytes (= cyanocytes). The major fatty acids were saturated C16:0 and C18:0. More than ten times as much sterol was present as was found in the small cell fractions (IV), with the major sterol being the side chain saturated 1 (Fig. 4). This is another deviation from the 1:1 ratio that the sterols exhibit on whole sponge analysis. In these cell fractions, unsaturation in the membrane lipids is predominantly found in the sterols. A fatty acid of nonbacterial origin, 4,8,12-trimethyltridecanoic acid, is present in fraction VIc, which is consistent with the composition of cells internally located in the sponge (46). This fatty acid was not present in any of our cultured symbionts. In terms of cell and lipid content, fraction VIb represents a transition between Fractions V and VIc.

CONCLUSIONS

This study demonstrates that the cells of a sponge can be separated and differentiated by combined EM and fatty acid analysis, while retaining cell viability, thus setting the stage for a new era of biosynthetic studies in sponges. At least in this study, the cells could be separated not only by size, but also by function, because the surface cells were much smaller than the

internal symbiont-infested archeocytes (cyanocytes). The surface cells (choanocytes, pinacocytes) collectively had a higher concentration of long chain fatty acids and a lower concentration of sterols than the internally located archeocytes. This was not surprising because the function of a surface cell, primarily as insulation from the marine environment, is different than that of the internal archeocytes, where growth, reproduction and other regulation are handled. The knowledge that sterols are present in high concentration in these internal cells, and that the sterol nucleus is central to cell regulation in higher forms of life, moves us closer to unlocking the mystifying role that the complex and diverse array of sterol structures present in sponges must play.

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Effects of Cholesterol Oxidation Derivatives on Cholesterol Esterifying and Cholesteryl Ester Hydrolytic Enzyme Activity of Cultured Rabbit Aortic Smooth Muscle Cells

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The effects of 5 μg/ml of 25-hydroxycholesterol; cholestane-3β,5α,6β-triol; and cholesterol on acyl CoA cholesterol acyltransferase, acid cholesteryl ester hydrolase and neutral cholesteryl ester hydrolase was studied in cultured rabbit aortic smooth muscle cells. After 1 hour incubation, 25-hydroxycholesterol resulted in a fourfold stimulation of acyl CoA cholesterol acyltransferase activity. No stimulation by 25-hydroxycholesterol was noted before 15 minutes or after 5 hours of incubation. Neither cholestane-3β,5α,6β-triol nor cholesterol influenced acyl CoA cholesterol acyltransferase activity at any time interval. No significant effects of any of the sterols were noted on acid cholesteryl ester hydrolase or neutral cholesteryl ester hydrolase activity. The imbalance between acyl CoA cholesterol acyl transferase and hydrolase activities induced by 25-hydroxycholesterol could result in cholesteryl ester accumulation by arterial smooth muscle cells, which may be associated with atherosclerosis.
Lipids 24, 217-220 (1989).

Administration of oxidized derivatives of cholesterol to rabbits has resulted in arterial endothelial injury (1) and, to pigeons, has produced a greater degree of atherosclerosis than cholesterol feeding alone (2). In vitro, these cholesterol oxidation products have been shown to be toxic to arterial smooth muscle cells (3-5) and fibroblasts (6) and to induce a number of alterations in membrane structure and function (7-9). In cultured mouse L fibroblast cells, incubation with 25-hydroxycholesterol (25-OH) or cholestane-3β,5α6β-triol (Triol) resulted in intracellular lipid accumulation (6). The cholesteryl ester content of cultured heptocytes from rabbits fed oxidized cholesterol derivatives was greatly increased as compared with hepatocytes from control or rabbits fed pure cholesterol (10). Atherosclerosis is characterized by a marked increase in the content of cholesteryl esters in the arterial wall. Cholesteryl esters in the cell are in dynamic equilibrium, and are continuously synthesized by esterifying enzymes and degraded by hydrolytic enzymes. The balance between these processes could determine the extent of cholesteryl ester accumulation. Most cholesteryl esters originate from esterification of plasma lipoprotein-derived free cholesterol by the action of the microsomal enzyme acyl CoA:cholesterol acyltransferase (ACAT) (EC 2.3.1.26). The activity of this enzyme is increased up to 70-fold during experimental atherosclerosis (11,12). Hydrolysis of arterial cholesteryl esters by a lysosomal acid cholesteryl ester hydrolase (ACEH) (EC 3.1.1.13) and a cytosolic neutral cholesteryl ester hydrolase (NCEH) (EC 3.1.1.13) may regulate the amounts of cholesteryl esters accumulating in the arterial wall. In the present study, the effects of 25-OH and Triol on ACAT, ACEH and NCEH were studied in cultured rabbit aortic smooth muscle cells to explore the mechanisms for the lipid accumulation and atherogenesis previously induced by these agents.

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Abbreviations: ACAT, acyl CoA cholesterol acyl transferase; ACEH, acid cholesteryl ester hydrolase; HDL, high density lipoprotein; HMGCoA, hydroxy methyl glutaryl coenzyme A; NCEH, neutral cholesteryl ester hydrolase; SVE, sucrose containing 1.0 mM EDTA and 1% ethanol; triol, cholestane-3β,5α,6β-triol; 25-OH, 25-hydroxycholesterol.

MATERIALS AND METHODS

Aortas of New Zealand white, young adult rabbits (2-3 kg) were aseptically removed using the ventral approach. After dissection of adventitial fat, aortic segments were immersed in basal culture medium and cut into ca. 1-mm^2 pieces. All fragments were treated with 0.2% collagenase, incubated at 37°C for 15 min to remove endothelium, washed 2× with growth medium and, then, transferred into 30-ml Falcon flasks. These explants were nourished in Eagle's medium supplemented with 10% fetal calf serum and antibiotics. Medium in the flasks was changed 3× a week. After growth to confluence (3 wk), the cells were washed with calcium- and magnesium-free phosphate-buffered saline solution. Two ml of 0.20% trypsin was added to each flask and, then, decanted off 2 min later. After incubation for 10 min, with occasional agitation, all cells were detached from the flasks. The remaining small amount of trypsin was inactivated by the addition of 10 ml of culture medium containing 10% fetal calf serum. The cells were transferred into new 75-cm^2 Falcon flasks. Confluent monolayers of aortic smooth muscle cells developed within 2 wk. Cell cultures were incubated in lipoprotein-deficient media [sera delipidized as described by Brown et al. (13)] for 24 hr prior to ACAT assay. The cell cultures were then incubated with ethanol, cholesterol, 25-OH or Triol at 5 μg/ml final concentration for 15 min, 1 hr or 5 hr. Cells were then washed 3× with media, the media discarded and the cells then scraped with a rubber policeman into 0.15 M Tris-HCl buffer, pH 7.4, containing 0.01 M mercaptoethanol and 0.5 mg/ml defatted bovine serum albumin. Cells were then completed homogenized using a glass Potter-Elvehjem homogenizer (Baxter Scientific Products, McGraw Park, IL).

Assay of ACAT activity was done using a modification of the method described by Hajjar et al. (14), using exogeneous cholesterol and radioactive fatty acyl CoA (50 mCi/mmol, New England Nuclear, Boston, MA) in phospholipid liposomes. Unilamellar liposomes were prepared by mixing 10 μCi 1-^{14}C oleyl CoA, 0.25 μmol cholesterol, 0.47 nmol oleic acid and 25 μmol

egg lecithin (Sigma Chemical Co., St. Louis, MO) in 2.0 ml methanol: methylene chloride. The solvents were removed by evaporation under N_2 followed by lyophilization. The mixture was resuspended by mechanical rotational agitation in 6.0 ml of 100 mM KCl containing 10 mM Tris-HCl buffer, pH 7.0, and 3.1 mM sodium azide. The suspension was then sonicated at 45°C for 12 min at 100 watts in a Branson sonifier, and then centrifuged at 30,000 rpm in a Beckman 50Ti rotor (Palo Alto, CA) to remove particulate matter. For the ACAT reaction aliquots of the cell homogenates were mixed with 0.25 ml of the liposomal substrate, 0.5 ml 0.15M Tris-HCl buffer, pH 7.4, containing 0.01 M mercaptoethanol and 0.05% defatted bovine serum albumin. Incubations were done in a shaking water bath at 37°C for 30 min. The reactions were stopped by addition of chloroform: methanol, 2:1, containing unlabeled cholesteryl oleate as a carrier. The lipids were extracted by the Folch procedure (15) and separated by thin layer chromatography on silica gel 250-μ plates using hexane/ethyl ether/glacial acetic acid (100:8:2) as the developing solvent. The lipids were visualized by spraying with Rhodamine 6G in phosphate buffer, the cholesteryl ester zones scraped into vials containing Aquasol-2/water (10:1) and counted in a Beckman Liquid Scintillation spectrometer (Fullerton, CA). Quenching was corrected by automated external standardization.

Assay of NCEH was done using a mixed micellar substrate (16) prepared as follows: 10 μCi of cholesteryl [1-^{14}C] oleate, (55 mCi/mmol, Amersham, Arlington Heights, IL) was freed of contaminating fatty acids by liquid-liquid partitioning using a methanol/water (1:1) phase and a chloroform/heptane (5:4) phase (17). The purified radioactive substrate was added to chloroform containing 3.8 μmol of phosphatidylcholine and 0.8 μmol of unlabeled cholesteryl oleate. The solvent was evaporated under N_2 at 37°C and the lipids suspended in 8.0 ml of 0.1 M potassium phosphate buffer, pH 7.0, containing 2.0 μmol sodium taurocholate. This was sonicated at 46°C in a Branson Sonifier (Danbury, CT) using 100 watts, then centrifuged at 30,000 rpm for 15 min in a Beckman 50Ti rotor to remove particulate matter. Incubation mixtures were prepared by adding 50 μl of the micellar cholesteryl [1-^{14}C] oleate substrate to 0.8 ml of 0.1 M phosphate buffer, pH 7.0, with 0.05% bovine serum albumin and 0.15 ml cell homogenate in the same buffer. After incubation for 60 min at 37°C, 16.3 ml of methanol/chloroform/heptane (1.4:1.3:1.0) and 5.3 ml of 50 mM potassium carbonate/50 mM potassium borate buffer, pH 10.0, was added. The mixture was agitated by mechanical rotation for 5 min, shaken for 30 min and centrifuged for 10 min at 2500 rpm to separate the phases. Radioactivities in aliquots of the upper aqueous phases containing the 1-^{14}C oleate product were counted in an Aquasol-2 scintillation solution. Micellar 1-^{14}C oleic acid standards were incubated, processed and counted in an identical manner to monitor recovery.

Assay of ACEH was done by a modification of the Haley, et al. method (18) using a micellar cholesteryl [1-^{14}C) oleate substrate, prepared as previously described for the NCEH assay. After sonication, one part of the prepared lecithin dispersion was added to 4 parts 0.125 M Na acetate buffer, pH 3.9, containing 5.0 mM Na taurocholate. Cells were homogenized with a glass mortar and pestle in 0.25 M sucrose containing 1.0 mM EDTA and 0.1% ethanol (SVE) and centrifuged for 10 min at 1,000 rpm. The pellets were rehomogenized in SVE and recentrifuged, and the combined supernatants were used as the enzyme source. These were adjusted to 0.1% digitonin and incubated for 10 min at 0°C. Equal parts of the enzyme and substrate solution were mixed and incubated at 37°C for 60 min. The reactions were stopped and the substrates and products separated, as previously described for the NCEH assay. The radioactivities of the 1-^{14}C oleate in the aqueous phase were counted, also as previously described.

Protein content of the cell homogenates was determined by the Lowry method (19).

RESULTS AND DISCUSSION

Effects of cholesterol oxides on cholesterol esterifying activity. Incubation of rabbit aortic smooth muscle cells for 1 hr with 25-OH at 5 μg/ml resulted in a ca. 4-fold increase in ACAT activity (Table 1). Cholesterol and Triol under similar conditions had no significant effects on ACAT. The effect of incubation time on ACAT enzyme activities as influenced by cholesterol and its oxidation products is indicated in Table 2. The stimulation of ACAT by 25-OH was not manifest at 15 min, was maximal at 1 hr and disappeared after 5 hr of incubation. Incubation with 25-OH for 24 hr also did not result in any stimulatory effect. Cholesterol

TABLE 1

Effect of Cholesterol Oxides on Cholesterol-Esterifying and Cholesteryl Ester Hydrolytic Enzyme Activity[a]

	Control	Chol	25-OH	Triol
ACAT	2.71 ± 1.75	2.28 ± 0.538	10.6 ± 3.09	3.10 ± 1.15
ACEH	58.4 ± 11.2	63.8 ± 19.8	47.4 ± 8.98	65.7 ± 17.2
NCEH	4.55 ± 0.61	4.46 ± 2.72	4.00 ± 0.13	4.49 ± 0.38

[a]p moles substrate esterified or hydrolyzed/mg protein/min. Cultured rabbit aortic smooth muscle cells were preincubated for 1 hr with ethanol control, cholesterol, 25-OH or Triol at 5 μg/ml. Each represents the mean of 4-6 experiments ± S.D.

[b]Significantly different than control and Triol at $p < 0.01$.

Abbreviations: ACAT, acyl CoA cholesterol acyltransferase; ACEH, acid cholesteryl ester hydrolase; NCEH, neutral cholesteryl ester hydrolase; Chol, cholesterol; 25-OH, 25 hydroxy-cholesterol; Triol, cholestane-3β, 5α, 6β-triol.

TABLE 2

Effect of Incubation Time with Cholesterol Oxides on Cholesterol-Esterifying and Cholesteryl Ester-Hydrolyzing Activity of Rabbit Aortic Smooth Muscle Cells[a]

Incubation time	15 min	1 hr	5 hr
ACAT			
Chol	66 ± 41	84 ± 37	124 ± 35
25-OH	102 ± 48	389 ± 182[b]	97 ± 34
Triol	76 ± 55	114 ± 99	84 ± 27
ACEH			
Chol	87 ± 25	109 ± 27	100 ± 24
25-OH	82 ± 25	81 ± 15	107 ± 46
Triol	97 ± 29	113 ± 25	96 ± 10
NCEH			
Chol	122 ± 61	106 ± 39	112 ± 50
25-OH	81 ± 13	90 ± 15	93 ± 22
Triol	81 ± 21	102 ± 21	112 ± 53

[a]Each value represents mean percentage of control ± S.D. of 4-6 experiments. Abbreviations are defined in Table 1.
[b]Significantly different than controls at $p < 0.01$.

and Triol had no significant effects on ACAT activity at any time interval. Stimulation of ACAT by 25-OH has been observed previously in human fibroblasts (13), mouse L fibroblast cells (20) and in rat hepatocytes (21,22). Cholesteryl ester synthesis in bovine adrenal cortical cells and microsomal preparations was not affected by 25-OH (23).

The influence of 25-OH on ACAT activity in arterial smooth muscle cells has not been studied previously. The time course of 25-OH stimulation of ACAT in this preparation differs from that in previous experiments on other cells. In human fibroblasts, the rate of cholesterol esterification increased linearly from 0 to 24 hr and was correlated with the degree of suppression of hydroxy methyl glutaryl coenzyme A (HMGCoA) reductase activity by 25-OH (13). ACAT activity after 5 hr of incubation with 25-OH was increased 8-fold over control levels. In cultured hepatocytes, stimulation of ACAT by 25-OH was maximal after 15 min, less at 4-6 hr and had disappeared after 18 hr (22). The inhibition of HMGCoA reductase by 25-OH, however, persisted for at least 22 hr. One proposed mechanism of action of 25-OH on ACAT activity is that suppression of HMGCoA reductase leads to decreased microsomal membrane cholesterol content and a reduced cholesterol/phospholipid ratio in these membranes (20). This could make exogenous cholesterol more accessible to the active sites of ACAT. According to this theory, a lag phase in 25-OH action would be expected until HMGCoA had been sufficiently suppressed. This could account for the lack of stimulation observed at 15 min in the present experiments. In arterial smooth muscle cells, suppression of HMGCoA reductase by 25-OH increases exponentially to a maximum of about 80% suppression at 1 hr (4). This time of maximum suppression correlates with the maximum stimulation of ACAT activity observed at 1-hr incubation with 25-OH in the present experiments. At later time intervals, there is possibly an adaptive effect similar to that previously observed in rat hepatocytes (22).

Effects of Cholesterol Oxides on Cholesteryl Ester Hydrolytic Activity. As seen in Tables 1 and 2, there were no significant effects of cholesterol, 25-OH or Triol on either ACEH or NCEH activity after incubation with the arterial smooth muscle cells for 15 min, 1 hr or 5 hr. Previous experiments using acetone-butanol extracts of pig aortas have shown a 4-fold stimulation of ACEH by 26-hydroxycholesterol and a 9-fold stimulation by Triol (24). It was postulated that this was due to a dispersing effect of the added sterols, improving access of the enzyme to the cholesteryl esters. The divergent results of the present experiments may be attributable to the different preparations used (intact cells vs extracts) or to the different sterol concentrations used (5 μg/ml vs 50 μg/ml) in the previous experiment.

The observed 25-OH induced stimulation of ACAT activity in arterial smooth muscle cells without a corresponding increase in cholesteryl ester hydrolase activity may result in a net accumulation of cholesteryl esters, leading to initiation or progression of atherosclerosis. Another effect of 25-OH, however, is to increase high density lipoprotein (HDL) receptor sites on bovine vascular cells (25). This may result in increased reverse cholesterol transport by HDLs, which could modulate the effect of 25-OH on ACAT by decreasing substrate availability. Determination of the net effect of 25-OH on atherogenesis, therefore, will require further experiments using in vivo models.

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METHODS

An Improved Procedure for Bile Acid Extraction and Purification and Tissue Distribution in the Rat

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Two bile acid extraction procedures were compared using endogenously radiolabeled tissues and feces. The method of Setchell et al. (*J. Lipid Res. 24,* 1085-1100, 1983) resulted in essential complete extraction, whereas that of Manes and Schneider (*J. Lipid Res. 6,* 376-377, 1971) gave recoveries between 56-82%. The time requirement for the method of Setchell et al. could be drastically reduced with no loss in extraction efficiency. Using extracts from endogenously labeled material, a purification procedure using C18 solid-phase extraction cartridges was developed that recovers >90% of bile acids. The distribution of bile acids within the intestinal tract and liver of the rat was determined.
Lipids 24, 221-223 (1989).

Bile acid metabolism has gained increasing attention in recent years in part due to the purported role of these compounds in the regulation of cholesterol synthesis (1) and the etiology of colon cancer (2), as well as their proven efficacy in gallstone dissolution (3). However, studies of bile acid metabolism have been hampered by the difficulty of extracting and purifying bile acids from biological material quantitatively. Although many extraction procedures have been described in the literature (ref. 4), it has been our experience that few of them result in greater than 80% recovery when recovery is tested by use of an added standard. Previously, one of us (DDG) had found nearly complete recovery of an added bile acid standard from rat intestinal contents using the acidified ethanol extraction procedure of Manes and Schneider (5). However, due to the reported difficulty of extracting bile acids from feces and the possibility of the acidic conditions of this procedure causing structural changes in the bile acids, we chose to explore other bile acid extraction procedures. Our objective was to develop a procedure to extract and partially purify a large number of samples in a minimum amount of time with minimum loss for later analysis of bile acids by high performance liquid chromatography. To test recoveries, we chose to use tissues and fecal material endogenously labeled with bile acids, because it has been reported that fecal samples labeled exogenously do not give an accurate measure of bile acid recovery (6,7).

MATERIALS AND METHODS

Materials. All solvents were of analytical grade and redistilled. [24-^{14}C]Chenodeoxycholic acid (sp. act. 50 mCi/mmol) was obtained from E.I. Du Pont de Nemours & Co., Biotechnology Systems Division (Boston, MA). [24-^{14}C]-Cholic acid (sp. act. 50 mCi/mmol) was obtained from Research Products International Corp. (Mount Prospect, IL). The radiopurity of each bile acid was checked by thin layer chromatography and found to be >98% for chenodeoxycholic acid and >97% for cholic acid. Both bile acids were used without further purification.

Endogenous labeling of tissue and fecal samples. ^{14}C-Cholic and ^{14}C-chenodeoxycholic acid, 0.625 uCi of each, were added to 10 ml of methanol and thoroughly mixed into 15 g of a semipurified diet. The diet was similar to the AIN-76 diet (8), except that cornstarch was the sole carbohydrate source and no cellulose was added. The diet was dried in a vacuum oven at ca. 30°C to evaporate the methanol. Following a 24 hr fast, 3 g of labeled diet was offered to each of 4 male weanling rats for 2 hr. Forty-eight hours after presentation of the labeled meal the rats were anesthesized by exposure to ethyl ether, laparotomized, and the small intestine, cecum, colon, stomach and liver removed and weighed. A blood sample was taken by cardiac puncture and plasma collected after centrifugation. Plasma volume of the animals was taken as 3.28% of body weight (9). Fecal pellets were collected from the time of feeding of the labeled diets. The collected tissues and fecal pellets were homogenized (Tissumizer, Tekmar Co., Cincinnati, OH) in distilled water, freeze-dried, weighed and stored at −20°C until bile acid extraction.

Bile acid extraction. Two different bile acid extraction methods were investigated using the endogenously labeled tissues and feces. Method 1 was the procedure described by Manes and Schneider (5). Two ml of 0.5 N HCl in absolute ethanol was added to 100 mg of lyophilized tissue in screw cap tubes. The mixture was incubated at 37°C in a shaking water bath for 1 hr, with vortexing every 15 min. Tubes were centrifuged at 1500 × *g* for 10 min and the supernatants collected. The pellet was resuspended in 2 ml of the acidified ethanol, incubated and centrifuged as above. Supernatants were pooled and evaporated with nitrogen gas at low heat (55°C).

Method 2 was the procedure of Setchell et al. (6). Two ml of absolute ethanol was added to 100 mg of sample in 20 I.D. × 125 mm screw cap tubes. Tubes were tightly capped and sonicated in a bath sonicator for 30 min. Following sonication, the tubes were placed in a Reactitherm heating block (Pierce Chemical Co., Rockford, IL) and refluxed at 100°C. Reflux times of 2 hr, 1 hr, and 15 min were tested. After refluxing, tubes were centrifuged at 1500 × *g* for 10 min, and supernatants were removed and retained. The pellet was resuspended in 2 ml 80% ethanol, refluxed and centrifuged as before. Supernatants were pooled with the first. The pellet was resuspended a third time in 2 ml chloroform/methanol (1:1, v/v) and again refluxed and centrifuged and the supernatant was decanted and pooled. The pellet was washed once by adding 2 ml of chloroform/methanol (1:1, v/v), followed by

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Abbreviations: DPM, disintegration per minute; DW, distilled water.

centrifugation and removal of the supernatant as before.

Pooled extracts and the residues remaining after extraction were quantitatively transferred to liquid scintillation vials and dried under nitrogen gas. One ml of tissue solubilizer (TS-2, Research Products International, Mount Prospect, IL) and 200 μl of distilled water were added to each vial and the vials placed on a rotary shaker for 18 hr. Two drops of glacial acetic acid and 15 ml of liquid scintillation cocktail (Safety-Solve, Research Products International, Mount Prospect, IL) were added to each vial and radioactivity was determined by liquid scintillation, counting with quenching corrected by use of an external standard. Recovery was calculated as the ratio of disintegrations per minute (DPM) in the extract to the DPM in the extract plus residue, and was expressed as a percentage.

Partial purification. Reversed-phase C18 bonded-phase cartridges (Sep-Pak, Waters Assoc., Milford, MA) were used for purification of the bile acid extracts. Recovery of bile acids from various purification procedures was tested using labeled bile acids obtained from the method 2 extraction procedure. Losses at each stage of the purification procedure were evaluated by a counting of the cartridge eluant.

Cartridges were initially solvated with 4 ml of methanol, followed by 4 ml of distilled water. To tubes containing the dried bile acid extract, 0.6 ml of methanol was added and the tubes vortexed to solubilize bile acids. Next, 2.4 ml of distilled water was added, the tubes vortexed and the solution loaded onto the cartridges at a rate of 1 ml/min using a vacuum pump. The tubes containing the extract were washed twice with 4 ml of methanol/water (20:80, v/v), with each wash subsequently loaded onto the cartridges. Combinations of distilled water (DW), hexane and methanol were used to remove water soluble and lipid impurities. Purification procedure 1 was that described (10), which was subsequently modified. The wash sequences (4 ml of each solution) for each purification procedure were as follows: (1) DW, hexane, DW, methanol/DW (40:60) washes; methanol/DW (75:25) to elute bile acids. (2) DW, hexane, methanol/DW (40:60) washes; methanol to elute bile acids. And, (3) DW, hexane washes; methanol to elute bile acids.

Bile acid distribution. One of the animals ate only a small quantity of the labeled diet, expressed almost no fecal material over the following 2 days and acted lethargic. Thus, the results from this animal were not used in determining tissue distribution.

The tissue distribution of endogenously labeled bile acids was calculated by extraction of the bile acids from small intestine, cecum, liver, colon contents and plasma, using the method 2 extraction procedure, and by determining the total amount of radioactivity in each sample.

RESULTS AND DISCUSSION

Bile acid extraction. Table 1 shows a comparison of the two extraction methods used and the effect of reducing the refluxing time in method 2. The results show that with the method of Setchell et al. (method 2) (6), almost complete extraction of bile acids was obtained from all samples tested. In contrast, the procedure of Manes and Schneider (method 1) (5) led to incomplete and variable extraction. In both methods, the poorest recovery of bile acids was from liver.

TABLE 1

Comparison of Bile Acid Extraction Methods Using Endogenously Radiolabeled Rat Tissues and Feces

	% Bile acids recovered[a]			
		Method 2 Minutes of reflux		
Sample	Method 1	120	60	15
Small intestine	81.6[b] (5.2)	99.2 (0.2)	99.2 (0.3)	99.2 (0.3)
Cecum	75.9 (5.7)	98.5 (1.2)	98.2 (0.6)	98.3 (0.9)
Liver	55.7 (4.1)	93.8 (7.5)	96.1 (3.0)	95.1 (2.5)
Feces	79.5 (5.0)	97.9 (2.1)	97.5 (0.5)	97.6 (0.3)

[a] Recovery calculated as radioactivity in extract/radioactivity in extract and residue remaining. Method 1 is that of Manes and Schneider (4); method 2 is that of Setchell et al. (6), with reflux times varied. See text for details of extraction procedure.

[b] Each value is the mean (SD) of duplicate determinations from 4 animals, except for method 2 fecal samples, where samples from 2-3 animals were used.

One of us (DDG) had previously found that recovery of an added taurocholate standard to rat intestinal contents was essentially 100% using method 1 (unpublished results). However, using endogenously labeled small intestinal tissue, we have found recovery, using this procedure, to be only about 80% complete. These results are similar to those of Setchell et al. (6) who found that after sonification and extraction with 90% ethanol, recovery of added standards from human feces ranged between 90 and 100%, whereas the recovery of endogenous radiolabeled bile acids averaged 82%. Thus, the use of a single bile acid standard to represent the entire spectrum of bile acids, which vary considerably in their hydrophobicity and metabolism, cannot be relied on to give an accurate measure of the completeness of extraction of all bile acids.

As bile acids are taken up by the cells of the gastrointestinal tract, sonicating the samples to disrupt these cells would seem important and may partially explain the completeness of the recovery in method 2. However, we questioned the necessity of the long reflux times, as originally described by Setchell et al. (6). As shown in Table 1, it is possible to reduce the refluxing times from 2 hr to 15 min with no loss in recovery. This greatly reduces the time of the method and allows many more samples to be extracted in a day.

Bile acid purification. The effectiveness of three different purification procedures is shown in Table 2. Procedure 1 had previously been found to give 93% recovery from small intestinal contents when added radiolabeled taurocholate was used to measure recovery (10). In the present study, using endogenously radiolabeled samples, this procedure gave only 42% recovery. Considerable losses were found in both the methanol/water wash and upon resolvating the cartridge with methanol. As in procedure 2, using methanol instead of a methanol/water solution to elute the bile acids improved recovery considerably. Most of the losses in these two procedures were

TABLE 2

Comparison of Bile Acid Extract Purification Procedures Using C18 Reversed Phase Cartridges

Procedure[a]	Sample loading	Washes				Eluants		Resolvation
	MeOH:DW (20:80)	DW	Hexane	DW	MeOH:DW (40:60)	MeOH:DW (75:25)	MeOH	MeOH
	% radioactivity recovered in each fraction							
1	4.10[b] (2.08)	1.52 (1.43)	1.56 (1.50)	6.04 (3.71)	27.05 (20.54)	41.93 (5.76)	—	17.79 (22.44)
2	3.33 (0.76)	0.26 (0.24)	0.83 (0.78)	—	23.41 (18.06)	—	72.15 (19.03)	0
3	3.87 (2.34)	1.69 (2.35)	0.38 (0.34)	—	—	—	93.93 (3.71)	0.12 (0.14)

[a]Cartridges were solvated with 4 ml of methanol, followed by 4 ml of distilled water. Four ml of each solution was drawn through the cartridge by vacuum at a rate of 1 ml/min. MeOH, methanol; DW, distilled water.

[b]Each value represents the mean (SD) of 6 determinations.

in methanol/water washes, apparently due to the solubility of certain polar bile acids in this concentration of methanol. Elimination of methanolic solutions as a wash and using it solely to elute the bile acids (as in method 3) increased recovery to ca. 94% and greatly reduced the variability of recovery. Because ca. 4% of the radiolabel is lost during sample loading, some bile acids do not adhere to the C18 packing material of the Sep-Pak even in only 20% methanol.

To our knowledge, this is the first report of a bile acid purification procedure using endogenously labeled material. The finding that a procedure previously thought to give 93% recovery of bile acids (10) in actuality gives a recovery of only 42% during purification indicates that the use of a single bile acid as a marker for all the bile acids is inappropriate. When developing a purification procedure using only a single bile acid as a marker of recovery, it is clear that one can only be confident of the recovery of that single bile acid. Bile acids of greater and lesser polarity may be lost by the procedure. As a result, optimization based on recovery of a single marker may greatly overestimate the recovery of bile acids as a group.

Bile acid distribution. From analysis of the quantity of radiolabel in each tissue sample we have calculated the distribution of bile acids in the intestinal tract and liver. The small intestine contained 73.0 ± 2.8% (mean ± SD) of the bile acids, the cecum 10.4 ± 3.5%, the colon contents 13.4 ± 3.2% and the liver 3.3 ± 0.5%. These results are in general agreement with the finding of Uchida et al. (11) who reported that, of the bile acids in the intestinal tract and liver, 87% were in the small intestinal contents and 10% in the large intestinal contents. Although we find slightly fewer bile acids in the small intestine and more in the large intestine than Uchida et al. (11), the combined amount in these two tissues of 96.7% (SD 0.5%) is virtually identical to that found by Uchida et al. (11). The quantity of bile acids within the plasma was extremely small, accounting for <0.1% of the radiolabel consumed by each rat. Thus, determination of the bile acid content of the intestinal tract would be a good estimate of bile acid pool size.

Fecal excretion over the 2 days was 12.1% (SD 2.4%) of the radiolabel fed. Combining the tissue label with that excreted in the feces, 86.2% (SD 2.6%) of the radiolabel can be accounted for. The remainder is presumably located in other soft tissues. Bile acids have been isolated from several soft tissues in the rat (12). As the ^{14}C-labels were located in the side chain, a position that is refractory to biological removal, it is unlikely that any label was lost due to degradative reactions of the gut microflora.

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Comparative Study of Methods For Measuring Cholesterol in Biological Fluids

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A method combining enzymatic and electrochemical detection of cholesterol in biological fluids was compared with conventional detection methods: two chromatographic methods—gas chromatography or high performance liquid chromatography—and two enzymatic methods with colorimetric detection either by kinetic measurement or at the end of the reaction. For serum, enzymatic determination is accurate with both colorimetric detection methods; but for bile, colorimetric detection is difficult to perform due to interference from bile pigments. Enzymatic cholesterol determination, combined with electrochemical detection, is simpler and gives results in good agreement with those of chromatographic methods.
Lipids 24, **224–228 (1989).**

Accurate determination of cholesterol levels in biological fluids is of great importance in routine medical testing. The enzymatic method has proven to be an effective technique for measuring cholesterol in serum and plasma (1–4), but few methods have been developed for other fluids such as bile (5,6). This study was undertaken partly in view of this fact.

Quantitative determination of cholesterol level in bile is necessary because of the relationship between this factor and gallstone formation. However, it is difficult for several reasons: Bile is a complex medium containing certain constituents such as bile salts that are structurally analogous to cholesterol. Moreover, bile pigments are dark green, thus causing very high colorimetric interference in the 300–600 nm wavelength zone.

The first techniques used for routine cholesterol determination were chemical methods followed by colorimetric reactions (7). However, because these reactions lack strict specificity, great care was required in applying them to complex media such as plasma or bile.

Use of enzymes in second generation methods has enabled more specific recognition of the substrate, but because the enzyme reaction is followed by colorimetric detection (8), interference is still high (9). The catalytic action of cholesterol oxidase is measured colorimetrically by a coupled system involving oxidation of either methanol in the presence of catalase or phenol and 4-aminoantipyrine in the presence of peroxidase. These reactions present certain disadvantages—lengthy reaction time and interfering substances during H_2O_2 production in the first step of the reaction or during the colorimetric reaction (10). Some authors (9) have also mentioned the potential reaction of bilirubin during procedures using H_2O_2.

Because our aim was to choose a cholesterol determination method applicable to a variety of fluids, the association of an enzyme reaction and electrochemical detection seemed to be a method of choice, to avoid colorimetric interference and take advantage of the specificity of the enzyme. Chromatographic methods—gas chromatography (GC) (11) and high performance liquid chromatography (HPLC) (12,13)—were used to separate cholesterol from the other lipids and allow cholesterol determination using calibration. Chromatographic results were compared with those obtained by enzymatic reaction and electrochemical detection with an O_2 selective electrode.

Electrochemical measurement was performed by voltammetry using an oxygen electrode, and cholesterol level was calculated from the rate of oxygen consumption (14). An O_2 selective electrode was calibrated and the decrease of oxygen level was monitored during the catalytic action of cholesterol oxidase on its substrate. Once the conditions of the use of this electrode in reactive media were determined, the accuracy of the results, obtained with enzymatic determination with electrochemical detection, was evaluated by comparing them with results obtained by four other methods, namely: enzymatic determination combined with two kinds of colorimetric detections, GC on capillary column and HPLC.

MATERIALS AND METHODS

Reagents. Pseudomonas cholesterol oxidase: EC 1.1.3.6, 18 IU/mg protein; pseudomonas cholesterol esterase: EC 3.1.1.13, 400 IU/mg protein; aqueous cholesterol calibrators 2.59 mM, 5.18 mM and 10.36 mM; standards for chromatography: ergosterol, cholestanol, cholesterol and cholesteryl esters were obtained from Sigma Chemical Co. (St. Louis, MO). Serum standard TQC chemistry calibrator 1 from Technicon (Geneva, Switzerland) and all other chemicals and solvents were of at least reagent grade.

Treatment of samples. Human gallbaldder biles were obtained from patients by preoperative puncture of the gallbladder. They were stored at 4°C and used within a few days. Human hepatic biles were obtained by cannulation of the common bile duct.

When enzymatic methods were used, the bile and serum samples were not submitted to any prior preparation. Chromatographic methods required extraction of lipids according to Folch's method (15), followed by a filtration on a Sep-Pak (16) to remove phospholipids from the neutral lipids containing cholesterol. HPLC allowed simultaneous measurement of cholesterol and its esters; on the other hand, with GC, only total cholesterol could be determined, and that, only after hydrolysis of esters using methanolic potassium hydroxide (17).

Enzymatic determination followed by colorimetric detection. The CHOD-PAP method (8) from Boehringer Mannheim (Meylan, France) was used for colorimetric detection. The colorimetric reaction was read at 500 nm with a spectrophotometer (Jobin Yvon 220, Longjuneau, France). This method cannot be applied to bile samples at the end of the reaction step because of the presence

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Abbreviations: GC, gas chromatography; HPLC, high performance liquid chromatography.

of color interference (18). For kinetic measurement, the method described by Deeg and Ziegenhorn (8) was used to obtain quantitative data.

Gas chromatography (11). A Girdel chromatograph (Suresnes, France) was used under the following conditions: carrier gas nitrogen inlet pressure, 0.6 bar; fused silica column, length 30 m, diameter 0.5 mm; stationary phase OV 17., oven temperature: 240°C; Ross evaporator injector temperature: 270°C; flame ionization detector; and internal standard, cholestanol.

The sample is mixed, volume for volume, with the internal standard at 1 g l^{-1}. The same total volume of silylation reagent (1 ml pyridine, 0.9 ml hexamethyl disilazane and 0.6 ml trimethyl chlorosilane) is added and the solution is incubated 10 min at 110°C. After drying and recovery by 1 ml of hexane, 1 or 2 μl are injected.

Liquid chromatography. A Waters chromatograph system (720 system controller, WISP 710A automatic injector and M 6000A pump, Waters, Milford, MA) was employed with a Hibar column (250 mm × 4 mm, Merck, Darmstadt, FRG), packed with a stationary phase (LiChrosorb RP-18 [7 μm]Merck, Darmstadt, FRG).

The chromatographic conditions were optimized in the laboratory. The mobile phase was acetonitrile/isopropanol (50:50, v/v) and the flow rate 1.5 ml/min. The detection wavelength was 214 nm. The internal standard was ergosterol for free cholesterol and cholesterol myristate for esterified cholesterol. Five μl of the internal standard solution at 2 g l^{-1} were added to 50 μl of the sample solution and made up to 200 μl with the mobile phase; 50 μl were injected. The results of chromatographic (HPLC) separation of cholesterol and cholesteryl esters in biological media are given in Figures 1 and 2.

Enzymatic determination, followed by electrochemical detection. A Beckman Cholesterol Analyzer II (Beckman Instruments, Fullerton, CA), modified to measure current intensity after enzymatic reaction, was used. It was equipped with a voltage generator and an amperemeter with a sensitivity of ca. one nanoampere. This instrument was connected to an oxygen electrode coated with a Teflon membrane. The oxygen electrode is a "polarographic" electrode in that it measures current limited by the diffusion of oxygen through the Teflon membrane towards a rhodium cathode (5 mm diam.). This cathode is maintained at a potential of −550 mV in relation to a metallic silver anode. The cathode and the anode were connected via an electrolyte gel containing potassium chloride. A uniform thickness of electrolyte gel was maintained between the membrane toward a rhodium cathode by a nylon mesh disc, inserted whenever the electrode was charged. A Teflon membrane (0.0254 mm diam.) was in contact with the test solution. The amount of oxygen that diffuses through the membrane is proportional to the oxygen concentration in the solution. Free cholesterol is oxidized by cholesterol oxidase to cholest-4-en-3-one with the simultaneous consumption of oxygen. The rate of oxygen consumption measured by the analyzer is directly proportional to the cholesterol concentration in the solution.

The electrode was immersed in a cell with a constant temperature of 37°C and containing 5 ml of phosphate buffer. This cell was placed on a magnetic shaker until the current intensity stabilized, at which time 0.6 Units of cholesterol oxidase in 10 μl distilled water were added, followed 1 min by 40 μl of the test solution. If the sample contained esterified cholesterol, 0.06 IU of

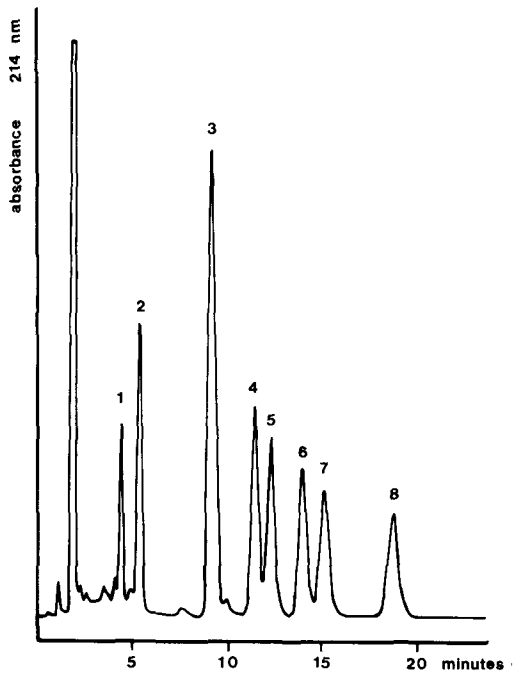

FIG. 1. HPLC separation of cholesterol and cholesteryl esters of serum samples. Chromatographic conditions: column RP 18 (7μ) LiChrosorb Merck; eluent solvent: acetonitrile-isopropanol (50:50); flow: 1.5 ml.min^{-1}. Peaks: 1, ergosterol; 2, cholesterol; 3, cholesterol arachidonate; 4, cholesterol linoleate; 5, cholesterol myristate; 6, cholesterol oleate; 7, cholesterol palmitate; and 8, cholesterol stearate.

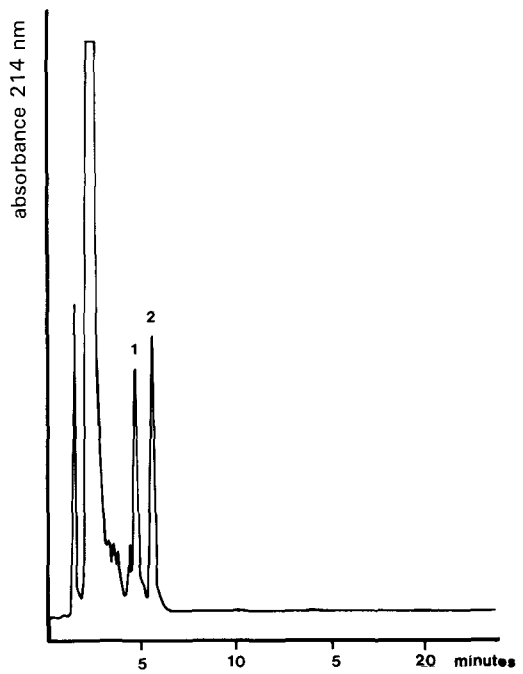

FIG. 2. HPLC separation of cholesterol and cholesteryl esters of bile samples. Chromatographic conditions: column RP 18 (7μ) LiChrosorb Merck; eluent solvent: acetonitrile-isopropanol (50:50); flow: 1.5 ml.min^{-1}. Peaks: 1, ergosterol; 2, cholesterol; 3, cholesterol arachidonate; 4, cholesterol linoleate; 5, cholesterol myristate; 6, cholesterol oleate; 7, cholesterol palmitate; and 8, cholesterol stearate.

cholesterol esterase in 10 µl distilled water were added with cholesterol oxidase. The reaction was complete within 5 min and the response of the instrument was then read. To obtain reproducible results, all solutions were added by a microsyringe.

DEVELOPMENT OF THE EXPERIMENTAL PROCEDURE

Composition of the buffer solution. Trials with phosphate buffer and Tris buffer showed that the response of the oxygen electrode was better with the latter. It was also observed that a surfactant was needed to properly stabilize cholesterol in both standard solutions and biological samples. The effect of Triton X 100 concentration on the response of the oxygen electrode (Fig. 3) was studied. On the basis of these preliminary trials, the following buffer mixture was finally selected: Tris buffer, 0.1 M; sodium cholate, 3 mM; and Triton X100, 0.05% in volume.

Effect of the pH. The buffer was tested at various pH in order to select the level of maximum cholesterol oxidase activity. The pH was adjusted with HCl solutions. Although variations were slight, 7.5 seemed to be the optimal level (Fig. 4).

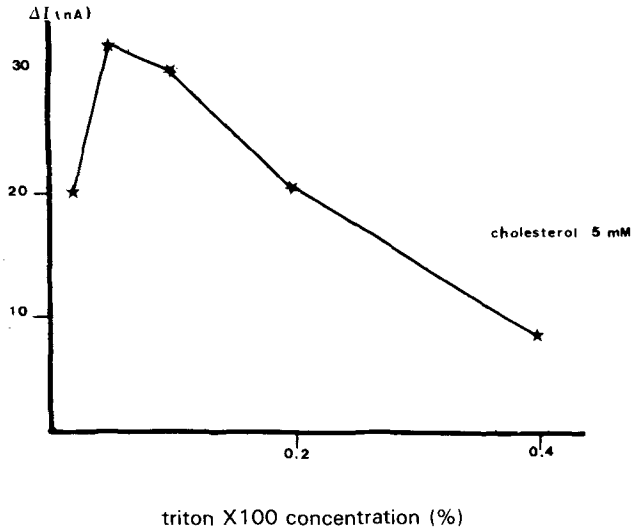

FIG. 3. Effect of Triton X100 concentration on the electrode response.

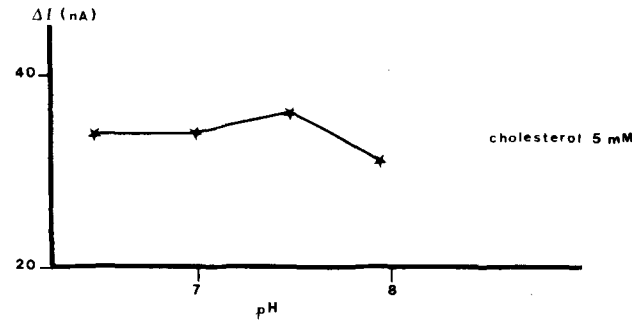

FIG. 4. Effect of the pH on the reaction rate of oxygen consumption.

Effect of temperature. Under identical experimental conditions, measurements were taken at 25°C, 37°C and 40°C. at 37°C (temperature selected), the electrode was appreciably more sensitive, because the measured response was ca. 50% higher. At 40°C, diffusion of O_2 was accelerated, but the enzyme was less stable.

Evaluation of the electrochemical detection—sensitivity. The smallest test sample giving a reliable reading is 100 µl of a solution containing 0.5 mmol l^{-1} cholesterol. This corresponds to the level of cholesterol in rat hepatic bile.

Linearity. Calibration was achieved with aqueous standard solutions of cholesterol (aqueous cholesterol calibrators, Sigma Chemical Co.). For 0.3 IU, the variation in current intensity measured at the end of the reaction was proportional to the quantity of cholesterol up to 8 mmol l^{-1}. For 0.6 IU, the interval increased to 12.5 mmol l^{-1} (Fig. 5).

Reproducibility. The day-to-day reproducibility of the standard curve was checked for a 10 mmol l^{-1} cholesterol standard solution. The variations observed over a 13-day period were identical to those observed in serial measurements on the same day.

The number of determinations performed was 13, the mean was 60 nA, the SD was ±2 nA, and CV was 3%. The lifetime of the electrode was estimated to be 3–4 wk.

Recalibration was necessary every time the electrode was charged and was valid only under the following conditions: Tris/cholate/Triton X100 buffer; pH 7.5; temperature 37°C; and 0.6 IU of cholesterol oxidase.

Solutions higher than 12.5 mmol l^{-1} of cholesterol must be diluted to be in the linear range of the calibration curve.

Because bile is a viscous medium, a microsyringe must be used to inject samples in order to minimize volume error and apply the calibration curve.

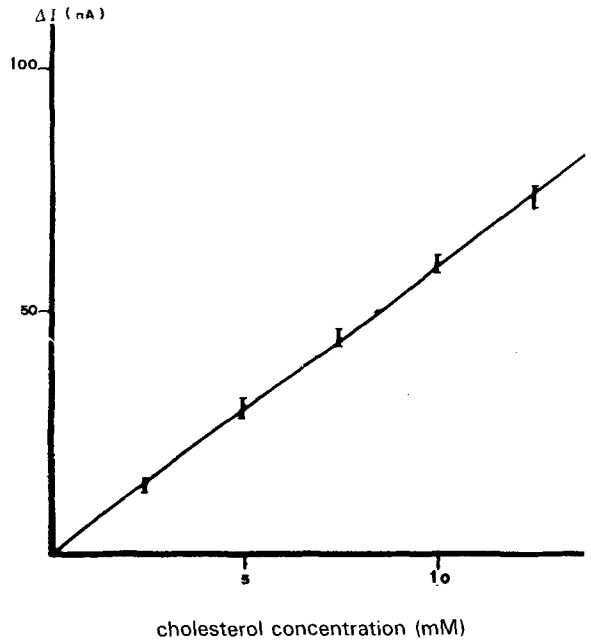

FIG. 5. Calibration curve expressed the variation of the response (ΔI) of the electrode following the consumption of oxygen in the medium due to the cholesterol oxidase activity.

Interfering substances (sodium taurocholate, sodium glycodeoxycholate, sodium cholate). To test their effects on electrode response, bile salts were added to the standard cholesterol solution. These substances had no effect on cholesterol oxidase activity, as shown by Noma et al. (19).

COMPARISON OF THE FOUR METHODS OF DETERMINATION

Using the same serum standard, the results of the electrochemical enzymatic method were compared with those obtained with four other procedures (Table 1). Serum rather than bile was used, because bile standard is not commercially available. Each value is the mean of three measurements. In all cases and especially for the enzymatic methods, the results obtained were very similar and close to the manufacturer's values (4.85 mmol l^{-1} for total cholesterol and 1.20 mmol l^{-1} for free cholesterol). The electrochemical detection method was also tested with different bile samples and the results compared with those obtained with other methods.

One gallbladder bile sample was chosen to compare values obtained by the enzymatic method with electrochemical detection, those obtained by the enzymatic method with kinetic studies and to test HPLC and GC. Because bile pigments perturb the final colored reaction, colorimetric enzymatic methods were inappropriate for measuring cholesterol in gallbladder bile. The dispersion data recorded with four different techniques applied to two biological fluids are presented in Table 2. The variations in results were smaller for enzymatic procedures than for chromatographic techniques. The chromatograms in Figures 1 and 2 are given as examples of HPLC separation.

DISCUSSION AND CONCLUSION

Enzymatic determination in association with electrochemical detection is a reliable and reproducible technique for measuring cholesterol in various biological fluids (serum and bile). Once the calibration curve has been established, measurement is rapid (5 min, as the mean) with no pretreatment of samples.

TABLE 1

Comparison of the Results Obtained With Serum and Bile From Five Techniques

	Enzymatic methods			Chromatographic methods	
Samples	Electrochemical detection	Colorimetric final point	Kinetic detection	GC	HPLC
Serum					
Free cholesterol	1.17	1.24	1.20	1.04	1.09
Total cholesterol	4.79	4.97	4.85	—	5.45
Bile					
1	7.00	—	4.00	7.50	6.50
2	18.20	—	6.10	19.40	17.40
3	6.20	—	6.10	5.70	6.50
4	6.70	—	6.00	5.70	6.50
5	2.85	—	2.10	2.90	2.10

For each sample, three measurements are performed. The mean is expressed in mmol l^{-1}. No value is reported for bile cholesterol in the case of colorimetric final point, because the interference of bile pigments leads to erratic value.

TABLE 2

Reproducibility of Measurements of Free Cholesterol Determination in One Human Bile Sample With the Four Methods

Methods	Number of determinations	Mean mmol l^{-1}	SD mmol l^{-1}	CV%	SEM
Enzymatic electrochemical detection	10	6.20	0.25	4	0.08
Enzymatic kinetic colorimetric detection	14	6.09	0.15	2.5	0.03
GC	10	5.70	0.50	9	0.15
HPLC	10	6.48	0.50	8	0.15

One gallbladder bile was chosen and 10 determinations were performed with the same sample in the case of enzymatic electrochemical detection, GC and HPLC, respectively, and 14 determinations in the case of enzymatic procedure associated with a kinetic colorimetric detection.

The initial difficulties encountered with the electrode were easily overcome. Oxygen diluted in a buffer containing surfactant diffused well in all the fluids studied. Several tests were required to optimize the composition of the buffer solution in which the enzymatic reaction must be carried out. For proper calibration, it is essential that the standard cholesterol be totally solubilized under the same conditions as the sample. Provided this precaution is taken, the method is effective, quick and reliable for routine determination of cholesterol in biological fluids. Comparison of results obtained with those from other techniques showed it to be a particularly high-performance technique that eliminates interferences associated with colorimetric detection.

Besides convenience and rapidity, this method for determining cholesterol is very sensitive. The results obtained by electrochemical detection are readily reproducible and, given its excellent sensitivity, this method can be used for less concentrated samples, e.g., hepatic bile. Either the kinetic rate or the steady state method can be used to assay the free cholesterol. The selectivity of the electrode can be enhanced by a Teflon membrane. The use of electrochemical detection instead of colorimetric determination after enzyme treatment of cholesterol lowers the level of interferences especially with bile samples. Furthermore, under our conditions, bilirubin reaction with H_2O_2 cannot be a source of interferences in electrochemical determination of the cholesterol oxidase activity, because the reaction is estimated from the oxygen consumption rather than from H_2O_2 formation.

These findings contrast with the very marked interferences due to bile pigments observed when colorimetric determination in association with enzymatic reactions is applied to bile. The use of colorimetry after enzymatic reaction is very difficult and only the method described by Deeg and Ziegenhorn (8) has been used effectively, although it requires great caution.

As stated by Noma and Nakayama (10), ascorbic acid does not affect oxygen consumption and may be electrochemically neutral at the potential used. These authors reported comparable results with respect to the range of linearity and the sensitivity threshold for the cholesterol determination in bile and serum. GC and HPLC confirmed, as well, the quantitative results obtained by enzymatic method associated with electrochemical detection in the absence of interferences or inhibition observed in this method.

The lesser accuracy of chromatographic methods, when compared with the other methods (9), may stem from differences in the way that samples are prepared. Pretreatment of samples, especially saponification of esterified cholesterol, and losses during sample injection in the case of GC may be largely responsible for these discrepancies between GC and HPLC. HPLC is the method of choice to separate and identify cholesteryl esters; however, the quantification of these esters is difficult in the case of a sample containing triglycerides that interfere in the quantitative determination. Regardless of the detection procedure, the cholesterol levels measured after enzymatic reaction are similar in both bile and serum. Inaccuracy with these techniques is minimal. In conclusion, it can be said that, given its precision and convenience, electrochemical detection should be considered a valid alternative for determination of cholesterol.

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COMMUNICATIONS

2-Hydroxy Fatty Acids From Marine Sponges 2. The Phospholipid Fatty Acids of the Caribbean Sponges *Verongula gigantea* and *Aplysina archeri*

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The α-hydroxy fatty acids 2-hydroxy-eicosanoic (1) acid, 2-hydroxyheneicosanoic (2) acid, 2-hydroxydocosanoic (3) acid, 2-hydroxytetracosanoic (4) acid, 2-hydroxy-23-methyl-tetracosanoic acid and 2-hydroxypentacosanoic (5) acid were isolated from the Caribbean sponges *Verongula gigantea* and *Aplysina archeri*. The very long chain fatty acids 5,9-nonacosadienoic acid (29:2) and 5,9,23-tricontatrienoic acid (30:3) were also identified together with the *iso*-prenoid fatty acid 3,7,11,15-tetramethylhexadecanoic (phytanic) acid that seems to be common in the Aplysinidae. *A. archeri* contained an extremely long chain fatty acid tentatively characterized as dotricontaenoic (32:1) acid. These acids were found to occur in phosphatidylserine, phosphatidylinositol, phosphatidylethanolamine, phosphatidylcholine and traces of phosphatidylglycerol. *Lipids 24,* 229–232 (1989).

Phospholipid fatty acids from marine sponges have been the subject of numerous research efforts in the search for unique structures with unusual membrane functions (1). One interesting group of fatty acids, namely the 2-hydroxy acids, have not been recognized to exist in the phospholipids of marine sponges until just recently when we reported, for the first time, the isolation of the interesting 2-hydroxydocosanoic and 2-hydroxytricosanoic acids from the Caribbean sponge *Amphimedon compressa* (2). To our surprise, the latter α-hydroxy acids were found in an unusual abundance of more than 50% of the total phospholipid fatty acid composition of *A. compressa*. These α-hydroxy acids have been found to occur mainly in brain sphingolipids and in a few other sources such as influenza virus (3), but our recent report is the first time that these interesting acids have been found to occur in the phospholipids of a marine sponge. Cerebrosides have been quite generally present in marine invertebrates and have also been known to be a rich source of α-hydroxy fatty acids. Karlsson and Björkman (4) have isolated a cerebroside fraction from the sea star *Asterias rubens* and characterized it by degradation. The cerebroside was found to contain glucose, saturated α-hydroxy fatty acids (C16–C26) and uncommon long-chain basis. Schmitz and McDonald (5) reported the isolation of a cerebroside mixture from the marine sponge *Chondrilla nucula*. They reported that these cerebrosides contained a mixture of homologous C16 to C26 saturated straight-chain αhydroxy acids plus traces of saturated C25 *iso* α-hydroxy acid. In light of the above findings, we have undertaken a search for α-hydroxy acids in other marine sponges, to better understand their role in these invertebrates. In this paper we wish to report the isolation of yet another series of α-hydroxy fatty acids from the Caribbean sponges *Verongula gigantea* and *Aplysina archeri*, both belonging to the family Verongidae. Sponges from the family Verongidae have been particularly interesting because they have been shown to possess, as a characteristic feature, the rare sterols aplysterol and 24,28-didehydroaplysterol (6). *V. gigantea* has also been the subject of previous scrutiny by several researchers because, as other members of the Aplysinidae (= Verongidae), it has been found to be a rich source of brominated metabolites, in particular racemic and levorotatory Aeroplysinin-1 (7).

EXPERIMENTAL PROCEDURES

Verongula gigantea and *Aplysina archeri* were collected June 8, 1987, at 75 feet near the Shelf Edge of La Parguera, Puerto Rico. The sponges were washed in sea water, carefully cleaned of all nonsponge debris and cut into small pieces. Immediate extraction with chloroform/methanol (1:1, v/v) yielded the total lipids. The neutral lipids, glycolipids and phospholipids were separated by column chromatography on ammonium hydroxide-treated silicic acid (100–200 mesh) using the procedure of Privett et al. (8). The phospholipid classes were investigated by preparative thin layer chromatography (TLC) using silica gel and chloroform/methanol/water (65:25:4, v/v/v) as solvent. The phospholipid mixture was also studied by means of ^{31}P NMR. In a typical run, phospholipids (20–30 mg) were dissolved in 3 ml of deuterated chloroform/methanol 2:1 (v/v) containing as internal reference triphenyl phosphine. The run was performed at 22°C on a GN 300 FT-NMR spectrometer at 121.6 MHz. Approximately 1000 accumulations were obtained before Fourier transformation of the free induction decay. 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/ether (1:1, v/v). The resulting methyl esters were analyzed by gas chromatography–mass spectrometry (GC–MS) using a Hewlett-Packard 5995 A gas chromatograph–mass spectrometer equipped with a 30 m × 0.32 mm fused silica column coated with SE-54. 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 (1 hr at 100°C), followed by ethereal extraction from

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Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; PtO_2, platinum oxide; ECL, equivalent chain length; GC, gas chromatograph(ic,y); GLC, gas liquid chromatography; MS, mass spectrometry; PLC, preparative layer chromatography; TLC, thin layer chromatography.

the acidified solution and purification by preparative layer chromatography (PLC). Hydrogenations were carried out in 10 ml of absolute methanol and catalytic amounts of platinum oxide (PtO_2). Mass spectral data of key fatty acids for this discussion, not reported before by us, are presented here:

2-Hydroxy-eicosanoic acid methyl ester. MS *m/z* (rel intensity) 342 (M^+, 12), 310 (1), 283 ($M^+ - 59$, 13), 281 (3), 264 (2), 159 (2), 145 (4), 141 (2), 139 (3), 127 (6), 125 (6), 111 (12), 103 (8), 90 (23), 83 (39), 81 (29), 71 (32), 69 (59), 57 (100).

2-Hydroxyheneicosanoic acid methyl ester. MS *m/z* (rel intensity) 356 (M^+, 14), 297 ($M^+ - 59$, 15), 295 (3), 278 (3), 159 (3), 145 (5), 141 (5), 127 (5), 125 (5), 111 (13), 109 (6), 103 (11), 97 (28), 90 (27), 83 (38), 81 (25), 71 (38), 69 (49), 57 (100), 55 (81).

2-Hydroxytetracosanoic acid methyl ester. MS *m/z* (rel intensity) 398 (M^+, 15), 339 ($M^+ - 59$, 11), 283 (4), 145 (4), 141 (2), 140 (4), 139 (5), 125 (7), 124 (6), 111 (12), 109 (8), 103 (5), 97 (26), 90 (19), 83 (29), 81 (23), 71 (36), 69 (44), 57 (100), 55 (78).

2-Hydroxy-23-methyltetracosanoic acid methyl ester. MS *m/z* (rel intensity) 412 (M^+, 10), 353 ($M^+ - 59$, 4), 127 (12), 125 (10), 113 (11), 111 (21), 109 (16), 103 (9), 97 (40), 90 (42), 85 (28), 83 (44), 71 (47), 69 (55), 57 (100), 55 (76).

2-Hydroxypentacosanoic acid methyl ester. MS *m/z* (rel intensity) 412 (M^+, 5), 353 ($M^+ - 59$, 4), 127 (11), 125 (10), 113 (10), 111 (15), 109 (9), 103 (9), 97 (28), 90 (30), 85 (19), 83 (41), 71 (31), 69 (41), 57 (100), 55 (61).

Dotricontaenoic acid methyl ester. MS *m/z* (rel intensity) 492 (M^+, 6), 135 (8), 126 (9), 125 (13), 112 (57), 111 (28), 98 (27), 97 (41), 95 (18), 87 (57), 85 (18), 84 (24), 83 (58), 81 (21), 74 (100), 71 (33), 70 (17), 69 (77), 67 (13), 59 (22), 57 (61), 56 (27), 55 (86).

TABLE 1

Identified Phospholipid Fatty Acids From *Verongula gigantea* and *Aplysina archeri*

Fatty acid	Abundance (%) *Verongula gigantea*	Abundance (%) *Aplysina archeri*
Tetradecanoic (14:0)	8.6	2.8
Methyltetradecanoic (br-15:0)	—	14.2
Pentadecanoic (15:0)	7.4	—
Methylpentadecanoic (br-16:0)	7.8	2.3
Hexadecanoic (16:0)	5.5	8.2
Methylhexadecanoic (br-17:0)	7.9	5.1
Heptadecanoic (17:0)	1.9	—
3,7,11,15-Tetramethylhexadecanoic (20:0)	5.6	5.0
Methylheptadecanoic (br-18:0)	—	4.6
Octadecanoic (18:0)	4.6	5.2
Methyloctadecanoic (br-19:0)	5.8	12.1
Docosatetraenoic (20:4)	—	6.9
Nonadecanoic (19:0)	0.7	—
Eicosanoic (20:0)	2.6	2.3
Methyleicosanoic (br-21:0)	—	1.4
Heneicosanoic (21:0)	—	0.4
2-Hydroxy-eicosanoic (h20:0)	8.5	0.8
Docosanoic (22:0)	5.7	0.3
2-Hydroxyheneicosanoic (h21:0)	2.4	—
2-Hydroxydocosanoic (h22:0)	5.3	—
Tetracosanoic (24:0)	1.7	0.9
Methyltetracosanoic (br-25:0)	1.1	5.6
Pentacosanoic (25:0)	2.1	0.8
Hexacosanoic (26:0)	—	1.5
2-Hydroxytetracosanoic (h24:0)	1.4	0.7
2-Hydroxy-23-methyltetracosanoic (h25:0)	—	1.0
2-Hydroxypentacosanoic (h25:0)	—	1.0
5,9-Nonacosadienoic (29:2)	1.4	6.2
5,9,23-Tricontatrienoic (30:3)	10.8	7.5
Dotricontaenoic (32:1)	—	1.5

Most branched acids (denoted by br) are *iso-anteiso* pairs.

RESULTS

Our results are presented in Table 1, where the phospholipid fatty acid compositions of *Verongula gigantea* and *Aplysina archeri* are presented. The phospholipid fatty acid composition of these sponges basically presented a series of saturated fatty acids (70% of the total fatty acid composition of *V. gigantea* and 75% of *A. archeri*) with big amounts of *anteiso* and *iso* acids. Interesting to point out in the mixtures is the presence of the acid 3,7,11,15-tetramethylhexadecanoic (phytanic) in ca. 5.6% abundance in *V. gigantea* and 5% abundance in *A. archeri*. The mass spectrum of phytanic acid methyl ester was very characteristic because it presented a base peak at *m/z* 101 (100%) and peaks at *m/z* 171 (15%) and *m/z* 241 (2%), immediately revealing branching and confirming its identity (10). A series of very long chain α-hydroxy fatty acids were also characterized in these sponges. In *V. gigantea* the α-hydroxy fatty acids identified were 2-hydroxy-eicosanoic (1), 2-hydroxyheneicosanoic (2), 2-hydroxy-docosanoic (3) and 2-hydroxytetracosanoic (4), which together accounted for 18% of the total fatty acids of this sponge. On the other hand, *A. archeri* contained the acids 2-hydroxy-eicosanoic (1), 2-hydroxytetracosanoic (4), 2-hydroxy-23-methyltetracosanoic and 2-hydroxypentacosanoic (5), which accounted for 3.5% of the total fatty acids from *A. archeri*. The characterization of these fatty acids (see below) was achieved by means of capillary GC–MS. Instrumental in their characterization was the presence of, in the mass spectrum of these acids, a prominent M^+-$COOCH_3$ ion strongly suggesting α-substitution, which was further confirmed by other diagnostic peaks. Lack of peaks at *m/z* 74 and 104, but presence of fragmentation ions at *m/z* 90 and *m/z* 103, arising from the characteristic McLafferty rearrangement (11), suggested immediately the presence of α-hydroxy substitution instead of, for example, α-methoxy. In fact, after literature comparison (11), it became obvious that we were dealing with a complete family of 2-hydroxy acids. In Figure 1 we present the gas liquid chromatographic (GLC) region of the α-hydroxy fatty acids methyl esters from *Verongula gigantea*. From the chromatogram it can be readily seen that the α-hydroxy fatty acids had a longer retention time than their analogous nonhydroxylated acids. Figure 2 shows a plot of retention time of α-hydroxy acids methyl esters from *Verongula gigantea* as a function of acid carbon-chain length. The linearity of the acids in question is clear from the plot.

Capillary GC and GC–MS analysis of the mixtures also revealed the presence of two very long chain phospholipid fatty acids displaying base peaks at *m/z* 81, a value diagnostic of acids possessing the Δ5,9-unsaturation pattern typical of "demospongic acids" (12). On the basis of capillary GC retention times and equivalent chain length

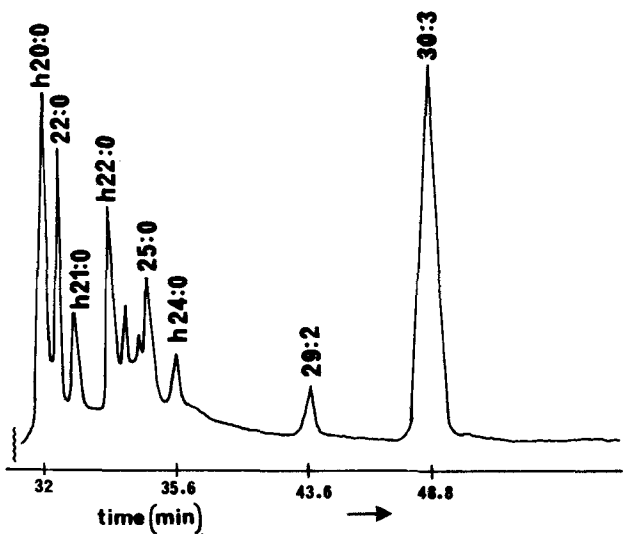

FIG. 1. GLC of the region of the α-hydroxy fatty acids methyl esters from *Verongula gigantea*. Note the longer retention time of the α-hydroxy fatty acids as compared with the analogous nonhydroxylated acids.

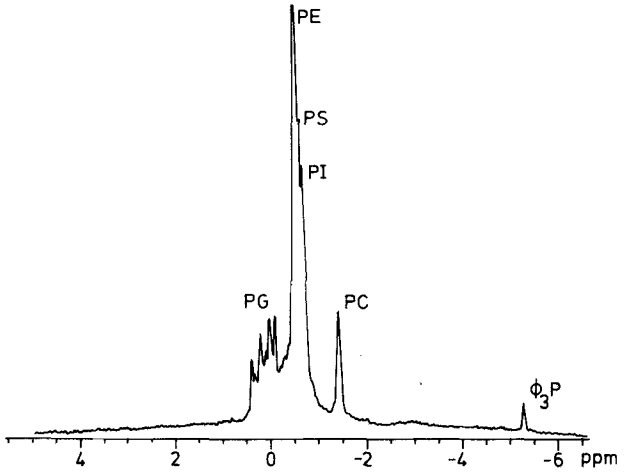

FIG. 3. The complete ^{31}P NMR spectrum of the phospholipids from *Verongula gigantea*. The abbreviations are PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PG, phosphatidylglycerol; PC, phosphatidylcholine; ϕ_3P, triphenylphosphine. Some minor peaks were not identified.

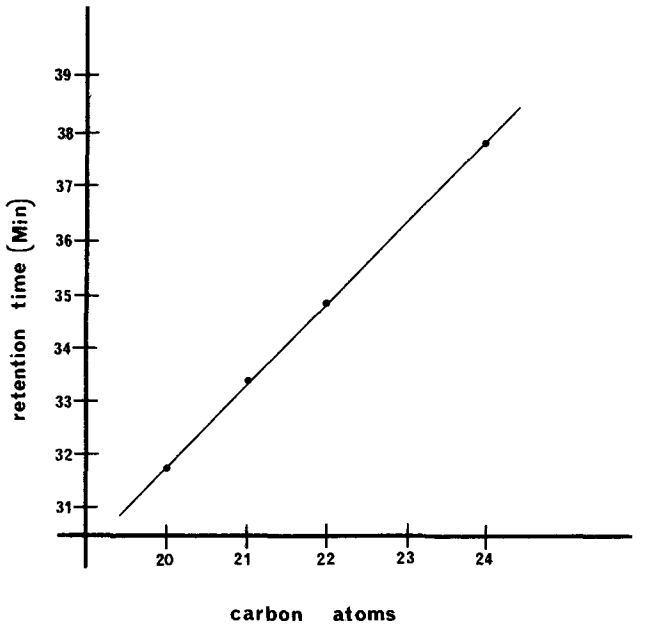

FIG. 2. Plot of retention time of α-hydroxy fatty acid methyl esters from *Verongula gigantea* as a function of acid carbon-chain length.

(ECL) values, together with mass spectral comparisons, these acids were characterized as 5,9-nonacosadienoic ($\Delta^{5,9}$-29:2) with ECL of 28.44 and 5,9,23-tricontatrienoic ($\Delta^{5,9,23}$-30:3) with ECL of 29.43, which were present in *V. gigantea* in 1.4 and 10.8% abundance, respectively, whereas *A. archeri* contained the same acids in 6.2 and 7.5% abundance, respectively (Table 1). These fatty acids have been previously reported to occur in marine sponges (12). *A. archeri* also contained another very long chain unsaturated fatty acid methyl ester that presented a molecular ion peak at m/z = 492, a base peak at m/z = 74 and fragmentation peaks at m/z = 69 and m/z = 55. On the basis of the mass spectrum and ECL values, the isolated compound could only correspond to a dotricontaenoic (32:1) fatty acid methyl ester. However, the small amounts of this acid prevented us from unequivocally locating the double bond position. It is very likely for the double bond of this acid to be located far from the carbonyl group because the base peak at m/z = 74 is still prevailing (Normally, monounsaturated acids with double bonds near the carbonyl group have base peaks at m/z = 55.) and likely possibilities are the familiar ω3 and ω6 types of unsaturation.

The main phospholipids of *Verongula gigantea* and *A. archeri* were separated and analyzed by preparative TLC and these were mainly characterized as phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylethanolamine (PE) and lesser amounts of phosphatidylglycerol (PG) and phosphatidylcholine (PC). Sphingomyelin was not observed in the mixture. The ^{31}P NMR spectrum of the total phospholipids from *V. gigantea* is presented in Figure 3.

DISCUSSION

The α-hydroxy fatty acids isolated in this work are 2-hydroxy-eicosanoic (1), 2-hydroxyheneicosanoic (2), 2-hydroxydocosanoic (3), 2-hydroxytetracosanoic (4), 2-hydroxy-23-methyl-tetracosanoic and 2-hydroxypentacosanoic (5). Also, taking into account the 2-hydroxytricosanoic acid, which we previously isolated from *Amphimedon compressa*, we can say that, overall, α-hydroxy fatty acids to be found in the phospholipids of marine sponges range in length between C20:0 and C25:0. As the prior case of the marine sponge *A. compressa*, it seems that 2-hydroxy fatty acids tend to exist in the phospholipids of these sponges together with their analogous saturated long-chain acids. However, it is more

interesting to notice that very long chain polyunsaturated fatty acids such as 5,9,23-tricontatrienoic (30:3) and 5,9-nonacosadienoic (29:2) have been found also to be present in these phospholipids when we have isolated the α-hydroxy acids. The latter is a very interesting observation because polyunsaturated fatty acids are conspicuously absent from sphingolipids, but in these marine phospholipids they seem to be a major constituent. It then seems reasonable to assume that these α-hydroxy fatty acids and the 30:3 and 29:2 acids play a significant membrane function in these sponges. The α-hydroxy fatty acids isolated in this work have been previously found to occur in brain cerebrosides, sphingomyelins and lecithins arising from the cerebral cortex, cerebral white matter, diencephalon and midbrain in amounts ranging between 10 and 15% (13). Some of the α-hydroxy acids isolated in this work also have been detected in monohexosylceramides, lactosylceramides and oligohexosylceramides from A_2-Asia and A_2-England Influenza Virus (3). However, the publication more related to our work is the report, by Schmitz and McDonald (5), of the isolation of α-hydroxy acids from cerebrosides isolated from the sponge *Chondrilla nucula.* These investigators reported the presence of α-hydroxy acids ranging in length from C16 to C26. The most interesting α-hydroxy acid in their mixture was the saturated C25 *iso* α-hydroxy acid, because it seems to be present only in marine sponges. In fact, we do not know of any other report for this acid, apart from the publication by Schmitz and McDonald and this report. The interesting observation of our present work is that we have encountered the α-hydroxy acids in the phospholipids rather than in cerebrosides. We discard the presence of cerebrosides in our phospholipid mixtures due to our rigorous isolation procedure. Cerebrosides normally elute in the acetone fraction on column chromatography with silica gel, in contrast to phospholipids that elute with the more polar methanol fraction. Naturally occurring α-hydroxy fatty acids are known to possess the R configuration, and we believe that the acids isolated in this work also possess the R configuration at carbon 2.

It will be interesting to find out the mode of biosynthesis of the α-hydroxy fatty acids in *Verongula gigantea* and *A. archeri.* One good possibility is α-oxidation of their corresponding very long chain saturated fatty acids such as docosanoic (22:0) and tetracosanoic (24:0), because these are found in relatively good amounts in *V. gigantea.* This possibility has been demonstrated in the case of brain sphingolipids, by Mead and Levis (14), who established that α-oxidation is the preferred pathway for the biosynthesis of the 2-hydroxytetracosanoic acid (4), better known as cerebronic acid. Still another interesting question is if the hydroxyl moiety is introduced before the fatty acid is coupled to the phospholipid backbone or if it is introduced once the fatty acid is linked to the glycerol backbone. Work in progress may elucidate the role of α-hydroxy acids in sponges.

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Effect of Dietary Fat on Individual Long-Chain Fatty Acyl-CoA Esters in Rat Liver and Skeletal Muscle

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The effect of dietary fat on the long-chain acyl-CoA ester profile of liver and skeletal muscle was investigated by feeding weanling rats 12%-fat diets composed of high-linoleic safflower oil (73% 18:2n-6), high-oleic safflower oil (70% 18:1n-9) or olive oil (70% 18:1n-9) for six and ten weeks. Approximately 50% of both hepatic and skeletal muscle acyl-CoA esters comprised linoleoyl-CoA or oleoyl-CoA with high-linoleic or oleic feeding, respectively. Total hepatic acyl-CoA ester concentration was 40% higher ($p < 0.05$) in rats fed 12% fat compared with controls fed a 4%-fat diet. These data demonstrate that the long-chain acyl-CoA ester profile of liver and skeletal muscle reflects the dietary fatty acid profile.
Lipids 24, 233–235 (1989).

Long-chain acyl-CoA esters are key substrates for lipid biosynthesis and oxidation and are effectors for many other important enzymatic reactions. Current information demonstrates that they have site-specific interactions with enzyme systems (1), suggesting that long-chain acyl-CoA esters play a role in the regulation of cell metabolism (2). The fatty acid composition of mammalian membranes can be altered by changing the proportion of the different classes of fatty acids in the diet (3), which may subsequently affect the activity of specific lipid enzyme systems and cellular function (4). The effects of dietary fat on the composition of individual long-chain acyl-CoA esters have not been reported. This communication describes the effects associated with ingestion of diets composed predominantly of linoleic acid or oleic acid on the composition of individual long-chain acyl-CoA esters in rat liver and skeletal muscle.

MATERIALS AND METHODS

Male weanling, Sprague-Dawley rats weighing 45–50 g (Sasko King, Inc., Omaha, NE) were fed ad libitum one of three 12%-fat diets composed predominantly of linoleic or oleic acids, or a stock laboratory diet (Wayne Rodent Blox, Chicago, IL) composed of 4% fat. The 12%-fat diets contained (percentage by weight): sucrose, 50.0; casein, 20.0; oil, 12.0; cornstarch, 8.0; cellulose, 5.0; AIN 76 mineral mix, 3.5; AIN 76A vitamin mix, 1.0; DL-methionine, 0.3; choline dihydrogen citrate, 0.2; and butylated hydroxy toluene, 0.02 (5,6). Fat was provided exclusively from high-linoleic safflower oil (U.S. Biochemical Corp., Cleveland, OH), high-oleic safflower oil (California Fats and Oils, Richmond, CA) or 100% pure cold-pressed olive oil (U.S. Biochemical Corp.). The fatty acid composition of the 12%-fat diets was determined by gas chromatography after total lipid extraction (7) and transmethylation of the fatty acids with methanolic HCl (8). The resultant fatty acid methyl esters were separated on a Hewlett-Packard 5830A gas chromatograph equipped with a packed glass column and a flame ionization detector (9), identified by comparison of retention times with standards and expressed as percentage distribution of fatty acid methyl esters. The fatty acid distribution of the diets is given in Table 1.

After feeding for 6 and 10 weeks, 6–9 animals from the 12%-fat dietary groups and 3 animals from the stock-fed 4%-fat control group were fasted overnight and anesthetized with CO_2 before collection of tissues. Samples of liver and hind limb skeletal muscle were removed immediately, frozen in liquid nitrogen and kept at −70°C until analyzed by reverse-phase high performance liquid chromatography (HPLC) for individual long-chain fatty acyl-CoA esters (10,11).

Results were analyzed by two-way analysis of variance (ANOVA) in a 4-diets × 2-lengths-of-feeding design, using the General Linear Model (GLM) technique (12). Means were compared with the least significant difference technique (13) at 5% level of probability and reported with pooled standard error.

RESULTS AND DISCUSSION

Ingestion of diets composed predominantly of linoleic or oleic triacylglycerols resulted in clear differences in the long-chain acyl-CoA ester composition of both liver and skeletal muscle (Tables 2 and 3). The fatty acyl-CoA ester profile reflected the fatty acid profile of the diets such that ca. 50% of total liver or muscle acyl-CoA esters were composed of linoleoyl-CoA in rats fed the high-linoleic safflower oil diet (73% 18:2n-6), or oleoyl-CoA in rats fed the high-oleic safflower or olive oil diets (70% 18:1n-9). This effect is consistent with changes in the fatty acid composition of membranes, serum, adipose tissue or tumors in response to variation in dietary fatty acid intake (3,14,15). In addition, a higher concentration of palmitoleoyl-CoA was noted in both liver and skeletal muscle from animals fed oleic versus linoleic oils, although the palmitic acid content of the diets was not appreciably different (Table 1). Despite wide differences in linoleic acid

TABLE 1

Fatty Acid Distribution of Diets[a]

Fatty acid	High-linoleic safflower oil	High-oleic safflower oil	Olive oil	Control-stock
14:0	0.2	0.2	0.1	1.5
16:0	7.1	5.7	11.8	15.1
16:1	—	—	1.6	2.3
18:0	1.9	2.2	2.4	3.8
18:1n-9	16.3	70.0	70.3	20.8
18:2n-6	72.8	19.3	10.8	44.9

[a]Expressed as % distribution of fatty acid methyl esters. Only the major fatty acids are reported, therefore, the total does not equal 100.

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Abbreviations: ANOVA, analysis of variance; GLM, general linear model; HPLC, high performance liquid chromatography.

TABLE 2

Long-Chain Acyl-CoA Levels in Hepatic Tissue From Rats Fed Oleic or Linoleic-rich Oils for 6 and 10 Weeks (nmol/g wet wt)

Diets	12:0	14:1	14:0	16:1	18:2	16:0	18:1	18:0	20:4	Total
6 weeks										
Control, n = 3	0.75^{d}	0.26^{b}	0.05^{c}	1.08^{c}	$14.25^{b,c}$	2.76^{b}	7.25^{b}	0.87^{b}	$0.06^{a,b}$	27.3^{b}
Saff linoleic, n = 6	1.66^{c}	0.09^{b}	$0.14^{d,c}$	1.14^{c}	25.96^{a}	3.08^{b}	4.93^{b}	1.40^{b}	ND	38.4^{a}
Saff oleic, n = 6	$1.50^{d,c}$	0.02^{b}	$0.19^{d,c}$	$2.76^{a,b}$	$10.75^{c,d}$	1.95^{b}	22.06^{a}	1.22^{b}	$0.15^{a,b}$	40.6^{a}
Olive oil, n = 6	$1.36^{d,c}$	0.21^{b}	$0.32^{d,c}$	$2.22^{b,c}$	$8.35^{d,e}$	2.70^{b}	17.84^{a}	1.19^{b}	$0.38^{a,b}$	$34.6^{a,b}$
10 weeks										
Control, n = 3	1.92^{c}	1.87^{a}	$0.59^{b,c}$	$1.37^{b,c}$	$6.84^{d,e}$	4.81^{a}	4.22^{b}	3.83^{a}	0.41^{a}	27.8^{b}
Saff linoleic, n = 7	2.97^{a}	0.38^{b}	$0.96^{a,b}$	0.79^{c}	16.44^{b}	5.46^{a}	5.73^{b}	3.41^{a}	$0.34^{a,b}$	$35.9^{a,b}$
Saff oleic, n = 9	$2.01^{b,c}$	0.23^{b}	1.07^{a}	3.92^{a}	6.56^{e}	5.04^{a}	19.68^{a}	4.16^{a}	$0.32^{a,b}$	43.2^{a}
Olive oil, n = 8	$2.68^{a,b}$	0.17^{b}	1.35^{a}	3.76^{a}	4.71^{e}	5.06^{a}	17.75^{a}	3.07^{a}	$0.33^{a,b}$	38.9^{a}
Two-way ANOVA										
Diet	0.01	.001	.01	.001	.001	NS	0.001	NS	NS	.002
Week	0.001	.001	.001	NS	.001	.001	NS	.001	.02	NS
Diet × week	NS	.001	NS	NS	NS	NS	NS	NS	NS	NS
Pooled SE	0.27	0.21	0.21	0.37	0.6	0.33	0.74	0.33	0.19	0.92

Mean values in a column with a different superscript (a–e) are significantly different, $p < 0.05$.
ND, not detected; NS, not significant.

TABLE 3

Long-Chain Acyl-CoA Levels in Skeletal Muscle From Rats Fed Oleic or Linoleic-rich Oils for 6 and 10 Weeks (nmol/g wet wt)

Diets	12:0	14:1	14:0	16:1	18:2	16:0	18:1	18:0	20:4	Total
6 weeks										
Control, n = 3	$0.26^{a,b,c}$	$0.15^{a,b,c}$	0.06^{d}	0.25^{c}	1.30^{b}	0.55^{c}	$1.30^{c,d}$	0.40^{b}	0.04^{b}	4.3^{b}
Saff linoleic, n = 6	$0.33^{a,b}$	$0.21^{a,b,c}$	$0.20^{b,c,d}$	0.16^{c}	1.88^{a}	0.42^{c}	0.096^{d}	$0.48^{a,b}$	$0.10^{a,b}$	4.6^{b}
Saff oleic, n = 7	$0.15^{b,c}$	$0.13^{b,c}$	$0.12^{c,d}$	0.26^{c}	$0.79^{c,d}$	0.51^{c}	2.22^{b}	0.31^{b}	0.05^{b}	4.4^{b}
Olive oil, n = 7	$0.28^{a,b,c}$	$0.13^{b,c}$	$0.23^{b,c}$	0.27^{c}	0.48^{d}	0.35^{c}	2.04^{b}	$0.50^{a,b}$	0.04^{b}	4.2^{b}
10 weeks										
Control, n = 3	0.40^{a}	0.09^{c}	0.30^{b}	$0.30^{b,c}$	$1.02^{b,c}$	1.31^{b}	1.06^{d}	$0.69^{a,b}$	0.20^{a}	5.4^{b}
Saff linoleic, n = 7	$0.17^{b,c}$	$0.26^{a,b,c}$	0.48^{a}	0.19^{c}	2.02^{a}	1.86^{a}	$1.72^{b,c}$	0.87^{c}	$0.13^{a,b}$	7.9^{a}
Saff oleic, n = 9	$0.23^{b,c}$	0.34^{a}	$0.21^{b,c,d}$	$0.48^{a,b}$	0.54^{d}	1.18^{b}	3.27^{a}	$0.59^{a,b}$	0.04^{b}	6.8^{a}
Olive oil, n = 8	$0.21^{b,c}$	$0.31^{a,b}$	$0.26^{b,c}$	0.54^{a}	0.42^{d}	1.24^{b}	3.50^{a}	$0.57^{a,b}$	$0.09^{a,b}$	7.1^{a}
Two-way ANOVA										
Diet	NS	NS	.002	0.002	.001	.005	.001	NS	NS	NS
Week	NS	.04	.001	0.006	NS	.001	.001	.005	NS	.001
Diet × week	.02	NS	.03	NS	NS	.002	.004	NS	NS	NS
Pooled SE	0.04	0.13	0.12	0.14	0.20	0.17	0.24	0.19	0.11	0.35

Mean values in a column with a different superscript (a–d) are significantly different, $p < 0.05$.
NS, not significant.

intake, the concentrations of arachidonyl-CoA in liver and skeletal muscle were similar among linoleic and oleic groups, as previously noted in serum and tumors from rats (14). This response may reflect the inhibitory action of excess dietary linoleic acid on the Δ^6-desaturase reaction (16) and subsequent conversion of linoleic acid to arachidonic acid and differences in the selective incorporation of arachidonic acid into membrane phospholipids. Hepatic palmityl-CoA concentrations were similar; but, after 10 weeks of feeding, skeletal muscle palmityl-CoA was higher in rats fed high-linoleic safflower oil compared with both oleic-rich diets. The stearyl-CoA concentration of liver and skeletal muscle was not affected by diet.

The total concentration of long-chain acyl-CoA esters in liver (per g wet wt) was ca. 6-fold higher than in skeletal muscle. In addition, the total concentration of acyl-CoA esters in liver and skeletal muscle differed in response to diet and length of feeding. Hepatic acyl-CoA content was not affected by the length of feeding; however, feeding for 10 weeks resulted in a 50% higher concentration of total acyl-CoA esters in skeletal muscle compared with feeding for 6 weeks. This observation may reflect an increase in the adipose content of skeletal muscle due to aging, whereas hepatic triglyceride content was similar in animals fed for 6 and 10 weeks (17).

Total concentration of liver acyl-CoA esters was 40% higher in animals fed the 12%-fat diets compared with controls fed a stock diet containing 4% fat, although skeletal muscle total acyl-CoA ester levels were not different from controls. This suggests that hepatic tissue

is more responsive to the quantity of dietary fat than skeletal muscle. In addition, total hepatic acyl-CoA ester content is 2- to 3-fold higher with fasting compared with the fed state, reflecting an increase in beta oxidation with fasting (10). Thus, the conditions of fasting and high-fat feeding, as used in this study, both resulted in an increase in hepatic acyl-CoA ester content.

Distinctive changes in the pattern of individual hepatic acyl-CoA esters were noted due to length of feeding in both control and 12%-fat groups, $p < 0.001$ (Table 2). These changes were not observed in skeletal muscle and were independent of the effect of diet. Hepatic concentrations of 12:0, 14:0, 16:0 and 18:0 acyl-CoA esters increased with time, whereas the concentration of 18:1 acyl-CoA was not affected by the length of feeding. The concentration of 18:2 acyl-CoA decreased by ca. 40% from 6 to 10 weeks with a concomitant increase in 20:4 acyl-CoA ester content. A similar decrease in the linoleic acid content of hepatic microsomal and mitochondrial membranes has been noted in aging rats (18). Changes in the proportion of hepatic 18:2 and 20:4 acyl-CoA esters may reflect a greater conversion of linoleic to arachidonic acid (16) with maturation.

These data demonstrate the sensitivity of both hepatic and skeletal muscle long-chain acyl-CoA ester composition to dietary fatty acid intake, and the potential utility of measuring tissue concentrations of individual and total long-chain acyl-CoA esters during different metabolic conditions.

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Vitamin E. Deficiency Increases the Synthesis of Platelet-Activating Factor (PAF) in Rat Polymorphonuclear Leucocytes

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Vitamin E deficiency was found to stimulate FMLP (N-formyl-L-methionyl-L-leucyl-L-phenylalanine)-induced biosynthesis of PAF (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) in polymorphonuclear leucocytes (PMN) from rat peritoneum. In three separate experiments each, the amounts of PAF synthesized during 6 min and 12 min incubation of PMN cells from control, vitamin E-supplemented, and vitamin E-deficient rats were 129–240, 131–227 and 248–354 pmol/10^6 cells, respectively. The activity of the acetyl-transferase, which transfers the acetyl moiety of [^{3}H]acetyl-CoA to 2-lysoPAF (1-*O*-alkyl-*sn*-glycero-3-phosphocholine) to form [^{3}H]PAF, was higher in PMN homogenates from vitamin E-deficient rats (2.28 ± 0.07 nmol/min/mg protein) than in those from E-supplemented rats (1.06 ± 0.10 nmol/min/mg protein). However, there was no difference between the two groups in the activity of acetylhydrolase (4.26 ± 0.71 and 4.26 ± 0.06 nmol/min/mg protein, respectively), measured as degradation of [^{3}H]PAF to [^{3}H]lysoPAF. *In vitro* addition of α-tocopherol did not inhibit the increased activity of acetyl-transferase in vitamin E-deficient rats, indicating that the enzyme in vitamin E-supplemented rats was not directly inhibited by α-tocopherol. The acetyl-transferases of the two groups showed similar Km values for acetyl-CoA, but different Vmax values (225 μM and 6.4 nmol/min/mg protein in vitamin E-deficient rats, and 216 μM and 3.6 nmol/min/mg protein in vitamin E-supplemented rats), suggesting that the enzyme was not activated but increased in amount in vitamin E deficiency. *Lipids 24,* 236–239 (1989).

Platelet aggregability is reported to be elevated in vitamin E-deficient animals (1,2). Vitamin E, which functions as a physiological membrane lipid antioxidant, has been shown to inhibit platelet aggregation and platelet serotonin release (3), and to reduce endogenous thromboxane and malondialdehyde formation in stimulated platelets (4,5). The mechanism of this effect of vitamin E, however, is not known.

Platelet-activating factor (PAF) is a potent phospholipid mediator that causes stimulation of leukocytes, vascular hypotension and broncho-constriction besides aggregation of platelets (6). However, no evidence has been reported of a biological relation between vitamin E and PAF.

In the present paper we report the influence of dietary and in vitro addition of vitamin E on the biosynthesis and catabolism of PAF in rat polymorphonuclear leukocytes (PMN).

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Abbreviations: FMLP, N-formyl-L-methionyl-L-leucyl-L-phenylalanine; lysoPAF, 1-*O*-alkyl-*sn*-glycero-3-phosphocholine; PAF, platelet-activating factor, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; PMN, polymorphonuclear leucocytes; PMSF, phenylmethylsulfonyl-fluoride.

MATERIALS AND METHODS

Chemicals. [^{3}H]PAF (1-*O*-[^{3}H]octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine, 80 Ci/mmol) was purchased from Amersham Corp. Unlabeled PAF and lysoPAF (1-*O*-octadecyl-*sn*-glycero-3-phosphocholine) were obtained from Feinchemikalien. [^{3}H]Acetyl-CoA (1.6 Ci/mmol) was from New England Nuclear. Unlabeled acetyl-CoA, FMLP (N-formyl-L-methionyl-L-leucyl-L-phenylalanine) and cytochalasin B were from Sigma Chemical Co. PMSF (phenylmethylsulfonyl fluoride) was from Nakarai Chemicals. Percoll was from Pharmacia Co. Silica gel-60 plates were purchased from Merck. α-Tocopherol was supplied by Eisai Co. Ltd.

Animals. Male Wistar strain rats, initially weighing 30–50 g, were divided into a control group, vitamin E-deficient group and vitamin E-supplemented group, and given rat chow described below containing all-*rac*-α-tocopheryl acetate at 2 IU, less than 0.1 IU and 50 IU per 100 g of diet, respectively, for 16–20 weeks. The chow consisted of 36% corn starch, 25% vitamin-free casein, 10% α-starch of wheat, 8% powdered filter paper, 6% salt mixture, 5% granulated sugar, 2% vitamin mixture and 10% stripped corn oil (Eastman Kodak Chemicals). The salt mixture provided Na, 270 mg; K, 692 mg; P, 579 mg; Ca, 411 mg; Mg, 86 mg; Fe, 41 mg; Zn, 0.4 mg; Mn, 1.3 mg, and I, 7.7 mg per 100 g of diet. The vitamin mixture provided vitamin A, 1000 IU; D_2, 200 IU; E, less than 0.1 IU; B_1, 2.4 mg; B_2, 8 mg; B_6, 1.6 mg; B_{12}, 0.001 mg; C, 60 mg; K, 10.4 mg; biotin, 0.04 mg; folic acid, 0.4 mg; Ca-panthenate, 10 mg; para-aminobenzoic acid, 10 mg; niacin, 12 mg; inositol, 12 mg, and choline-Cl, 4000 mg per 100 g of diet. Rats weighing about 230–280 g were used for experiments.

Determination of α-tocopherol content. α-Tocopherol was determined by the method of Abe and Katsui (7) as follows. α-Tocopherol was extracted with 7 ml of water/ethanol/hexane (1:1:5, v/v/v) from 0.2-ml samples of sera, and its content in the hexane layer was measured fluorometrically.

Preparation of PMN cells. Rat peritoneal PMN were obtained from male Wistar rats 15–16 hr after intraperitoneal injection of sodium caseinate as described by Cunningham et al. (8). The cells were separated by centrifugation at 300 g for 5 min, and a highly purified preparation of neutrophils (>95%) was obtained by Percoll gradient centrifugation.

Biosynthesis of PAF in PMN cells. Reaction mixtures containing 1×10^7 PMN cells/ml, Krebs Ringer phosphate buffer (KRP), and 2 mM PMSF, a serine hydrolase inhibitor, were incubated at 37°C for 15 min. The mixture was then centrifuged at 120 g for 2 min at 4°C to remove PMSF, and the PMN were resuspended in the same volume of fresh KRP. Then 3-ml portions (3×10^7 cells) were stirred with 10^{-6} M FMLP, 0.5 μg/ml cytochalasin B, and 1 mM $CaCl_2$ for the indicated times. Reactions were terminated by the addition of 10 ml of chloroform/methanol (1:2), and lipids were extracted by

the method of Bligh and Dyer (9). After evaporation of the solvent, the residues were dissolved in 100 μl of chloroform/methanol (2:1), and subjected to TLC on silica gel-60 plates with chloroform/methanol/water (65:35:7) as solvent. Bands of PAF were scraped off and extracted by the method of Bligh and Dyer (9). The solvent was evaporated, and the concentration of PAF was determined by assay of its ability to aggregate washed rabbit platelets (10). Student's t-test was used to calculate the significance of differences.

Assay of acetyltransferase. The activity of lysoPAF: acetyl-CoA acetyltransferase (EC 2.3.1.67) was assayed by a modification of the method of Albert and Snyder (11). The acetylation reaction was carried out at 37°C in basic medium containing 60 mM Tris-HCl (pH 7.5), 0.2% fatty acid-free bovine serum albumin, 10 μM $CaCl_2$, 2 mM PMSF (an inhibitor of acetylhydrolase), 12 μM lysoPAF, 2 μCi of [^{3}H]acetyl-CoA, 130 μM acetyl-CoA and about 0.2 mg protein of cell lysate in a final volume of 2 ml. The reaction was started by the addition of [^{3}H]acetyl-CoA. After incubation, radioactive PAF was extracted and purified chromatographically using silica gel-60 TLC plates as described above, and its radioactivity was assayed in a liquid scintillation counter.

Assay of acetylhydrolase. The activity of PAF acetylhydrolase (EC 3.1.1.48) was determined by the method of Blank et al. (12) with minor modifications. The reaction mixture consisted of 60 mM Tris-HCl (pH 7.5), 80 μCi of [^{3}H]PAF, 50 μM PAF, 0.8% fatty acid-free bovine serum albumin, 0.1 mM EDTA (to inhibit acetyltransferase) and about 0.2 mg protein of cell lysate in a final volume of 2 ml. The reaction was carried out at 37°C and then radioactive PAF and lysoPAF were extracted, purified and measured in a liquid scintillation counter.

RESULTS AND DISCUSSION

The mean α-tocopherol contents of the serum of the control, vitamin E-supplemented and vitamin E-deficient groups were 8.63 ± 0.6 μg/ml (n = 20), 13.1 ± 0.9 μg/ml (n = 20) and less than 0.2 μg/ml (n = 20), respectively.

PMN are one of the main types of blood cells in which PAF is synthesized. Table 1 shows the effect of the dietary vitamin E level on FMLP-induced biosynthesis of PAF by PMN from rat peritoneum. The amount of PAF production in the vitamin E-deficient group was about twice that in the control and vitamin E-supplemented groups. That is, the only marked difference was in the vitamin E-deficient group.

Acetylation of lysoPAF by acetyl-transferase [1] is the main pathway for the biosynthesis of PAF, and deacetylation of PAF by acetyl-hydrolase [2] is the first step in its conversion to biologically inactive lysoPAF in PMN.

$$\text{lysoPAF} + \text{acetyl-CoA} \xrightarrow{\text{acetyltransferase}} \text{PAF} \quad [1]$$

$$\text{PAF} \xrightarrow{\text{acetylhydrolase}} \text{lysoPAF} + CH_3COOH \quad [2]$$

Next we examined whether the increase of PAF production in vitamin E deficiency was attributable to increased synthesis and/or decreased catabolism of PAF. As shown in Figure 1, when lysoPAF and [^{3}H]acetyl-CoA were incubated with PMN homogenates at 37°C, [^{3}H]PAF production by the preparation from vitamin E-deficient rats was about twice that of the preparation from vitamin E-supplemented rats. However, there was no difference between the activities of acetyl-hydrolase in the two groups, measured as degradation of PAF and formation of lysoPAF on incubation of [^{3}H]PAF with the PMN homogenates (Fig. 2). Little formation of 1-*O*-[^{3}H]-alkyl-2-acyl-*sn*-glycero-3-phosphocholine was observed during 45 min incubation (data not shown). The extent of increase in acetyltransferase activity in homogenates

TABLE 1

Effect of Dietary Vitamin E on PAF Production in FMLP-Activated PMN of Rats

		PAF (pmol/10^6 cell)		
		Control	E-Supplemented	E-Deficient
Exp. 1	6 mn	80	93	280
	12 min	156	153	354
Exp. 2	6 min	78	75	154
	12 min	129	131	248
Exp. 3	6 min	101	136	273
	12 min	240	227	345

PMN obtained from three or four rats in each experiment were preincubated at 37°C for 15 min in 3 ml of KPR solution containing 6 μmol PMSF, and then incubated with 3 nmol FMLP and 1.5 μg cytochalasin B for 6 min and 12 min. After incubation, PAF in the whole incubation mixtures was extracted and analyzed as described under Materials and Methods. The values for vitamin E-deficient rats at 6 min and 12 min were significantly different ($p < 0.01$) from those of the controls.

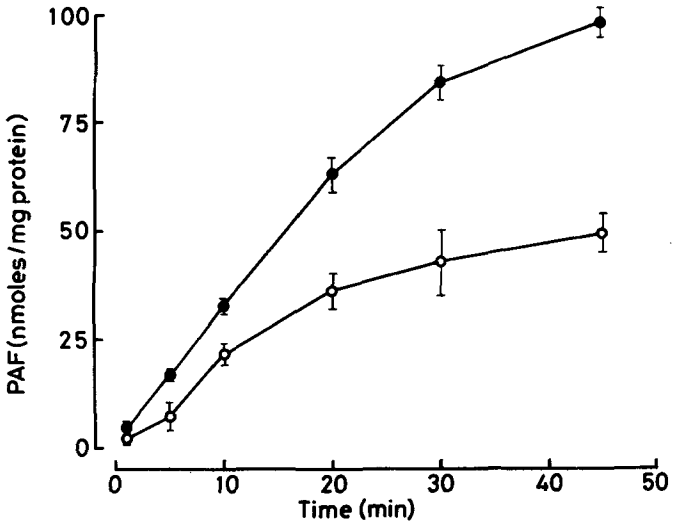

FIG. 1. Time course of incorporation of [^{3}H]acetyl-CoA into lysoPAF by PMN homogenates prepared from vitamin E-deficient (●) and vitamin E-supplemented (○) rats. Cell homogenates were incubated at 37°C for the indicated times in 2 ml of Tris-HCl (pH 7.5) media containing 260 nmol [^{3}H]acetyl-CoA and 24 nmol lysoPAF. Radioactive PAF in the whole incubation mixtures was measured as described in Materials and Methods. Points and bars are means ± S.E. for four experiments. The values for the two groups were significantly different ($P < 0.001$) at all time points.

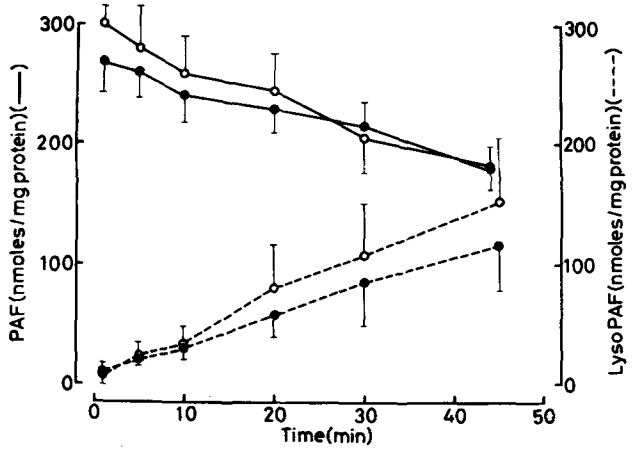

FIG. 2. Time course of conversion of [^{3}H]PAF to [^{3}H]lysoPAF by PMN homogenates from vitamin E-deficient (●) and vitamin E-supplemented (○) rats. Homogenates were incubated at 37°C for the indicated times in 2 ml of Tris-HCl (pH 7.5) medium containing 100 nmol [^{3}H]PAF. Remaining [^{3}H]PAF (—) and [^{3}H]lysoPAF formed (– – –) in the whole incubation mixtures were measured as described in Materials and Methods. Points and bars are means ± S.E. for four experiments.

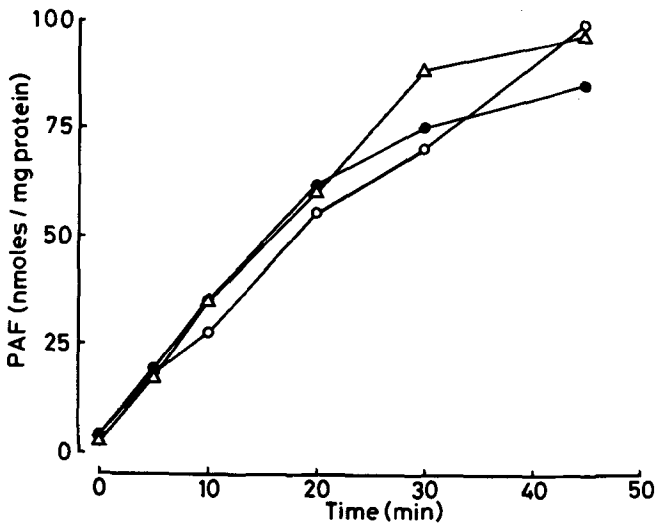

FIG. 3. Effect of in vitro addition of α-tocopherol on the activity of PAF:acetyl-CoA acetyltransferase in PMN homogenates prepared from vitamin E-deficient rats. α-Tocopherol dissolved in 2 μl of DMSO was added to 2 ml of incubation mixtures. The final concentrations of α-tocopherol were 0 ng/ml (△), 40 ng/ml (○) (normal level, and 400 ng/ml (●). Incubation conditions and measurement of radioactive PAF were as for Fig. 1.

of vitamin E-deficient animals was parallel to that of the amount of PAF produced in PMN cells, strongly suggesting that the elevated level of PAF in vitamin E-deficient cells was due mainly to increase in acetyltransferase activity.

Phospholipid metabolism is modulated by the vitamin E status of the animal (13–15). In vitamin E-deficient

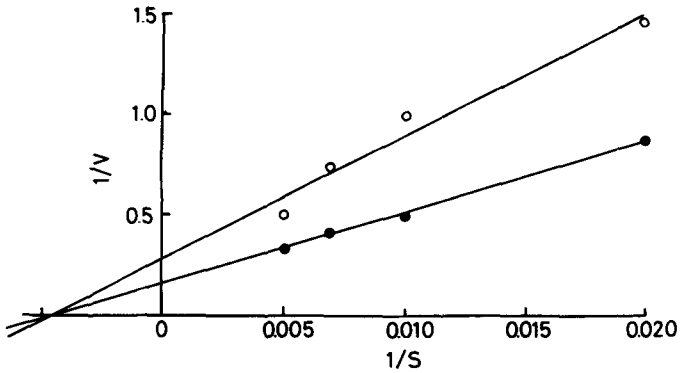

FIG. 4. Double reciprocal plot of PAF:acetyl-CoA acetyl-transferase activity of vitamin E-deficient (●) and vitamin E-supplemented (○) rats. Incubation conditions were as described for Fig. 1. V, PAF (nmol/min/mg protein); S, acetyl-CoA (μM).

rats, both biosynthesis and catabolism of phosphatidylcholine, the major phospholipid in mammalian tissues, are reported to be increased through acylation and deacylation at its *sn*-2 position. The decreased deacylation induced by dietary vitamin E is though to be due to its direct inhibition of phospholipase A_2, because the enzyme is inhibited *in vitro* by addition of α-tocopherol (14,15). As the lower activity of acetyltransferase in vitamin E-supplemented rats could also be attributed to direct inhibition of the enzyme by α-tocopherol, we examined the effect of added α-tocopherol on the activity of acetyltransferase *in vitro*. For this, a physiological concentration of α-tocopherol (40 ng/ml of incubation medium) and a 10-fold higher concentration (400 ng/ml) were added to samples of homogenate of PMN from vitamin E-deficient rats, and incubated with lysoPAF and [^{3}H]-acetyl-CoA. As shown in Figure 3, no difference was found in the time course of [^{3}H]PAF production with and without added α-tocopherol, indicating that the acetyltransferase was not directly inhibited by α-tocopherol.

Kinetic data on the acetyltransferases from the two groups are shown in Figure 4. The Km and Vmax values of the enzymes for acetyl-CoA were determined from double reciprocal plots. The Km values of the vitamin E-deficient and -sufficient groups were similar (225 and 216 μM, respectively), but their Vmax values were different (6.4 and 3.6 nmol/min/mg protein). These results suggest that vitamin E deficiency increased the amount of the acetyltransferase.

PAF was first characterized by its platelet aggregating activity (16), but later it was shown to have a wide spectrum of biological activities on physiological and pathological processes (6). For example, a direct involvement of PAF has been shown in ischemic cellular damage (17,18), which is protected by vitamin E (19,20). Our findings should be helpful in further studies on other effects of PAF besides elevated platelet aggregation in vitamin E-deficient animals.

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The Effect of Enhanced Intake of Linoleic Acid on the Fatty Acid Composition of Tissue Polar Lipids of Post-Smolt Atlantic Salmon (*Salmo salar*)

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Duplicate groups of Atlantic salmon (*Salmo salar*) post smolts were given diets in which the lipid component was either fish oil or a mixture of corn oil and lard. This difference in the dietary lipid did not significantly affect growth over a period of sixteen weeks.

Proportions of docosahexaenoic acid [22:6(n-3)] and total (n-3) fatty acids in the polar lipids of liver and white muscle were unaffected by this difference in dietary lipid component over the time period used. Fish given the diet containing corn oil and lard had significantly higher levels of 20:2(n-6), 20:3(n-6) and 20:4(n-6) in the polar lipids of these tissues than were present in the tissues of the fish given diets containing fish oil. These results suggest that linoleic acid [18:2(n-6)] undergoes elongation and desaturation to arachidonic acid [20:4(n-6)] in post-smolt Atlantic salmon.
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Marine fish species such as turbot (*Scophthalmus maximus*) have an absolute requirement for the highly unsaturated fatty acids (HUFA), 20:5(n-3) and 22:6(n-3), (1) and probably 20:4(n-6) as well. This requirement arises because they are unable to elongate and desaturate the 18-carbon precursors of these fatty acids (2). Some freshwater fish, however, can satisfy their HUFA requirement when given an adequate dietary supply of 18:2(n-6) and 18:3(n-3) (3,4). The freshwater parr of anadromous salmonid fish, e.g., rainbow trout (*Salmo gairdneri*) and chum salmon (*Oncorhynchus keta*), are also capable of elongation and desaturation of both (n-3) and (n-6) 18-carbon precursors (5,6). However, very little is known about fatty acid metabolism during the stressful period of parr-smolt transformation in Atlantic salmon (*Salmo salar*) or during the time of marine ongrowing, although Ackman and Takeuchi (7) observed that hatchery-reared fish undergoing this transformation appeared unable to convert 18:2(n-6) to 20:4(n-6).

With sources of marine oil becoming limited due to demands for human pharmaceutical products and a worldwide expansion in aquaculture, there is interest in using increasing levels of cheaper, more readily available plant oils in salmonid feeds (8,9). It is important, therefore, that we know the consequences of this for membrane fatty acid composition and metabolism, as well as overall carcass composition. In this experiment, post-smolt Atlantic salmon were given practical-type diets containing either fish oil (FO) or a mixture of lipids, mainly corn oil and lard (CO/L).

MATERIALS AND METHODS

Six hundred Atlantic salmon S1 smolts (mean wt, ca. 34 g) were obtained from Sea Farm (Kerry) Ltd, Gairloch, Scotland, distributed into 4 tanks of 2000 l capacity each and supplied with sea-water at a rate of 26 l/min. The temperature over the experimental period (July-November) was 12-9°C and the fish were subjected to natural photoperiod. The FO and CO/L diets were given by automatic feeders which were activated for a few seconds every 15 min (during daylight hours) and adjusted to supply 25 g/kg biomass per day, 7 days each week. Fish were bulk weighed every 28 days and the ration adjusted accordingly.

Both the FO and CO/L diets were formulated to meet the nutritional requirements of salmon (10) (Table 1). A quantity of canning oil (a deodorised fish oil) was added to the CO/L diet to provide adequate (n-3) fatty acids. Salmonid fish require ca. 1% of their diet as (n-3) lipid (3). The major fatty acid components of the two diets are shown in Table 2. After 16 weeks, samples of liver and muscle were removed from at least 4 fish per group. Samples were frozen immediately in liquid N_2 and stored at −80°C until analyzed.

Extraction of lipid, separation of polar and neutral lipid and analyses of purified fatty acid methyl esters were carried out as described by Cowey et al. (11). Significance of difference between treatments was determined by Students t-test.

Samples of heart, kidney, liver, gills, pyloric caeca (with pancreas) and red and white muscle were fixed in 8% formaldehyde in buffered saline prior to light microscopy. Parrafin wax-embedded sections were cut at 5 μm and stained with haematoxylin and eosin (H+E).

RESULTS AND DISCUSSION

Good growth was achieved (32-37 g to 150-180 g in 16 weeks) with both experimental diets and no significant differences in weight gain were observed. Tables 3 and 4 show fatty acid compositions from the polar lipid fractions of liver and muscle from one group from each treatment. No significant differences were observed between duplicate groups of the same dietary treatment. No histological lesions were observed.

Polar lipids from the livers of fish given the CO/L diet showed increased proportions of arachidonic [20:4(n-6)], dihomogammalinolenic [20:3(n-6)], eicosadienoic [20:2(n-6)] and linoleic [18:2(n-6)] acids compared with those fish given the FO diet. Compensatory decreases were observed in 14:0, 16:0, 20:1(n-9), 20:5(n-3), 22:5(n-3) and 24:1 fatty acids. The presence of high levels of 20-carbon (n-6) fatty acids in membrane lipids indicates appreciable Δ6- and Δ5-desaturase activity in liver

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Abbreviations: HUFA, highly unsaturated fatty acid; CO/L, corn oil and lard; FO, fish oil.

TABLE 1

Dietary Composition (g/kg dry diet)

Fish oil diet		Corn oil/lard diet	
Fishmeal	589.6	Fishmeal	500
Starch[1]	150	Soybean meal	265
Fish oil[2] (Fosol)	100	Starch	92
Vitamin mix[3]	28	Corn oil	35
Mineral mix[1]	24	Lard (Additive free)	45
αTocopheryl acetate 50%	200 mg	Canning oil[2]	10
Rovimex A + D[4]	74 mg	Vitamin mix[3]	28
Ascorbic acid	500 mg	Mineral mix[1]	24
α-cellulose	108	Rovimix A + D[4]	74 mg
		Ascorbic acid	500 mg
		αTocopheryl acetate 50%	200 mg

[1]Supplied per kg diet: KH_2PO_4 22.0 g, $FeSO_4 \cdot 7H_2O$, 1.0 g $\cdot ZnSO_4 \cdot 7H_2O$ 6.5 g, $MnSO_4 \cdot 4H_2O$ 52.8 mg. $CuSO_4 \cdot 5H_2O$ 12.0 mg, $CoSo_4 \cdot 4H_2$ 2.0 mg, KI 2.0 mg.
[2]Marfleet Ltd., Hull, UK.
[3]Supplied per kg diet: thiamin hydrochloride 50 mg, riboflavin 200 mg, pyridoxine HCI 50 mg, nicotinic acid 750 mg, calcium pantothenate 500 mg, myo-inositol 2 g, biotin 5 mg, folic acid 15 mg, choline bitartrate 9 g, menaphthone 40 mg, cyanocobalamin 0.09 mg.
[4]Roche Products Ltd., Dunstable, Beds., UK; supplied 11.1 mg retinol, 185 μg cholecalciferol/kg diet.

TABLE 2

Fatty Acid Composition of Diets (wt %)

Fatty acid	Fish oil	Corn oil/lard
14:0	5.10	3.66
16:0	15.33	18.86
16:1(n-7)	5.94	3.64
18:0	4.31	5.25
18:1(n-9)	9.81	25.00
18:1(n-7)	2.52	2.31
18:2(n-6)	1.59	20.55
18:3(n-3)	0.92	1.32
18:4(n-3)	2.93	1.37
20:1(n-9)	12.37	3.85
20:3(n-6)	0.08	0.05
20:4(n-6)	0.40	0.25
20:4(n-3)	0.51	0.27
20:5(n-3)	8.55	3.44
22:1(n-11)	14.46	4.22
22:1(n-9)	1.20	—
22:5(n-3)	1.02	0.32
22:6(n-3)	10.40	3.25
total n-3	24.02	9.97
total n-6	2.07	20.85
n-3/n-6	11.60	0.48

TABLE 3

Fatty Acid Composition (wt %) of Polar Lipids From Livers of Atlantic Salmon Post-Smolts

	FO diet		CO/L diet		Significance of difference
Fatty acid	Mean	SEM	Mean	SEM	P>
14:0	0.93	0.06	0.28	0.04	0.001
16:0	17.53	0.44	16.15	0.16	0.05
16:1(n-7)	1.24	0.15	0.81	0.12	NS
18:0	6.11	0.32	5.73	0.16	NS
18:1(n-9)	11.95	0.85	11.27	0.66	NS
18:1(n-7)	2.01	0.25	1.97	0.10	NS
18:2(n-6)	0.71	0.15	8.55	0.20	0.001
18:4(n-3)	trace		trace		
20:1(n-9)	3.47	0.02	0.88	0.09	0.001
20:2(n-6)	0.19	0.07	1.07	0.01	0.001
20:3(n-6)	0.31	0.04	2.81	0.65	0.02
20:4(n-6)	2.16	0.20	5.07	0.46	0.01
20:4(n-3)	0.62	0.03	0.40	0.02	0.01
20:5(n-3)	6.94	0.46	4.03	0.64	0.05
22:5(n-3)	2.45	0.13	1.61	0.23	0.05
22:6(n-3)	34.46	0.82	34.68	1.57	NS
24:1	3.99	0.28	2.33	0.09	0.01
total n-3	44.47	0.45	40.72	2.16	NS
total n-6	3.27	0.22	17.50	1.04	0.001
n-3/n-6	13.60	0.82	2.33	0.26	0.001

[1]Given either fish oil (FO) or corn oil/lard (CO/L) diets. SEM, Standard error of the mean; NS, not significant.

cells. No further elongation and Δ4-desaturation to 22:4(n-6) and 22:5(n-6) was observed.

The presence of 20:2(n-6) shows significant "dead-end" chain elongation from 18:2(n-6). Hagve et al. (12) had similar findings from experiments in which isolated hepatocytes from rainbow trout were incubated with [1-^{14}C] 18:2(n-6). This dead-end elongation product is normally incorporated preferentially into the neutral lipid of rainbow trout (13).

In white muscle there was a similar fatty acid profile to that in liver polar lipids. The muscle polar lipids of CO/L fish showed increased 18:2(n-6), 20:2(n-6), 20:3(n-6) and 20:4(n-6) with compensatory decreases in 14:0, 16:1(n-7), 20:1(n-9), 20:4(n-3), 22:1(n-11) and 24:1. These results indicate that the flesh content and relative proportions of (n-3) and (n-6) fatty acids may be manipulated to some extent by dietary means.

Recent work by Ackman and Takeuchi (7) on Atlantic salmon, and Dosanjh et al. (9) on chinook salmon (*O. tshawytscha*), both undergoing parr-smolt change, indicated that little or no elongation and desaturation of 18:2(n-6) to 20-carbon (n-6) HUFA occurred although substantial amounts of 18:2(n-6) were present in the tissue. It is possible that at the time of the change to smolts, when body lipids are changing to an even more preponderantly long-chain (n-3) HUFA spectrum, some inhibition by dietary fatty acids of the desaturases that metabolise 18:2(n-6) may occur.

However, Hardy et al. (8), in an experiment in which Atlantic salmon of over 1.5 kg wt were given a diet that provided a level of 18:2(n-6) similar to that used here, did not find any significant increase of 20:3

TABLE 4

Fatty Acid Composition (wt %) of Polar Lipids From White Muscle of Atlantic Salmon Post-Smolts[1]

Fatty acid	FO diet		CO/L diet		Significance of difference
	Mean	SEM	Mean	SEM	P>
14:0	0.79	0.09	0.23	0.01	0.01
16:0	13.13	0.46	12.51	0.27	NS
16:1(n-7)	1.47	0.08	0.41	0.04	0.001
18:0	5.41	0.39	5.17	0.16	NS
18:1(n-9)	7.03	0.23	6.64	0.17	NS
18:1(n-7)	2.23	0.08	2.02	0.11	NS
18:2(n-6)	0.57	0.01	6.25	0.13	0.001
18:3(n-6)	0.15	0.02	0.14	0.003	NS
18:4(n-3)	0.58	0.09	0.18	0.008	0.001
20:1(n-9)	2.85	0.05	0.44	0.05	0.001
20:2(n-6)	0.16	0.01	0.84	0.04	0.001
20:3(n-6)	0.12	0.01	1.25	0.10	0.001
20:4(n-6)	1.19	0.03	2.01	0.03	0.001
20:4(n-3)	1.03	0.03	0.84	0.04	0.02
20:5(n-3)	7.04	0.29	6.64	0.14	NS
22:1(n-11)	0.82	0.10	0.16	0.01	0.01
22:5(n-3)	2.50	0.14	2.42	0.05	NS
22:6(n-3)	46.58	0.29	47.08	0.51	NS
24:1	1.45	0.17	0.87	0.03	0.05
total n-3	57.14	0.20	56.98	0.39	NS
total n-6	2.19	0.03	10.46	0.24	0.001
n-3/n-6	26.13	0.37	5.46	0.16	0.001

[1]Given either fish oil (FO) or corn oil/lard (CO/L) diets. SEM, Standard error of the mean; NS, not significant.

(n-6) and 20:4(n-6) in muscle lipids. They did observe a significant increase in the dead-end product 20:2(n-6) when compared with diets containing entirely fish oils.

It is of interest that the proportions of 20:4(n-6) and 20:5(n-3) in liver polar lipids sum to 9.1% in both treatments (2.16 + 6.94 for FO; 5.07 and 4.03 for CO/L). The corresponding values for white muscle are 8.25% and 8.65%, again very similar. It seems possible, therefore, that in the structural sense these two fatty acids are interchangeable—one able to substitute for the other. Data from the CO/L diet that contained a sufficiency of both 20:4(n-6) and 20:5(n-3) indicate that dietary 20:5(n-3) is the preferred polar lipid fatty acid, if adequate in amount, but that 20:4(n-6) is an acceptable substitute if some of the 20:5(n-3) is used instead for elongation to 22:6(n-3).

20:3(n-6) is present at ca. tenfold greater proportion in tissue polar lipids of fish given CO/L diet compared with those given FO. Because dietary 20:3(n-6) was very low in both diets it seems likely that this increase must be a result of elongation and desaturation of 18:2(n-6). The accumulation of 20:3(n-6) and 20:2(n-6) reflects the rate limiting activity of the desaturases compared with the much more rapid reaction rates of the elongases. Hagve et al. (12) demonstrated that Δ5-desaturation of 20:3(n-6) in rainbow trout hepatocytes was only a quarter of that in rat hepatocytes.

It may be noted that (n-6) fatty acids cannot be substituted entirely for (n-3) fatty acids in artificial diets for fish (5,14); nor was this the intention. The inclusion of (n-6) fatty acids in feed for salmonids would however reduce the amount of dietary fish oil [(n-3) fatty acids] necessary in culture of these fish without any effects on tissue pathology or growth rate.

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Fatty Acid Hydroperoxide Isomerase in *Saprolegnia parasitica*: Structural Studies of Epoxy Alcohols Formed From Isomeric Hydroperoxyoctadecadienoates

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The major part (80%) of the fatty acid hydroperoxide isomerase activity present in homogenates of the fungus, *Saprolegnia parasitica*, was localized in the particle fraction sedimenting at 105,000 × *g*.

13*(S)*-Hydroperoxy-9*(Z)*,11*(E)*-octadecadienoic acid and 9*(S)*-hydroperoxy-10*(E)*,12*(Z)*-octadecadienoic acid were both good substrates for the particle-bound hydroperoxide isomerase. The products formed from the 13*(S)*-hydroperoxide were identified as an α,β- and a γ,δ-epoxy alcohol, i.e., 11*(R)*,12*(R)*-epoxy-13*(S)*-hydroxy-9*(Z)*-octadecenoic acid and 9*(S)*,10*(R)*-epoxy-13*(S)*-hydroxy-11*(E)*-octadecenoic acid, respectively. The 9*(S)*-hydroperoxide was converted in an analogous way into an α,β-epoxy alcohol, 10*(R)*,11*(R)*-epoxy-9*(S)*-hydroxy-12*(Z)*-octadecenoic acid and a γ,δ-epoxy alcohol, 12*(R)*,13*(S)*-epoxy-9*(S)*-hydroxy-10*(E)*-octadecenoic acid.

9*(R,S)*-Hydroperoxy-10*(E)*,12*(E)*-octadecadienoic acid and 13*(R,S)*-hydroperoxy-9*(E)*,11*(E)*-octadecadienoic acid were poor substrates for the *S. parasitica* hydroperoxide isomerase. Experiments with 13*(R,S)*-hydroperoxy-9*(Z)*,11*(E)*-octadecadienoic acid showed that the 13*(R)*-hydroperoxy enantiomer was slowly isomerized by the enzyme. The major product was identified as α,β-epoxy alcohol 11*(R)*,12*(R)*-epoxy-13*(R)*-hydroxy-9*(Z)*-octadecenoic acid.

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A pathway for sequential degradation of certain polyunsaturated fatty acids in the primitive fungus, *Saprolegnia parasitica*, was identified in recent work (1-3). Thus, arachidonic acid added to homogenates of the fungus was converted into 15*(S)*-HPETE by a lipoxygenase. The hydroperoxide did not accumulate, but was rapidly converted by action of a hydroperoxide isomerase into a pair of epoxy alcohols—11*(S)*,12*(R)*-epoxy-15*(S)*-hydroxy-5*(Z)*,8*(Z)*,13*(E)*-eicosatrienoic acid and 13(*R*),14(*R*)-epoxy-15(*S*)-hydroxy-5(*Z*),8(*Z*),11(*Z*)-eicosatrienoic acid. The two epoxy alcohols possessed an allylic epoxide group and therefore underwent spontaneous hydrolysis into a number of isomeric trihydroxyeicosatrienoates. Interestingly, relatively large amounts of trihydroxyeicosatrienoates and trihydroxyoctadecenoates could be isolated from the culture medium of the fungus, thus showing that the lipoxygenase/hydroperoxide isomerase pathway is active in the growing organism (1).

The present paper describes further studies of the hydroperoxide isomerase of *S. parasitica* using a number of isomeric hydroperoxy-octadecadienoates derived from linoleic acid as substrates.

Abbreviations: 9*(S)*-HPOD, 9*(S)*-hydroperoxy-10*(E)*,12*(Z)*-octadecadienoic acid; 9*(R,S)*-HPOD, 9*(R,S)*-hydroperoxy-10*(E)*,12*(Z)*-octadecadienoic acid; 13*(S)*-HPOD, 13*(S)*-hydroperoxy-9*(Z)*,11*(E)*-octadecadienoic acid; 13*(R,S)*-HPOD, 13*(R,S)*-hydroperoxy-9*(Z)*,11*(E)*-octadecadienoic acid; 15*(S)*-HPETE, 15*(S)*-hydroperoxy-5*(Z)*,8*(Z)*,11*(Z)*,13*(E)*-eicosatetraenoic acid; 8*(R)*-HETE, 8*(R)*-hydroxy-5*(Z)*,9*(E)*,11*(Z)*,14*(Z)*-eicosatetraenoic acid; I_{9S}, methyl 12*(R)*,13*(S)*-epoxy-9*(S)*-hydroxy-10*(E)*-octadecenoate; II_{9S}, methyl 10*(R)*,11*(R)*-epoxy-9*(S)*-hydroxy-12*(Z)*-octadecenoate; I_{13S}, methyl 9*(S)*,10*(R)*-epoxy-13*(S)*-hydroxy-11*(E)*-octadecenoate; II_{13S}, methyl 11*(R)*,12*(R)*-epoxy-13*(S)*-hydroxy-9*(Z)*-octadecenoate; MC, (−)-menthoxycarbonyl; CC, cyclic carbonate; Me_3Si, trimethylsilyl; GC-MS, gas chromatography-mass spectrometry; GLC, gas liquid chromatography; HPLC, high performance liquid chromatography; TLC, thin layer chromatography.

MATERIALS

13*(S)*-[1-^{14}C]HPOD (specific radioactivity, 1.4 kBq/μmol) was prepared by incubation of [1-^{14}C]linoleic acid with soybean lipoxygenase as previously described (4). 9*(S)*-[1-^{14}C]HPOD (specific radioactivity, 1.3 kBq/μmol) was prepared by incubation of [1-^{14}C]linoleic acid with whole homogenates of tomato, essentially as described by Matthew et al. (5). II_{13S} and its diastereomer, methyl 11*(S)*,12*(S)*-epoxy-13*(S)*-hydroxy-9*(Z)*-octadecenoate, were prepared by epoxidation of the methyl ester of 13*(S)*-HPOD using vanadium oxyacetylacetonate as catalyst (6). Methyl *erythro*- and *threo*-12,13-dihydroxyoctadecanoates were prepared by OsO_4- and performic acid hydroxylations, respectively, of methyl 12*(Z)*-octadecenoate (6). Methyl *erythro*- and *threo*-9,10-dihydroxyoctadecanoates were prepared, in a similar way, starting with methyl oleate.

9(R,S)*-Hydroperoxy-10*(E),*12*(E)*-octadecadienoic acid and 13*(R,S)*-hydroperoxy-9*(E),*11*(E)*-octadecadienoic acid.* Linoleic acid (100 mg) was kept under O_2 at 37°C for 15 hr. The product was subjected to silicic acid chromatography (column, 5 g). Elution with diethyl ether/hexane (2:8, v/v) yielded a mixture of hydroperoxyoctadecadienoates (ca. 16 mg, according to UV spectrometric analysis). This material was subjected to preparative reversed-phase high performance chromatography (HPLC) using a column of Polygosil C_{18} 5 μ (300 × 8 mm) and acetonitrile/water/acetic acid (68/32/0.02, v/v/v) at a flow rate of 1.5 ml/min. Three peaks of material showing strong absorption at 230-235 nm appeared, i.e., I (38.9-41.9 ml effluent), II (42.3-44.1 ml) and III (44.6-46.5 ml). The UV spectra of material forming peaks II and III showed an absorption band with λ_{max} = 231 nm, indicating an *E,E* diene structure. Further proof of the presence of one pair of conjugated *E,E* double bonds in compounds forming peaks II and III was provided by the IR spectra which showed an absorption band at 995 cm^{-1} (7). Catalytic hydrogenation of II and III afforded 13-hydroxy- and 9-hydroxyoctadecanoic acids, respectively. Thus, material present in peak II was identical to 13*(R,S)*-hydroperoxy-9*(E)*,11*(E)*-octadecadienoic acid, whereas peak III was due to 9*(R,S)*-hydroperoxy-10*(E)*,12*(E)*-octadecadienoic acid.

9(R,S)*-HPOD and 13*(R,S)*-HPOD.* Material forming peak I was subjected to straight-phase HPLC using a column of Nucleosil 5 μ (250 × 4.6 mm) and isopropanol/

hexane/acetic acid (1.5:98.5:0.02, v/v/v) at a flow rate of 3 ml/min. Two peaks appeared, i.e., 13*(R,S)*-HPOD (23.4–26.1 ml effluent) and 9*(R,S)*-HPOD (34.5–37.2 ml). Catalytic hydrogenation, which afforded 13-hydroxyoctadecanoic acid from the former hydroperoxide and 9-hydroxyoctadecanoic acid from the latter, demonstrated the positions of the hydroperoxy groups. The presence of a conjugated *E,Z* diene structure in the two compounds was shown by UV spectrometry, which showed an absorption band with λ_{max} = 234 nm, and by IR spectrometry, which showed bands at 990 and 955 cm^{-1} (ratio of intensities, 0.9:1) (7).

Methyl 10(R),*11*(R)*-epoxy-9*(S)*-hydroxy-12*(Z)*-octadecenoate.* The methyl ester of 9*(S)*-[1-^{14}C]HPOD (3 mg) was treated for 1 hr at room temperature with 3 ml of hexane saturated with vanadium oxyacetylacetonate. Thin layer radiochromatography showed two peaks of radioactivity due to a pair of diastereomeric α,β-epoxy alcohols (6). The more polar epoxy alcohol (R_f = 0.49; solvent system ethyl acetate/hexane [3:7, v/v]) was collected and characterized as follows: UV spectrometry did not show any specific absorption. The infrared spectrum showed bands at 3620–3350 cm^{-1} (hydroxyl), 1735 cm^{-1} (ester carbonyl) and 890 cm^{-1} (*trans* epoxide). No absorption band appeared in the region 950–1000 cm^{-1}. Thus, the presence of *E* double bond(s) could be excluded. Analysis of the trimethylsilyl (Me_3Si) derivative by gas chromatography–mass spectrometry (GC–MS) showed a peak with a retention time corresponding to C 22.5. The mass spectrum showed prominent ions at *m/e* 383 (M − 15, 4%), 367 (M − 31, 1), 308 (M − 90, 1), 259 (Me_3SiO^+=CH-$[CH_2]_7$-$COOCH_3$, 100), 212 (M − 186, [tentatively ascribed to migration of Me_3Si from C-9 to epoxide oxygen followed by elimination of OHC-$\{CH_2\}_7$-$COOCH_3$], 13) and 155 (OHC-$(CH_2)_7$-C≡O^+, 35), indicating a methyl octadecenoate carrying a hydroxyl at C-9, as well as a second oxygen function (epoxide according to the IR analysis). $KMnO_4$ oxidation performed on the MC derivative yielded the MC derivative of 2*(S)*-hydroxysebacate, demonstrating that the hydroxyl group of the epoxy alcohol had the *S* configuration. Catalytic hydrogenation yielded a major component that was identified as methyl *threo*-9,10-dihydroxyoctadecanoate by thin layer chromatography (TLC) carried out with $NaAsO_2$-impregnated silica gel using the authentic *threo* (R_f = 0.72) and *erythro* (R_f = 0.63) isomers as references. The presence of a hydroxyl group at C-9 (mass spectrometry, $KMnO_4$ oxidation), coupled with the results of the catalytic hydrogenation, located the epoxide group at C-10/C-11. The absolute configurations of C-10 and C-11 followed from the established configuration of C-9 (*"S"*) coupled with the findings that the relative configuration of C-9/C-10 was *threo* and that the configuration of the C-10/C-11 epoxide group was *trans*. On basis of these data, the epoxy alcohol was assigned the structure methyl 10*(R)*,11*(R)*-epoxy-9*(S)*-hydroxy-12*(Z)*-octadecenoate.

METHODS

Enzyme preparation. Homogenates of *S. parasitica* in 0.1 M potassium phosphate buffer pH 7.4 (1:2, w/v) (2) were centrifuged at 750 × *g* for 10 min. The supernatant was further centrifuged at 105,000 × *g* for 60 min. The sediment thus obtained was resuspended in potassium phosphate buffer (if not otherwise indicated, protein concentration was adjusted to 0.16 mg/ml). Protein was determined according to Bradford (8).

Spectrophotometric assay of hydroperoxide isomerase activity. Suspensions of the sediments obtained by centrifugation at 750 × *g* and 105,000 × *g*, as well as the clear 105,000 × *g* supernatant, were diluted with potassium phosphate buffer as needed. Samples of 2 ml were transferred to cuvettes and treated with 10–20 μg of the different hydroperoxides (temperature, 20°C). The absorbance at 236 nm was followed, vs time. The initial slopes (0–30 sec) of the decrease in absorbancy were taken as a measure of hydroperoxide isomerase activity.

Incubations and isolation of epoxy alcohols. [1-^{14}C]-Linoleic acid, 13*(S)*-[1-^{14}C]HPOD and 9*(S)*-[1-^{14}C]HPOD (0.25 mg) were shaken with suspensions of the 105,000 × *g* particle fraction (5 ml; 0.16 mg of protein/ml) at 22°C for 30 min. The mixtures were acidified to pH 3 and rapidly extracted with two portions of diethyl ether. The residue obtained after evaporation of the solvent was treated with diazomethane and subjected to thin layer chromatography (TLC) (solvent system, ethyl acetate/hexane [3:7, v/v]). With the solvent system used, the R_f values of methyl 11*(S)*,12*(S)*-epoxy-13*(S)*-hydroxy-9*(Z)*-octadecenoate, II_{13S}, and II_{9S} were 0.63, 0.54 and 0.49, respectively.

Linoleic acid and 13*(S)*-HPOD were found to yield a common pair (ratio, ca. 3:1) of epoxy alcohols. The R_f values of the methyl esters of the major and minor epoxy alcohols were 0.54 (II_{13S}) and 0.42 (I_{13S}), respectively. Incubation of 9*(S)*-HPOD resulted in the formation of another pair (ratio, ca. 2:1) of epoxy alcohols. The R_f values of their methyl esters were 0.49 (II_{9S}, major epoxy alcohol) and 0.39 (I_{9S}, minor epoxy alcohol). Unlabeled 13*(R,S)*-HPOD (0.25 mg) was incubated in the same way, although in this case 10 ml of enzyme preparation was used. Analysis of the esterified product by TLC showed two major spots, i.e., epoxy alcohols II_{13S} (R_f = 0.54) and II_{13R} (R_f = 0.63), as well as fainter spots of more polar material that was not further characterized.

Methods for structure determination. TLC was carried out with precoated plates (Kieselgel 60, 0.25 mm) from E. Merck (Darmstadt, FRG). Analysis of diastereomeric methyl 9,10- and 12,13-dihydroxyoctadecanoates was carried out using plates coated with silica gel G-$NaAsO_2$ (9:1, w/w) and methanol/chloroform (1:99, v/v) as solvent. Material was located by spraying with 2′,7′-dichlorofluorescein and viewing under UV light. Gas liquid chromatography (GLC) was performed with an F&M Biomedical gas chromatograph model 402 using a column of 5% QF-1 on Supelcoport. GC–MS was carried out with an LKB 9000S instrument equipped with a column of 3% OV—210 on Supelcoport. The electron energy was set at 22.5 eV, and the trap current was 60 μA. Ultraviolet spectra were recorded with a Hewlett-Packard model 8450A UV/VIS spectrophotometer. Radioactivity was determined with a Packard Tri-Carb series 4000 liquid scintillation counter. A Berthold Dünnschichtscanner II was used for determination of radioactivity on TLC plates.

Methods for catalytic hydrogenation (2), oxidative ozonolysis (9), $KMnO_4$ oxidation (3), and preparation and analysis of MC (9) and CC (3) derivatives were as previously described.

RESULTS

Localization and specificity of hydroperoxide isomerase activity. The major part (80%) of the hydroperoxide isomerase activity of homogenates of *S. parasitica* was present in the particle fraction sedimenting at 105,000 × *g*, whereas ca. 10% of the activity remained in the cytosol (Table 1).

13*(S)*-HPOD and 9*(S)*-HPOD were both good substrates for the particle-bound isomerase. No significant difference between the initial rates of isomerization of these hydroperoxides could be detected (Fig. 1). On the other hand, the *E,E* hydroperoxides, 9*(R,S)*-hydroperoxy-10*(E)*,12*(E)*- and 13*(R,S)*-hydroperoxy-9*(E)*,11*(E)*-octadecadienoates, were isomerized slowly (rates relative to those of the *E,Z* hydroperoxides, 10% or less (Fig. 1). 13*(R,S)*-HPOD was incubated in order to examine the stereospecifical requirements of the enzyme. If the enzyme did utilize only the 13*(S)*-hydroperoxide in the mixture of enantiomers it would be expected, with the spectrophotometric assay used, that the decrease in absorbancy at 235 nm would level off at 50% of the starting value. Interestingly, this was not found (Fig. 2). The curve obtained suggested that the 13*(R)*-hydroperoxide acted in a dual way, i.e., as a (poor) substrate for and as a (competitive) inhibitor of the isomerase. That 13*(R)*-HPOD was indeed isomerized by the enzyme was supported by the finding that preincubation with 10^{-4} M *p*-hydroxymercuribenzoate, an inhibitor of the *S. parasitica* hydroperoxide isomerase (2), resulted in complete inhibition of isomerization of 13*(R,S)*-HPOD, and further proved by the isolation of a specific hydroperoxide isomerase product formed from 13*(R)*-HPOD.

TABLE 1

Hydroperoxide Isomerase Activity in Fractions of Homogenates of *S. parasitica*[a]

Fraction	Total activity (%)	Specific activity (μmol/min·mg protein)
750 × *g* sediment	11	0.28
105,000 × *g* supernatant	9	0.06
105,000 × *g* sediment	80	0.45

[a]Fractions indicated were diluted with 0.1 M potassium phosphate buffer pH 7.4. Two ml of the dilutions were transferred to a cuvette and treated with 25 μM 13*(S)*-HPOD (temperature, 20°C). Hydroperoxide isomerase activity was calculated from the initial slopes (0–30 sec) of the decrease in absorbancy at 236 nm.

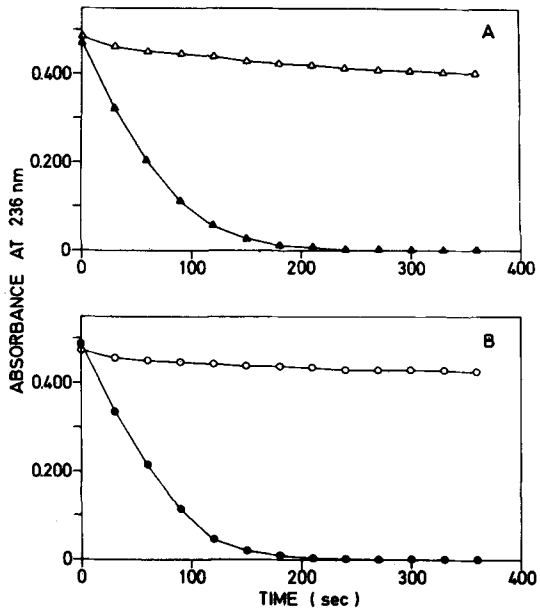

FIG. 1. Spectrophotometric assay of hydroperoxide isomerase activity. The blank and sample cuvettes contained 2 ml of a suspension of the particle fraction sedimenting at 105,000 × *g* (protein concentration, 27 μg/ml). Hydroperoxides (11 μg) were added to the sample cuvette in 5 μl of ethanol. Temperature, 20°C. (A) 9*(S)*-HPOD (-▲-▲-) and 9*(R,S)*-hydroperoxy-10*(E)*,12*(E)*-octadecadienoic acid (-△-△-). (B) 13*(S)*-HPOD (-●-●-) and 13*(R,S)*-hydroperoxy-9*(E)*,11*(E)*-octadecadienoic acid (-○-○-).

Structures of epoxy alcohols formed from linoleic acid and 13(S)-HPOD. II_{13S}, the methyl ester of the major epoxy alcohol formed from 13*(S)*-HPOD and linoleic acid (Fig. 3), was found to be identical to methyl 11*(R)*,12*(R)*-epoxy-13*(S)*-hydroxy-9*(Z)*-octadecenoate as shown by comparison with authentic material prepared by vanadium-catalyzed epoxidation of the methyl ester of 13*(S)*-HPOD. Criteria for identity included identical infrared spectra, identical C values and mass spectra, as well as identical results obtained on $KMnO_4$ oxidation and catalytic hydrogenation. Oxidation of the MC derivative (which yielded the MC derivative of 2*(S)*-hydroxyheptanoic acid) and the mass spectrum recorded on the Me_3Si derivative (which showed a base peak at *m/e* 173 due to $Me_3SiO^+ = CH\text{-}C_5H_{11}$) demonstrated the presence of an alcohol group at C-13, and thus excluded isomeric epoxy alcohols such as 12,13-epoxy-11-hydroxy-9-octadecenoate, 12,13-epoxy-9-hydroxy-10-octadecenoate, 9,10-epoxy-11-hydroxy-12-octadecenoate, and 10,11-epoxy-9-hydroxy-12-octadecenoate.

UV spectrometric analysis of I_{13S}, the methyl ester of the minor epoxy alcohol formed from 13*(S)*-HPOD and linoleic acid (Fig. 3), did not show any specific absorption

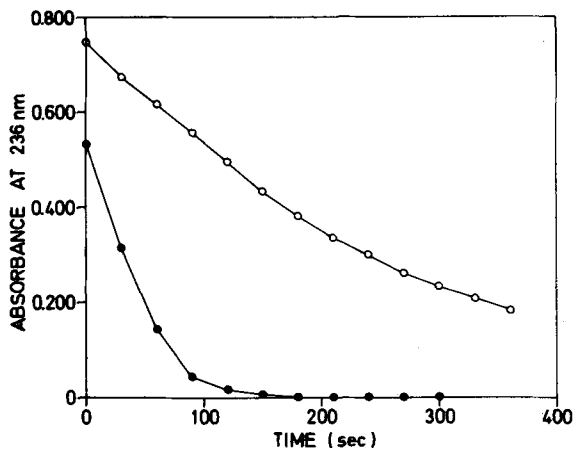

FIG. 2. Spectrophotometric assay of hydroperoxide isomerase activity. The blank and sample cuvettes contained 2 ml of a suspension of the particle fraction sedimenting at 105,000 × *g* (protein concentration, 40 μg/ml). 13*(S)*-HPOD (12 μg; -●-●-) and 13*(R,S)*-HPOD (17 μg; -○-○-) were added to the sample cuvette in 5 μl of ethanol. Temperature, 20°C.

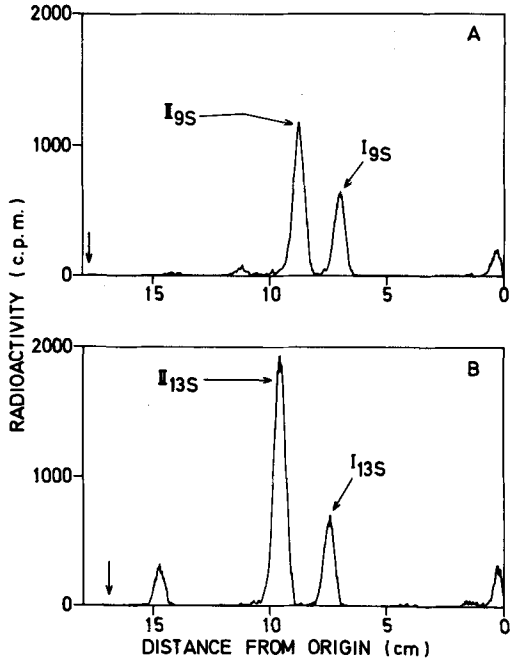

FIG. 3. Thin layer radiochromatograms of esterified products obtained after incubation of 0.25 mg of 9(S)-[1-^{14}C]HPOD (A) and 0.25 mg of [1-^{14}C]linoleic acid (B) with 5 ml of suspension of 105,000 × g particle fraction at 22°C for 30 min. Solvent fronts are indicated by arrows. Solvent system, ethyl acetate/hexane (3:7; v/v).

band. Infrared spectrometry showed bands inter alia at 3620–3350 cm^{-1} (hydroxyl), 1735 cm^{-1} (ester carbonyl) and 970 cm^{-1} (*E* double bond). No absorption band was observed in the region 850–900 cm^{-1}, thus excluding the presence of a *trans* epoxide group. The C-value of the Me_3Si derivative of I_{13S} was 23.9 and the mass spectrum showed prominent ions at *m/e* 398 (M, 1%), 383 (M − 15, 3), 327 (M − 71 [loss of $^{\bullet}C_5H_{11}$], 80), 308 (M − 90, 9), 298 (M − 100 [rearrangement with loss of $OHC-C_5H_{11}$], 17), 241 (M − 157 [loss of $^{\bullet}\{CH_2\}_7-COOCH_3$], 14), 237 (327 − 90, 40), 199 (M − 199 [cleavage at C-10/C-11 with charge retention in the fragment C-1 to C-10 and/or C-11 to C-18], 13), 185 (probably $O^+\equiv C-[CH_2]_7-COOCH_3$, 100) and 173 ($Me_3SiO^+=CH-C_5H_{11}$, 80). This spectrum indicated a methyl octadecenoate carrying a hydroxyl group at C-13, as well as a second oxygen function. Oxidative ozonolysis performed on the MC derivative of I_{13S} yielded the MC derivative of 2(*S*)-hydroxyheptanoic acid demonstrating the presence in I_{13S} of a double bond at Δ^{11} and a hydroxyl group at C-13 (*S* configuration). Catalytic hydrogenation of I_{13S} yielded comparable amounts of three components, i.e., methyl 9,13-dihydroxyoctadecanoate, methyl 9-hydroxyoctadecanoate, and methyl 13-hydroxyoctadecanoate, the identities of which were established by GC–MS. The absence of ketoesters in the hydrogenation product indicated that the nonhydroxylic oxygen function of I_{13S} detected by mass spectrometry was, as expected, an epoxide group.

Conclusive evidence for the presence of an allylic epoxide group at C-9/C-10 was provided by the finding that I_{13S} underwent spontaneous hydrolysis into isomeric trihydroxyesters (2,10–12). Thus, I_{13S} (1 mg) in methanol (0.5 ml) was treated with glass-distilled water (50 ml) at room temperature for 20 hr and the product subjected to thin layer radiochromatography (solvent, ethyl acetate). Two major peaks of radioactivity appeared, i.e., peak I (28%, R_f = 0.35) and peak II (60%, R_f = 0.48). Analysis by GC–MS of the Me_3Si derivatives of material forming peak I showed the presence of comparable amounts of a methyl 9,12,13-trihydroxy-10-octadecenoate (ion due to loss of $^{\bullet}CH_3$ [M − 15] at *m/e* 545, base peak at *m/e* 173 due to $Me_3SiO^+=CH-C_5H_{11}$) and a methyl 9,10,13-trihydroxy-11-octadecenoate (M − 15 ion at *m/e* 545, base peak at *m/e* 259 due to $Me_3SiO^+=CH-[CH_2]_7-COOCH_3$) (see refs. 3 and 6 for detailed mass-spectrometric data of trihydroxyoctadecenoates and trihydroxyeicosatrienoates). Oxidative ozonolysis performed on the MC derivatives of material present in peak I yielded the MC derivatives of 2(*S*)-hydroxysebacate and 2(*S*)-hydroxyheptanoate (from the 9,12,13- and 9,10,13-trihydroxyoctadecenoates, respectively). Ozonolysis of the cyclic carbonate (CC) derivatives of material present in peak I yielded the CC derivative of *erythro*-2,3-dihydroxyoctanoate (from the 9,12,13-trihydroxyoctadecenoate) and the CC derivative of a 2,3-dihydroxyundecanedioate (from the 9,10,13-trihydroxyoctadecenoate). The configuration of the latter product could not be established because of lack of the appropriate reference compounds. From these data it followed that peak I was due to a methyl 9(*S*),12,13-trihydroxy-10-octadecenoate which had the *erythro* configuration at C-12/C-13, and a methyl 9,10,13(*S*)-trihydroxy-11-octadecenoate. In the same way, peak II was found to be due to a methyl 9(*S*),12,13-trihydroxy-10-octadecenoate that had the *threo* configuration at C-12/C-13 (15%), and a methyl 9,10,13(*S*)-trihydroxy-11-octadecenoate (85%).

The two methyl 9(*S*),12,13-trihydroxy-10-octadecenoates present in peaks I and II were formed from I_{13S} by solvent attack at C-12 followed by isomerization of the Δ^{11} double bond into the Δ^{10} position and opening of the epoxide ring (Fig. 4). The fact that the carbon-oxygen bond at C-9 remains intact during this conversion, and the fact that the two trihydroxyesters formed had both the "*S*" configuration at C-9, demonstrated that C-9 of

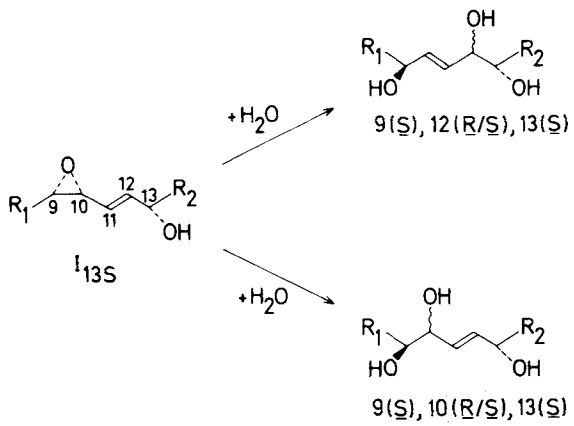

FIG. 4. Stereochemistry in the hydrolysis of γ,δ-epoxy alcohol I_{13S}. Formations of methyl 9(S),12(R,S),13(S)-trihydroxy-10-octadecenoate and methyl 9(S),10(R,S),13(S)-trihydroxy-11-octadecenoate by solvent attack at C-12 and C-10, respectively, are shown. R_1 = $(CH_2)_7-COOCH_3$, R_2 = $(CH_2)_4-CH_3$.

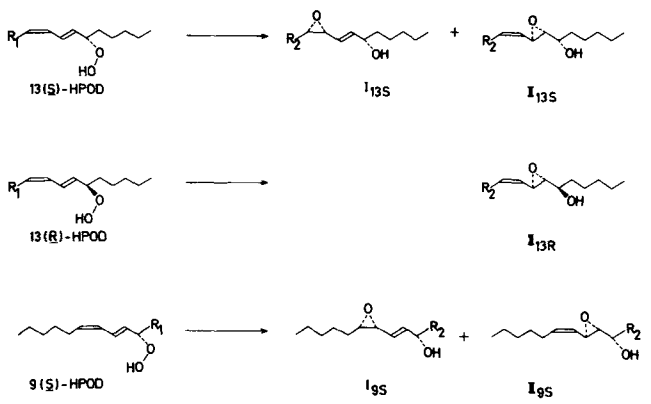

FIG. 5. Structures of epoxy alcohols formed from 13*(S)*-HPOD, 13*(R)*-HPOD and 9*(S)*-HPOD by action of *S. parasitica* hydroperoxide isomerase. $R_1 = (CH_2)_7$-COOH, $R_2 = (CH_2)_7$-$COOCH_3$.

epoxy alcohol I_{13S} had the "*S*" configuration. Furthermore, the absence of an absorption band typical of a *trans* epoxide in the infrared spectrum of I_{13S} showed that the C-9/C-10 epoxide group had the *cis* configuration. Accordingly, epoxy alcohol I_{13S} was assigned the structure methyl 9*(S)*,10*(R)*-epoxy-13*(S)*-hydroxy-11*(E)*-octadecenoate (Fig. 5).

Structures of epoxy alcohols formed from 9(S)*-HPOD.* II_{9S}, the methyl ester of the major epoxy alcohol formed from 9*(S)*-HPOD, was identical to methyl 10*(R)*,11*(R)*-epoxy-9*(S)*-hydroxy-12*(Z)*-octadecenoate, as shown by comparison with the authentic compound by UV and IR spectrometry, mass spectrometry, chemical degradation of the MC derivative, as well as catalytic hydrogenation. Formation of the MC derivative of 2*(S)*-hydroxysebacate upon oxidation with $KMnO_4$, and the mass spectrum of the Me_3Si derivative, showing a base peak at *m/e* 259 (Me_3SiO^+=CH-$(CH_2)_7$-$COOCH_3$), both demonstrated the presence of an alcohol group at C-9. This eliminated possible isomeric epoxy alcohols such as 9,10-epoxy-11-hydroxy-12-octadecenoate, 9,10-epoxy-13-hydroxy-11-octadecenoate, 12,13-epoxy-11-hydroxy-9-octadecenoate and 11,12-epoxy-13-hydroxy-9-octadecenoate.

The UV spectrum of I_{9S}, the methyl ester of the minor epoxy alcohol formed from 9*(S)*-HPOD, did not show any specific absorption band. The IR spectrum showed absorption bands at 3620-3350 cm^{-1} (hydroxyl), 1735 cm^{-1} (ester carbonyl) and 970 cm^{-1} (*E* double bond). The absence of absorption band in the region 850-900 cm^{-1} showed that no *trans* epoxide group was present in I_{9S}.

The C-value of the Me_3Si derivative of I_{9S} was 23.9 and the mass spectrum showed prominent ions at *m/e* 398 (M, 1%), 383 (M − 15, 3), 367 (M − 31, 1), 327 (M − 71 [loss of $^{\cdot}C_5H_{11}$], 3), 308 (M − 90, 4), 285 (M − 113 [cleavage at C-11/C-12 with elimination of C-12 to C-18], 4), 259 (Me_3SiO^+=CH-$\{CH_2\}_7$-$COOCH_3$, 35), 241 (M − 157 [loss of $^{\cdot}\{CH_2\}_7$-$COOCH_3$], 100), 212 (M − 186 [tentatively ascribed to migration of Me_3Si to epoxide oxygen followed by elimination of OHC-$\{CH_2\}_7$-$COOCH_3$], 9), 155 (OHC-$[CH_2]_7$-$C{\equiv}O^+$, 13), 151 (241 − 90, 24), and 99 ($O{\equiv}C$-C_5H_{11}, 82). This spectrum was similar to that of the Me_3Si derivative of a methyl 12,13-epoxy-9*(R,S)*-hydroxy-10-octadecenoate previously isolated in a study of the hemoglobin-promoted degradation of 13*(S)*-HPOD (11).

Oxidative ozonolysis performed on the MC derivative of I_{9S} yielded the MC derivative of 2*(S)*-hydroxysebacate demonstrating the presence in I_{9S} of a hydroxyl group at C-9 (*S* configuration). Catalytic hydrogenation of I_{9S} yielded comparable amounts of three components, i.e., methyl 9,13-dihydroxyoctadecanoate, methyl 13-hydroxyoctadecanoate and methyl 9-hydroxyoctadecanoate.

Thin layer radiochromatographic analysis of the hydrolysis product of I_{9S} showed two major peaks, i.e., peak I (27%, R_f = 0.35) and peak II (65%, R_f = 0.48). GC-MS analysis and steric analysis demonstrated that peak I was due to comparable amounts of a methyl 9,10,13*(S)*-trihydroxy-11-octadecenoate and a methyl 9*(S)*,12,13-trihydroxy-10-octadecenoate, the latter of which had the *erythro* configuration at C-12/C-13. Peak II was due to a methyl 9,10,13*(S)*-trihydroxy-11-octadecenoate (ca. 15%), and a methyl 9*(S)*,12,13-trihydroxy-10-octadecenoate having the *threo* configuration at C-12/C-13 (ca. 85%).

The two methyl 9,10,13*(S)*-trihydroxy-11-octadecenoates present in peaks I and II were formed from I_{9S} by solvent attack at C-10 followed by isomerization of the Δ^{10} double bond into the Δ^{11} position and opening of the epoxide group (Fig. 4). The carbon-oxygen bond at C-13 was not affected by this conversion. This fact, coupled with the finding that the configuration of C-13 of the two trihydroxyesters was "*S*," showed that the parent epoxy alcohol I_{9S} had the "*S*" configuration at C-13. Furthermore, from the fact that the C-12/C-13 epoxide group of I_{9S} had the *cis* geometry (IR spectrometry), the complete structure of epoxy alcohol I_{9S} could be deduced, i.e., methyl 12*(R)*,13*(S)*-epoxy-9*(S)*-hydroxy-10*(E)*-octadecenoate (Fig. 5).

Structures of epoxy alcohols formed from 13(R,S)*-HPOD.* The methyl esters of two epoxy alcohols were isolated following incubation of 13*(R,S)*-HPOD. The more polar one (R_f = 0.54) was identified as II_{13S} by comparison with the authentic compound using IR spectrometry, mass-spectrometric analysis of the Me_3Si derivative, chemical degradation and catalytic hydrogenation.

The methyl ester of the less polar epoxy alcohol (II_{13R}; R_f = 0.63) cochromatographed with authentic methyl 11*(S)*,12*(S)*-epoxy-13*(S)*-hydroxy-9*(Z)*-octadecenoate (6). Analysis by GC-MS showed that the C value (22.5) and mass spectra of the Me_3Si derivatives of the two compounds were identical. The infrared spectra of the compounds were also identical and showed absorption bands at 3620-3350 cm^{-1} (hydroxyl), 1735 cm^{-1} (ester carbonyl) and 890 cm^{-1} (*trans* epoxide). No absorption appeared in the region 950-1000 cm^{-1} demonstrating the absence of *E* double bonds. Catalytic hydrogenation of II_{13R} yielded a major product that was identified as methyl *erythro*-12,13-dihydroxyoctadecanoate by $NaAsO_2$-TLC (references: methyl *erythro*-12,13-dihydroxyoctadecanoate, R_f = 0.69; methyl *threo*-12,13-dihydroxyoctadecanoate, R_f = 0.79). Oxidation of the MC derivative of II_{13R} with $KMnO_4$ gave rise to the MC derivative of 2*(R)*-hydroxyheptanoate, as well as methyl hydrogen azelate. From these data it was apparent that II_{13R} was an 11,12-epoxy-13*(R)*-hydroxy-9*(Z)*-octadecenoate. The absolute configurations of C-11 and C-12 were

both *"R"* as deduced from the *erythro* relationship between C-12/C-13, coupled with the configuration of C-13 (*"R"*) and the geometry of the C-11/C-12 epoxide group *(trans)*.

The stereochemistry of epoxy alcohol II_{13R} was studied in an independent way, i.e., by steric analysis of trihydroxyesters formed by hydrolysis. Thus, II_{13R} (1 mg) in methanol (0.5 ml) was treated with glass-distilled water (50 ml) at room temperature for 20 hr. Analysis of the hydrolysis product by TLC (solvent, ethyl acetate) showed two major peaks in a 1:1 ratio (R_f = 0.36 and 0.45), as well as two minor peaks (R_f = 0.57 and 0.71) (6). The two major compounds were subjected to GC–MS analysis as the Me_3Si derivatives and found to be diastereomeric methyl 9,12,13-trihydroxyoctadecenoates. Their stereochemistry was established in the following way. As was previously shown, e.g., by ^{18}O labeling experiments, hydrolysis of 11,12-epoxy-13-hydroxy-9-octadecenoates into 9,12,13-trihydroxy-10-octadecenoates occurs by solvent attack at C-9, isomerization of the Δ^9 double bond into the Δ^{10} position and opening of the epoxide group (6). Thus, the hydroxyl group at C-13 of the epoxy alcohol does not participate in the hydrolysis and its configuration will remain unchanged in the trihydroxyesters formed (in the present case, *"R"* configuration). Oxidative ozonolysis performed on the CC derivative of the two trihydroxyesters yielded *erythro*-2,3-dihydroxyoctanoic acid. This finding, coupled with the fact that the trihydroxyesters had the *"R"* configuration at C-13, showed that the hydroxyl group at C-12 of the trihydroxyesters had the *"S"* configuration. Oxidative ozonolysis performed on the MC derivative of the two trihydroxyesters yielded the MC derivative of 2*(R)*-hydroxysebacate and 2*(S)*-hydroxysebacate from the more polar and the less polar trihydroxyester, respectively. The two trihydroxyesters could thus be identified as methyl 9*(R)*,12*(S)*,13*(R)*-trihydroxy-10-octadecenoate and methyl 9*(S)*,12*(S)*,13*(R)*-trihydroxy-10-octadecenoate. The former diastereomer, in which the two allylic hydroxyl groups lie on the same side with respect to the plane of the double bond, was the more polar diastereomer, in agreement with results recently obtained with related trihydroxyesters (3,6). The fact that the two major trihydroxyesters obtained from II_{13R} were enantiomeric to the corresponding trihydroxyesters formed by hydrolysis of methyl 11*(S)*,12*(S)*-epoxy-13*(S)*-hydroxy-9*(Z)*-octadecenoate (6) gave further proof for II_{13R} being identical to methyl 11*(R)*,12*(R)*-epoxy-13*(R)*-hydroxy-9*(Z)*-octadecenoate (Fig. 5).

DISCUSSION

Recent experiments showing that trihydroxyeicosatrienoates and trihydroxyoctadecenoates accumulate in the medium of growing cultures of *Saprolegnia parasitica* indicated the presence of a lipoxygenase pathway in this fungus (1). Subsequent studies, in which arachidonic acid was incubated with homogenates of the fungus, led to the identification of a lipoxygenase that catalyzed oxygenation of arachidonic acid into 15*(S)*-HPETE and a hydroperoxide isomerase that catalyzed conversion of the hydroperoxide into two epoxy alcohols (2,3). The mechanism of the latter conversion consisted of an intramolecular epoxidation of either of the two conjugated double bonds by the distal hydroperoxide oxygen and thus differed from that of previously described nonenzymatic and pseudoenzymatic conversions of fatty acid hydroperoxides into epoxy alcohols (10–13).

Parts of the lipoxygenase and hydroperoxide isomerase activities of homogenates of *S. parasitica* remained in the supernatant fraction following centrifugation at 105,000 × *g*. These soluble enzyme activities have been characterized and found to be associated with a common protein having a molecular weight of 145,000–150,000 (14).

In the present study of hydroperoxide isomerase from *S. parasitica* using isomeric hydroperoxyoctadecadienoates as substrates, the particle fraction sedimenting at 105,000 × *g* was used as the enzyme source. This fraction was found to contain the major part (about 80%) of the hydroperoxide isomerase activity present in homogenates of *S. parasitica*. Experiments in which linoleic acid was found to be efficiently converted into epoxy alcohols in the presence of the particle fraction (Fig. 3) showed that this fraction also contained lipoxygenase activity, however, it is not known whether the particle-bound lipoxygenase and hydroperoxide isomerase activities, like the soluble ones, are due to the same protein.

Among the different hydroperoxyoctadecadienoates tested, 13*(S)*-HPOD and 9*(S)*-HPOD both served as good substrates for the particle-bound hydroperoxide isomerase. The product in each case consisted of an α,β-epoxy alcohol, in which the epoxide group had the *trans* configuration, and a γ,δ-epoxy alcohol, in which the epoxide group had the *cis* configuration. The structures and stereochemistry of epoxy alcohols formed from 13*(S)*-HPOD and 9*(S)*-HPOD were analogous to those formed from arachidonic acid (2,3), however, the ratio between α,β- and γ,δ-epoxy alcohols differed. When arachidonic acid and 15*(S)*-HPETE were used as precursors, α,β- and γ,δ-epoxy alcohols were formed in a ratio of 0.5–1:1 (2), whereas the corresponding ratio was 2–3:1 when linoleic acid, 13*(S)*-HPOD and 9*(S)*-HPOD were the precursors.

Incubation of racemic 13-HPOD showed that, in addition to 13*(S)*-HPOD, the unnatural 13*(R)*-hydroperoxy isomer was slowly isomerized by the enzyme. The α,β-epoxy alcohol formed from 13*(R)*-HPOD was identified as 11*(R)*,12*(R)*-epoxy-13*(R)*-hydroxy-9*(Z)*-octadecenoic acid. Stereochemical analysis of α,β-epoxy alcohols formed from 13*(S)*-HPOD, 13*(R)*-HPOD and 9*(S)*-HPOD showed that in each case the configuration of the epoxide group was *R,R*. These results thus indicated that the steric course of the enzymatic epoxidation of the *E* double bond of the substrate was directed by the hydroperoxide isomerase and did not bear any relationship to the position or configuration of the hydroperoxy group of the substrate. The *E,E*-hydroperoxides, 9*(R,S)*- and 13*(R,S)*-hydroperoxy-*E,E*-octadecadienoates, were very poor substrates for the isomerase, indicating that the presence of an *E,Z* conjugated diene structure is an important feature of hydroperoxy acids serving as good substrates for the isomerase.

A number of recent studies indicate the importance of lipoxygenase-derived products for regulation of various cell functions in lower organisms. For example, 10,11,12-trihydroxy-5*(Z)*,8*(Z)*,14*(Z)*,17*(Z)*-eicosatetraenoic acid, a lipoxygenase product derived from 5,8,11,14,17-eicosapentaenoic acid, has been identified as a hatching factor in the barnacle, *Balanus balanoides* (15), and 8*(R)*-HETE in low concentrations has been found to induce starfish

oocyte maturation (16). The existence, in *S. parasitica*, of a lipoxygenase pathway that is in operation during active growth (1) may indicate a role of lipoxygenase products in the regulation of growth and reproduction of the fungus.

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Three New and Bioactive Icosanoids from the Temperate Red Marine Alga *Farlowia mollis*

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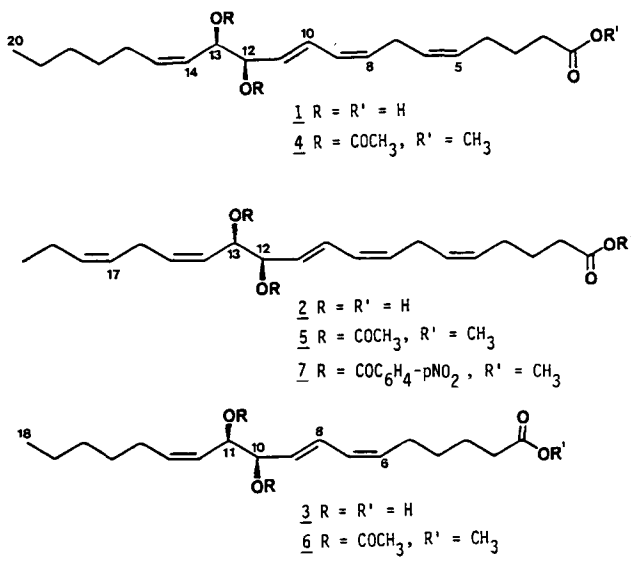
Three new dihydroxyicosanoids, 12(R),13(R)-dihydroxyicosa-5(Z),8(Z),10(E),14(Z)-tetraenoic acid, 12(R),13(R)-dihydroxyicosa-5(Z),8(Z),10(E),14(Z),17(Z)-pentaenoic acid and 10(R*),11(R*)-dihydroxyoctadeca-6(Z),8(E),12(Z)-trienoic acid, have been isolated from a previously unstudied temperate red marine alga, *Farlowia mollis* (Cryptonemiales, Rhodophyta). The structures of these new metabolites have been deduced from detailed nuclear magnetic resonance and mass spectrometry analyses on stabilized diacetate-methyl esters and stereochemistry deduced by ^{1}H NMR couplings and CD analysis of a dibenzoate derivative. Collectively, these new natural products modulate fMLP-induced superoxide anion generation in human neutrophils, inhibit the conversion of arachidonic acid to lipoxygenase products by human neutrophils, and inhibit the functioning of the dog kidney Na^+/K^+ ATPase.
Lipids 24, 256-260 (1989).

Recently, we have reported on the isolation of several new icosanoids from the red seaweeds *Ptilota filicina, Murrayella periclados* and *Platysiphonia miniata* (1-4). Our continued survey of Oregon seaweeds for new natural products with potential biomedicinal application has now identified another red alga, *Farlowia mollis* (Harv. & Bail.) Farl. & Setch., as a rich source of structurally novel and physiologically active icosanoids. The complete structures of these unstable diols (**1,2,3**) were efficiently solved by application of 2D-nuclear magnetic resonance (NMR) and CD methodologies on stabilized derivatives. Furthermore, a metabolite recently isolated from *P. filicina*, 5(Z),7(E),9(E),14(Z),17(Z)-icosapentaenoic acid, as well as all Z 5,8,11,14,17-icosapentaenoic acid (EPA), were also present in the organic extract of this temperate seaweed.

EXPERIMENTAL METHODS

General. Ultraviolet spectra were recorded on an Aminco DW-2a UV-Vis spectrophotometer and infrared spectra (IR) were recorded on a Nicolet 5 DXB FT 15 spectrophotometer. Circular dichroism was measured on a Jasco 41A spectropolarimeter. NMR spectra were recorded on a Bruker AM 400 NMR spectrometer and all shifts are reported relative to an internal TMS standard. Low resolution mass spectra (LRMS) were obtained on a Varian MAT CH7 spectrometer, whereas high resolution mass spectra (HRMS) were obtained on a Kratos MS 50 TC. High performance liquid chromatography (HPLC) was done using a Waters M-6000 pump, U6K injector and R 401 differential refractometer, whereas thin layer chromatograms (TLC) were made using Merck aluminum-backed TLC sheets (silica gel 60 F_{254}). All solvents were distilled from glass prior to use.

Collection, extraction and isolation. Farlowia mollis was collected from exposed intertidal pools (−0.5 to +0.5 m) at Cape Perpetua on the Oregon coast in August 1986. The seaweed was preserved by freezing until workup, at which time the defrosted alga (103 g dry wt) was homogenized in warm $CHCL_3$/MeOH (2:1, v/v). The mixture was filtered and the solvents were removed *in vacuo* to yield 600 mg of a dark green tar which displayed antibiotic activity to *S. aureus* and *E. coli.* The extract was fractionated by silica gel chromatography in the vacuum mode (10 cm × 9 cm, Merck TLC-grade Kieselgel), and metabolites were progressively eluted with increasingly polar mixtures of isooctane and ethyl acetate. The known compounds, 5(Z),7(E),9(E),14(Z),17(Z)-icosapentaenoic acid and all Z 5,8,11,14,17-icosapentaenoic acid (EPA), eluted in 20-40% EtOAc/isooctane (ca. 2-3% of the lipid extract each) and were identified by comparison of their 400 MHz ^{1}H NMR features with authentic standards. Those eluting with 50% EtOAc/isooctane were a mixture of fatty acids and contained diols **1, 2** and **3**. Treatment of a portion of these fractions with CH_2N_2 afforded a mixture of methyl esters that was subsequently chromatographed on normal phase HPLC (μ-Porasil Z-module, 65% EtOAc/isooctane) which removed residual pigments from the fatty acid mixture. After observations using NMR, the fatty acid mixture was acetylated using excess acetic anhydride/pyridine (1/1) and the resulting mixture was separated by normal phase HPLC (2 × 3.9 mm × 25 mm μ-Porasil, 10% EtOAc/isooctane) to yield 10 mg of **4**, 15 mg of **5** and 4 mg of **6**.



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Abbreviations: DMAP, dimethylaminopyridine; EPA, icosapentaenoic acid; SOD, superoxide dismutase; HPLC, high performance liquid chromatography; HRMS, high resolution mass spectra; IR, infrared spectra; LR EIMS, low resolution electron impact mass spectrometry; LRMS, low resolution mass spectra; TLC, thin layer chromatogram.

Methyl 12(R),13(R)-diacetoxy 5(Z),8(Z),10(E),14(Z)-icosatetraenoic acid (4). Compound 4 was a colorless oil showing the following: UV (MeOH) λ_{max} 236 nm (log ε = 4.532); IR ($CHCL_3$) 3022, 3019, 2931, 1734, 1372, 1246, 1222, 1217, 1210, 1026 cm^{-1}; $[\alpha]_D^{25}$ = +2.24° (c = 0.63, CCL_4); low resolution electron impact mass spectrometry (LR EIMS) *m/z* (rel. intensity) 374 (M^+ − AcOH, 9.1), 314 (M^+ − 2 AcOH, 4.4), 265 (26.1), 225 (22.0), 223 (82.7), 205 (36.7), 191 (20.5), 173 (12.8), 169 (22.6), 163 (17.3), 131 (14.5), 127 (62.9), 95 (11.2), 91 (14.4), 83 (15.3), 81 (23.3), 79 (16.6), 67 (20.2), 57 (20.2), 55 (20.6), 43 (100); HR EIMS *m/z* obs. 374.24768 (M^+ − AcOH, $C_{23}H_{34}O_4$ requires 374.24757; for ^1H NMR and ^{13}C NMR data see Table 1.

Methyl 12(R),13(R)-diacetoxy-5(Z),8(Z),10(E),14(Z),17(Z)-icosapentaenoic acid (5). Compound 5 was also isolated as a colorless oil and showed UV (MeOH) λ_{max} 236 nm (log ε = 4.432); IR ($CHCL_3$) 3020, 2965, 2955, 1734, 1434, 1372, 1223, 1220, 1208, 1025 cm^{-1}; $[\alpha]_D^{25}$ = +3.72° (c = 1.13, CCL_4); LR EIMS *m/z* (rel. intensity) 372 (M^+ − AcOH, 6.6), 312 (M^+ − 2AcOH, 4.7), 265 (29.6), 237 (17.0), 224 (15.5), 223 (100), 205 (48.8), 191 (28.0), 173 (21.0), 163 (28.6), 147 (13.7), 145 (20.2), 141 (16.6), 135 (10.1), 131 (28.9), 129 (11.9), 125 (32.5), 121 (13.2), 109 (14.7), 107 (93.0), 105 (19.0), 97 (10.1), 95 (24.0), 93 (20.3), 91 (36.3), 83 (22.8), 81 (35.0), 80 (12.5), 79 (53.2), 77 (13.2), 71 (16.3), 69 (15.6), 59 (13.1), 57 (20.3), 55 (37.1); for ^1H NMR and ^{13}C NMR data see Table 1.

Methyl 10(R),11(R*)-diacetoxy 6(Z),8(E),12(Z)-octadecatrienoic acid (6).* Compound 6 was also isolated as a colorless oil showing the following: UV (MeOH) λ_{max} 234 nm (log ε = 4.224); IR (neat) 3014, 2954, 2930, 2858, 1742, 1457, 1437, 1371, 1241, 1226, 1168, 1114, 1026 cm^{-1}; LR EIMS *m/z* (rel. intensity) 348 (M^+ − AcOH, 13.5), 239 (25.4), 237 (10.7), 223 (15.9), 198 (14.6), 197 (73.5), 180 (12.5), 179 (100), 169 (24.4), 165 (62.4), 147 (25.0), 137 (10.8), 127 (63.1), 119 (19.4), 109 (21.1), 107 (13.7), 105 (11.7), 95 (10.8), 93 (10.6), 91 (14.6), 83 (10.8), 81 (22.4), 79 (14.3), 67 (18.9), 57 (19.4), 55 (16.9), 43 (78.9); for ^1H NMR and ^{13}C NMR data see Table 1.

Formation of methyl 12(R),13(R)-bis(p-nitrobenzoyl)-5(Z),8(Z),10(E),14(Z),17(Z)-icosapentaenoic acid (7) from diol 2. A portion of crude extract (1.2 g) was treated with CH_2N_2 and chromatographed over silica gel in the vacuum mode (EtOAc/isooctane). Fraction 3 (30% EtOAc/isooctane) contained mainly methyl ester derivatives of diols 1-3 and was refluxed overnight with p-nitrobenzoyl chloride in CH_2Cl_2 and pyridine (1:1) with catalytic amounts of dimethylaminopyridine (DMAP). The reaction was terminated with the addition of ice and then H_2O and the products extracted with Et_2O. The Et_2O was sequentially washed with portions of saturated $NaHCO_3$ (2 × 25 ml), 5% HCl (2 × 25 ml), and H_2O (1 × 25 ml). A rapid vacuum chromatography of this material gave a fraction enriched in 7, which was further purified over HPLC (Alltech RSIL 10 μ 50 cm × 10 mm, 15% EtOAc/isooctane, 1.3 mg). Pure derivative 7 showed the following: UV (MeOH) λ_{max} = 240 nm (log ε = 4.874); $[\alpha]_D^{25}$ = +21.0° (MeOH); CD (MeOH) $\Delta\varepsilon$ = −19.8, +34.6 (λ_{max} = 235, 256 nm); ^1H NMR (bz-d-6, 400 MHz) δ 0.97 (t, 3H, J = 7.7 Hz, $C\underline{H}_3CH_2$-), 1.70 (p, 2H, J = 7.4 Hz), 2.09 (m, 4H), 2.31 (t, 2H, J = 7.4 Hz), 2.93 (t, 2H, J = 6.9 Hz), 3.03 (bt, 2H, J = 6 Hz), 3.66 (s, 3H), 5.30-5.49 (m, 5H), 5.52 (m, 3H), 5.88 (dd, 1H, J = 3.8, 8.2 Hz), 6.03 (t, 1H, J = 11.0 Hz), 6.17 (dd, 1H, J = 3.8, 9.5 Hz), 6.80 (dd, 1H, J = 14.7, 11.0 Hz), 8.16 (d, 2H, J = 8.7 Hz, Ar-$\underline{H}$), 8.18 (d, 2H, J = 8.0, Hz, Ar-$\underline{H}$), 8.28 (d, 2H, J = 8.7 Hz, Ar-$\underline{H}$), 8.30 (d, 2H, J = 8.0 Hz, Ar-$\underline{H}$).

Evaluation of superoxide anion production by human neutrophils stimulated with the diols 1-3. Ca. 150 to 200 ml of whole blood (ACD anticoagulant) was drawn from various donors by certified members of the Red Cross or Good Samaritan Hospital (Corvallis, Oregon). The blood was immediately subjected to dextran precipitation (6% Dextran, M.W. = 70,000, Pharmacia). Ca. 60 ml of whole blood were mixed with 40 ml 6% Dextran, and the cells were allowed to stand for 1 hr, undisturbed, in a 100-ml plastic graduated cylinder. After this time, most of the RBCs had settled to the bottom leaving a straw-colored leukocyte-rich plasma on top. This plasma was drawn off and centrifuged at 120 × *g* for 20 min at 4°C. The supernatant was discarded, and the cells were shocked with ice-cold distilled H_2O (ca. 10% the volume of the original plasma suspension), and after 30 sec their isotonicity restored by the addition of 0.6 M NaCl (33% the volume of added H_2O. The cells were washed with PBS (Sigma, w/o Ca^{+2}), and then subjected to a discontinuous gradient centrifugation using Ficoll-Paque (Pharmacia). Ca. 3×10^7 cells in 8 ml PBS were layered onto 4 ml Ficoll-Paque in a 15-ml glass centrifuge tube and then centrifuged at 250 × *g* for 20 min at 4°C. The pellet of cells at the bottom of the tube contained ca. 99% PMNLs by microscopic examination. These cells were washed with PBS and then resuspended using Hank's buffered saline solution (Sigma, with Ca^{+2}, w/o phenol red (HBSS)) at a concentration of 6.0×10^6 cells/ml (5). The cells were counted using a hemocytometer and their viability (>95% for all assays) determined using a trypan blue (0.5% in PBS) exclusion assay in which only damaged cells take up the dye.

The following protocol was used to measure the production of superoxide anion by isolated human neutrophils (6,7). Isolated neutrophils (1-2 hr old, 3-4 × 10^6/0.5 ml Hank's buffered saline solution (HBSS)) were incubated for 10 min at 37°C. Cytochalasin B (5 μg/ml) was added to the cells and the mixture was incubated for 3 min, or if no cytochalasin B was used, the incubation time of 3 min was maintained. Test lipids (0.10-10 μM) were then added to the cells, and the mixture was incubated at 37°C for 2 min. Cytochrome C was then added to the mixture (final conc. 10 mM in HBSS), and followed immediately by fMLP or LTB_4, if they were to be tested. The final assay volume of 2.0 ml was then incubated at 37°C for 15 min and then stopped by the addition of 10 μl of superoxide dismutase (1 mg/0.3 ml HBSS). The cells were pelleted by centrifugation (200 × *g* for 10 min) and the supernatant was measured for absorbances between 500-570 nm (reduced cytochrome C λ_{max} = 550 nm). Blanks were used to obtain baseline values and controls produced by the addition of 10 μl of superoxide dismutase (SOD) before cytochalasin B, fMLP or LTB_4 were added to the cells.

RESULTS AND DISCUSSION

The temperate red alga *Farlowia mollis* was originally collected in our survey efforts from the Oregon coast in 1986 and the lipid extract showed antimicrobial activity to

several human pathogens and the occurrence of potentially novel compounds by TLC (blue char upon acidification at 0.13 R_f). Furthermore, the crude extract of *F. mollis* displayed good inhibitory activity to dog kidney Na^+/K^+ ATPase (IC_{50} = 38 μg/ml) and hog gastric mucosa H^+/K^+ ATPase (IC_{50} = 9.4 γ/ml). Thus, larger collections were made in August 1986, May 1987 and June 1987, all of which contained the same unusual appearing blue-charring compounds. Bioautography against *E. coli* indicated that polar compounds in the extract were responsible for the antimicrobial activity. Hence, the material collected in August of 1986 was vacuum chromatographed over silica gel to rapidly yield mixtures enriched in these polar blue-charring diols (1–3).

In order to stabilize and more easily separate compounds 1-3, they were first treated with CH_2N_2 and later with acetic anhydride in pyridine, to produce diacetate-methyl ester derivatives. This mixture of derivatives was then easily separated by HPLC to yield (in order of their polarity) a purple-charring compound, (4), a blue-charring compound, (5), and a grey-blue-charring compound, (6).

The diacetate-methyl ester derivative of 4 was a colorless and optically active oil which gave a measurable M^+ − acetate peak by HR EIMS affording a molecular formula of $C_{25}H_{38}O_6$ (7° unsaturation). The IR spectrum for 4 showed an intense carbonyl stretch for multiple esters ($\gamma_{C=0}$ = 1734 cm^{-1}), the protonic consequences of which were readily observed in the ^1H NMR (Table 1) and defined two acetates and one methyl ester. By ^{13}C NMR, the remaining four degrees of unsaturation presented as olefinic bonds (Table 1), and two of these formed a conjugated system (λ_{max} = 236 nm).

The overall structure of derivative 4 was readily approachable by ^1H-^1H COSY experiments and lead to the generation of two partial structures which accounted for all of the atoms in the molecule. The first partial structure began with a sharp 2H triplet at δ 2.09, which was readily identified from comparisons with model compounds as belonging to the C-2 protons of a fatty acid, in this case in the form of a methyl ester. Sequential correlations between these C-2 protons and those at C-3, C-4 and C-5 defined a normal Δ 5 unsaturated fatty acid (Table 1). The other proton of the Δ 5 olefin was located at δ 5.29 and was additionally correlated to a bisallylic methylene at δ 2.78 (H_2-7). The C-7 methylene was a triplet and was therefore coupled to one other proton, located at δ 5.38 from the COSY experiment. The C-8 proton was coupled by 11.0 Hz to its olefin partner (δ 5.98) and, thus, defined a *cis* geometry for this double bond. This latter proton was coupled to another olefin proton at δ 6.83 (H-10), which in turn was correlated to its olefin partner (δ 5.78, H-11) by 15.0 Hz and, therefore, was of

TABLE 1

NMR Data for the Methyl Ester Diacetate Derivatives of Three Icosanoid Natural Products From *Farlowia mollis*[a]

	Compound 4				Compound 5				Compound 6			
	^1H			^{13}C[b]	^1H			^{13}C[c]	^1H			^{13}C[d]
C#	δ	J	(Hz)	δ	δ	J	(Hz)	δ	δ	J	(Hz)	δ
1	—	—	—	173.18	—	—	—	173.18	—	—	—	—[e]
2	2.09	t	7.3	33.32	2.09	t	7.3	33.31	2.01	t	7.4	33.85
3	1.57	tt	7.3,7.3	25.03	1.57	tt	7.3,7.3	25.02	1.43	tt	7.4,7.6	24.65
4	1.91	dt	7.3,7.3	26.75	1.91	dt	7.3,7.3	26.75	1.15	tt	7.6,7.2	29.13
5	5.25	m	—	129.86	5.25	m	—	129.86	1.92	dt	7.2,7.2	27.60
6	5.29	m	—	128.16	5.29	m	—	—[e]	5.31	td	7.2,11.0	134.44
7	2.78	bdd	7.3,7.3	26.43	2.77	bdd	7.2,7.2	26.42	5.97	bdd	11.0,11.0	127.93
8	5.38	td	7.3,11.0	132.44	5.40	m	—	132.48	6.75	dd	11.0,15.0	130.91
9	5.98	bdd	11.0,11.0	127.93	5.97	bdd	11.2,11.2	127.90	5.78	dd	15.0,8.1	127.04
10	6.83	dd	11.0,15.0	130.75	6.82	dd	11.2,15.2	130.84	5.87	dd	8.1,3.7	75.61
11	5.78	dd	15.0,8.2	127.38	5.76	dd	15.2,8.2	127.23	6.21	dd	3.7,8.2	70.65
12	5.87	dd	8.2,3.7	75.58	5.85	dd	8.2,3.8	75.47	5.60	m	—	124.02
13	6.20	dd	3.7,8.0	70.61	6.19	dd	3.8,8.5	70.54	5.60	m	—	136.91
14	5.59	m	—	124.02	5.55	m	—	124.13	2.21	m	—	28.40
15	5.59	m	—	136.93	5.59	m	—	135.02	1.30	m	—	29.46
16	2.22	m	—	28.40	3.03	m	—	26.75	1.25	m	—	31.71
17	1.29	tt	6.9,6.9	29.47	5.40	m	—	126.31	1.22	m	—	22.87
18	1.23	m	—	31.72	5.40	m	—	133.04	0.89	t	6.8	14.22
19	1.23	m	—	22.89	2.03	dq	7.3,7.3	20.88				
20	0.86	t	6.8	14.22	0.91	t	7.3	14.38				
OMe	3.65	s	—	50.98	3.65	s	—	50.99	3.65	s	—	50.98
OAc	1.70	s	—	20.64	1.70	s	—	20.69	1.70	s	—	20.65
OAc	1.75	s	—	20.70	1.75	s	—	20.69	1.70	s	—	20.65
C=0	—	—	—	169.51(2C)	—	—	—	169.46(2C)	—	—	—	169.46(2C)

[a] Chemical shift values in ppm relative to TMS as an internal standard operating at 9.398 T. All spectra obtained in deuterated benzene.

[b] Assignments made from a ^1H-^{13}C heteronuclear 2D shift correlation spectroscopic experiment and by comparison with model compounds[1,2].

[c] Assignments made by comparing with (4) and with model compounds[1,2].

[d] Assignments made by comparing with (4), (5) and with model compounds[1,2].

[e] Peak not observed in spectrum.

trans geometry. The H-11 proton was coupled to a proton α to an acetate located at δ 5.87 (H-12, 75.58 ppm from HETCOR) and this was adjacent to another such α-acetoxy proton at δ 6.20 (H-13, 70.61 ppm). This partial structure terminated with a correlation between the H-13 proton and a two proton olefin multiplet at δ 5.59 (H-14,15).

The second partial structure in derivative 4 began with a poorly defined methyl triplet at δ 0.86 which showed correlations to a 4H multiplet at δ 1.23 (H_2-19, H_2-18). The multiplet was further coupled to a 2H multiplet at δ 1.29 (H_2-17), itself correlated to an allylic doublet of triplets at δ 2.22 (H_2-16). Finally, this latter band was also correlated to the 2H olefin multiplet at δ 5.59 (H-15,14).

Combination of these spin system-derived partial structures accounted for all of the atoms in the molecule and could be put together in only a single manner, thus giving the constitutive structure for diol 4. The stereochemistry of the C5-6 olefin was given by the ^{13}C NMR chemical shift of the adjacent *bis*-allylic methylene. A value of δ 26.43 for this carbon atom (assigned from the HETCOR experiment) defines both adjacent olefins as possessing Z stereochemistries (8,9). Similarly, the shift of δ 28.40 for C-16 defines a Z stereochemistry for the C14-15 olefin. The relative configurations at C-12 and C-13 were defined as threo from the diagnostic couplings (J = 3.7 Hz) between these protons (see discussion which follows for 5) (10). The absolute stereochemistry was deduced as 12R, 13R by virtue of this threo relationship and the comparable optical rotations of derivatives 4 and 5. Determination of the absolute stereochemistry of diol 2 and, thus, of derivative 5, was by CD as will be discussed. Hence, the structure of diol 1 was deduced as 12(R),13(R)-dihydroxyicosa-5(Z),8(Z),10(E),14(Z)-tetraenoic acid.

The spectroscopic features (IR, UV, optical rotation and NMR) of the diacetate-methyl ester derivative 5 were similar to those obtained for the same derivative of diol 4 (Table 1). Further, the LR EIMS of derivative 5 showed a highly analogous pattern of cleavage relative to compound 4. However, several significant peaks (i.e., M+ − HOAc) in the mass spectrum of 5 were 2 amu units less than in 4 and indicated that it contained an additional olefin. All of the proton and carbon resonances ascribable to protons and carbons at C-1 through C-15 were nearly identical in compounds 4 and 5. However, two additional olefin resonances and one additional *bis*-allylic methylene resonance were observed in the ^1H NMR spectrum of derivative 5 relative to derivative 4. Further, the terminal methyl group was slightly further down field and was coupled to an allylic methylene at C-19, positioning the new olefin between C-17 and C-18. This olefin was of the Z stereochemistry as revealed by characteristic carbon shifts of δ 26.75 for C-16 and δ 20.88 for C-19 (1,8,9).

In a simple diol such as 5 (i.e., without the confounding effects of 1–3 interactions from additional substituents at C-10, C-11, C-14 or C-15), the C-12-C-13 rotomer with antioriented alkyl groups will predominate as the lowest energy conformation (10). Hence, a threo arrangement between protons at C-12 and C-13 was indicated by measurement of a diagnostically small coupling constant (J = 3.8 Hz) (10). The *bis*-(p-nitrobenzoate) derivative 7 showed a bisignate CD curve with a positive maximum at 256 nm and a negative maximum at 235 nm and, thus, could be assigned to heterochromophoric exciton coupling between the C-8 to C-11 diene and p-nitrobenzoate at C-12 (11,12). This positive split Cotton effect indicates a right-hand screwness between these groups and defines the stereochemistry at 12 as R (12–15). The 12R, 13R stereoisomer is the only one consistent with (a) the threo arrangement of protons at C-12 and C-13, (b) the anti arrangement of alkyl chains and (c) a righthand screwness between benzoate and diene chromophores (Fig. 1). Hence, diol 2 was the ω-3 analog of compound 1, or 12(R),13(R)-dihydroxyicosa-5(Z),8(Z),10(E),14(Z),17(Z)-pentaenoic acid. The R stereochemistry at C-12 correlates to the same relative arrangement of atoms as found in 12-(S)-HETE and 12-(S)-HEPE, hydroxyicosanoids we have recently isolated from other red marine algae (3,4).

Derivative 6 of diol 3 was isolated from related chromatography fractions and again showed very similar spectroscopic (IR, UV, LR EIMS and NMR) features to derivatives 4 and 5. The mass spectrum of 6 gave analogous peaks as for 4, however, they occurred at 26 amu lower, indicating that it was derived from an 18-carbon fatty acid. Derivative 6 showed nearly identical ^1H and ^{13}C NMR bands for atoms at C-6 to C-18, as assigned to C-8 to C-20 in derivative 4 (Table 1). From ^1H-^1H COSY data, sequential correlations were observed between methylene groups at C-2 to C-5, as well as a correlation from H_2-5 to an olefin proton at δ 5.31 (C-6). Although the threo relative stereochemical relationship between protons at C-12 and C-13 was intact in 6 (J = 3.7), correlation to the absolute stereochemistry in

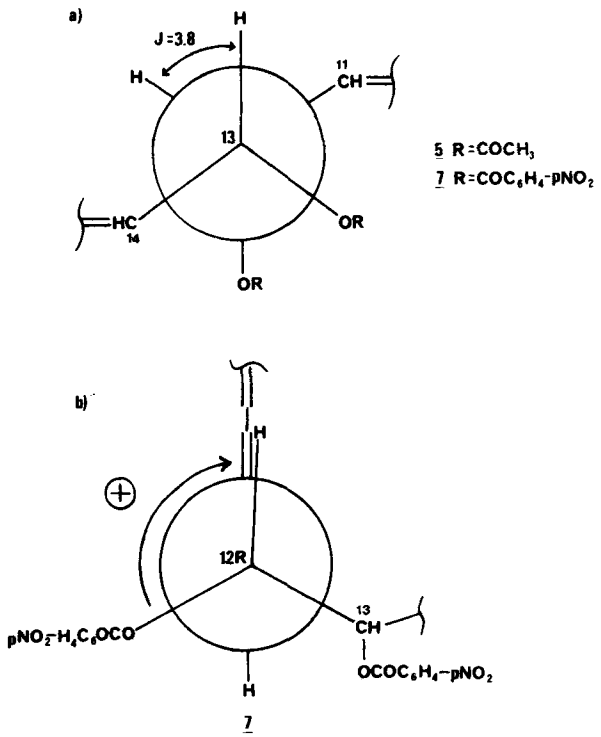

FIG. 1. Newman projection of (a) C-13-C-12 in derivatives 5 and 7 showing threo relationship of protons based on their coupling constant, and (b) C-12-C-11 in derivative 7 showing the clockwise (positive split Cotton effect) relationship of the C-12 p-nitrobenzoate chromophore and the C-8 to C-11 diene chromophore.

TABLE 2

Superoxide Anion Production in Human Neutrophils Stimulated by a 1:1.5 Mixture of Diols 1 and 2[a]

	Donor 1[b]	Donor 2[b]	Donor 3[c]
Control (SOD,fMLP) w/o CytoB	ND	ND	ND
Control (SOD,fMLP) w/CytoB	ND	ND	ND
Control (SOD,LTB_4	ND	ND	ND
Control (cells,no SOD)	—	—	ND
fMLP (20^{-7}M)	20.8	20.4[c]	14.6
1+2 (10^{-5}M)	19.4	2.1	5.5
1+2 (10^{-6}M)	22.2	ND	2.7
1+2 (10^{-7}M)	23.7	1.4	2.6
1+2 (10^{-5} fMLP (10^{-7}M)	17.5	17.1	9.5
1+2 (10^{-6}M) fMLP (10^{-7}M)	24.7	—	—
1+2 (10^{-7}M) fMLP (10^{-7}M)	21.8	21.8	16.7

[a] 3.0×10^6 cells per assay, O_2^- production expressed as total nmoles/3.0×10^6 cells.

[b] Single measurement.

[c] Average of two replicates.

ND, none detected; —, experiment not performed.

derivative 5 was precluded as the compound decomposed before rotational data could be recorded. Hence, 3 was an 18-carbon analog (10(R*),11(R*)-dihydroxyoctadeca-6(Z),8(E),12(Z)-trienoic acid) of diol 1, in which the olefin and hydroxy functionalities were in the same positions in the two compounds relative to their methyl termini.

A mixture of diols 1 and 2 (1:1.5) were evaluated for several pharmacological properties expected in analogs of leukotriene, lipoxin and diHETE-type natural products. In Table 2, values are reported for the production of superoxide anion by human neutrophils stimulated under various experimental conditions. The mixture appears to be a weak primary stimulator of superoxide anion production. However, at 10^{-5} M, it inhibited fMLP-stimulated superoxide anion production in ranges between 15% and 34% of control values for three donors. This inhibitory activity is comparable with the activities of the prostaglandins PGE_1 and PGI_2 at equivalent concentrations (7).

The 1:1.5 mixture of diols 1 and 2 showed weak activity in preliminary testing for inhibition of 5-lipoxygenase activity in A23187-stimulated human polymorphonuclear leukocytes (38% inhibition at 10^{-4} M). Other dihydroxy-icosanoids are known inhibitors of lipoxygenase activity (16). Further, the mixture was only moderately inhibitory to the dog kidney Na^+/K^+ ATPase preparation (54% inhibition at 10^{-4} M, 35% at 10^{-5} M).

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Polyunsaturated Fatty Acid Changes Suggesting a New Enzymatic Defect in Zellweger Syndrome

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The fatty acid composition of red blood cells, fibroblasts, forebrain, liver and kidney were studied in a 3-month-old infant who died from Zellweger Syndrome, and the results were compared with those of age-matched controls. Besides a typical increase in the very long chain fatty acids 26:0 and 26:1 and a great reduction in the plasmalogen levels, confirming the diagnosis of Zellweger Syndrome, some striking changes in the polyunsaturated fatty acid patterns were discovered. The most important was a very drastic decrease in the values of 22:6ω3 and 22:5ω6, the two products of Δ4-desaturation. In the kidney, the level of 22:6ω3 fell below that of 26:0. Consequently, the ratio 26:0/22:6ω3 (and 26:1/22:6ω3) was most useful in emphasizing the fatty acid anomalies, especially in renal tissue, where the 26:0/22:6ω3 ratio increased to almost 200 times the normal values. Other significant, although less consistent fatty acid alterations were increases in 18:2ω6, 18:3ω6, 20:3ω6, 18:4ω3 and 20:4ω3, and a decrease in 20:4ω6 in some tissues. The existence is proposed of a new enzyme defect in peroxisomal disorders, involving the desaturase system of long chain polyunsaturated fatty acids.
Lipids 24, 261-265 (1989).

Zellweger Syndrome is a rapidly progressive, rare hereditary disorder that involves the central nervous system, liver, kidney and bones. Affected patients die within the first months of life with severe nervous system dysfunction. A total absence of peroxisomes in hepatocytes and renal proximal tubules has been reported (1) in these patients, in association with severe defects in certain enzymes known to be located in peroxisomes. These include the long chain fatty acid β-oxidation system and dihydroxyacetone phosphate acyltransferase (EC 2.3.1.42), a crucial enzyme in plasmalogen synthesis (2). As a result of these enzyme defects, an accumulation of very long chain fatty acids (3) and a drastic decrease in plasmalogen concentration (4) have been described in tissues of Zellweger patients. No other anomalies in the fatty acid composition of affected tissues have been reported, and no data exist for the polyunsaturated fatty acid (PUFA) patterns of any of the affected tissues (See references 5-7 for recent reviews on the subject.).

This paper reports some hitherto undescribed, very significant changes in the PUFA composition of tissues from a patient with Zellweger syndrome. A preliminary account of this work has appeared elsewhere (8).

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Abbreivations: BHT, butyl hydroxytoluene; DMA, dimethyl acetal; EFA, essential fatty acid; EP, ethanolamine plasmalogen; FAME, fatty acid methyl ester; GPC, glycerophosphocholine; GPE, glycerophosphoethanolamine; GPL, glycerophospholipid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PUFA, polyunsaturated fatty acid; FID, flame ionization detector; GLC, gas liquid chromatography; TLC, thin layer chromatography.

MATERIALS AND METHODS

A 2-month-old infant with Zellweger syndrome was studied for the total fatty acid and plasmalogen composition of plasma, red blood cells and cultured fibroblasts. The neurological picture and the X-ray findings were typical of the disease. Biochemically, the patient could be diagnosed in vivo by significant increases in the ratios 26:0/22:0 and 26:1/22:0, most marked in fibroblasts, and by important decreases in total plasmalogens and in the 18:0 dimethyl acetal/methyl stearate ratio and 16:0 dimethyl acetal/methyl palmitate ratio, mainly in red blood cells. After death, which occurred at 3 months of age, the fatty acid and plasmalogen composition of the patient's cerebrum, liver and kidney were studied. The cerebrum, liver and kidney of 5 control infants, neurologically normal and with ages ranging from 30 prenatal weeks to 7 postnatal months, were also studied. Total forebrain homogenates were generally used, but in addition, the cerebral cortices of the patient and one of the controls were studied. The bodies were refrigerated at 4°C immediately after death, and autopsy was carried out within 24 hr postmortem.

All the solvents were of highest commercial quality (Merck, Darmstadt, West Germany) and contained 50 mg/l of butyl hydroxy toluene (BHT) (Fluka, Buchs, Switzerland) to prevent lipoperoxidation. In order to study the total fatty acid and plasmalogen composition of tissues, the fatty acid methyl esters (FAME) and aldehyde dimethyl acetals (DMA) of all the samples (about 100 mg of tissues and packed red blood cells or 1 mg of protein, in the case of fibroblasts) were obtained by direct transesterification without lipid extraction, according to the method of Lepage and Roy (9). Suitable amounts of 13:0 and 19:0 (500 μg for tissues, 100 μg for packed erythrocytes and 50 μg for fibroblasts) were added before transesterification, as internal standards.

Given the enormous alterations found in the total fatty acid patterns, the fatty acid composition of ethanolamine and choline glycerophospholipids was also studied. Total lipids were extracted with 20 volumes of chloroform/methanol/water (50:50:15, v/v/v), and separated into neutral and acidic lipids by DEAE-Sephadex column chromatography (10). Ethanolamine and choline glycerophospholipids (from 15 μg of lipid phosphorus) were separated by thin layer chromatography (TLC) from neutral lipids, with chloroform/methanol/water (60:25:4, v/v/v) as the solvent system. The spots were made visible with bromothymol blue, scraped off, transferred to methanolysis tubes (Corning, New York, NY) and transesterified by the same method (9), after addition of 50 μg of 13:0 and 19:0.

The FAME and DMA were separated by capillary column gas-liquid chromatography (GLC). A Hewlett-Packard 5890 gas chromatograph was used, equipped with a 30 m long, 0.25 mm i.d., SP-2330 fused silica capillary column (Supelco, Bellefonte, PA) and flame ionization detectors (FID). A two-step temperature program was used (140-180°C, at 4°C/min; 180-210°C, at

2°C/min) in order to optimize separation of all the peaks. This program allowed a very clean separation of 26:0 and 26:1 from the two artifactual peaks coming from cholesterol esters (marked 1 and 2 in Fig. 1), without the need for using TLC for methyl ester purification, a step that always leads to losses of PUFA. The carrier gas was helium, at a column pressure of 17 psi (flow rate, about 1 ml/min), and the split ratio varied between 10:1 and 50:1, depending on the amount of FAME injected. Injector and detector temperatures were 200°C and 220°C, respectively.

Identification of the peaks was carried out by comparison with authentic standards, whenever possible, and/or with mixtures of known fatty acid and aldehyde composition. Otherwise, fatty acids were identified by the equivalent chain length approach (11). The peak areas were measured with a Hitachi D-2000 integrator (Hitachi, Ltd., Tokyo, Japan) and quantitated on a molar basis. Because nanomoles of DMA directly gave nanomoles of alkenyl-acyl compounds (plasmalogens), the figures for FAME and DMA were used to calculate the absolute amounts of the corresponding diacyl glycerophospholipids (GPL), phosphatidylethanolamine (PE) and phosphatidylcholine (PC), by using the following formula:

$$\text{nmol of diacyl GPL (PE or PC)} = \frac{\text{nmol FAME} - \text{nmol DMA}}{2}$$

All computations and statistical analyses were carried out with a Compucorp electronic calculator-programmer, Model 445 Statistician (Computer Design Corp., Los Angeles, CA). The values for grouped data were the means ± SEM.

RESULTS

Tables 1 and 2 show the total fatty acid and plasmalogen composition, respectively, of the tissues and cells studied in absolute, quantitative terms. The decreases in total plasmalogens and in the 16:0 dimethyl acetal/palmitic acid (16DMA/16:0) ratio and the 18:0 dimethyl acetal/stearic acid (18DMA/18:0) ratio were evident in all samples, especially in the kidney and red blood cells, whereas the increase in the very long chain fatty acids 26:0 and 26:1 was most pronounced in fibroblasts and

TABLE 1

Total Fatty Acids in Several Tissues of a Case of Zellweger Syndrome Compared With Controls

	Cerebrum		Liver		Kidney		Erythrocytes		Fibroblasts	
	ZS	Controls (n = 5)	ZS	Controls (n = 5)	ZS	Controls (n = 5)	ZS	Controls (n = 6)	ZS	Controls (n = 6)
14:0	643	860 ± 116	1108	1072 ± 232	714	584 ± 154	97	28 ± 2	7	7 ± 0.4
16:0	17822	18533 ± 1366	31755	30083 ± 3134	10990	13972 ± 1959	2708	1547 ± 100	154	136 ± 6
16:1ω7	1011	532 ± 44	2203	3735 ± 929	776	1416 ± 607	99	26 ± 7	5	8 ± 0.7
18:0	16048	12392 ± 3389	10869	12873 ± 945	8026	7715 ± 901	1126	1040 ± 53	135	116 ± 6
18:1ω9	10035	8362 ± 1568	12534	19579 ± 2782	7663	9981 ± 896	1228	764 ± 35	87	96 ± 5
18:1ω7	3762	2557 ± 431	1408	3593 ± 776	1040	1939 ± 360	109	75 ± 8	27	28 ± 2
18:2ω6	1028	364 ± 82	18842	13842 ± 3878	7079	5967 ± 1690	973	533 ± 52	11	11 ± 1
18:3ω6	39	23 ± 6	116	149 ± 13	43	34 ± 5	13	2 ± 1	0.4	0.7 ± 0.1
18:3ω3	10	12 ± 7	149	225 ± 99	18	30 ± 8	8	5 ± 1	0.4	0.4 ± 0.1
18:4ω3	246	9 ± 5	26	14 ± 5	23	6 ± 2	ND	ND	0.4	0.1 ± 0.1
20:0	225	162 ± 50	118	266 ± 32	426	332 ± 43	25	32 ± 3	1.7	2.1 ± 0.2
20:1ω9	761	262 ± 71	318	202 ± 29	379	195 ± 30	41	16 ± 2	3.4	1.9 ± 0.2
20:3ω9	818	310 ± 62	284	846 ± 479	205	192 ± 66	33	15 ± 4	1.2	1.2 ± 0.3
20:3ω6	2379	736 ± 148	1845	1432 ± 188	1116	809 ± 77	333	97 ± 9	8	8 ± 1
20:4ω6	7314	6802 ± 736	7174	9880 ± 954	5768	8060 ± 816	619	853 ± 53	85	92 ± 6
20:4ω3	26	6 ± 4	24	16 ± 6	37	14 ± 3	5	1 ± 1	0.6	0.2 ± 0.1
20:5ω3	58	34 ± 11	335	174 ± 29	134	130 ± 31	18	29 ± 7	0.6	5 ± 0.4
22:0	127	116 ± 69	152	513 ± 58	464	625 ± 86	68	97 ± 11	4	7 ± 1
22:4ω6	2020	3904 ± 760	406	443 ± 42	466	599 ± 135	159	183 ± 11	38	15 ± 2
22:5ω6	181	1823 ± 170	32	619 ± 106	26	225 ± 50	28	58 ± 7	2.4	2 ± 0.1
22:5ω3	102	134 ± 28	252	338 ± 146	132	172 ± 40	43	65 ± 18	19	16 ± 2
22:6ω3	1478	4943 ± 633	183	3188 ± 281	58	933 ± 174	32	283 ± 34	17	22 ± 2
24:0	72	265 ± 185	354	576 ± 57	1110	845 ± 110	102	243 ± 16	16	13 ± 1
24:1ω9	351	422 ± 275	535	611 ± 115	577	666 ± 136	285	246 ± 13	12	14 ± 1
26:0	12	33 ± 21	72	29 ± 6	305	28 ± 5	27	18 ± 2	4.1	0.3 ± 0.1
26:1ω9	52	67 ± 42	107	26 ± 6	123	35 ± 9	29	15 ± 1	3.3	0.4 ± 0.1
TFA[a]	68422	70633 ± 8771	92504	105805 ± 8181	48751	56392 ± 5213	8481	6406 ± 294	673	635 ± 27
22:6ω3/22:5ω3	14.44	46.26 ± 15.05	0.73	21.57 ± 7.77	0.44	7.29 ± 2.15	0.73	5.40 ± 1.23	0.89	1.39 ± 0.12
22:5ω6/22:4ω6	0.09	0.49 ± 0.06	0.08	1.40 ± 0.19	0.05	0.38 ± 0.04	0.18	0.32 ± 0.03	0.06	0.14 ± 0.02
20:4ω6/20:3ω6	3.07	10.49 ± 1.59	3.89	7.25 ± 1.06	5.17	10.01 ± 0.56	1.86	9.14 ± 1.03	11.21	12.66 ± 1.16
26:0/22:6ω3	0.01	0.02 ± 0.01	0.39	0.010 ± 0.002	5.28	0.03 ± 0.01	0.84	0.07 ± 0.02	0.25	0.02 ± 0.01
26:1/22:6ω3	0.04	0.01 ± 0.01	0.59	0.009 ± 0.002	2.13	0.04 ± 0.01	0.93	0.06 ± 0.01	0.20	0.02 ± 0.003

[a]Total fatty acids. Other minor constituents not reported in this table, such as 12:0, 20:2ω9, 20:2ω6, etc., are included in the figures for tissue total fatty acids. The fatty acid values are given in nmols/g of wet tissue for forebrain, liver and kidney, in namols/ml of packed cells for erythrocytes, and in nmols/mg of protein for fibroblasts. Values for grouped data are means ± SEM.

ZS, Zellweger Syndrome.

TABLE 2

Alkenyl Composition of Total Plasmalogens in Several Tissues of Zellweger Syndrome Compared With Controls

	Cerebrum		Liver		Kidney		Erythrocytes		Fibroblasts	
	ZS	Controls (n = 5)	ZS	Controls (n = 5)	ZS	Controls (n = 5)	ZS	Controls (n = 6)	ZS	Controls (n = 6)
16DMA	424	2043 ± 400	32	330 ± 26	38	1481 ± 179	0.9	123 ± 12	7.1	20 ± 1.2
18DMA	238	2690 ± 677	58	298 ± 39	7	781 ± 123	6.4	188 ± 16	3.4	11 ± 0.4
18:1ω9DMA	18	307 ± 126	11	48 ± 6	ND	178 ± 42	4.9	48 ± 2	1.5	4 ± 0.2
18:1ω7DMA	90	434 ± 168	ND	31 ± 8	ND	126 ± 41	ND	12 ± 1	1.4	6 ± 0.2
TP[a]	770	5474 ± 1354	102	706 ± 43	46	2566 ± 328	12	371 ± 24	13	42 ± 1.1
16DMA/16:0	0.02	0.11 ± 0.01	0.001	0.012 ± 0.002	0.004	0.114 ± 0.017	0.0004	0.080 ± 0.005	0.05	0.15 ± 0.004
18DMA/18:0	0.02	0.14 ± 0.02	0.005	0.023 ± 0.001	0.001	0.102 ± 0.016	0.006	0.181 ± 0.013	0.02	0.10 ± 0.007

[a]Total plasmalogens. DMA, dimethyl acetal; ZS, Zellweger Syndrome. The values for total plasmalogens and alkenyl groups are given in nmols/g of wet tissue for forebrain, liver and kidney, in nmols/ml of packed cells for erythrocytes, and in nmols/mg of protein for fibroblasts. Control values are means ± SEM.

kidney. The most striking and consistent finding, however, was a drastic decrease in the amount of 22:6ω3 (docosahexaenoic acid), as well as 22:5ω6 (docosapentaenoic acid), particularly in the kidney and liver. The decrease in docosahexaenoic acid in renal tissue was such that it fell below that of cerotic acid (26:0), as could be seen by just looking at the chromatogram (Fig. 1). This inversion of the normal situation—in which 26:0 is only a trace constituent at that age, whereas 22:6ω3 accounts for about 2% of the kidney total fatty acids—was much emphasized by using the ratio 26:0/22:6ω3. In all tissues, the ratio 26:0/22:6ω3 (and 26:1/22:6ω3) is normally much less than 0.1, whereas in the patient's kidney, this ratio was 5.3.

The ratios 22:6ω3/22:5ω3 and 22:5ω6/22:4ω6 were clearly decreased in the tissues studied, indicating that the desaturation reactions 22:5ω3 → 22:6ω3 and 22:4ω6 → 22:5ω6 were probably defective in the patient. To a lesser extent, the reactions 20:3ω6 → 20:4ω6 and 20:4ω3 → 20:5ω3 were also diminished in most tissues, as indicated by a decreased 20:4ω6/20:3ω6 ratio, mainly in erythrocytes, and by an augmentation of the minor component, 20:4ω3, in the forebrain and kidney. The main constituent of the ω6 series, arachidonic acid (20:4ω6), was within normal limits in the cerebrum and slightly decreased in erythrocytes, liver and kidney. The parent ω6 fatty acid, linoleic acid (18:2ω6), was very significantly increased in the forebrain and red blood cells; and the minor precursor, 18:3ω6, was only slightly increased in erythrocytes. The trace ω3 precursor, 18:4ω3, was significantly increased in the cerebrum. These small changes suggest that the elongation of fatty acids may also be slightly impaired in some tissues. The two minor ω9 fatty acids, 20:1ω9 and 20:3ω9, tended to increase in most tissues, but only significantly in the forebrain. It is worthwhile noticing that fibroblasts were almost normal in PUFA composition, although the two ratios, 22:6ω3/22:5ω3 and 22:5ω6/22:4ω6, were significantly decreased in the Zellweger fibroblasts compared with controls.

Figure 2 shows the absolute values of glycerophosphoethanolamines (GPE) and glycerophosphocholines (GPC) in the cerebral cortex, liver and kidney of the patient compared with a control. This figure shows that there was no decrease in the absolute amounts of the major two

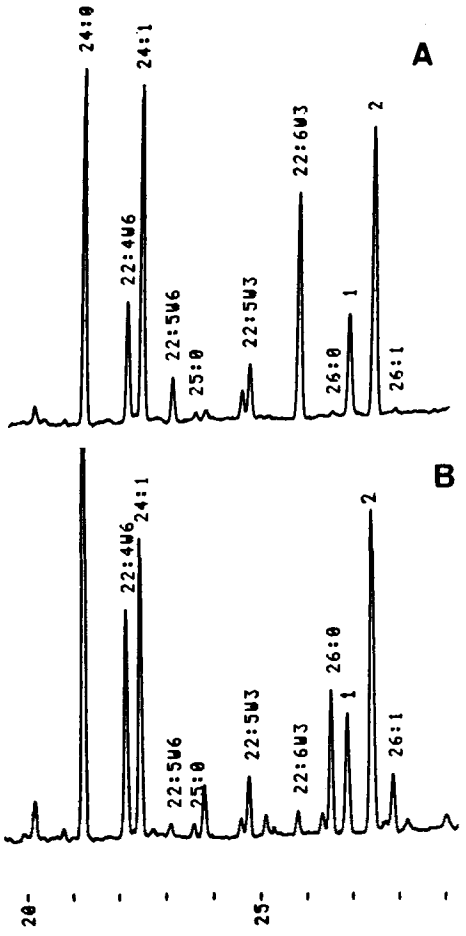

FIG. 1. Part of two gas chromatograms showing the long chain fatty acid methyl esters of renal tissue. (A) Normal profile in a 3-month old infant. (B) Altered profile in the Zellweger patient.

phospholipids of the patient, but rather an increase. In agreement with data by Heyman et al. (4), the diacyl glycerophosphoethanolamines (GPE) were increased in the Zellweger patient, in contrast to the drastic decrease in alkenylacyl glycerophosphoethanolamines, the ethanolamine plasmalogens (EP).

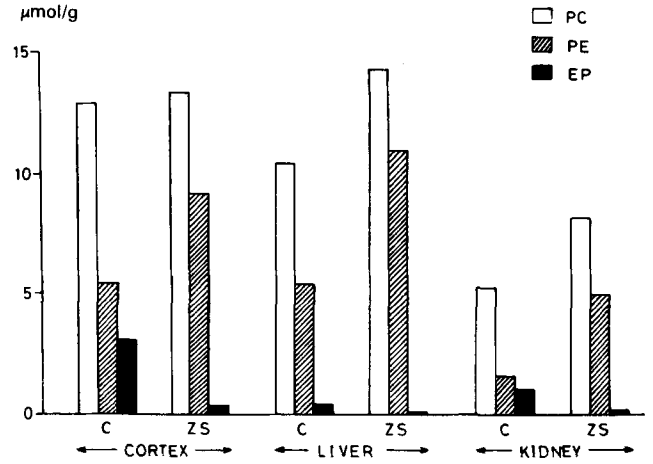

FIG. 2. Absolute amounts of phosphatidylcholine (PC), phosphatidylethanolamine (PE) and ethanolamine plasmalogen (EP) in the cerebral cortex, liver and kidney of the infant with Zellweger Syndrome (ZS), compared with a neurologically normal control (C).

Tables 3 and 4 show the fatty acid patterns of GPE and GPC, respectively, in the three tissues studied, expressed as molar percentages. Again, a very important decrease in the two long chain PUFA, 22:6ω3 and 22:5ω6, can be discerned as the major change in both phosphoglycerides. It can also be seen that the proportion of linoleic acid was significantly increased in GPE of all three tissues. In GPC, the increase in 18:2ω6 was only significant in the cerebrum and liver. The proportion of 20:4ω6 was very significantly decreased in cerebral and renal GPC.

DISCUSSION

The marked decrease in the tissue levels of the two long chain PUFA, 22:6ω3 and 22:5ω6, as reported in this paper, has not been described before in reference to Zellweger Syndrome and simply cannot be explained by an altered distribution and/or a decrease in the lipid content of tissues. Figure 2 shows that the total levels of ethanolamine and choline glycerophospholipids were not decreased in the patient, and the normal amount of total fatty acids excludes any significant loss of lipids, such as is found in PUFA lipidosis (12). Although the total amount of GPE was not decreased in the patient, this very unsaturated phospholipid was remarkably poor in 22:6ω3 and 22:5ω6—a finding consistent with a defect in the synthesis of these two fatty acids.

A dietary effect on the PUFA patterns can be excluded, because even in the most extreme cases of essential fatty acid (EFA) deficiency or imbalance, the proportion of 22:6ω3 never decreases below 50% of the normal values (13,14). The patient had been fed since birth with maternal milk and commercial milk formulas with ω3/ω6 ratios ranging from 1:10 to 1:26. Although these low ω3/ω6 ratios could partly explain the increase in 18:2ω6 and the decrease in 20:4ω6 in some tissues, they could never explain a reduction in the absolute amount of 22:6ω3 by a factor of almost 20 in the liver and kidney and by a factor of 4 in the brain, leading to extremely low proportions of docosahexaenoate in GPE of all tissues. The triene fatty acid, 20:3ω9—a marker for EFA deficiency (15)—

TABLE 3

Fatty Acid Composition of Glycerophosphoethanolamines in Zellweger Syndrome Compared With Controls

	Cerebrum		Liver		Kidney	
	ZS	Controls (n = 5)	ZS	Controls (n = 5)	ZS	Controls (n = 5)
14:0	0.2	0.4 ± 0.1	0.4	0.6 ± 0.1	0.5	0.8 ± 0.1
16:0	9.8	7.7 ± 0.5	23.6	22.6 ± 1.2	12.5	14.9 ± 1.6
16:1ω7	0.9	0.6 ± 0.2	1.1	1.0 ± 0.3	1.1	0.8 ± 0.1
18:0	29.5	28.5 ± 0.9	21.0	23.8 ± 0.6	25.9	24.7 ± 1.7
18:1ω9	13.0	7.6 ± 0.8	7.8	6.9 ± 1.0	17.7	15.2 ± 1.0
18:1ω7	5.7	2.2 ± 0.1	0.6	2.0 ± 0.6	2.0	2.0 ± 0.5
18:2ω6	1.2	0.4 ± 0.1	21.0	8.2 ± 2.5	10.8	5.7 ± 1.6
20:3ω9	2.7	1.0 ± 0.1	0.7	0.8 ± 0.5	0.6	0.5 ± 0.2
20:3ω6	4.4	2.1 ± 0.4	2.6	1.9 ± 0.1	2.7	2.2 ± 0.2
20:4ω6	20.9	16.3 ± 0.2	17.0	19.7 ± 2.3	22.2	25.1 ± 3.1
20:5ω3	0.1	0.4 ± 0.1	0.9	0.5 ± 0.1	0.5	0.6 ± 0.1
22:4ω6	5.5	13.3 ± 0.4	0.9	0.8 ± 0.1	2.1	2.0 ± 0.6
22:5ω6	0.4	5.2 ± 0.6	0.1	1.2 ± 0.2	0.1	1.0 ± 0.2
22:5ω3	0.2	0.4 ± 0.1	0.7	0.8 ± 0.3	0.4	0.3 ± 0.1
22:6ω3	2.8	12.9 ± 0.8	0.7	8.4 ± 1.8	0.4	3.1 ± 0.7

ZS, Zellweger Syndrome. The values are molar percentage (mean ± SEM). Other fatty acids, present in small proportions (16:1ω9, 20:1ω9, etc.) have been omitted from this table but were included in the percentage computation. There wre only trace amounts of 18:3ω3 and the minor precursor 18:4ω3 was present only in the cerebrum of the Zellweger patient, accounting for 0.6% of GPE fatty acids.

TABLE 4

Fatty Acid Composition of Glycerophosphocholines in Zellweger Syndrome Compared With Controls

	Cerebrum		Liver		Kidney	
	ZS	Controls (n = 5)	ZS	Controls (n = 5)	ZS	Controls (n = 5)
14:0	1.9	2.3 ± 0.5	0.8	0.7 ± 0.1	1.8	1.0 ± 0.1
16:0	51.2	50.1 ± 1.3	39.2	36.2 ± 0.8	37.8	32.8 ± 2.0
16:1ω7	2.2	1.1 ± 0.2	2.0	2.7 ± 0.7	1.7	1.7 ± 0.3
18:0	6.3	11.1 ± 0.8	9.3	12.4 ± 0.7	11.2	11.2 ± 0.4
18:1ω9	21.4	19.6 ± 2.8	12.8	14.4 ± 1.6	20.6	19.4 ± 0.7
18:1ω7	5.6	4.9 ± 0.2	0.6	2.7 ± 0.6	3.1	3.4 ± 0.7
18:2ω6	1.4	0.5 ± 0.1	22.9	11.9 ± 3.0	12.6	8.6 ± 2.3
20:3ω9	0.3	0.2 ± 0.1	0.3	0.8 ± 0.4	0.2	0.3 ± 0.1
20:3ω6	1.6	0.9 ± 0.1	3.1	2.2 ± 0.2	2.6	1.9 ± 0.2
20:4ω6	3.5	5.0 ± 0.4	6.9	10.3 ± 1.6	5.7	14.6 ± 1.4
20:5ω3	0.1	0.2 ± 0.1	0.4	0.3 ± 0.1	0.2	0.5 ± 0.3
22:4ω6	0.3	1.2 ± 0.4	0.3	0.4 ± 0.1	0.6	1.2 ± 0.3
22:5ω6	tr	0.5 ± 0.1	0.1	0.5 ± 0.1	0.1	0.3 ± 0.1
22:5ω3	tr	tr	0.3	0.4 ± 0.1	0.2	0.4 ± 0.1
22:6ω3	0.2	1.3 ± 0.3	0.1	3.1 ± 0.6	0.1	1.7 ± 0.4

ZS, Zellweger Syndrome. The values are molar percentage (mean ± SEM). Only the main fatty acids are listed in this table.

was significantly increased only in the brain, and was within normal limits in the liver and kidney, especially in GPC. Furthermore, it must be taken into account that 20:3ω9 not only increases in EFA deficiency but is higher in the immature than in the mature human brain (16), and it could also be higher in the cerebrum of a patient with a disease producing mental retardation. Last and most

important, the pentaene fatty acid, 22:5ω6—the other product of Δ4-desaturation, together with 22:6ω3—is known to increase in ω3 deficiency (17). However, in the Zellweger patient this fatty acid was, like 22:6ω3, far below normal.

Therefore, the enormous decrease in the two products of Δ4-desaturation, 22:6ω3 and 22:5ω6, found in the Zellweger patient very strongly suggests a Δ4-desaturase defect in this infant. The existence of an enzyme defect in the long chain PUFA desaturase system in a peroxisomal disorder would be consistent with the view that peroxisomes are derived from the endoplasmic reticulum (18). In this connection, it is interesting to point out that smooth endoplasmic reticulum was reported to be very scarce in the original description of peroxisomal defects in Zellweger patients by Goldfischer et al. (1). However, more recent studies have failed to reveal true connections between peroxisomes and endoplasmic reticulum (19), and the polypeptide compositions of these two organelles seem to be substantially different (20). The endoplasmic reticulum anomalies originally described (1) have not been considered a generalized finding in Zellweger Syndrome. Therefore, should the present data be substantiated by enzyme studies, it is probably better to assume that Δ4-desaturase is simply one of the many enzymes that are being considered genuinely peroxisomal.

The fact that the PUFA composition of fibroblasts was nearly normal in the patient deserves some comment and must be further investigated in other patients with Zellweger Syndrome. It must be taken into account that fibroblasts are ex vivo cells, cultured in an artificial medium, and this could partly account for the difference compared with in vivo grown tissues. Cultured cells preferentially use preformed fatty acids (21,22), and fetal bovine serum is relatively rich in 22:6ω3 and 22:5ω3 (23,24), so the exogenous PUFA in the medium could partly mask the enzyme defect. In these conditions, Zellweger fibroblasts, which seem to contain some peroxisomes (25), could display a PUFA composition much nearer the normal than the more severely affected tissues grown in vivo.

Although only one case has been studied so far, the significance of the reported findings is such that it urges investigators of peroxisomal disorders to study the desaturase system in these patients. If confirmed, the existence of the proposed Δ4-desaturase defect would greatly contribute to understanding the pathogenesis of Zellweger Syndrome. Indeed, so drastic a decrease in the long chain PUFA docosahexaenoic acid, a fatty acid mainly enriched in synaptic membranes and the retina, might explain the mental deterioration and visual impairment in Zellweger syndrome, especially when such a decrease cannot be compensated for by any increase in the other long chain PUFA, 22:5ω6.

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Fish Oil Prevents Change In Arachidonic Acid and Cholesterol Content in Rat Caused by Dietary Cholesterol

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Rats were fed diets high in either saturated fat (beef tallow) or α-linolenic acid (linseed oil) or eicosapentaenoic and docosahexaenoic acids (fish oil) with or without 2% cholesterol supplementation. Consumption of linseed oil and fish oil diets for 28 days lowered arachidonic acid content of plasma, liver and heart phospholipids. Addition of 2% cholesterol to diets containing beef tallow or linseed oil lowered 20:4ω6 levels but failed to reduce 20:4ω6 levels when fed in combination with fish oil. Feeding ω3 fatty acids lowered plasma cholesterol levels. Addition of 2% cholesterol to the beef tallow or linseed oil diet increased plasma cholesterol concentrations but not when fish oil was fed. Feeding the fish oil diet reduced the cholesterol content of liver, whereas feeding the linseed oil diet did not. Dietary cholesterol supplementation elevated the cholesterol concentration in liver in the order: linseed oil > beef tallow > fish oil (8.6-, 5.5-, 2.6-fold, respectively). Feeding fish oil and cholesterol apparently reduced 20:4ω6 levels in plasma and tissue lipids. Fish oil accentuates the 20:4ω6 lowering effect of dietary cholesterol and appears to prevent accumulation of cholesterol in plasma and tissue lipids under a high dietary load of cholesterol.
Lipids 24, 266-270 (1989).

Consumption of marine oils rich in eicosapentaenoic (20:5ω3) and docosahexaenoic (22:6ω3) acids decreases plasma levels of cholesterol and triacylglycerols in healthy (1-3) and hyperlipidemic subjects (4-6). While the hypotriglyceridemic effect of fish oil exceeds the capacity of most available drugs, the hypocholesterolemic effects are still doubtful except when the excess cholesterol is present in very low density lipoprotein (4,5). Consumption of fish oil also lowers serum levels of thromboxane (TxA_2) and thus has anti-aggregatory effects on the manifestation of thrombosis (7-9). While the exact mechanisms for the fish oil-induced decrease in plasma lipid levels are not clear, the platelet anti-aggregatory effect is most likely mediated via changes in the balance of eicosanoids formed from their common precursor, arachidonic acid (20:4ω6) (7-9). Arachidonic acid is usually prsent in the SN-2 position of membrane phospholipids (10). Eicosapentaenoic acid present in fish oil competes with 20:4ω6 for incorporation into phospholipid (11,12) and subsequently competes with 20:4ω6 at the level of cyclo-oxygenase (13) in the formation of eicosanoids such as prostacyclin, prostaglandins, thromboxane and leukotrienes. In a recent study we demonstrated that fish oil reduces synthesis of 20:4ω6 by inhibiting the Δ^6-desaturase enzyme (14), a rate-limiting enzyme in the 20:4ω6 biosynthetic pathway (15,16).

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Abbreviation: ACAT, acyl coenzyme A:cholesterol acyltransferase.

Dietary cholesterol has also been shown to interfere with metabolism of essential fatty acids (17-19). Cholesterol supplementation produces changes in fatty acid composition of plasma and tissue lipids, similar to that found in essential fatty acid deficiency (17-19), i.e., decreases in 20:4ω6 and 22:6ω3 content and increases in monounsaturated fatty acids (16:1ω7 and 18:1ω9) and their long chain polyunsaturated (20:3ω9) metabolites. In the present study we examined the interaction of dietary cholesterol with ω3 fatty acids of linseed oil (18:3ω3) or fish oil (20:4ω6 and 22:6ω3) in the modification of the cholesterol content and fatty acid composition of rat plasma, liver, heart and adipose tissue lipids.

MATERIALS AND METHODS

Fish oil concentrate (S-28GA) was obtained from Nissho Iwai American Corp., New York; beef tallow was supplied by Canada Packers Inc., Edmonton, Alberta, Canada, and linseed oil was purchased from a local health food store. All solvents were redistilled before use.

Animals and diets. Weanling male Sprague-Dawley rats weighing 50-60 g were housed individually in hanging stainless steel cages in a well ventilated room maintained at 22 ± 2°C on a 12/12 hr light-dark cycle. Animals were randomly divided into 3 groups of 12 rats each such that the average weight per group was similar. Experimental diets were prepared by adding one of the following fat mixtures to 800 g of fat-free basal diet mix described previously in detail (20): 180 g beef tallow plus 20 g safflower oil (beef tallow group); 160 g linseed oil plus 40 g beef tallow (linseed oil group); 200 g fish oil (fish oil group). Half of the animals in each group were fed the same diet supplemented with 20 g cholesterol/kg of diet. The fatty acid composition of the diets has been presented (Table 1). The beef tallow diet contained mainly saturated and monounsaturated fatty acids (83% w/w of total fatty acids). Both the linseed oil and fish oil diets were enriched with the same amount of ω3 fatty acids (approximately 40% of total fatty acids), but qualitatively, the linseed oil diet was high in α-linolenic acid (18:3ω3), whereas the fish oil diet was enriched with 20:5ω3 and 22:6ω3. Addition of 2% cholesterol to the fat-supplemented diets had no effect on the fatty acid composition of the diets. Diets were prepared weekly and stored at −20°C. Food and water were provided ad libitum for 28 days.

Lipid analysis. After the 4-week experimental period, the rats were killed by decapitation between 0800 and 1000 hr; livers, hearts and epidydimal fat pads were excised immediately and rinsed with ice-cold physiological saline. Following decapitation, blood was collected with a heparinized funnel and plasma was separated by centrifuging (1500 × g for 10 min). Lipids were extracted from plasma and tissues by homogenizing with chloroform/methanol (2:1, v/v) (21) followed by washing with saline. Total and free cholesterol content were determined using an enzymatic method (22). The difference between

TABLE 1

Fatty Acid Composition of The Experimental Diets[a,b]

Fatty acid	Beef tallow	Linseed oil	Fish oil
14:0	0.6	0.1	6.1
16:0	26.3	10.3	11.3
16:1ω7	3.0	0.7	7.6
17:0	1.3	0.3	2.0
18:0	17.2	6.3	6.2
18:1ω9	31.9	23.5	7.8
18:1ω7	2.6	1.1	2.8
18:2ω6	10.3	16.2	1.6
18:3ω3	0.3	39.9	0.2
20:4ω6	—	—	1.2
20:5ω3	—	0.1	27.5
22:5ω3	—	0.1	2.2
22:6ω3	—	—	8.9
Total			
Saturated	45.4	17.0	21.9
Monounsaturated	37.5	25.3	21.9
ω6	10.2	—	2.8
ω3	0.3	40.1	44.6

[a] Addition of 2% cholesterol to beef tallow, linseed oil or fish oil diets had no effect on the fatty acid composition of the diets.

[b] At least four random samples from each diet were analyzed.

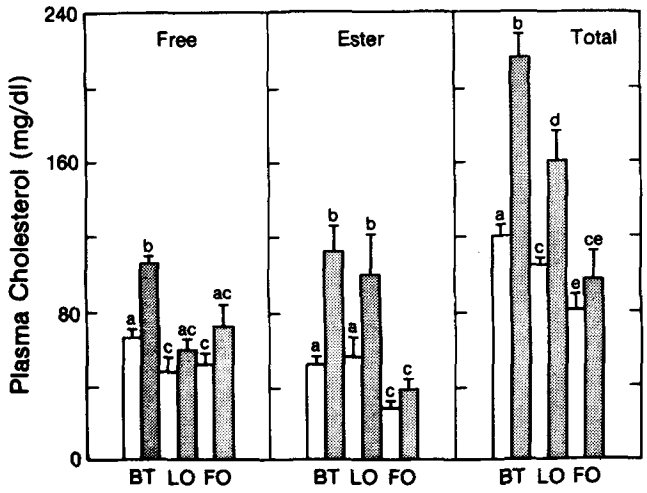

FIG. 1. Effect of dietary cholesterol and/or dietary ω3 fatty acid on cholesterol content of rat plasma. Values are the mean ± standard deviation of 6 animals (n = 6). Values without a common superscript are significantly different ($p < 0.05$). Open bars indicate low cholesterol diets, and shaded bars represent high cholesterol (2%) diets. BT, beef tallow diet; LO, linseed oil diet; FO, fish oil diet.

the total and free cholesterol content gave a measure of the esterified cholesterol content.

Total phospholipids were separated from neutral lipids by thin layer chromatography on Silica Gel G plates using a solvent system comprised of hexane/diethyl ether/acetic acid (80:20:1, v/v/v) (23). The areas corresponding to phospholipids were scraped off the plates and methylated using BF_3-methanol (14%, w/w) reagent, at 100°C for one hr (24). Fatty acid composition was determined by automated gas liquid chromatography (Varian, Model 6000) of methyl esters using a fused silica capillary column (BP20, bonded phase) as reported previously (25).

STATISTICAL ANALYSIS

Results are presented as the mean ± standard deviation. The effect of dietary fat treatments was evaluated by analysis of variance procedures. Comparison between individual diets was made using Duncan's multiple range test (26).

RESULTS

Food consumption, body weight, liver weight, heart weight and liver weight/body weight ratio data for animals fed the lipid-supplemented diets have been presented elsewhere (13). Animals fed the fish oil diet with or without cholesterol supplementation grew at a slower rate than those fed the beef tallow or linseed oil diet. The amount of food consumed per day, liver weights, heart weights and liver weight/body weight ratios remained unchanged with dietary fatty acid and/or cholesterol treatment (13).

Cholesterol content of plasma, liver, heart and adipose tissue. Cholesterol content of plasma was significantly reduced by feeding either the linseed oil or fish oil diet (Fig. 1). The reduction was greater with fish oil (32.8%) than with linseed oil (12.6%). The decrease in plasma cholesterol content was found to be mainly in the free cholesterol content following consumption of the linseed oil diet, whereas the fish oil diet lowered both the free and esterified cholesterol content. Addition of 2% (w/w) cholesterol to the beef tallow or linseed oil diet increased the plasma level of cholesterol; however, cholesterol supplementation with fish oil had no significant effect (Fig. 1). Dietary cholesterol elevated both the free and esterified cholesterol content of plasma when fed with beef tallow, and elevated only the esterified cholesterol content of plasma when the diet containing linseed oil was fed (Fig. 1.).

In liver tissue, feeding the fish oil diet decreased the cholesterol level whereas feeding the linseed oil diet had no significant effect (Fig. 2). The decrease associated with feeding the fish oil diet was observed in the cholesterol ester fraction while the free cholesterol content remained unaffected. Addition of 2% (w/w) cholesterol increased cholesterol concentrations in liver in the following order: linseed oil > beef tallow > fish oil groups. Both the free and esterified cholesterol content was elevated following feeding of cholesterol-enriched diets (Fig. 2). The level of cholesterol in heart and adipose tissue was not affected by dietary fatty acid and/or cholesterol treatments (data not shown).

Fatty acid composition of plasma and tissue lipids. Feeding either the linseed oil or fish oil diet, without added cholesterol, decreased the 20:4ω6 content of rat plasma, liver and heart phospholipids (Fig. 3). In liver and heart the decrease was greater after feeding the fish oil than after feeding the linseed oil diet when compared with the control animals fed diets containing beef tallow. Feeding diets containing linseed oil increased the proportion of 18:3ω3, 20:5ω3, 22:5ω3 and 22:6ω3 in liver, heart and adipose tissue lipids (Table 2, Fig. 3). Animals fed the diet containing fish oil exhibited larger increases in

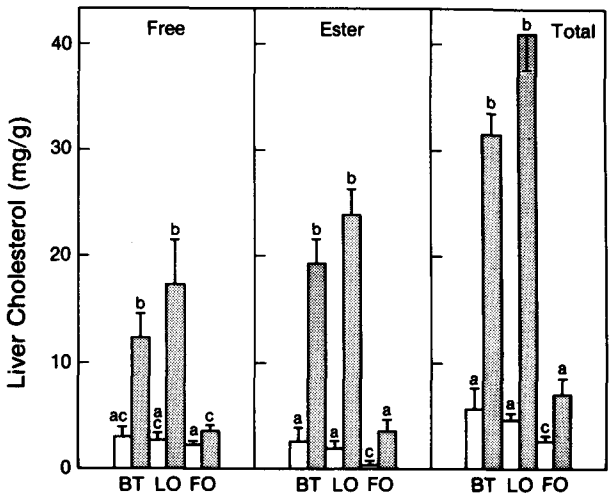

FIG. 2. Effect of dietary cholesterol and/or dietary ω3 fatty acid on cholesterol content of rat liver. Values are the mean ± standard deviation of 6 animals (n = 6). Values without a common superscript are significantly different (p < 0.05). Open bars indicate low cholesterol diets and shaded bars represent high cholesterol (2%) diets. BT, beef tallow diet; LO, linseed oil diet; FO, fish oil diet.

the proportion of 20:5ω3 and 22:6ω3 in phospholipids of plasma, liver and heart and in the triacylglycerol fraction from adipose tissue. The 18:2ω6 content of plasma, liver and heart phospholipids and that of adipose tissue triacylglycerol was significantly increased by feeding linseed oil and decreased by feeding the fish oil diet (Table 2, Fig. 3).

Addition of 2% (w/w) cholesterol to either the beef tallow or linseed oil diet reduced the 20:4ω6 content of plasma and tissue phospholipids (Fig. 3) with an accompanying increase occurring in 18:2ω6 content (Fig. 3). Cholesterol supplementation in combination with fish oil failed to alter levels of 18:2ω6 or 20:4ω6 in plasma and tissue phospholipids. The fatty acid composition of the triacylglycerol fraction in the adipose tissue remained unchanged by dietary cholesterol treatments (Table 2).

DISCUSSION

Reports from our laboratory and others have demonstrated that dietary ω3 fatty acids, particularly those of fish oils (20:5ω3 and/or 22:6ω3), and cholesterol enrichment of the diet independently alter metabolism of

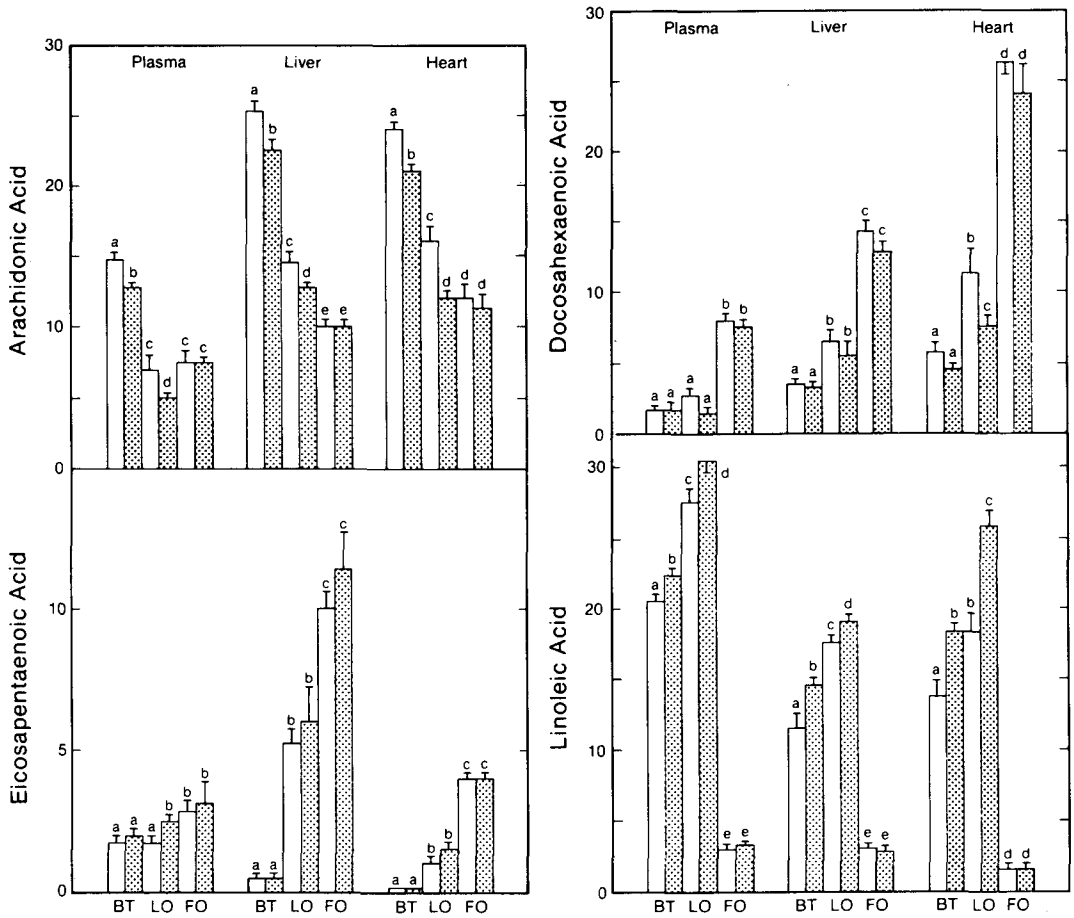

FIG. 3. Effect of dietary cholesterol and/or dietary ω3 fatty acid on polyunsaturated fatty acid 6 content (area percent) of rat plasma, liver and heart phospholipids. Values are the mean ± standard deviation of 6 animals (n = 6). Values without a common superscript are significantly different (p < 0.05). Open bars indicate low cholesterol diets and shaded bars represent high cholesterol (2%) diets. BT, beef tallow diet; LO, linseed oil diet; FO, fish oil diet.

TABLE 2

Effect of Feeding Lipid Supplemented Diets on Fatty Acid Composition (area percent) of Triacylglycerols in Rat Adipose Tissue

Fatty acid	Beef tallow	Beef tallow + chol.	Linseed oil	Linseed oil + chol.	Fish oil	Fish oil + chol.
14:0	5.0 ± 0.7^a	4.9 ± 0.1^a	2.6 ± 0.2^b	2.6 ± 0.2^b	7.7 ± 0.7^c	8.9 ± 0.8^c
16:0	31.2 ± 0.8^a	32.1 ± 0.4^a	21.0 ± 0.7^b	21.9 ± 0.5^b	33.2 ± 4.4^a	29.7 ± 2.6^a
16:1ω7	8.4 ± 1.0^a	9.0 ± 0.5^a	4.5 ± 0.4^b	5.0 ± 0.3^b	14.0 ± 0.6^c	13.7 ± 0.7^c
17:0	0.6 ± 0.1^a	0.7 ± 0.0^a	0.3 ± 0.0^b	0.3 ± 0.0^b	0.1 ± 0.0^c	0.2 ± 0.1^{b,c}
18:0	4.4 ± 0.3^a	4.7 ± 0.2^a	3.4 ± 0.2^b	3.4 ± 0.2^b	3.2 ± 0.7^b	3.0 ± 0.3^b
18:1ω9	36.5 ± 0.7^a	36.7 ± 0.6^a	23.5 ± 0.5^b	23.8 ± 0.5^b	13.2 ± 2.0^c	12.5 ± 0.4^c
18:1ω7	1.0 ± 0.2^a	1.2 ± 0.2^a	0.7 ± 0.1^b	0.7 ± 0.1^b	2.1 ± 0.2^c	2.7 ± 0.5^c
18:2ω6	7.6 ± 1.0^a	7.1 ± 0.3^a	14.1 ± 0.5^b	13.7 ± 0.2^b	2.3 ± 0.5^c	2.7 ± 0.5^c
18:3ω6	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.0	0.3 ± 0.1	0.3 ± 0.1
18:3ω3	0.2 ± 0.1^a	0.2 ± 0.0^a	22.6 ± 1.0^b	22.4 ± 0.6^b	1.1 ± 0.2^c	1.3 ± 0.2^c
18:4ω3	—	—	—	—	3.7 ± 0.9	3.9 ± 0.2
20:1ω9	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.1
20:3ω9	0.2 ± 0.2	0.2 ± 0.0	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
20:4ω6	0.2 ± 0.1^a	0.3 ± 0.1^a	0.2 ± 0.1^a	0.2 ± 0.1^a	0.7 ± 0.1^b	0.6 ± 0.1^b
20:5ω3	—	—	0.4 ± 0.1^a	0.5 ± 0.1^a	12.3 ± 2.9^b	12.3 ± 0.9^b
22:4ω6	—	—	0.2 ± 0.1^a	0.1 ± 0.0^a	0.5 ± 0.2^b	0.6 ± 0.1^b
22:5ω3	—	—	0.3 ± 0.1^a	0.3 ± 0.1^a	1.3 ± 0.3^b	1.5 ± 0.4^b
22:6ω3	—	—	0.3 ± 0.1^a	0.3 ± 0.0^a	4.5 ± 0.9^b	5.3 ± 1.0^b

Values are the mean ± standard deviation of six rats (n = 6). Values without a common superscript are significantly different ($p < 0.05$).

essential fatty acids (11-14,17-19). Fish oil (27-29) or cholesterol (17-19) supplementation decreases 20:4ω6 content of plasma and tissue phospholipids and in turn alters the pattern of eicosanoids synthesized from 20:4ω6 (13). The present study was designed to examine the interaction of dietary cholesterol with ω3 fatty acids of linseed oil (18:3ω3) or fish oil (20:5ω3 and 22:6ω3) to modify cholesterol content and fatty acid composition of rat plasma and tissue lipids. Feeding the diets containing linseed oil or fish oil to rats for a 4-week period lowered plasma cholesterol level by 13% and 33%, respectively, compared with animals fed the beef tallow diet (Fig. 1). These results are in agreement with several previous reports that dietary ω3 fatty acids decrease blood cholesterol levels in normal animals and human subjects (2-6). Some authors have reported that fish oil may increase serum cholesterol levels in man (1). Because the rat is a species that has a very high density lipoprotein and low levels of low density lipoprotein under normal dietary conditions, the changes in the plasma cholesterol level may be related to disturbances in lipid transport and lipoprotein metabolism (4). Feeding the fish oil diet also provided protection against cholesterol accumulation in plasma that was apparent when 2% (w/w) cholesterol was fed with diets containing beef tallow or linseed oil. These results suggest that fish oil consumption may have beneficial effects against hypercholesterolemia even under a high dietary load of cholesterol. In this regard, feeding the fish oil diet lowered cholesterol content of liver tissue, while feeding the linseed oil diet had no effect (Fig. 2). Following feeding of low-cholesterol diets, rats have been shown to accumulate more liver cholesterol when ω6 fatty acids of corn oil or sunflowerseed oil are the fat sources, than when coconut oil is the fat source (18,30,31). The present study demonstrates that when a high fat diet is fed, the ω3 fatty acid of linseed oil (18:3ω3) does not affect the concentration of liver cholesterol, whereas the ω3 fatty acids of fish oil (primarily 20:5ω3 and 22:6ω3) reduce liver cholesterol levels, chiefly in the form of cholesterol esters. This decrease in esterified cholesterol content may occur because feeding fish oil may increase the flow of cholesterol toward bile formation (28). Therefore, liver cholesterol would be expected to be utilized preferentially for bile acid synthesis instead of being stored or transported as cholesterol esters (28). In contrast to this, other research has shown that feeding fish oil increases acyl coenzyme A:cholesterol acyltransferase (ACAT) activity (32) which is expected to increase the level of cholesterol esters in liver lipids. Explanation of these conflicting observations is not apparent but may relate to the difference in levels of 20:5ω3 and 22:6ω3 fed in this study (40% w/w of fatty acids) (14) and the previous study (25% w/w of total fatty acids) (32). As well, the fish oil used in the present study contained a different amount and type of monounsaturated fatty acid (primarily 16:1 and 18:1) than utilized in the previous study (primarily 16:1, 18:1, 20:1 and 22:1). Monounsaturated fatty acids are preferred substrates for liver ACAT activity and thus may also contribute to the difference in observations between the two studies. Addition of 2% (w/w) cholesterol to the diet containing beef tallow or linseed oil increased cholesterol content of the liver tissue; however, cholesterol feeding with fish oil appears to at least partially prevent accumulation of cholesterol in the liver. Dietary cholesterol and/or ω3 fatty acids of linseed oil or fish oil did not have any appreciable effects on heart and adipose tissue cholesterol concentrations. It is conceivable that entry and exit of cholesterol is tightly regulated in heart and adipose tissue as the cholesterol content remained unaffected even after a high load of dietary cholesterol. It has been shown previously (33) that heart and adipose tissue are capable of synthesizing a small amount of cholesterol *de novo*. However, the regulation of cholesterol biosynthesis by dietary changes in these tissues remains to be examined.

Feeding ω3 fatty acids of linseed oil or fish oil and cholesterol supplementation to beef tallow or linseed oil

decreased 20:4ω6 content of rat plasma, liver and heart phospholipids (Fig. 3). This observation is in agreement with previous reports suggesting that dietary cholesterol and ω3 fatty acids individually decrease 20:4ω6 content of tissue phospholipids (17-19,27-29). The decrease in 20:4ω6 content following cholesterol feeding has been attributed to its increased utilization for cholesterol ester formation for secretion into the circulation as very low density lipoproteins (34,35). Dietary cholesterol has also been shown to inhibit Δ^6- and Δ^5-desaturase activities in liver microsomes which are key enzymes in the 20:4ω6 biosynthetic pathway (17). Reduction of 20:4ω6 levels in tissue phospholipids following fish oil consumption may occur because: (i) 20:5ω3 present in fish oil has greater affinity than 20:4ω6 for the acyltransferase responsible for the synthesis of phospholipids (10). Thus, 20:5ω3 may replace some of the 20:4ω6 in the liver phospholipids; (ii) fish oil inhibits Δ^6-desaturase activity in the liver (14), which is a rate-limiting step in the synthesis of 20:4ω6 from 18:2ω6; and (iii) we have shown recently that some 20:4ω6 may shift from phospholipid pools to cholesterol esters or triacylglycerol pools following feeding of fish oil (Garg et al., unpublished data).

Consumption of cholesterol and fish oil together failed to produce any alteration in the 20:4ω6 content of tissue phospholipids. This unique ability to resist changes in 20:4ω6 content when feeding fish oil may be attributed to the observation that feeding fish oil can prevent changes in Δ^6-desaturase activity in rat liver microsomes (14) caused by cholesterol supplementation. The possibility cannot be ruled out that feeding diets containing a high content of fish oil without any added cholesterol reduces 20:4ω6 levels to the minimal point required by the body to maintain eicosanoid balance and/or membrane phospholipid integrity.

In summary, dietary cholesterol interacts with dietary ω3 fatty acids of linseed oil (18:3ω3) to alter cholesterol and fatty acid metabolism in rat plasma and tissue pools. Feeding diets containing fish oil appears to at least partially reduce the effect of dietary cholesterol supplementation on cholesterol accumulation in and fatty acid profiles of tissue lipid. The mechanisms determining the unique effect of feeding fish oil on cholesterol levels and tissue fatty acid composition even after a high dietary load of cholesterol merit further investigation.

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Developmental Regulation of Sterol Biosynthesis in *Cucurbita maxima* L.

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Twenty-two sterols were identified by capillary gas chromatography and capillary gas chromatography/mass spectroscopy in *Cucurbita maxima* grown under greenhouse conditions. Both whole plants and individual tissues (leaves, stems, roots, cotyledons, flowers) were analyzed at weekly intervals during the 12-week development of the plant. In whole plants, sterol accumulation parallels plant growth except for a period in the mid-life cycle where there is a reduction in the amount of sterol accumulated on a total sterol/plant and mg sterol/g dry wt basis. This reduction in the amount of sterol is coincident with the visual onset of flowering. During development, the percent contribution of each class of sterol (Δ^5-, Δ^7-, Δ^0-sterols) remains relatively constant. However, the percent contribution of an individual sterol species varies depending on the tissue examined and the developmental period selected for analysis. While the young plant (<2 weeks) possesses elevated levels of sterols with the $\Delta^{25(27)}$-double bond, the trend was toward a reduction in the amounts of these sterols with development. Leaves and stems accumulate large quantities of 24ζ-ethyl-5α-cholesta-7,22-dien-3β-ol (7,22-stigmastadienol) and 24ζ-ethyl-5α-cholest-7-en-3β-ol (7-stigmastenol), while roots accumulate only 7,22-stigmastadienol as their principal sterol. Male flowers and roots were found to contain elevated levels of Δ^5-sterols.
Lipids 24, 271–277 (1989).

The seeds and seedlings of some Cucurbitaceae contain primarily Δ^7-sterols possessing the $\Delta^{25(27)}$ side chain unsaturation (1,2). In higher plants this phenomenon has been reported in only a small number of other groups (4–6), and from biosynthetic considerations the $\Delta^{25(27)}$ double bond is associated with the β-configuration at C-24 (5,7–10). This is unusual because previously, sterols with the C-24 β-ethyl configuration were associated with lower organisms, especially the algae (11,12). In higher plants, sterols with the C-24 ethyl substituents and lacking the $\Delta^{25(27)}$ unsaturation were shown to have the α-configuration (7,9). However, there is some evidence that in seeds and seedlings from *Cucurbita pepo* and some species of *Cucurbita maxima*, $\Delta^{7,22}$-stigmastadienol may be an epimeric mixture at C-24 (1,13–16). The only other case in plants where a C-24 ethylsterol exists as an epimeric mixture is in the bryophytes (17,18). In the mature tissues of Cucurbitaceae analyzed, the C-24 α-isomers predominate (4,19,20) and are comprised mainly of the C-24 α-isomer of $\Delta^{7,22}$-stigmastadienol, i.e., spinasterol, and slightly lesser amounts of Δ^7-stigmastenol. Further complicating the composition of these tissues was the finding that Δ^7- and Δ^5-sterols co-exist in members of this plant group (19,21,22). The discovery that the beet (23), while having Δ^7-sterols as its major component, also contained significant quantities of Δ^5-sterols, has led researchers to examine more closely plants known to contain Δ^7-sterols. Co-existence of Δ^7- and Δ^5-sterols has been found to be rather common in the Caryophyllales (24,25). Examination of *Cucurbita maxima* (True Hubbard squash) showed that Δ^5-sterols were present but disappeared about two weeks after germination (19).

It is apparently not uncommon that different sterols predominate in different stages of plant development or in different plant parts. Previous experiments in our laboratory have shown that during the life cycle of *Cucurbita maxima* L. (var. Fordhook zucchini) and *Glycine max* L. (var. Sussex), sterol accumulation is reduced during periods of reproductive processes (16). Studies conducted by Heupel et al. showed a similar occurrence in *Sorghum bicolor* (26). Examination of the leaves, shoot apex, leaf sheath and stems, from *Lolium temulentum* during flower initiation and floral development revealed fluctuations in their sterol quality and quantity (27). Sterols or their metabolites have been suggested to be involved in reproductive processes in plants (28–31), but the exact role is not understood.

Both qualitative and quantitative sterol fluctuations during nonreproductive periods in a plant's life cycle have been witnessed also. In the 1960's Kemp et al. performed developmental studies on maize seedlings during germination and reported stage dependent changes (32). Yoshida and Kajomoto have since demonstrated developmental changes in sterol composition in cotyledons (33), and in root and axis (34) of germinating soybeans. In oats, stigmasterol represents only a few percent of the total sterol in the roots, whereas it is one of the major components in the leaves of the same plant (35). Whether these fluctuations are indicative of a metabolic or some other developmental role of endogenous sterols in plants awaits complete understanding of complex patterns of synthesis (and/or degradation).

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Abbreviations: Cycloartenol, 4,4,14-trimethyl-9,19-cyclo-5α-cholest-24-en-3β-ol; 24-methylene cycloartenol, 4,4,14-trimethyl-9,19-cyclo-5α-campest-24(28)-en-3β-ol; obtusifoliol, 4α,14α-dimethyl-5α-campesta-8,24(28)-dien-3β-ol; 4α-methyl-7-stigmastenol, 4α-methyl-5α-stigmast-7-en-3β-ol; citrostadienol, 4α-methyl-5α-stigmasta-7,24(28)-dien-3β-ol; codisterol, 24β-methyl-cholesta-5,24-dien-3β-ol; 24-methylenecholesterol, 24-methylenecholesta-5,24(28)-dien-3β-ol; campesterol, 24ζ-methyl-cholest-5-en-3β-ol; campestanol, 24ζ-methyl-5α-cholesta-3β-ol; stigmasterol, 24α-ethylcholesta-5,22-dien-3β-ol; 22-dehydrostigmastenol, 24ζ-ethyl-5α-cholest-22-en-3β-ol; clerosterol, 24β-ethyl-cholesta-5,25-dien-3β-ol; sitosterol, 24α-ethyl-cholest-5-en-3β-ol; stigmastanol, 24ζ-ethyl-5α-cholest-3β-ol; gramisterol, 4α-methyl-5α-campesta-7,24(28)-dien-3β-ol; 7-campestenol, 24ζ-methyl-5α-cholest-7-en-3β-ol; 7,22,25-stigmastatrienol, 24β-ethyl-5α-cholesta-7,22,25-trien-3β-ol; 7,22-stigmastadienol, 24ζ-ethyl-5α-cholesta-7,22-dien-3β-ol; 7,25-stigmastadienol, 24β-ethyl-5α-cholesta-7,25-dien-3β-ol; 7-stigmastenol, 24ζ-ethyl-5α-cholest-7-en-3β-ol; 7-avenasterol, 24-ethylidine-5α-cholesta-7,24(28Z)-dien-3β-ol; 7,24(28)-campestadienol, 24-methylene-5α-cholesta-7,24(28)-dien-3β-ol; TMSE, trimethyl silyl ether; FID, flame ionization detection; GC, gas chromatography; MS, mass spectroscopy; RRT, relative retention times.

Though developmental studies have been conducted, most have involved only seeds to seedling stages or have utilized both extremes in the life cycle (19,32-37). Other studies have focused on segregated points of development (26,27). Since there exist so many diversities between plant groups and even within a given plant, it was of interest to follow sterol changes during development of a single plant species to provide a complete account of the kinds and amounts of sterols therein. The study reported here represents the first examination of sterol profiles of a plant taken at regular intervals, beginning with the seed and continuing until mature seed of the next generation are produced. Sterol composition in the Fordhook variety of squash *Cucurbita maxima* was qualitatively and quantitatively monitored on a weekly basis from seed germination to seed production. Both whole plants and organs were analyzed. Our results indicate developmental regulation of sterol accumulation, and quantitative differences in sterol profiles from various organs.

MATERIALS AND METHODS

Cucurbita maxima L. (var. Fordhook zucchini) seeds purchased from Burpee Seed Co. (Warminster, PA) were planted under greenhouse conditions on March 9, 1984 and in a duplicate experiment on October 1, 1984. Seeds were germinated in flats 12″ × 8″ and plants of equal size were harvested weekly for 12 weeks with day 7 (after planting) taken as week 1. Plants remaining after 3 weeks were transferred from flats to pots ranging from 5-12 inches in diameter, and each pot contained one plant. This growth period produced all stages of development except senescence. At each harvest a known number of plants was washed and oven dried. Other plants from that same week's harvest were dissected into their respective roots, stems, leaves (and/or cotyledons), flowers and pericarp. In cases where the pericarp was used, care was taken to assure that seeds were completely separated from the fruits.

Plant material was ground and extracted in a Soxhlet apparatus with $CHCl_3$:MeOH 2:1 (v/v/v) following standard laboratory methods (11,18). After saponification of the total lipid, the mixture was exhaustively extracted with Et_2O and the extracts pooled and dried *in vacuo* with a rotary evaporator. Sterols were recovered after alumina chromatography. Total sterol referred to in this work actually is a combination of free sterols and sterol esters. Steryl glycosides previously have been analyzed over the squash life cycle and were found to be present in small, constant amounts (16).

Trimethyl silyl ether (TMSE) derivatives of sterols (38) were identified and quantitated by gas chromatography (GC) with cholesterol TMSE as an internal standard. The instrumentation consisted of a Varian 3700 or a Varian 3400 gas chromatograph both equipped with flame ionization detection (FID) systems, and a Varian 8000 autosampler. Data was processed on a Vista 401 data system. Sterols were analyzed on two types of capillary columns. One was a nonpolar fused silica column (SPB-1; bonded methyl silicone, Supelco Inc., Bellefonte, PA) 30 m × 0.32 mm i.d., 0.25 μ film thickness operated under the following conditions: oven temp, 230°C isothermal; injector, 290°C; detector, 300°C; split ratio, 10:1; average linear carrier gas velocity (helium), 80 cm/sec; 33 psi column head pressure. The other column was a polar fused silica column (SP-2330; cyanopropyl silane, Supelco Inc.) 30 m × 0.25 mm i.d.; 0.25 μ film thickness operated at: oven temp, 200°C isothermal; injector, 290°C; detector, 300°C, split ratio, 10:1; average linear carrier gas velocity (helium), 40 cm/sec; 26 psi column head pressure.

GC/mass spectroscopy (MS) was performed on a Finnigan Model 4510 mass spectrometer fitted with a fused silica capillary column (DB-1; bonded methyl silicone, J&W Scientific) 30 m × 0.32 mm i.d., 0.25 μm film thickness. The column was operated at 255°C with 13 psi head pressure of helium. Electron ionization (70 eV) spectra were obtained at a source temperature of 120°C, and processed with an Incos data system.

Total sterols were separated into fractions containing "methyl" sterols (here defined as sterols containing one or two methyl groups at C-4), Δ^5-sterols (also stanols) and Δ^7-sterols by alumina column chromatography on grade III alumina, eluted with increasing concentrations of Et_2O in hexane (39). Sterols were introduced to the column dissolved in hexane and eluted with 17%, 20% 23%, a second 23% (23%′), then 100% Et_2O. Following each separation, columns were thoroughly flushed with Et_2O to assure complete removal of sterols.

RESULTS AND DISCUSSION

Squash developmental stages were as follows: seeds were referred to as week 0; week 1, seedlings with cotyledons but no true leaves; weeks 2-4, seedlings with cotyledons and true leaves; week 5, cotyledons disappear; week 6, first male flowers appear; week 8, first female flowers appear; weeks 8-10, small fruits appear; weeks 10-12, plants with mature fruits and seed.

Squash plants grown in the spring were slightly larger at a comparable age than those in the fall, and development of the fall plants appeared to be delayed by one week. The trends in sterol composition were similar between the two experiments.

GC/MS performed on each fraction from the alumina column provided the identity of the components. In most cases spectra were compared to authentic sterols; otherwise, data were compared to those in the literature. Representative ions characteristic of specific sterols (40,41) are given, and the data are grouped according to the elution pattern from the alumina column.

Sterols eluting in 20% Et_2O in hexane. Cycloartenol: m/z 426$^+$ (M^+, 4%), 411 (5%), 408 (7%), 315 (1%), 286 (13%); 24-methylenecycloartanol: m/z 440$^+$ (M^+, 4%), 422 (6%), 407 (7%), 315 (2%), 300 (12%). Along with sterols in this fraction, there were compounds yielding fragmentation patterns consistent with those produced by pentacyclic triterpenoids. Characteristic of some pentacyclic triterpenoids is a prominent ion at m/z 218$^+$ upon cleavage, and these compounds are known constituents of many plant groups (37,42-44).

23% Et_2O in hexane. Obtusifoliol: m/z 426$^+$ (M^+, 15%), 411 (35%), 327 (7%), 285 (5%), 245 (45%); 4α-methyl-7-stigmastenol: m/z 426$^+$ (M^+, 60%), 411 (55%), 285 (70%), 245 (8%); citrostadienol: 426$^+$ (M^+, 40%), 411 (60%), 327 (30%), 285 (70%), 245 (5%).

23′% Et_2O in hexane. Codisterol: m/z 398$^+$ (M^+, 30%), 383 (3%), 365 (2%), 355 (1%), 314 (10%), 299 (10%), 271

(24%); 24-methylenecholesterol: m/z 398$^+$ (M$^-$, 13%), 383 (2%), 314 (20%), 271 (20%); campesterol: m/z 400$^+$ (M$^+$, 60%), 385 (10%), 382 (12%), 367 (8%), 315 (25%), 289 (30%), 273 (18%), 261 (5%); campestanol: m/z 402$^+$ (M$^+$, 60%), 315 (10%); stigmasterol: m/z 412$^+$ (M$^+$, 20%), 369 (3%), 351 (3%), 351 (5%), 300 (14%), 271 (60%), 255 (57%); 22-dehydrostigmastenol: m/z 414$^+$ (M$^+$, 54%), 371 (8%), 353 (15%), 301 (73%), 273 (40%), 257 (39%); clerosterol: m/ 412$^+$ (M$^+$, 80%), 397 (5%), 379 (10%), 314 (30%), 299 (39%); sitosterol: m/z 414$^+$ (M$^+$, 45%), 399 (10%), 396 (13%), 329 (18%), 303 (22%), 2783 (15%); stigmastanol: m/z 416$^+$ (M$^+$, 60%), 233 (85%); gramisterol: m/z 412$^+$ (M$^+$, 60%), 397 (30%), 314 (80%), 299 (21%), 285 (85%).

100% Et_2O. 7-Campestenol: m/z 400$^+$ (M$^+$, 65%), 385 (5%), 273 (6%), 255 (25%); 7,22,25-stigmastatrienol: m/z 410$^+$ (M$^+$, 40%), 395 (8%), 381 (10%), 300 (10%), 271 (35%), 255 (15%); 7,22-stigmastadienol: m/z 412$^+$ (M$^+$, 72%), 397 (20%), 369 (28%), 300 (33%), 285 (6%), 271 (56%), 255 (18%); 7,25-stigmastadienol: m/z 412$^+$ (M$^+$, 60%), 397 (59%), 299 (18%), 271 (69%), 255 (17%); 7-stigmastenol: m/z 414$^+$ (M$^+$, 98%), 399 (20%), 273 (16%), 255 (75%); 7-avenasterol m/z 412$^+$ (M$^+$, 33%), 397 (45%), 314 (40%), 271 (100%).

Akihisa reported a similar number of sterols in the Duchense variety of squash including 22-dehydrostigmastenol which was not previously reported (20). The sterol designated codisterol in the True Hubbard squash (45) was identified in this variety also. The sterols' gas chromatographic relative retention times (RRT) relative to cholesterol on SPB-1 are comparable to those reported for the packed SE-30 column (46). It is important to note that more than one column phase may be needed for identification. For example, two frequently reported cucurbit sterols; $\Delta^{7,22}$-stigmastadienol and $\Delta^{7,22,25}$-stigmastatrienol, virtually co-elute on the SPB-1, but are well separated on the polar SP-2330 column. The reverse situation exists for campesterol and stigmasterol.

Whole plant analyses. There is a gradual accumulaton in the amount of sterol per plant and mg sterol/g dry weight during the first few weeks of growth (16), after which there is a rapid increase, a decrease, and another rapid increase. One could attempt to explain the decline in sterol accumulation by a rapid increase in plant dry matter so that the level of sterol would appear to decrease proportionally. Such a phenomenon may not be the case since rapid growth is seen both before and after the period of sterol decline. No known sterol metabolic function can account for the magnitude of sterol decrease seen in this study. Nonetheless, the possibility of metabolism should not be discarded. It could be that multiple events exist related to the flowering process that are additive in terms of affecting the total quantity of sterol. As an example, accompanying flower formation in squash is the synthesis of copious amounts of carotenoids, as observed in the highly pigmented flowers. This magnitude of carotenoid synthesis may reduce the pool of sterol precursors since both pathways utilize many common intermediates (47).

In whole plants the composition of each class of sterol is relatively constant when expressed as a percent of the total sterol (Fig. 1). As expected, the Δ^7-sterols comprised the largest amount. These were followed by the Δ^5-sterols or methyl-sterols (depending on the stage). Previously in True Hubbard squash the Δ^5-sterols were shown to be higher in the seeds and to disappear soon after germination (19). In the Fordhook variety our results show that the Δ^5-sterols are higher in the seeds but their presence persisted throughout the developmental period. In order to see if there were interspecific differences, True Hubbard seeds were germinated and seedlings harvested 17 days after germination. After a qualitative analysis of sterols by our methods, it was seen that Δ^5-sterols were present during the entire period of growth (data not shown).

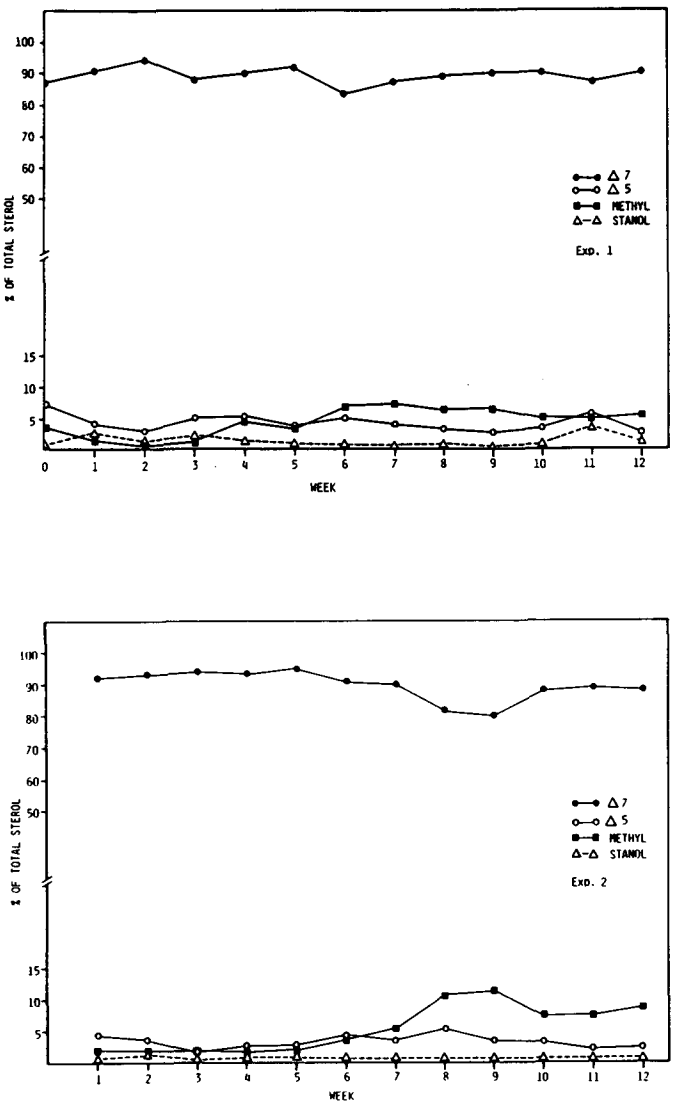

FIG. 1. Comparison of Δ^7, Δ^5, methyl-sterols and stanols as percentages of the total sterol in whole squash plants during development.

Squash plants accumulate two principal sterols during development, $\Delta^{7,22}$-stigmastadienol and Δ^7-stigmastenol (Table 1). In the seeds and in week 1, sterols with the $\Delta^{25(27)}$-unsaturated side chain make up a large proportion of the sterol fraction. The percentages of these sterols begin to decline between weeks 2 and 3 such that by the later weeks they are minor constituents. There is also a gradual increase in Δ^7-avenasterol as the plant develops. This sterol has been suggested as the precursor to the C-24 α-ethyl sterols found in the Cucurbitaceae (20). Since $\Delta^{25(27)}$-unsaturated sterols are associated with the C-24

TABLE 1

Percentages[a] of the Major Sterols from Whole Squash Plants During Development

Sterol	Seeds	Week 1	2	3	4	5	6	7	8	9	10	11	12
7,22-Stigmastadienol	27	37	54	47	47	49	43	43	35	39	39	37	39
7,22,25-Stigmastatrienol	27	26	15	6	8	5	4	5	5	4	5	3	4
7,25-Stigmastadienol	26	17	6	5	6	5	5	5	6	6	7	6	5
7-Stigmastenol	2	8	16	23	24	24	21	24	23	24	24	27	30
7-Avenasterol	4	2	1	4	4	3	8	8	10	9	10	10	7
7-Campestenol	2	2	2	3	3	4	5	3	4	4	4	4	3
Sitosterol	2	2	2	3	2	1	2	2	2	1	2	2	1
Cycloartenol	1	tr[b]	ND[c]	1	1	1	3	3	5	6	3	3	3
Total sterol (μg/g dry wt)	620	568	590	450	608	751	922	735	485	420	568	808	778

[a]Percent of the total sterol including minor components.

[b]Trace.

[c]Not detected.

TABLE 2

Percentages[a] of the Major Sterol from Squash Leaves

Sterol	Week 2	3	4	5	6	7	8	9	10	11	12
7,22-Stigmastadienol	43	43	42	41	41	38	30	40	35	33	36
7,22,25-Stigmastatrienol	7	6	3	4	5	4	9	4	4	3	3
7,25-Stigmastadienol	5	6	4	6	6	5	9	4	4	4	6
7-Stigmastenol	31	29	31	29	26	26	23	31	29	29	30
7-Avenasterol	tr[b]	4	6	5	12	15	11	10	11	10	8
7-Campestenol	3	3	4	3	4	3	5	3	3	2	3
Sitosterol	3	2	2	2	1	1	2	2	1	2	1
Cycloartenol	tr	tr	1	3	3	5	4	4	7	5	5
Total sterol (μg/g dry wt)	790	975	577	673	968	702	276	532	469	640	568

[a]Percent of the total sterol including minor components.

[b]Trace.

β-ethyl configuration, a detailed study was conducted on the configurations at C-24 of $\Delta^{7,22}$-stigmastadienol and 7-stigmastenol over the course of development. The results from those experiments will be reported in a future publication. Sitosterol was the major Δ^5-sterol and comprised between 1.0% and 3.0% of the total sterol of the whole plant. None of the stanols were found above 1% of total sterols at any time. The major methyl sterol was cycloartenol and, as Table 1 shows, its percentage gradually increased with the age of the plant.

Leaves. The true leaves emerge during the second week of growth. All leaves on a plant were analyzed together. No effort was made to compare younger to older leaves. Within these organs all of the different classes of sterols were found. Upon their emergence the true leaves contained a lower percentage of sterols containing the $\Delta^{25(27)}$ double bond than was expected as compared to the seed (Tables 1 and 2). These lower levels may indicate that from the outset, leaves are synthesizing primarily C-24 α-oriented sterols. The amount of Δ^7-stigmastenol approaches that of $\Delta^{7,22}$-stigmastadienol, beginning at leaf emergence and throughout the life cycle. Sitosterol was the major Δ^5-sterol and was found to be higher in the leaves of younger plants than in those of older plants. As in the whole plant analysis, cycloartenol was the major methyl sterol and showed an increase with age of the plant.

Roots. Only one sterol, $\Delta^{7,22}$-stigmastadienol, was found to dominate the sterol profile in the roots (Table 3). This was surprising, especially since Δ^7-stigmastenol usually accompanies $\Delta^{7,22}$-stigmastadienol in significant quantities in the leaves. In the root tissue from maize seedlings, stigmasterol, the Δ^5 analog of $\Delta^{7,22}$-stigmastadienol, was also found to increase with time (32). The percentage of Δ^5-sterols was higher in the root than in the other organs throughout the squash life cycle. The Δ^5-sterols were composed mainly of sitosterol and clerosterol. Stigmastanol was also found at 1% to 3% of the total sterol. Recently, it has been suggested that a relationship exists between cholesterol and embryonic tissues (27). Embryonic regions of the root apices were not analyzed separately in the present work, but it is interesting that roots which also contain a very active primary meristem contain higher levels of Δ^5-sterols.

Stems. Unlike the whole plant, leaves and roots, stems do not show a sterol increase during the mid-life

TABLE 3

Percentages[a] of Major Sterols from Squash Roots During the Life Cycle

Sterol	Week											
	1	2	3	4	5	6	7	8	9	10	11	12
7,22-Stigmastadienol	50	68	54	65	65	70	70	61	63	60	65	70
7,22,25-Stigmastatrienol	19	12	9	10	9	10	9	9	6	5	6	6
7,25-Stigmastadienol	12	3	4	5	4	4	5	5	5	4	4	4
7-Stigmastenol	7	2	3	4	3	3	4	5	5	6	6	5
7-Campestenol	1	1	2	2	1	1	1	2	3	2	2	2
Sitosterol	4	2	3	3	5	2	2	3	6	5	4	2
Clerosterol	2	5	6	3	3	3	3	3	3	3	3	3
Stigmastanol	2	2	2	2	1	1	1	1	2	3	1	1
Cycloartenol	tr[b]	2	tr	1	2	2	1	4	1	2	2	1
Total sterol (μg/g dry wt)	1186	903	580	695	462	1012	793	504	522	231	470	841

[a]Percent of the total sterol including minor components.
[b]Trace.

developmental period. There is an early accumulation of sterol, followed by a decrease to a relatively constant low level (Fig. 2). This may be a combination of pith loss during maturation and an increase in the amount of fibrous tissue. These would cause a reduction of the amount of membrane, therefore a reduction in the amount of total sterol. In the tomato a similar observation has been made (48). In terms of their sterol composition, stems were similar to the leaves in that they contain primarily 7,22-stigmastadienol and 7-stigmastenol as their major Δ^7-sterols and sitosterol as their major Δ^5-sterol (Table 4).

Cotyledons, flowers and pericarp. The cotyledons were analyzed for the first 4 weeks of growth after which they senesced and were no longer included in these analyses. In the cotyledons the percentage of sterols with the $\Delta^{25(27)}$ double bond do not show as rapid a decrease as seen in the other organs (Table 5). Although the levels of $\Delta^{7,22}$-stigmastadienol and Δ^7-stigmastenol increase, they do not dominate the sterol profile. During the first two weeks of growth the cotyledons are very similar to the seeds in their sterol composition (Tables 1 and 5), which was expected since the seeds are composed largely of two massive cotyledons. The cotyledons contain approximately 5% Δ^5-sterols comprised mainly of sitosterol.

Squash plants are monecious but imperfect (male and female reproductive organ on the same plant but not in the same flower), with male flowers produced first. In these studies the male flowers were harvested at week 6 in the first experiment (week 8 in the second), and female flowers were harvested at week 8 in the first experiment (week 10 in the second). Male flowers have relatively less $\Delta^{7,22}$-stigmastadienol and Δ^7-stigmastenol than female flowers or the pericarp. This is due to the significantly higher percentages of other sterols such as cycloartenol, 7-campestenol, 24-methylenelathosterol, stigmastanol, sitosterol and clerosterol. Sitosterol was the major Δ^5-sterol found in each of the reproductive structures and was similar in quantity in the pericarp and the flowers. These results show a slightly higher relative amount of Δ^5-sterols in the male flowers than female flowers, which had slightly more than the pericarp. Knights also analyzed squash flowers and reported the presence of Δ^5-sterols (21).

Throughout development Δ^7-sterols predominate the sterol profile in the Fordhook variety of *Cucurbita maxima* as in other Cucurbitaceae. There are significant

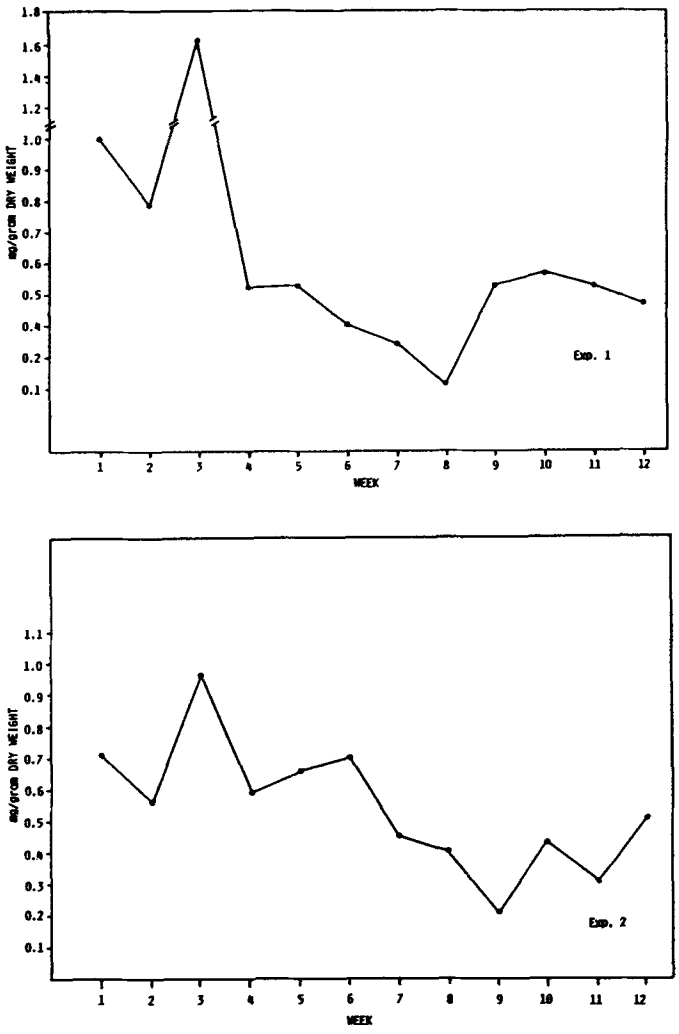

FIG. 2. Total sterol present in *Cucurbita maxima* stems on a mg/g dry wt basis over the life cycle.

TABLE 4

Percentages[a] of Major Sterols from Squash Stems

Sterol	Week											
	1	2	3	4	5	6	7	8	9	10	11	12
7,22-Stigmastadienol	47	59	56	59	60	54	57	49	43	49	43	48
7,22,25-Stigmastatrienol	27	14	7	4	4	6	4	7	6	6	7	5
7,25-Stigmastadienol	11	6	6	6	4	5	6	6	6	7	8	7
7-Stigmastenol	11	14	18	18	21	20	25	26	30	24	32	33
7-Avenasterol	tr[b]	tr	3	5	2	1	1	3	4	4	4	tr
7-Campestenol	3	3	3	4	3	2	2	2	3	2	2	3
Sitosterol	3	2	1	2	2	3	1	1	2	3	1	1
Total sterol (mg/g dry wt)	856	641	1299	562	599	556	401	311	371	506	420	462

[a] Percent of the total sterol including minor components.
[b] Trace.

TABLE 5

Percentage[a] of Major Sterols From Cotyledons, Flowers and Pericarp in Squash Plants

Sterol	Cotyledons				Male flowers	Female flowers	Pericarp
	Week 1	Week 2	Week 3	Week 4			
7,22-Stigmastadienol	29	39	40	34	22	33	43
7,22,25-Stigmastatrienol	22	22	17	13	10	10	5
7,25-Stigmastadienol	18	11	9	8	7	9	5
7-Stigmastenol	10	16	16	18	10	26	36
7-Avenasterol	12	2	5	7	8	6	tr
7-Campestenol	2	2	2	2	12	2	3
Sitosterol	2	3	3	4	6	5	4
Clerosterol	tr[b]	1	2	2	2	2	1
Stigmastanol	1	3	1	1	6	1	tr
Cycloartenol	2	tr	1	4	6	1	tr
Total sterol (mg/g dry wt)	710	477	601	500	3140	862	926

[a] Percent of the total sterol including minor components.
[b] Trace.

quantitative changes in the kinds of sterols, and these changes appear to be organ specific. In whole squash plants or in any of the organs analyzed, $\Delta^{7,22}$-stigmastadienol was always a major component regardless of the stage of growth. The predominance of $\Delta^{7,22,25}$-stigmastatrienol and $\Delta^{7,25}$-stigmastadienol is more associated with embryonic tissues. Here we elect the term "embryonic tissues" since it was not determined if higher levels of $\Delta^{25(27)}$-sterols found during week 1 in stems and roots resulted from seed storage pools or *de novo* synthesis. However, the finding that true leaves which are not part of embryonic tissues never contained high levels of sterols possessing the $\Delta^{25(27)}$-double bond, and the early decline in the amounts of these sterols in maturing stems and roots, support the above observation. It should be pointed out that small quantities of these sterols did persist throughout development in each organ. It could likewise be suggested that Δ^{7}-stigmastenol is associated with pigmented vegetative tissue in these plants, since it was not a major constitutent of cotyledons or roots but was a major sterol in leaves, stems, flowers and pericarp.

The Δ^{7}-sterols were found in each organ. The seeds, roots and flowers were found to contain the largest quantities of these sterols. In some of the early literature there were speculations of root involvement in reproductive processes (49). Our data neither support nor disagree with such speculations, but the slightly higher levels of Δ^{5}-sterols in roots and flowers accompanied by the simultaneous increase of Δ^{5}-sterols in the stem during flower development may be significant. In this respect a selective transport mechanism would have to be assumed, but to date no confirmed mechanisms have been established. Interestingly, roots which are actively growing and seeds and flowers which undergo rapid morphologic changes both contain higher levels of Δ^{5}-sterols. These findings may suggest, as previously speculated (27,50), that meristematic cell division processes and Δ^{5}-sterols may be interconnected. Sitosterol, which is one of the more frequently reported sterols in many other plants, was also the major Δ^{5}-sterol found in this study. It appears that squash with its "atypical" sterol composition approaches "typical" higher plants with regard to its major Δ^{5}-sterol.

A project such as the one undertaken here serves to open doors to ultimately determining the roles of sterols in plants. The detailed analyses should be most useful as a means for designing future experiments. These data

indicate that each organ from this plant may be used separately to gain insight into such occurrences as site specificity of sterol synthesis, differences in regulation of sterol synthesis throughout the plant, or possible transport from one plant part to another. The low sterol accumulation during the life cycle when vigorous growth is taking place and reproductive organs are being produced can be exploited to determine if sterols are participating in developmental regulation.

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Effects of Dietary n-3 Polyunsaturated Fatty Acids on Phospholipid Composition and Calcium Transport in Mouse Cardiac Sarcoplasmic Reticulum

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The effects of dietary n-3 and n-6 polyunsaturated fatty acids on the fatty acid composition of phospholipid, Ca^{++}· Mg^{++}ATPase and Ca^{++} transport activities of mouse sarcoplasmic reticulum were investigated. Mice were fed a 2 weight percent fat diet containing either 0.5 weight percent ethyl esters of 18:3n-3, 20:5n-3 or 22:6n-3 as a source of n-3 polyunsaturated fatty acid or 0.5 weight percent safflower oil as a source of n-6 polyunsaturated fatty acid for 10 days. Olive oil (2 weight percent) was used as a control diet. Although feeding n-6 polyunsaturated fatty acid induced very little modifications of the phospholipid sarcoplasmic reticulum fatty acid composition, feeding n-3 polyunsaturated fatty acid altered it markedly. Inclusion of 18:3n-3, 20:5n-3 or 22:6n-3 in the diet caused an accumulation of 22:6n-3, which replaced 20:4n-6 and 18:2n-6 in phospholipid sarcoplasmic reticulum. The saturated fatty acids were significantly increased with a concurrent reduction of 18:1n-9. These changes in the fatty acid composition resulted in a decrease in the values of the n-6/n-3 polyunsaturated fatty acid ratio and a decrease in the ratio of 20 carbon to 22 carbon fatty acids esterified in the phospholipid sarcoplasmic reticulum. This was associated with a decrease in Ca^{++} uptake by n-3 polyunsaturated fatty acid enriched sarcoplasmic reticulum vesicles as compared with n-6 fatty acid and control diet sarcoplasmic reticulum vesicles. However, neither the affinity for Ca^{++} nor the maximal velocity of ATP hydrolysis activity of Ca^{++}·MG^{++}ATPase were altered by the different diets. The data suggest that the incorporation of 22:6n-3 and/or the decrease of 20:4n-6 plus 18:2n-6 in the phospholipid sarcoplasmic reticulum may affect the membrane lipid bilayer structure and make it more permeable to Ca^{++}.
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A high intake of saturated fatty acids (SAT) and cholesterol is a risk factor in coronary heart disease (1,2), whereas consumption of polyunsaturated fatty acids (PUFA) from the n-6 family (n-6 PUFA) (3) and n-3 family (n-3 PUFA) appear to have beneficial effects in reducing these diseases (4). Moreover, n-3 PUFA seem to be more effective in their antithrombotic and antiatherogenic properties than the corresponding n-6 PUFA (5,6). Dietary eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3), which are the main n-3 PUFA present in seafood and fish oil, influence several biochemical parameters described as risk factor in development of thrombosis and coronary heart diseases (4,5). Their first biological effect is related to plasma lipids and lipoproteins (6,7). The n-3 PUFA of fish oil reduce serum triglyceride, mainly through inhibition of hepatic very low density lipoproteins (VLDL) formation (8). Supplementation of human diet by fish oil also decrease plasma concentration of VLDL- and low density lipoprotein (LDL)-cholesterol (6,9). Dietary n-3 PUFA compete with arachidonic acid (20:4n-6) for acylation in the tissue phospholipids, thereby reducing the formation of vasoconstrictor, prothrombotic and proinflammatory eicosanoids derived from 20:4n-6 (10,11). They also compete directly for the enzymatic site of cyclooxygenase (12) and alter the activity of membrane-bound enzymes (13) and, possibly, receptor functions (14). Although the role of n-3 PUFA, in improving the cardiac functions, has been attributed to reduction in the development of vascular atheroma, recent evidence has indicated a direct effect of these fatty acids on myocardial performance (15,16).

Dietary modifications in cardiac lipids are associated with changes in cardiac functions (15-17). An increase in left ventricular work and coronary flow rate has been reported in isolated hearts of rats fed PUFA-enriched vs SAT fat-enriched diets (17).

Feeding n-3 PUFA as menhaden oil (16) or cod liver oil (18) causes an extensive alteration in fatty acid composition of cardiac lipids in rats, influences the basal cardiac function and the response of the heart to stress (19). Dietary menhaden oil supplementation significantly reduced infarct size in dogs, by mechanisms other than the inhibition of 20:4n-6 metabolism (20). McLennan et al. (15) have shown that the cardiac muscle contractility and the incidence of spontaneous tachyarrhytmias under isoprenaline-induced stress were reduced by feeding n-6 PUFA or n-3 PUFA-enriched diets vs SAT fats. Moreover, the extent of cardiac ischemic damage following coronary artery ligation in rats was significantly reduced by feeding menhaden oil vs corn oil (16). This was associated with an increase in the value of n-3/n-6 PUFA ratio of cardiac phospholipids. These changes may be mediated by inhibiting the formation of eicosanoids and/or by affecting different cellular processes (21).

Calcium (Ca^{++}) homeostasis plays an important role in the regulation of heart contraction and relaxation and its concentration in heart tissue is regulated by several membrane transport systems localized in the sarcolemma, the sarcoplasmic reticulum (SR), and to a lesser extent, mitochondria (22). Cardiac SR is a major site of regulation of intracellular Ca^{++} levels. The calcium-magnesium adenosine 5'-triphosphatase (Ca^{++}·MG^{++}ATPase) is a key enzyme in this regulation (22). During the contraction phases, the intracytoplasmic concentration of Ca^{++} rises, activating the Ca^{++}·Mg^{++}ATPase which pumps it back into the SR (23). This process, together with the extrusion of Ca^{++} out of the myocyte, through the

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Abbreviations: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LDL, low density lipoprotein; LNA, linolenic acid; OO, control diet; Pi, phosphate; PL, phospholipid; PS, phosphatidylserine; PUFA, polyunsaturated fatty acid; SAT, saturated fatty acid; SF, n-6 fatty acid; SR, sarcoplastic reticulum; TCA, trichloroacetic acid; VLDL, very low density lipoprotein; HPTLC, high performance thin layer chromatography; P/S, PUFA to SAT fatty acid ratio.

sarcolemma, reestablishes the basal cytoplasmic concentration which induces the relaxation phases (23). The activity of muscle Ca^{++}·Mg^{++}ATPase, reconstituted into synthetic membrane is dependent on surrounding phospholipids (PL), both by the nature of lipid head groups and the fatty acyl chains (24). Therefore, we have studied the ability of different n-3 PUFA to alter the SR fatty acid PL composition compared with n-6 PUFA and their subsequent effects on the specific activity of Ca^{++}·Mg^{++}ATPase and Ca^{++} uptake by SR vesicles from mouse heart.

MATERIALS AND METHODS

Animals. Male CD-1 white mice (Charles River, Wilmington, MA) weighing 18–20 g were housed at 22°C, 6 mice per cage with food and water ad libitum and a 12-hr dark/light cycle.

Diets. After 10 days on an essential fatty acid free diet, mice were randomly divided into 5 groups of 6 animals each and fed, for 10 days, a fat-free diet supplemented with 2% by wt of different combinations of olive oil and n-6 PUFA or n-3 PUFA. Olive oil 2 wt% served as a control diet (OO Diet); 1.5 wt% olive oil supplemented with 0.5 wt% safflower oil was used as a source of n-6 fatty acids (SF) and 1.5 wt% olive oil mixed with 0.5 wt% of one of the following ethyl ester fatty acids: linolenic (18:3n-3, LNA), eicosapentaenoic (20:5n-3, EPA and docosahexaenoic (22:6n-3, DHA)—were the n-3 fatty acids diets. Ethyl esters of LNA (99.5% pure), EPA (55% pure) and DHA (99% pure) were purchased from NuChek Prep (Elysian, MN). Commercial edible olive and safflower oils were obtained locally. The diets were thoroughly mixed after the addition of oils and small amounts were transferred to Whirlpak plastic bags, flushed with nitrogen and stored at 4°C in the dark. The fatty acid composition of the different diets determinated by gas chromatography (25) are shown in Table 1. The diet enriched with 20:5n-3 contained only 0.3 wt% of 20:5n-3 (Table 1) because of the presence of others fatty acids in the only available commercial source of n-3 PUFA. The mice received fresh diet every day. There was no oxidation of dietary fats as measured by thiobarbituric acid reactive materials (26).

Isolation of sarcoplasmic reticulum. The SR vesicles were prepared according to the method of MacLennan (27) as modified by Swanson et al. (28). Mice were anesthetized with diethyl ether and the heart quickly removed. Hearts from two mice were pooled, washed thoroughly in ice-cold isotonic saline, weighed and minced with a razor blade in ice-cold buffer containing 20 mM TRIS, 30 mM L-Histidine and 0.6 M KCl, pH 7.5 (Buffer 1). Minced tissues were homogenized into 25 ml of buffer 1 with a Thomas homogenizer using a glass-teflon pestle set at speed 4 with 5 slow up and down strokes. The homogenate was centrifuged at 1,600 *g* for 10 min at 4°C to remove nuclei and cell debris. The supernatant was saved and the pellet resuspended in 30 ml of buffer 1, homogenized by 2 up and down strokes and recentrifuged at 1,600 *g* 10 min. The supernatants were pooled, filtered through four layers of cheese cloth and centrifuged at 14,000 *g* for 15 min at 4°C. The resultant supernatant was filtered through 4 layers of cheese cloth and centrifuged at 45,000 *g* for 50 min at 4°C. The pellet was resuspended in 16 ml of 20 mM TRIS, 30 mM L-Histidine, 250 mM sucrose (pH 7.4) (buffer 2) using a glass-glass homogenizer and recentrifuged at 48,000 *g* for 50 min at 4°C. The SR pellet was resuspended in 2 ml of buffer 2 (pH 7.2).

Measurements of Ca^{++} transport. ATP-dependent Ca^{++} uptake by the membrane vesicles was measured isotopically on fresh material as described by Hack et al. (29). The reaction was started by adding 50–80 μg of fresh SR protein to a medium containing 50 mM TRIS·HCl, 100 mM KCl, 5 mM $MgCl_2$, 5 mM potassium oxalate, 30 μM $CaCl_2$, 50 nanocuries $[^{45}Ca]Cl_2$ (sp. act. 25 mCi/mg; Amersham, Arlington Heights, IL) and 2 mM Na-ATP (pH 7.5), in a final volume of 0.5 ml. After 15 min incubation at 30°C, samples were filtered through 0.45 μm Millipore buffer soaked filters to stop the reaction. The filters were washed 4 times with 1 ml of water prior to drying and the radioactivity remaining on the filters counted in 10 ml of scintillation liquid. The Ca^{++}-stimulated uptake was calculated by subtracting the values obtained without ATP in the medium from the total count obtained in presence of ATP.

Measurements of Ca^{++}·Mg^{++}ATPase activity. The ATPase activity was determined using the method described by Bonis et al. (30). Frozen SR was thawed and 15–25 μg of SR protein was incubated at 37°C for 10 min in a standard reaction mixture containing 50 mM TRIS·HCl, 100 mM KCl, 5 mM $MgCl_2$, 5 mM potassium oxalate, 2 mM Na-ATP (pH 7.1) to determine the ATP hydrolysis upon stimulation of the enzyme by Ca^{++}. For the determination of Ca^{++} affinity (Km) and maximum velocity (Vmax) of the enzyme, the free Ca^{++} concentrations in this medium were controlled in the range of 0.05 μM to 1.0 mM by the use of Ca^{++}-EGTA buffer according to Portzehl et al. (31). The Ca^{++}-activated ATPase activity was calculated by subtracting the values obtained without adding $CaCl_2$ and in the presence of 0.1 mM EGTA from those obtained in presence of $CaCl_2$. The enzyme affinity for ATP was measured in presence of 30 μM $CaCl_2$ and the appropriate amounts of Na-ATP. For each concentration of ATP, the "basal" ATPase activity value obtained in the presence of 0.1 mM EGTA

TABLE 1

The Fatty Acid Composition (Mol%) of the Five Diets Fed to Mice for 14 Days

Fatty acid	Dietary treatment				
	OO	SF	LNA	EPA	DHA
14:0	23.3	15.7	21.8	23.4	21.0
16:0	7.5	6.5	4.9	5.7	5.5
16:1	1.1	0.8	0.8	1.1	0.8
18:0	1.3	1.3	0.9	1.2	1.0
18:1	55.9	53.1	43.7	48.7	46.5
18:2n-6	9.6	21.9	7.0	7.3	7.2
18:3n-3	0.9	0.4	20.5	1.0	0.5
20:4n-6	0.0	0.0	0.0	0.3	0.0
20:5n-3	0.3	0.2	0.2	8.1	0.9
22:5n-6	0.6	0.1	0.1	0.2	0.0
22:5n-3	0.0	0.0	0.0	1.1	0.0
22:6n-3	0.0	0.0	0.1	1.9	16.5

The diet contained olive oil (OO); safflower oil (SF); linolenic acid (LNA); eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) as ethyl esters, respectively.

was subtracted from those obtained in presence of $CaCl_2$. The reaction was stopped by precipitating the proteins by 0.5 ml of ice-cold 5% trichloroacetic acid (TCA), and the phosphate (Pi) released was assayed by the colorimetric method of Lebel et al. (32).

Lipid analysis. Lipids were extracted from the SR by the method of Bligh and Dyer (33). Total phospholipids were purified on high performance thin layer chromatography (HPTLC) plates using chloroform/methanol (80:20, v/v), scraped from the plate and extracted from the silica gel by chloroform/methanol (1:1). The phospholipids were saponified with 0.5 N KOH in methanol at 85°C and the free fatty acids extracted and methylated with diazomethane. The fatty acid methyl esters were quantified by gas chromatography (25) (5880 A, Hewlett Packard, PA) using a 0.75 mm by 60 m SP2330 capillary column (Supelco, Inc., Bellefonte, PA) with hydrogen as a carrier gas. The oven temperature was programmed from 140° to 240°C at 5°C/min. Fatty acids were identified by retention time comparisons with fatty acid methyl ester standards as described (25).

Protein and phospholipid estimations. Protein was estimated according to Lowry et al. (34). Total phospholipids were estimated according to Stewart (35).

Statistical analysis. Values from the different groups were compared by the student-t test (MINITAB, Inc.).

RESULTS

The amount of diet consumed and the weight gain by the animals in the different groups were identical. The heart weights, the yield of protein SR and the amounts of total phospholipids were not significantly different between the five dietary treatments (Table 2). The SR contamination by mitochondria and lysosomes was less than 10% on a protein basis and did not differ significantly between the different groups (not shown).

Fatty acid modification. Feeding a dietary supplement containing n-3 or n-6 fatty acids or olive oil for a short term period di not change the total amount of SR phospholipids (Table 2). However, the fatty acid composition of SR from different groups was drastically altered by the diets (Table 3). Substituting 0.5 wt% SF oil for

TABLE 2

Heart Weight, Protein Yield and Total Phospholipid Contents of Cardiac Sarcoplasmic Reticulum From Mice Maintained on Diets for 14 Days

Dietary treatment	Gain weight (g)	Heart weight (mg)	Sarcoplasmic reticulum protein (mg/sample)	Total phospholipids (μg/mg protein)
SF	6.0	366 ± 25	1.90 ± 0.12	101.3 ± 8.3
OO	6.3	367 ± 42	1.81 ± 0.20	113.9 ± 7.3
LNA	5.8	363 ± 58	1.96 ± 0.23	101.0 ± 7.0
EPA	6.2	348 ± 11	2.10 ± 0.24	109.3 ± 4.1
DHA	6.2	363 ± 24	1.96 ± 0.56	100.7 ± 14.4

Gain weight is a mean for 6 animals per group; heart weight, sarcoplasmic reticulum protein and total phospholipids represent the mean ±SD of n = 3 (3 pools of 2 hearts per group).

TABLE 3

Effect of Short-term Feeding of n-6 or n-3 PUFA Lipid Supplements on the Fatty Acid Composition of the Total Phospholipid Fraction of Mouse Heart Sarcoplasmic Reticulum

Fatty acid	Dietary treatment				
	SF	OO	LNA	EPA	DHA
16:0	11.03 ± 1.55	9.58 ± 0.29	12.72 ± 0.22[a]	13.56 ± 0.13[b]	15.36 ± 0.21[a]
16:1	2.27 ± 0.11	1.55 ± 0.22	2.28 ± 1.07	2.28 ± 0.16[a]	1.15 ± 0.46
18:0	17.24 ± 1.74	16.71 ± 1.61	18.15 ± 1.46	18.63 ± 0.26	18.65 ± 1.25
18:1	21.64 ± 0.52[a]	26.61 ± 0.31	24.38 ± 0.19[b]	21.00 ± 0.05[a]	19.42 ± 0.48[a]
18:2n-6	15.33 ± 0.44[b]	12.24 ± 0.41	9.93 ± 0.70[c]	8.04 ± 0.07[a]	7.81 ± 0.30[a]
18:3n-3	nd	nd	1.10 ± 0.63	0.31 ± 0.28	nd
20:3n-9	2.02 ± 0.08[d]	3.38 ± 0.23	1.10 ± 0.13[a]	0.80 ± 0.045[b]	0.37 ± 0.07[b]
20:3n-6	1.99 ± 0.21	1.83 ± 0.07	2.05 ± 0.90	1.37 ± 0.52	0.89 ± 0.04[a]
20:4n-6	12.16 ± 0.84	11.29 ± 0.52	8.66 ± 0.20[d]	7.33 ± 0.26[b]	4.76 ± 0.23[a]
20:5n-3	nd	nd	0.70 ± 0.58	0.65 ± 0.05	0.16 ± 0.03
22:4n-6	0.55 ± 0.05	0.50 ± 0.04	0.19 ± 0.17	0.31 ± 0.13	nd
22:5n-6	3.45 ± 0.35	3.09 ± 0.17	1.65 ± 0.31[b]	1.66 ± 0.12[a]	1.71 ± 0.18[b]
22:5n-3	0.55 ± 0.05	0.55 ± 0.02	1.34 ± 0.07[b]	2.59 ± 0.04[a]	0.76 ± 0.01[a]
22:6n-3	11.64 ± 0.91	13.12 ± 2.44	15.84 ± 0.23[e]	21.61 ± 0.39[c]	28.92 ± 1.38[b]
SAT	28.28 ± 3.29	26.29 ± 1.32	30.87 ± 1.24[c]	32.19 ± 0.17[c]	34.01 ± 1.15[b]
MONOSAT	23.91 ± 0.62[d]	28.16 ± 0.76	26.66 ± 1.20	23.28 ± 0.12[d]	20.57 ± 0.55[a]
PUFA	47.70 ± 2.66	46.00 ± 1.27	42.55 ± 0.53[e]	44.56 ± 0.22	45.40 ± 1.51
PUFA/SAT	1.70 ± 0.31	1.75 ± 0.13	1.38 ± 0.065[e]	1.39 ± 0.015[e]	1.33 ± 0.09[c]
N-6/N-3	2.74 ± 0.09	2.17 ± 0.41	1.26 ± 0.06[e]	0.75 ± 0.02[e]	0.51 ± 0.02[c]
C20/C22	1.00 ± 0.04	0.95 ± 0.16	0.66 ± 0.14	0.39 ± 0.03[c]	0.19 ± 0.01[d]

Values are reported as mol% of total fatty acids and represent the mean ±SD of 3 different experiments.

[a] $p < 0.001$.

[b] $p < 0.005$.

[c] $p < 0.02$.

[d] $p < 0.01$.

[e] $p < 0.05$.

SAT, sum (16:0 + 18:0); MONOSAT, sum (16:1 + 18:1); PUFA, sum (all fatty acids with 2 or more double bonds); C20/C22, sum (all 20-carbon fatty acids)/sum (all 22-carbon fatty acids); nd, not detected.

olive oil increased the amount of 18:2n-6 acylated in SR phospholipids by 20%, whereas 20:4n-6, its desaturation-elongation product, was not significantly increased. Long chain n-3 fatty acids were decreased by 11%, and 18:1n-9 and 20:3n-9 were significantly decreased by 19% and 41%, respectively. The inclusion of 18:3n-3, 20:5n-3 or 22:6n-3 in the diet induced a 1.20-, 1.65- and 2.20-fold increase in the 22:6n-3 levels, respectively. The accumulation of this fatty acid was associated with the displacement of n-6 fatty acids from the phospholipids. The relative decrease in 20:4n-6 and of its long chain metabolites 22:4n-6 and 22:5n-6 was more pronounced than that observed for 18:2n-6 levels. Feeding DHA, as compared with OO induced a 58% reduction in arachidonic acid content and a 36% decrease in linoleic acid, indicating that 22:6n-3 replaced 20:4n-6 more effectively than 18:2n-6. The SR from LNA-fed mice contained relatively low amounts of 18:3n-3 and of its metabolic product, 20:5n-3, which increased from trace amounts (OO) to 1.0 and 0.70%, respectively. Similarly, feeding 20:5n-3 raised its concentration in phospholipids to only 0.65%, but induced a considerable increase in long chain n-3 fatty acids. The diet supplementation by either n-6 or n-3 PUFA vs olive oil induced a significant increase of SAT fats esterified in the phospholipids, whereas the level of monounsaturated fatty acids was significantly decreased mostly because of the fall in 18:1n-9, because 16:1 increased, except after feeding DHA. Overall, although the amount of total PUFA was only slightly modified by the different dietary supplements, there was a significant alteration in the n-6/n-3 ratio which varied from 2.74 (SF) to 0.51 (DHA). The ratio of 20 carbon fatty acids to 22 carbon fatty acids (C20/C22) was also markedly modified.

Ca^{++} uptake and ATPase activity. Isolated SR vesicles actively accumulated and retained Ca^{++} from a medium containing both Ca^{++} and ATP. Previous results from our laboratory have shown that the Ca^{++} uptake by mouse cardiac SR vesicles is a quick and saturable process which reaches maximum velocity at 5 min and plateaus for up to 20 min (28). Therefore, the maximum Ca^{++} uptake by SR from the different groups were compared after a 15-min incubation time. Comparable levels of Ca^{++} accumulation were obtained for SR from animals fed SF and OO diets; however, this was significantly decreased after feeding n-3 PUFA (Fig. 1). The Ca^{++} uptake in the SR vesicles of mice fed 20:5n-3 and 22:6n-3 was significantly decreased as compared with the uptake by SR vesicles from mice fed 18:3n-3 ($p < 0.03$ and $p < 0.0002$, respectively). The alteration in Ca^{++} accumulation was associated with the change in n-3/n-6 ratio, the uptake being decreased when this ratio was increased.

The maximum specific activity (Vmax) and affinity for Ca^{++} and ATP of Ca^{++}·Mg^{++}ATPase associated with Ca^{++} uptake were compared for SR obtained from mice fed SF, EPA and DHA. The Ca^{++} activation of the enzyme was essentially the same in the three groups with a maximum Ca^{++} activation between 5 to 10 μM $-Ca^{++}$, a plateau between 10-50 μM and an inhibition of the enzyme activity at 100 μM $-Ca^{++}$ (Fig. 2). The apparent Km and Vmax values derived from double-reciprocal plots for Ca^{++} in a 0.5-50 μM $-Ca^{++}$ range did not differ significantly between the diets (Fig. 3A, Table 4). The ATP dependence of the Ca^{++}·Mg^{++}ATPase

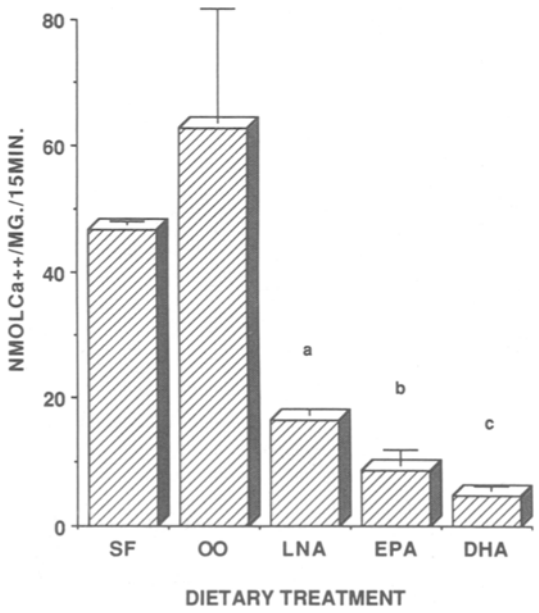


FIG. 1. The effect of different dietary unsaturated fatty acids on the maximum rate of Ca^{++} accumulation by mouse cardiac SR vesicles isolated from mice maintained on diets for 14 days. Results are mean ±SD, n = 3. The Ca^{++} accumulation in n-3 PUFA-enriched diets are significantly different than control (OO); a, $p < 0.05$, b, $p < 0.04$, c, $p < 0.03$. SF, Safflower oil; OO, olive oil; LNA, 18:3n-3; EPA, 20:5n-3; DHA, 22:6n-3.

activity was studied over a wide range of ATP concentrations (0.1-4 mM). A typical Lineweaver-Burk plot obtained over this range and using SR from mice fed SF shows a linear plot for the lower ATP concentrations (0.1-0.5 mM), whereas curvature is apparent at higher concentrations (Fig. 3B). The apparent Km values for ATP obtained at lower concentrations of ATP and in the presence of 30 μM Ca^{++} were 198, 157 and 190 μM, respectively, for the enzyme obtained from mice fed SF, EPA, DHA (average of 2 determinations).

DISCUSSION

All animals gained weight and grew normally, and there was no sign of essential fatty acid deficiency in the animals on the different dietary fatty acids throughout the period of the experiment, indicating that the 18:2n-6 level in the diet was adequate. The cardiac SR fatty acid composition was significantly modified by feeding n-6 or n-3 PUFA within a short period of time, confirming previous results from our laboratory (25,28). Feeding 18:2n-6 or one of the ethyl esters of n-3 PUFA altered the n-6/n-3 ratio in phospholipids in favor of the fatty acid family fed, although more profound modifications were obtained by feeding n-3 PUFA than by feeding n-6 PUFA. The level of n-6 PUFA was markedly lower and the level of n-3 PUFA higher in SR phospholipods of mice fed one of the three n-3 PUFA vs those fed SF or OO in accord with other studies (36-38). Previous results reported that dietary n-3 PUFA replaced 18:2n-6 more efficiently than they replaced 20:4n-6 in the rat cardiac total phospholipids (36-38). However, in the present experiment, 20:4n-6 was replaced more efficiently than 18:2n-6. It is

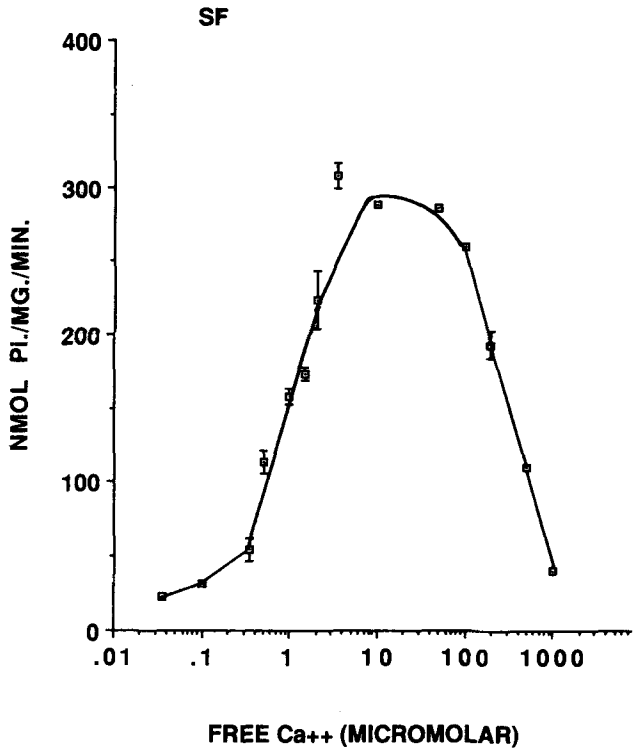


FIG. 2. (A) The dependence of mouse SR Ca^{++}·Mg^{++}ATPase activity on calcium concentration after feeding different n-6 PUFA- and n-3 PUFA-enriched diets. Reactions were carried out with 10 μg of SR protein in Ca^{++}-EGTA buffer and the reaction time adjusted to maintain initial reaction rate at low Ca^{++} concentrations. For Ca^{++} concentrations above 10 μM, only Ca^{++} was added in the medium. Mean ±SD of 3 determinations.

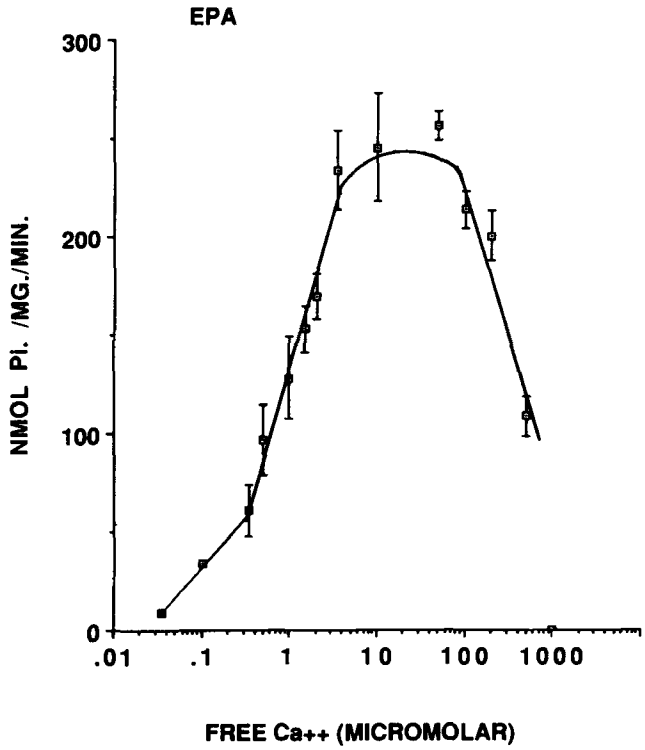


FIG. 2. (B) The dependence of mouse SR Ca^{++}·Mg^{++}ATPase activity on calcium concentration after feeding different n-6 PUFA- and n-3 PUFA-enriched diets. Reactions were carried out with 10 μg of SR protein in Ca^{++}-EGTA buffer and the reaction time adjusted to maintain initial reaction rate at low Ca^{++} concentrations. For Ca^{++} concentrations above 10 μM, only Ca^{++} was added in the medium. Mean ±SD of 3 determinations.

possible that the inhibitory effect of 22:6n-3 on Δ6-desaturase (39), together with the replacement of 20:4n-6 by 22:6n-3, contribute to the drastic decrease in phospholipid 20:4n-6 level from mice fed n-3 PUFA-enriched diets.

Feeding 18:3n-3 or 20:5n-3 resulted in a greater increase of 22:6n-3 in SR phospholipids than of these fatty acids themselves. Cardiac tissue possesses a very limited ability for desaturation (40); therefore, 22:5n-3 and 22:6n-3 acylated in SR phospholipids after feeding 18:3n-3 and/or 20:5n-3 must have been derived mainly from plasma lipids subsequent to their desaturation-elongation in the liver. This specific enrichment of 22-carbon PUFA in cardiac tissue may also reflect a stronger affinity of these fatty acids over 18- or 20-carbon chains fatty acids for the acylation enzymes of this tissues.

The mouse SR Ca^{++}·Mg^{++}ATPase showed Km values for Ca^{++} ranging from 0.8 μM to 1.46 μM. These values are slightly lower than previously reported for canine cardiac microsomes (4.7 μM) (41) and higher than obtained when determinated in absence of Ca^{++} precipitating agent (42). This might reflect a species difference. The double reciprocal plots of the Ca^{++}·Mg^{++}ATPase activity vs ATP concentrations yielded two Km values, as previously reported for ATPase from canine cardiac SR (41). None of these parameters were modified by n-3 PUFA enriched diets vs SF.

Abeywardena et al. (43) have shown that moderate modifications in the membrane fatty acid composition that occur after feeding sheep kidney fat vs sunflower oil to rats did not influence the Ca^{++}·Mg^{++}ATPase activity from cardiac microsomes. In the present experiment, although the membrane fatty acid composition was drastically altered by n-3 PUFA vs n-6 PUFA intake, the Ca^{++}·Mg^{++}ATPase was not altered by these modifications. It has been proposed that modifications in fatty composition of biological membranes result in changes in fluidity and bilayer thickness (24). The optimum activity for Ca^{++}·Mg^{++}ATPase in an enzyme-phosphatidylcholine bilayer reconstituted system is affected by fatty acid chain length (44). In the present study, we observed very little variations in 16- plus 18-carbon chain lengths in the mouse SR fed different diets; a decrease in 18-carbon chains being compensated by the increase in 16-carbon chains. However, the C20/C22 PUFA ratio varied from 1.0 (SF) to 0.19 (DHA) without affecting the enzyme activity. This is in agreement with the results reported by Lee et al. (24), showing that the enzyme is strongly stimulated when the chain length is increased from 12 to 16 carbons, with chains between 18 and 22 carbons supporting similar activities, whereas a further increase up to 24 carbons decreases it.

Membrane fluidity is also an important parameter which may modulate the membrane-bound enzyme activities (13). Electron spin resonance studies measuring the nitroxide-labeled cholestane motion in sonicated phosphatidylserine (PS) vesicles indicated that the hexaenoic

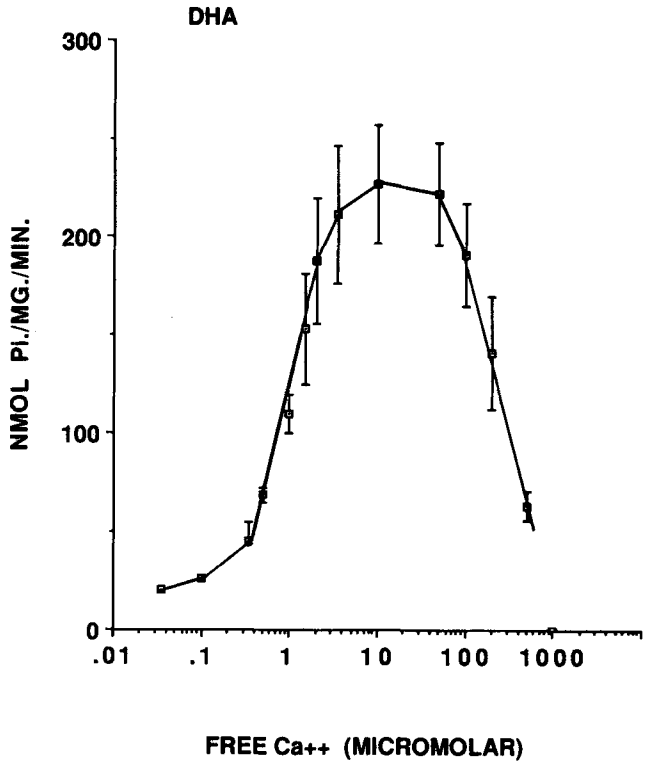


FIG. 2. (C) The dependence of mouse SR Ca^{++}·Mg^{++}ATPase activity on calcium concentration after feeding different n-6 PUFA- and n-3 PUFA-enriched diets. Reactions were carried out with 10 μg of SR protein in Ca^{++}-EGTA buffer and the reaction time adjusted to maintain initial reaction rate at low Ca^{++} concentrations. For Ca^{++} concentrations above 10 μM, only Ca^{++} was added in the medium. Mean ±SD of 3 determinations.

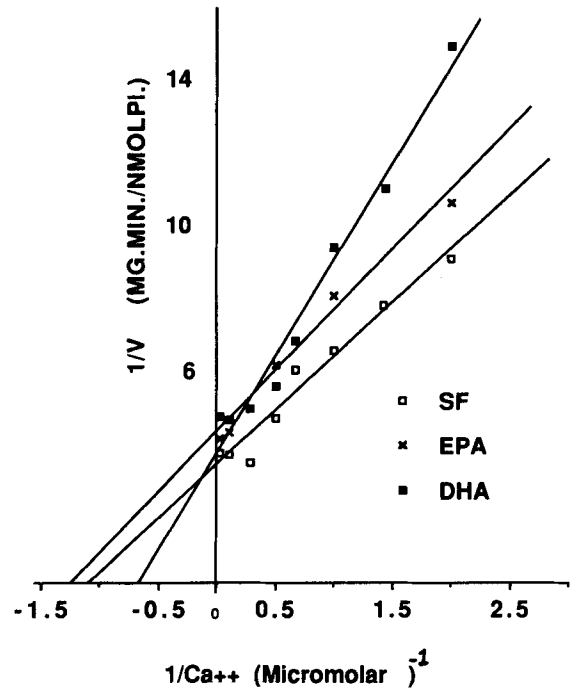
TABLE 4

Apparent Ca^{++} Affinity (Km Ca^{++}) and Maximum Velocity (Vmax) of Cardiac Mouse SR Ca^{++}·Mg^{++}ATPase[a]

Dietary treatment	SF	EPA	DHA
Vmax (nmolPi/mg/min)	315 ± 40	246 ± 45	283 ± 50
Km Ca^{++} (μM)	0.95 ± 0.30	0.81 ± 0.37	1.40 ± 0.47

Mean ±SD, n = 3.

[a]After feeding different dietary fatty acids for 14 days.



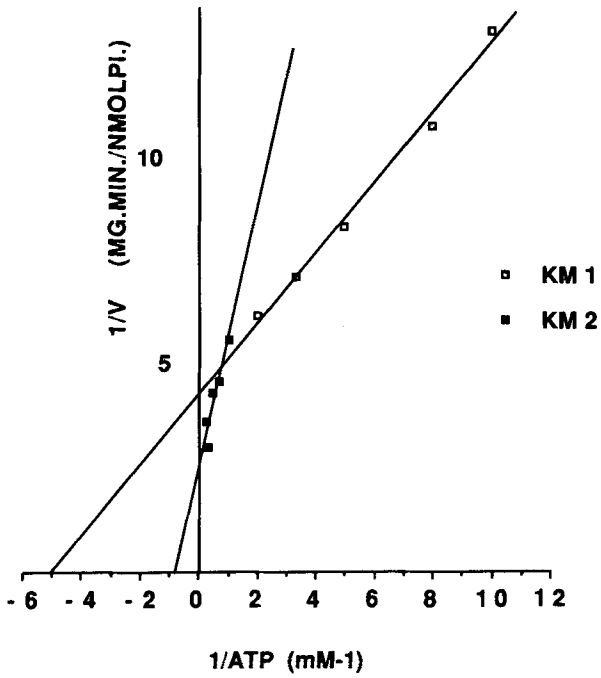


FIG. 3. (A) Lineweaver-Burk plot of the Ca^{++}·Mg^{++}ATPase of cardiac SR isolated from mice after feeding n-6 PUFA and n-3 PUFA diets for 14 days. Initial ATPase activity was measured at 37°C in presence of 2 mM ATP and 30 μg of SR protein. Each point is a mean of 3 determinations. (B) Typical Lineweaver-Burk plot of the Ca^{++}·Mg^{++}ATPase of cardiac SR isolated from mice fed safflower oil. The ATP concentration was varied between 0.1 and 4 mM. Initial ATPase activity was measured at 37°C in presence of 30 μM of Ca^{++}. Each point is an average of 2 determinations.

PS vesicles had the greater probe motion suggesting an increase in fluidity in these vesicles (45). However, in the present study, a 5-fold decrease in n-6/n-3 PUFA ratio in the SR phospholipids from mice fed SF to mice fed DHA did not alter the ATPase activity. In fact, Conroy et al. (46) recently reported that the whole intact membrane can effectively compensate for the decrease in the lipid bilayer order induced by 22:6n-3 in an in vitro model system. Moreover, when ATPase is reconstituted into phospholipids containing a variety of PUFA, the nature of PUFA is of little importance for ATPase activity as long as they maintained a liquid crystalline phase (24). We observed a high ratio of total PUFA to SAT fatty acids (P/S) in the membranes isolated from mice fed either n-6 or n-3 PUFA. These values, which varied from 1.70 (SF) to 1.33 (DHA) assure that lipids are in a liquid crystalline phase,

because the relative content of saturated fatty acids did not exceed 50% of the membrane fatty acid content (24).

Despite the similar ATPase activity, measured by ATP hydrolysis function between the different diets, we observed a significant decrease of Ca^{++} accumulation in n-3 PUFA—enriched vesicles as compared with n-9 PUFA- or n-6 PUFA—enriched vesicles. It is conceivable that a change in the membrane permeability to Ca^{++} is induced following SR enrichment with 22:6n-3 and/or 18:2n-6 plus 20:4n-6 depletion. Both saturated (47) and unsaturated fatty acids (48), when added exogenously to SR vesicles, inhibit Ca^{++} uptake and stimulate Ca^{++} release, although these effects are complex because, under some conditions they inhibited Ca^{++} release whereas they stimulated it under others (49). Fiehn and Hasselbach (50) have shown that phospholipase A_2 treatment of SR vesicles generated free fatty acids and lysophospholipids which remained trapped in the membrane, increased Ca^{++} permeability and inhibited active transport across this membrane without decreasing the ATPase activity. Subsequent removal of these molecules by binding to albumin reestablished the Ca^{++} uptake. It is possible that in the isolation procedure used herein an endogenous release of 22:6n-3 and lysophospholipids occurred which could have inhibited Ca^{++} uptake or promoted Ca^{++} release.

Furthermore, 22:6n-3 is the most oxidizable of the naturally occurring PUFA and generates oxidized products which could alter the membrane integrity of tissues highly enriched in this fatty acid (51). The decrease of Ca^{++} uptake observed after n-3 PUFA supplementation might reflect an increase in lipid oxidation in a membrane highly enriched in 22:6n-3, as it has been described for the retinal rod outer segment membrane (52). Recently, Swanson et al. (28) reported that cardiac SR from mice fed 12-wt% menhaden oil exhibit lower Ca^{++} uptake than SR from mice fed 12-wt% corn oil with a concomitant change of the ATPase activity (28). The present experiments show that decrease in Ca^{++} uptake after 22:6n-3 membrane enrichment can occur without modification in the enzymatic activity and, rather, could be the result of an alteration in the membrane integrity that contributes to decoupling of the enzyme activity and the Ca^{++} uptake by the vesicles. This hypothesis is supported by the demonstration that Ca^{++} permeability through bilayer lipid membranes is highly dependent on the amount of primary oxidation products of phospholipids in these membranes (53). It would also agree with the observation that vitamin E administration corrects the increase of catecholamine cardiotoxicity in rats fed cod liver oil (54).

In conclusion, cardiac mouse SR has a strong affinity for n-3 PUFA and extensively acylates 22:6n-3 into its phospholipids by replacing 20:4n-6 and, to a lesser extent, 18:2n-6. These alterations in fatty acid composition did not significantly modify the Ca^{++}·Mg^{++}ATPase activity, but strongly decreased the Ca^{++} transport by the SR vesicles in vitro, probably by affecting structural integrity of the membrane.

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Skeletal Lipid Depletion in Spawning Salmon

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Skeletal tissues in Pacific salmon are composed of significant amounts of lipid (which serves as an energy supply), that is then depleted during migration and freshwater spawning. The vertebral centra and neurocrania of ocean prespawning pink salmon *Oncorhynchus gorbuscha*, although composed respectively of 2.9–13% and 0.5–6.3% lipid (each by dry weight), amount only to 0–1.6% and 0–1.4% in river postspawning individuals. The skeletal lipids are 91–98% triacylglycerol with only small amounts of phospholipid and cholesterol. Triacylglycerols of carbon numbers 47, 49 and 51 are selectively depleted during spawning, whereas there are increases in the relative amounts of those of carbon numbers 55, 57, 59 and 61. The patterns of variation of the skeletal lipids of coho salmon, *O. kisutch*, chum salmon, *O. keta*, and sockeye salmon, *O. nerka* are similar to those of *O. gorbuscha*. *Lipids 24*, 286–289 (1989).

In an attempt to extend our understanding of the physiological changes that occur during spawning, we have explored the function of salmon skeleton as a lipid storage tissue. The work was prompted by the noval results of a number of studies in which it has been shown that, in fact, some skeletal tissues of marine fishes (the neurocranium and vertebral centra in particular) are composed of 24–90% (w/w) lipid (1–4). Moreover, the bone lipid in the majority of the 40 species examined to date is primarily triacylglycerol, which has been shown to be metabolized rapidly during starvation experiments (5).

Pacific salmon undergo starvation during the migrational spawning ritual (6–8), consuming in the process most of their body lipid (9–13). The blood calcium level, which is controlled by calcitonin secreted by the ultimobranchial gland, also drops by one half (14,15). Spawning salmon thereby lose calcium phosphate, which results, in turn, in the transformation of bone tissues into cartilage (often accompanied, as well, by grotesque deformations). It, therefore, seemed probable to us that the lipid content of the skeletal tissue also might be altered during spawning. As a result, we assayed salmon bone-lipid triacylglycerols by high-temperature capillary-column gas chromatography (GC) with the view of assessing, first, whether there might occur selective depletion of the triacylglycerols; and, second, if so, which compounds are lost during the spawning process.

MATERIALS AND METHODS

Ocean prespawning salmon were collected by purse seine near Alert Bay, British Columbia. Postspawning fish were taken by dip-net from the Ahta River, which empties into Bond Sound, BC. All were 2.0–2.2 kg, fresh weight. Seven prespawning specimens were selected for study and included three pinks, one chum, one sockeye and two coho. Four postspawning pink salmon were chosen for the work. There were both males and females in the prespawning and postspawning groups. All were dissected on the same day of collection, except for the sockeye salmon which was maintained without food for eight days in aquaria.

The neurocranial and vertebral centra skeletal tissues were dissected out of each fish, rigorously cleaned to remove flesh and brain or nerve cord, and then frozen at −20°C prior to transport. Lipids were extracted with 2:1 (v/v) chloroform/methanol. Tared bones were cut into small pieces and then macerated in a Waring Blendor. The dry weight of bone was taken following evaporation of residual solvent under N_2 or on a rotary evaporator. The lipids were isolated by silica-gel column chromatography, where the neutral species were eluted with chloroform, followed by elution of the polar lipids with methanol (4). All bone lipid examined in this work gave only phospholipid, cholesterol and triacylglycerol by thin-layer chromatography (silica adsorbent; petroleum ether/diethyl ether/glacial acetic acid eluent, 80:20:1).

The neutral lipids were assayed for cholesterol by the Liebermann-Burchard reaction (16). The amount of triacylglycerol was then determined by subtracting the amount found for cholesterol from the total weight of neutral lipids. The polar lipids were assumed to be largely phospholipid. Triacylglycerols were subsequently analyzed by GC (Carlo Erba Model 4160 GC: flame ionization detector, hydrogen carrier; 50:1 split injection). The column was a Model TAP fused-silica capillary obtained from Chrompack (Raritan, New Jersey) that contained a methylphenylsilicone stationary phase roughly equivalent to SE-52. The oven temperature was programmed from 300° to 355°C at 3° min^{-1}. Component triacylglycerols were identified by carbon number by comparison of the chromatograms with published spectra of margarine and palm-oil blends that were obtained with similar stationary phases (17–21). Quantitation was carried out manually by triangulation. The chromatograms of at least 50 samples from the eleven salmon specimens were taken; replicate analyses of those arising from the same tissue of a given fish differed by no more than 2–3%.

RESULTS

The results obtained for the assay of lipid in the neurocranial and vertebral centra tissues of several representative specimens of pre- and postspawning pink salmon, *O. gorbuscha*, are provided in Table 1. The data confirm, first, that skeletal lipid is indeed depleted during migration and spawning. For example, in prespawning specimens, 2.9–13% of the dry weight of the vertebral centra tissue was found to be lipid, as was 0.5–6.3% of the neurocranium; whereas, in postspawning salmon, the vertebral centra was composed of only 0.7–1.6% lipid and the neurocranium just 0–1.4%. (The neurocranium was found, in general, to contain less lipid as a percentage of the tissue dry weight than the vertebral centra.)

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Abbreviations: GC, gas chromatography; TLC, thin layer chromatography.

TABLE 1

Assay of Lipids in the Neurocranial (NC) and Vertebral Centra (VC) Skeletal Tissues of Representative Specimens of Pre- and Postspawning Pink Salmon *O. gorbuscha*

	Prespawning salmon			Postspawning salmon		
	Specimen 1	Specimen 2	Specimen 3	Specimen 1	Specimen 2	Specimen 3
% (w/w) Lipid of indicated skeletal tissue						
NC	6.3	2.9	0.5	0.2	1.4	tr
VC	2.9	13.	13.	1.6	1.3	0.7
% (w/w) Triacylglycerol of skeletal lipid						
NC	98.	96.	91.	78.	91.	nd
VC	97.	98.	98.	98.	95.	85.
% (w/w) Phospholipid of skeletal lipid						
NC	0.2	1.1	2.8	2.5	2.5	nd
VC	0.6	0.7	0.8	0.5	2.3	3.6
% (w/w) Cholesterol of skeletal lipid						
NC	2.2	2.9	6.1	20.	6.6	nd
VC	2.2	1.5	1.7	1.5	2.3	11.

nd, No data; tr, trace.

TABLE 2

Neurocranial (NC) and Vertebral Centra (VC) Skeletal Lipids in Three Species of Prespawning Pacific Salmon *Oncorhynchus*

	Coho salmon, *O. kisutch*	Chum salmon, *O. keta*	Sockeye salmon, *O. nerka*[a]
% (w/w) Lipid of indicated skeletal tissue			
NC	4.0	7.1	0.1
VC	15.	9.2	1.7
% (w/w) Triacylglycerol of skeletal lipid			
NC	98.	99.	77.
VC	98.	99.	95.
% (w/w) Phospholipid of skeletal lipid			
NC	0.6	0.1	1.4
VC	0.6	0.5	1.0
% (w/w) Cholesterol of skeletal lipid			
NC	1.6	0.4	22.
VC	1.8	0.5	4.3

[a]Fasted in aquaria for 8 days.

Second, and perhaps somewhat surprisingly, unlike the changes in the absolute amounts noted above, the relative quantities of each class of skeletal lipid were found to be virtually unaffected by spawning. For example, triacylglycerol comprised 91-98% of bone lipid in prespawning pink salmon and 78-98% in postspawning specimens. Also, prespawning phospholipid was 0.2-2.8% and cholesterol 1.5-6.1% of the total lipid content, whereas postspawning pink salmon contained substantially the same levels, i.e., 0.5-3.6% phospholipid and 1.5-20% cholesterol, respectively.

Interestingly, the skeletal lipid of prespawners was orange, the color presumably being due to the carotenoid astaxanthin (22). In contrast, that of postspawning pink salmon was nearly colorless.

The skeletal lipids of prespawning coho salmon, *O. kisutch*, and prespawning chum salmon, *O. keta*, were also analyzed (columns 2 and 3 of Table 2) and were found to parallel those of the prespawning pink salmon previously described. For example, the coho neurocranium was composed on average of 4% lipid, and the vertebral centra 15%. Similarly, the chum neurocranium lipid content averaged 7.1% and the vertebral centra lipid 9.2%. In contrast, the neurocranium of the starved sockeye salmon, *O. nerka*, showed only 0.1% lipid, whereas the vertebral centra had only 1.7%.

Parenthetically, we note that the total skeletal lipid averaged 98-99% triacylglycerol in the coho and chum salmon, yet was composed of only 77-95% in sockeye salmon. We have, as yet, no rationale for this difference.

Gas-chromatographic analysis also revealed selective depletion of component triacylglycerols from the skeletal tissues of postspawning pink salmon, as detailed in Table 3 (note that the triacylglycerol numbers include the three carbons of glycerol). The data obtained for the skeletal triacylglycerol lipids of prespawning coho, chum and sockeye salmon, are also presented in Table 4 for comparison. Triacylglycerols of carbon numbers 47, 49 and

TABLE 3

Average Wt % Composition of Triacylglycerol Lipid by Carbon Number[a]

Carbon number	Prespawning salmon		Postspawning salmon	
	Neurocranium	Vertebral centra	Neurocranium	Vertebral centra
37-46	2.8	5.1	8.4	9.2
47	7.2	6.0	4.1	3.9
49	18.	21.	9.6	19.
51	22.	23.	14.	13.
53	12.	9.8	13.	9.9
54-65	14.	16.	45.	44.

[a]Neurocranial and vertebral centra skeletal tissues of pre- and postspawning pink salmon, *O. gorbuscha*.

TABLE 4

Wt % Composition of Triacylglycerol Lipid by Carbon Number[a]

Carbon number		Coho	Chum	Sockeye
37-46	NC	5.3	8.4	27.
	VC	3.1	4.1	2.5
47	NC	7.5	9.4	4.4
	VC	6.6	8.6	8.9
49	NC	19.	20.	16.
	VC	17.	20.	22.
51	NC	18.	17.	13.
	VC	17.	18.	20.
53	NC	16.	11.	16.
	VC	16.	12.	11.
54-65	NC	23.	21.	19.
	VC	27.	22.	27.

[a]Neurocranial (NC) and vertebral centra (VC) skeletal tissues of prespawning coho salmon, *O. kisutch;* chum salmon, *O. keta;* and sockeye salmon, *O. nerka*.

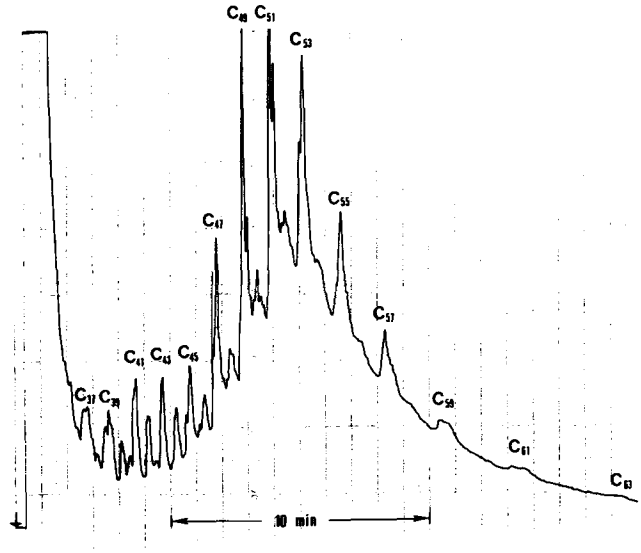

FIG. 1. Gas chromatogram of neurocranial lipid triacylglycerols of indicated carbon numbers (each including glycerol) obtained from ocean prespawning pink salmon, *O. gorbuscha*. Column: TAP fused-silica capillary (Chrompack). GC: Carlo Erba Model 4160 with FID detection and hydrogen carrier; split injections of ca. 50:1. Column temperature: programmed from 300-355°C at 3° min^{-1}.

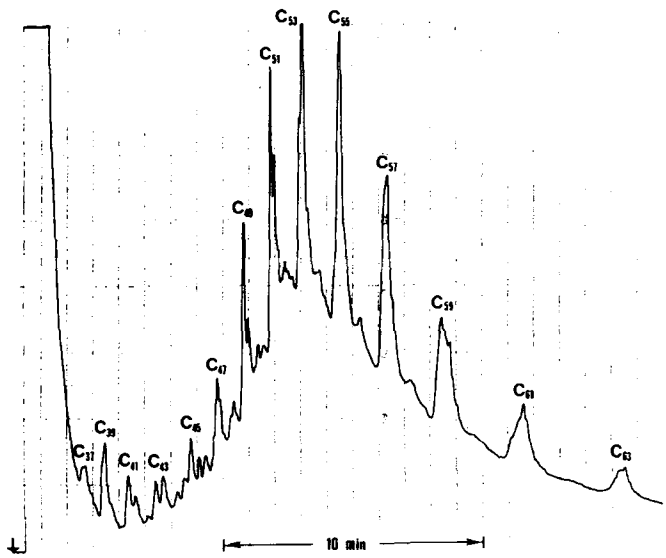

FIG. 2. Gas chromatogram of neurocranial lipid triacylglycerols of indicated carbon numbers (each including glycerol) obtained from river postspawning salmon. Column: TAP fused-silica capillary (Chrompack). GC: Carlo Erba Model 4160 with FID detection and hydrogen carrier; split injections of ca. 50:1. Column temperature: programmed from 300-355°C at 3° min^{-1}.

51 were the primary species lost, as shown in the chromatograms provided in Figures 1 and 2. Also, although those of carbon numbers 54-65 comprised, on average, only 14-16% of the total skeletal triacylglycerol content assayed in prespawners, they were enriched to 44-45% of the total in postspawning salmon. In addition, triacylglycerols of 37-46 carbons were enhanced, albeit slightly, from 2.8-5.1% in prespawners to 8.4-9.2% in postspawning specimens. (There is the possibility that minor amounts of wax esters or glyceryl ethers coeluted with the triacylglycerols; however, none of the former two classes of compounds was detected in replicate thin layer chromatography (TLC) analysis of the samples carried out prior to the GC assays.)

DISCUSSION

The demise of Pacific salmon following spawning is the result of a number of factors, including aging, starvation, atherosclerosis, arthritis and Cushing's syndrome (6), as well as cardiac infarction, liver degeneration and renal failure (23). These are, of course, important factors, as well, in human degeneration. Further, and in addition to the bone demineralization that occurs during the spawning ritual (14,15), lipid depletion in postspawning salmon can also mimic the symptoms of osteoporosis: lipid stored originally in spaces within the bone in prespawners is virtually depleted at its completion. Skeletal lipid is probably not membrane-bound, however, because the phospholipid content of bone tissue was found to be uniformly low (Table 1).

Skeletal-lipid depletion is not unique to spawning salmon. It has, for example, also been observed in controlled starvation studies recently completed on the tropical ocean surgeonfish, *Acanthurus bahianus* (24), in which neurocranium lipid was found to drop from 9-13% after 12 hr to 0.3-3.4% after 72 hr of food deprivation. Bone-lipid depletion has also been observed in the benthopelagic sablefish, *Anopoploma fimbria*, and the temperate shallow ocean sheepshead wrasse, *Semicossyphus pulchur*, following their starvation in aquaria for 96 hr (5). Further, upwards of 90% of the total body lipid of *S. pulchur* is skeletal.

The significance of bone lipid, particularly triacylglycerols, is probably identical to that of lipid in other tissues, i.e., a source of energy. For example, marrow fat cells are similar in composition to those found in adipose tissue. Moreover, in addition to supplying energy to osteocytes, bone lipid can also be used by a number of different cell types that are manufactured in bone tissue.

The evolution, as well as ecological significance, of the lipid content of the skeletal tissues of fish remains obscure, although the occurrence appears to be widespread. For example, oil-filled bones have been found in species that inhabit fresh water (25), midwaters (26), deep benthopelagic layers, (4), tropical marine regions (1,2) and various temperate aquatic climes (4). On the other hand, the representative elasmobranchs analyzed to date have been found to lack significant skeletal lipid. For example, the Chimaeran, *Hydrolagus collei*, has only 1.3% skeletal lipid (2). In mammals, lipid accounts for less than 1% of the dry weight of bone (27). (See, however, Slijper [28] for a discussion of the oil content of whale bones.) Reptiles and amphibians have yet to be assayed for skeletal lipids. Some isotope data suggest that variations encountered in the compositions of bone phosphate compounds in marine sediments might be due to differences extant in the lipid composition (as well as metabolism) of the living individuals (29,30).

Preferential utilization of the more highly unsaturated constituents of fat in starving fishes has been recognized for many years (31), which lends some credence to the selective triacylglycerol depletion from the skeletal lipid of starved postspawning salmon observed in this work. The individual fatty acids of specimen salmon triacylglycerols are, therefore, being assayed in work currently in progress, as are the number, kind, and distribution of positional isomers. The results may be of additional interest regarding heretofore-unrecognized sources of particular omega-3 and 6 fatty acids.

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Effects of Dietary Fish Oil on Human Mammary Carcinoma and on Lipid-metabolizing Enzymes

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The growth rate of a human mammary carcinoma, MX-1, was significantly reduced in athymic "nude" mice fed fish oil. Tumors from the fish oil-fed animals also showed a greater sensitivity to two anti-neoplastic agents, mitomycin C and doxorubicin. Mitochondria were isolated from control livers, host livers and tumors from fish oil- and corn oil-fed animals, and increased levels of 20:5n-3 and 22:6n-3 were found in mitochondrial lipids in all three tissues from the fish oil-fed animals. To investigate the effect of dietary n-3 fatty acids on lipid metabolism, the activity of the acyl-CoA:carnitine acyltransferase and three acyl-CoA desaturases were measured. Carnitine acyltransferase activity toward all four acyl-CoA substrates tested was markedly increased in mitochondria from liver by feeding fish oil. In mitochondria from tumors, feeding fish oil resulted in an increased activity toward only 18:3n-3. These data suggest that fish oil may induce an increase in the oxidation of fatty acids. The Δ^9-desaturase activity was decreased in microsomes from liver and tumor from fish oil-fed animals. However, both the Δ^6 and Δ^5 desaturases were increased in tumor and in control liver as a result of feeding fish oil. The Δ^5 desaturase was not altered in microsomes from the host animals. The effect of fish oil on the Δ^5 and Δ^6 desaturases may involve alterations to metabolism of specific polyunsaturated fatty acids especially in the tumor tissue.
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Several studies have shown that dietary lipid has marked effects on the growth of mammary tumors (1). High levels of dietary fat have been shown to promote the development of mammary tumors with polyunsaturated fats (PUFA) being found to be more effective than saturated fats (2–4). Mammary tumorigenesis in rats and mice has been shown to correlate positively with the content of 18:2 in the diet (5,6). In a recent study (7), a diet containing 14% more 18:2 than found in corn oil was shown to suppress tumorigenesis. This diet also contained 9% 18:3n-6. Supplementation with 18:3n-6 has been shown to inhibit growth in three malignant tumor cell lines (8–9), but pure 18:3n-6 given in amounts below that found in primrose oil (<9%) had no effect on tumor growth (10). Of the pure PUFA tested, only linoleate, n-6, has been shown to enhance tumor growth (11). Further, diets rich in fatty acids from the n-3 family (e.g., fish oils) reduce the size and growth of transplantable (12) and induced (13) mammary tumors. Thus, the type and amount of PUFA appear to be important with respect to tumor growth.

Although the mechanism for the effect of n-6 fatty acids on tumor growth has not been elucidated, the high concentration of prostaglandin found in both human (14) and experimental tumors (15) has implicated prostaglandins as tumor growth enhancers. Indomethacin, a cyclooxygenase inhibitor, reduced the mass of a transplantable mammary adenocarcinoma in the BALB/c mice by 50% (16), further supporting the involvement of prostaglandin. Alterations to the 20:3/20:4 ratio may alter tumor growth (17); this effect may be due to a resultant change in the concentration of the monoenoic prostaglandin, PGE_1. Furthermore, PGE_2 has been shown to suppress the immune response and is present in high levels in mammary tumors (18). Thus, promotion of tumorigenesis by n-6 fatty acids may be related to their role as precursors of certain prostaglandins. Dietary n-3 fish oil fatty acids may inhibit tumorigenesis by blocking the production of prostaglandins from the n-6 fatty acids, as the n-3 fatty acids can compete effectively with the n-6 fatty acids for the cyclooxygenase (19) and be converted into different prostaglandins possessing diminished tumor-promoting properties. However, PGI_3 was not detected after incubations of radiolabeled eicosapentaenoic acid (20:5n-3) with aortic tissue (20), which brings into question the role of n-3 acids as precursors of prostaglandins in all tissues. Although conversion of 20:5n-3 to PGI_3 has been demonstrated in homogenates of rat kidney, no PGI_3 was detected in the urinary products from these animals (21). Conversely, in other studies, this prostaglandin has been detected in the urine of humans who consumed 750 g of mackerel per day (22). These data suggest that the inhibition of tumorigenesis by n-3 fatty acids may be related to an altered production of prostaglandin, but considerable research must be done before a mechanism for the effects of the n-3 fatty acids on tumor growth can be established.

The research just described was conducted on tumors of animal origin; to date, no studies have been reported in which human mammary carcinomas have been used. There also have been no reported studies measuring the effect of an n-3-enriched diet on the sensitivity of tumors to anticancer drugs. Thus, we have fed diets rich in n-3 fatty acids to athymic "nude" mice inoculated with human mammary carcinoma, MX-1, and determined the growth rate of the tumor and its sensitivity to two anticancer drugs, doxorubicin and mitomycin C. Because alterations in dietary lipid have been shown to affect lipid metabolism, we also measured the activity of three of the acyl-CoA desaturases and the acyl-CoA:carnitine acyltransferase (CPT). The first three enzymes are involved in the biosynthesis of PUFA, and the latter is a regulatory enzyme in mitochondrial oxidation of fatty acids. A preliminary account of these studies has been published (23).

MATERIALS AND METHODS

Animals and diet: Young adult athymic (nude) mice (female heterozygous BALB/c nu/+) were used. The mice were housed at 27°C in a germ-free laminar-flow animal station and fed autoclaved rodent chow prepared by

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Abbreviations: CPT, carnitine acyltransferase; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); PG, prostaglandin; PUFA, polyunsaturated fatty acid; AIN, American Institute of Nutrition; T/C, percentage treated/control ratio; UI, unsaturation index.

Dyets, Inc., Bethlehem, PA. The basic diet was the American Institute of Nutrition (AIN) (24) diet in which the fat had been replaced with 10% by weight of either corn oil or MaxEPA (Seven Seas Health Care, Marfleet, Hull, England). The fat was mixed with the powdered diet every 1-3 days, and the final diet was kept in the freezer at −20°C until used. The diet was autoclaved prior to use and the fatty acid composition of the diet was determined (Table 1) before and after autoclaving (25). No differences in composition were observed. The MaxEPA was obtained from the R. P. Scherer North America Corp. (Troy, MI). In one set of experiments, the amounts of diet consumed were measured; the animals fed the corn oil diet consumed an average of 4.8 g/day and the animals fed the fish oil diet consumed 4.4 g/day. The data regarding tumor growth from this experiment were representative of the data collected from the other experiments. The animals were given sterile distilled water supplemented with Avitron, a liquid vitamin supplement from Lambert Kay (Cranbury, NJ).

Tumor implantation: The animals were fed their respective test diets for 10 days prior to inoculation with the tumor. The initial source of the MX-1 tumor was from Dr. W. Wara at the University of San Francisco, School of Medicine. This tumor line has been maintained in Reno, Nevada, since 1982. A tumor fragment (1-2 mm^3) of human mammary carcinoma (MX-1) was implanted subcutaneously on the left side of each nude mouse with a 15 gauge needle, 1 cm caudal to the scapula. Tumor dimensions were determined weekly or semiweekly by measuring 3 perpendicular diameters with vernier calipers. Tumor weights were estimated by the formula (A × B × C/2) where A, B and C represent the three perpendicular dimensions in millimeters (26). The tumor growth data were subjected to a one way analysis of covariance.

Doxorubicin (Adriamycin, Adria Laboratories, Inc., Columbus, OH) and mitomycin C (Mutamycin, Bristol Laboratories, Syracuse, NY) were purchased at a local pharmacy and reconstituted or diluted with sterile water to obtain an injection solution of 0.1 ml/20 g body weight. Doxorubicin was administered at a dose of 3 mg/kg body weight, iv, once a week for 3 weeks with the dosage beginning 7-10 days after the tumor implantation. Mitomycin C was administered at a dose of 0.5 mg/kg body weight, ip, once a week for 3 weeks, again beginning 7-10 days after tumor implantation. Tumor weights were estimated every 3-5 days as described above. The percentage treated/control (T/C) ratio was calculated (in mg) as the mean tumor weight of the treated group divided by the mean tumor weight of the appropriate dietary control group multiplied by 100. The criterion for antineoplastic activity was defined as having a percentage T/C ratio <42 (27).

Lipid and enzyme measurements: The acyl-CoA derivatives used in either the desaturase reactions or the CPT reactions were prepared by the procedure of Bergstrom and Reitz (28). Radioactive [1-^{14}C] oleate, [1-^{14}C] linolenate (n-3) and [1-^{14}C] eicosatrienoate (n-6) were obtained from Du Pont NEN Research Products (Wilmington, DE) and unlabeled acid chlorides of the other fatty acids were obtained from NuChek Prep, Inc. (Elysian, MN). The final specific radioactivities of the three desaturase substrates were 3600 dpm/nmol, 2265 dpm/nmol and 5475 dpm/nmol, respectively. Mitochondria and microsomes were prepared as previously described (29,30). CPT was measured using the 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) procedure described by Parker et al. (31), and the acyl-CoA desaturases were measured as previously described (32). The lipids were extracted by the procedure of Bligh and Dyer (33). The fatty acid methyl esters were prepared from the total lipid extracts by the procedure of Metcalf et al. (34). The total mitochondrial fatty acids were analyzed by gas chromatography (25), and the products of the desaturation reactions were analyzed by radio-high performance liquid chromatography (HPLC) using a LC-8 reverse phase column 15 cm × 4.6 mm with a 3μ particle size obtained from Supelco, Inc. (Bellefonte, PA). The sample was eluted with acetonitrile/water (90:10); slight variations in this solvent ratio were necessary to maximize separation of precursor and product from the three desaturase reactions. A Spectra-Physics SP8700 delivery system was used, and the effluent from the HPLC was directed into a Flo-One Beta model IC flow through scintillation counter from RadioAnalytic, Inc. (Tampa, FL) to quantitate the labeled substrate and product. The CPT-activity data were subjected to an analysis of variance, and the fatty acid compositional data and the desaturase data were analyzed using a Student's t test.

TABLE 1

Dietary Fatty Acid Composition

Fatty acid	Corn oil (% composition)	Fish oil (% composition)
14:0	—	6.0
16:0	11.1	14.2
16:1	0.1	8.6
18:0	2.0	4.1
18:1	25.8	13.2
18:2	58.9	1.6
18:3n-6	—	0.3
18:3n-3	1.6	0.8
18:4n-3	—	3.1
20:4n-6	—	1.3
20:4n-3	—	1.0
20:5n-3	—	17.1
22:1	—	2.1
22:4n-6	—	1.3
22:5n-3	—	2.7
22:6n-3	—	11.1
n-3/n-6	0.03	8.0

The fatty acid composition was determined as previously described (34).

RESULTS

Tumor growth studies: The data in Figure 1 demonstrate that feeding fish oil to the host animals significantly depressed the growth rate of human mammary carcinoma (MX-1), when compared to the corn oil-fed control group. At the end of the experiment, the tumors were excised and measured, and the weights were calculated (Table 2), rather than being weighted directly in order to keep the data consistant with the growth data shown in Figure 1. The mean weight of the tumors from the fish oil-fed group (896 mg) was less than half the mean weight of the corn

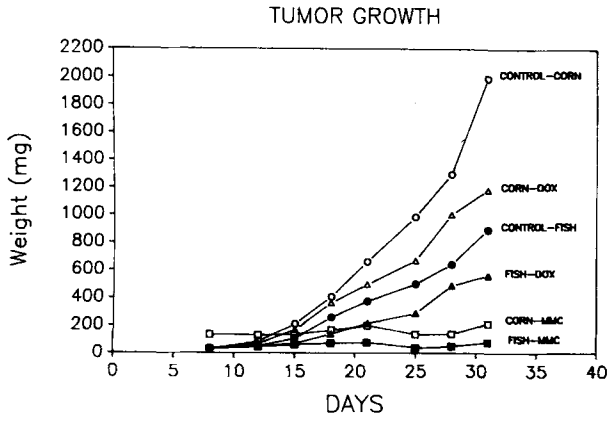

FIG. 1. Effects of corn oil, fish oil and the treatment of each of these two groups with mitomycin C and doxorubicin on the growth rate of transplantable human mammary carcinoma, MX-1, grown in athymic mice. Tumor weights were calculated using the formula (A × B × C/2) where A, B and C represent the three perpendicular dimensions of the tumor in millimeters (26). Each number represents the mean from 9–11 animals from two experiments. Using a one way analysis of covariance, the growth curves for the Control Fish Oil and the Control Corn Oil groups were statistically different with a $P < 0.001$.

TABLE 2

Anti-neoplastic Activity of Mitomycin C and Doxorubicin[a]

	Corn oil (tumor weight)		Fish oil (tumor weight)	
Treatment	mg ± SD	T/C	mg ± SD	T/C
Control	1847 ± 1104	—	896 ± 587	—
Mitomycin C	212 ± 232	12	80 ± 128[b]	9
Doxorubicin	1108 ± 484	60	565 ± 338[b]	63

[a]Towards MX-1 human mammary carcinoma grown in athymic mice fed different dietary lipids.

[b]Two of these animals were totally free of tumor at the end of the experiment. n = 11–12 for all groups.

oil-fed group (1847 mg). We also monitored the amount of diet consumed. The corn oil group ate an average of 4.8 gms/day, whereas the fish oil-fed groups ate 4.4–5.1 gms/day.

We next assessed the effect of modifying the dietary lipid on the chemotherapeutic response of the tumor to two antineoplastic agents, mitomycin C and doxorubicin (Table 2). The MX-1 human mammary carcinoma was sensitive to mitomycin C at a dose of 0.5 mg/kg body weight and demonstrated T/C ratios of 12 and 9 for the corn oil- and fish oil-fed groups, respectively. This is supported by the data in Figure 1. Conversely, when considering the T/C ratio in animals treated with doxorubicin (60 and 63 in corn oil- and fish oil-fed groups, respectively), it appeared that MX-1 was insensitive to this drug; however, the data in Figure 1 strongly suggest that feeding fish oil enhances the effect of doxorubicin. In both control and drug-treated groups, fish oil-fed animals had significantly smaller tumors than the corresponding corn oil-fed groups, suggesting that feeding fish oil enhances the chemotherapeutic effect of mitomycin C and doxorubicin. These findings are consistent with the findings of Burns and Spector (35), which showed that enrichment of L-1210 leukemic cells with PUFA increased their sensitivity towards the cytotoxic action of doxorubicin and hyperthermia. The extent to which dietary fish oil decreased the growth of MX-1 was similar to the extent to which doxorubicin decreased the growth of MX-1 in the corn oil-fed group. The mean tumor size of doxorubicin-treated corn oil-fed group was 1108 mg, whereas, the mean tumor size of the control fish oil-fed group was 896 mg. This finding implies that replacing dietary lipid with fish oil is at least as effective in treating MX-1 human mammary carcinoma in the athymic mouse as is chemotherapy with doxorubicin. It should be noted that, in our laboratory, doxorubicin has not been observed to reduce the size of the MX-1 tumor in mice fed regular pelleted rodent chow.

The data in Table 3 present the body weights from 5 separate experiments for the corn oil-fed and the fish oil-fed animals and from 2 separate experiments for the treatments of each of these two groups of animals with mitomycin C and doxorubicin. As can be seen from these studies there was no change in body weight in any of the groups of animals, indicating no toxic effect of either of the two drugs or of the fish oil.

Fatty acid composition of mitochondria: Table 4 shows that feeding fish oil results in marked increases in the content of both 20:5n-3 and 22:6n-3 in isolated mitochondria. The control animals were fed the AIN diet with the appropriate fat, and no tumors were inoculated into these animals. This increase appeared to be at the expense of the major n-6 fatty acids, 18:2 and 20:4. We also observed a significant increase in the amount of 16:0 present in the liver membranes after feeding fish oil. The mitochondria

TABLE 3

Mice Body Weights

Day	Corn oil	Corn oil + MMC	Corn oil + DOX
Corn oil			
2	22.7 ± 2.6		
8	23.5 ± 2.3	21.1 ± 0.7	22.3 ± 1.6
12	22.8 ± 2.5	23.1 ± 1.9	23.4 ± 1.7
15	22.9 ± 2.7	23.9 ± 2.8	23.5 ± 1.7
18	23.0 ± 3.1	21.3 ± 2.4	23.3 ± 1.5
21	24.4 ± 3.1	23.4 ± 2.2	23.1 ± 1.4
25	25.1 ± 2.7	23.4 ± 2.3	23.2 ± 1.4
29	25.0 ± 2.9	23.6 ± 2.4	23.6 ± 1.8
32	24.5 ± 3.1	24.2 ± 3.7	25.0 ± 2.3
Day	Fish oil	Fish oil + MMC	Fish oil + DOX
Fish oil			
2	23.2 ± 1.8		
8	22.7 ± 1.6	22.8 ± 1.4	24.5 ± 2.6
12	22.7 ± 2.1	23.6 ± 1.8	24.4 ± 2.3
15	22.4 ± 2.1	23.6 ± 1.5	24.3 ± 2.1
18	22.4 ± 2.8	21.5 ± 1.4	23.2 ± 1.5
21	23.6 ± 2.7	23.6 ± 1.6	24.8 ± 2.4
25	23.3 ± 2.7	24.0 ± 1.5	24.1 ± 2.5
29	24.1 ± 2.8	24.2 ± 1.8	23.5 ± 2.6
32	23.6 ± 2.4	23.9 ± 1.6	24.4 ± 2.5

Each value for the Fish Oil and Corn Oil groups represents the mean ± SD for 21–29 animals. All other values represent the mean ± SD for 6–11 animals. MMC, mitomycin C; DOX, doxorubicin.

TABLE 4

Mitochondrial Fatty Acids

Fatty acid	Control (nMol %)	Host (nMol %)	Tumor (nMol %)
Corn oil			
16:0	15.8 ± 1.4	19.6 ± 1.9	19.9 ± 1.0
16:1	1.0 ± 0.4	1.7 ± 0.9	3.6 ± 1.2
18:0	17.0 ± 2.3	16.2 ± 1.1	35.5 ± 4.0
18:1	15.5 ± 3.5	15.7 ± 1.7	6.6 ± 1.1
18:2	19.7 ± 1.6	17.8 ± 1.1	11.2 ± 1.3
20:3n-6	2.0 ± 0.8	2.3 ± 0.3	4.6 ± 2.3
20:4n-6	20.5 ± 2.4	17.6 ± 2.1	15.9 ± 3.1
22:4n-6	0.5 ± 0.4	0.5 ± 0.0	3.3 ± 0.3
22:5n-3	2.0 ± 0.5	2.3 ± 0.7	1.8 ± 0.6
22:6n-3	6.1 ± 0.7	4.9 ± 0.1	ND
UI-Index	190.2 ± 9.2	177.3 ± 8.7	134.6 ± 8.9
nMol/mg	526.0 ± 113.0	438.0 ± 117.0	575.0 ± 96.0
Fish oil			
16:0	23.6 ± 0.6[a]	23.6 ± 0.9[a]	19.2 ± 1.2
16:1	3.2 ± 0.4	3.2 ± 0.2	6.6 ± 0.5
18:0	13.6 ± 0.8	13.7 ± 0.7	16.8 ± 0.9[a]
18:1	18.6 ± 2.5	14.9 ± 4.1	28.7 ± 1.4[a]
18:2	3.1 ± 0.3[a]	4.3 ± 0.7[a]	3.7 ± 0.7[a]
20:3n-6	0.8 ± 0.3	1.0 ± 0.1	1.3 ± 0.3
20:4n-6	5.9 ± 0.4[a]	5.8 ± 0.4[a]	3.7 ± 1.0[a]
20:5n-3	8.4 ± 0.9[a]	7.3 ± 1.0[a]	5.5 ± 0.6[a]
22:5n-3	1.8 ± 0.1	1.9 ± 0.2	4.5 ± 1.2
22:6n-3	20.7 ± 1.2[a]	21.3 ± 0.7[a]	10.1 ± 2.4[a]
UI-Index	229.2 ± 0.7[a]	228.8 ± 5.6[a]	171.6 ± 14.5[a]
nMol/mg	604.0 ± 135.0	590.0 ± 66.0	625.0 ± 49.0

Each value represents the mean ± SD from 5 or 6 animals. The control animals were fed the AIN diets with the appropriate fat, and no tumors were inoculated into these animals.

[a]$P < 0.001$. ND, No data. Comparisons were made between corn oil and fish oil feedings. When comparisons were made between control and host within each dietary treatment group, no differences were noted; however, the fatty acid composition of the tumor mitochondria were quite different than either host or control.

TABLE 5

Effects of Fish Oil and Tumors on Carnitine Acyl Transferase Activity in Nude Athymic Mice

Fatty acid substrate	CPT activity (nMol/Min/Mg)		
	Fish oil diet	Corn oil diet	P
Tumor			
16:0	12.4 ± 3.8	10.1 ± 4.7	NS
18:1	9.8 ± 1.9	7.0 ± 2.9	NS
18:2n-6	19.7 ± 5.1	16.5 ± 6.5	NS
18:3n-3	31.0 ± 6.7	16.3 ± 7.9	<0.002
20:3n-6	19.5 ± 2.0	14.2 ± 5.9	NS
Host liver			
16:0	86.7 ± 18.4	36.7 ± 4.3	<0.001
18:1	85.6 ± 15.2	29.6 ± 6.5	<0.001
18:2n-6	85.7 ± 13.7	56.4 ± 7.5	<0.003
18:3n-3	103.1 ± 6.1	67.3 ± 7.9	<0.001
20:3n-6	247.7 ± 61.1	77.1 ± 14.8	<0.001
Control liver			
16:0	96.7 ± 20.8	63.3 ± 8.9	<0.01
18:1	121.4 ± 18.9	64.8 ± 14.8	<0.002
18:2n-6	236.5 ± 23.4	138.4 ± 22.4	<0.001
18:3n-3	266.7 ± 27.1	164.7 ± 26.5	<0.002
20:3n-6	400.5 ± 72.5	168.2 ± 36.2	<0.001

Each value represents the mean ± SD from 3-5 animals. The animals were fed the same diets as noted in Table 3. The statistical data resulted from an analysis of variance.

NS, Not significant.

TABLE 6

Effect of Diet and Tumor on Desaturase Activity

	Desaturase activity (nmol/min/mg protein)		
	Control liver	Host liver	Tumor
Δ^9 Desaturase			
Corn oil	2.17 ± 0.55	1.82 ± 0.38	0.21 ± 0.04
Fish oil	1.34 ± 0.52	1.16 ± 0.34	0.16 ± 0.02
P	<0.01	<0.01	<0.01
Δ^6 Desaturase			
Corn oil	0.13 ± 0.05	0.26 ± 0.06	0.08 ± 0.03
Fish oil	0.33 ± 0.07	0.44 ± 0.06	0.14 ± 0.06
P	<0.001	<0.001	<0.01
Δ^5 Desaturase			
Corn oil	0.14 ± 0.05	0.38 ± 0.08	0.07 ± 0.03
Fish oil	0.26 ± 0.03	0.37 ± 0.09	0.10 ± 0.01
P	<0.001	NS	<0.02

Each value represents the mean ± SD from 6-12 animals. Statistical comparisons (Students-t test) were made between the corn oil and the fish oil diets and the significance (P) is shown under the comparisons. Each determination from each animal was done in duplicate. The diets were the same as described in Table 3.

NS, Not significant.

from the tumor tissue had similar changes in their content of PUFA. In addition, there was a decrease in the amount of 18:0 and an increase in 18:1. The calculated unsaturation index (UI) was increased in the liver, as well as the tumor mitochondria, and this was largely the result of the increased amounts of the n-3 PUFA. No changes were observed in the nmol/mg tissue of the total fatty acids. Thus, feeding fish oil has markedly altered only the fatty acid composition of the mitochondrial membrane lipids.

Enzyme studies: Varying the lipid composition of the diet may affect the ability of the mitochondria to oxidize fatty acids after feeding fish oil; therefore, we measured activity of the CPT, the control enzyme for fatty acid oxidation (Table 5). Several different acyl-CoA substrates were used to determine whether fish oil would affect enzyme activity with any or all substrates. In the tumor tissue, feeding fish oil increased the activity with only one substrate, 18:3n-3. However, in the liver of either the host animals or the controls (no tumors), feeding fish oil markedly increased the activity of CPT, with all substrates tested. Interestingly, in the host liver, CPT activity was about half that observed in the control liver from animals that were not inoculated with tumor.

Another group of enzymes that are associated with membranes as integral proteins and which play a role in controlling the unsaturation level of the membrane fatty acids are the acyl-CoA desaturases. We measured the activity of the Δ^9, Δ^6 and Δ^5 desaturases in liver and tumor microsomes after feeding fish oil (Table 6). Feeding

fish oil significantly decreased the activity of the Δ^9 desaturase in liver from both control and host animals and in the tumor tissue. However, in all three groups of tissues, feeding fish oil increased the Δ^6 activity. The activity of the Δ^5 desaturase was increased in control liver and in tumor, but no difference was observed in activity from host liver. The activity of all three desaturases in the tumor tissue was considerably lower than the activities found in both control and host liver, and dietary fish oil significantly decreased the Δ^9-desaturase activity in tumors, whereas both the Δ^5 and Δ^6 desaturases were increased in tumors.

DISCUSSION

All of the previously reported studies involving the effects of dietary fats on the growth characteristics of tumors have been conducted on rodent tumors. The data presented in this report clearly demonstrate that growth of the transplantable human mammary tumor, MX-1, is decreased by dietary n-3 fatty acids or fish oil fatty acids. However, because mammary tumorigenesis has been shown to correlate with the content of dietary 18:2 (4,5), we cannot rule out the possibility that there may be a dual effect of low dietary 18:2 and high n-3 fatty acids. In addition, we have demonstrated that one of the key enzymes in the control of fatty acid oxidation, the acyl-CoA:carnitine acyltransferase, is markedly altered by feeding fish oil. Studies of the oxidation of fatty acids after feeding high-fat diets (15% by wt) have suggested that the enzymes of peroxisomal β-oxidation are selectively induced (36). Furthermore, it was concluded that, in addition to a general induction by high-fat diets, the extent of induction was determined by the fatty acid composition of the diet. No data were presented concerning mitochondrial fatty acid oxidation or any of the enzymes that control fatty acid oxidation. It is generally accepted that transport of fatty acids into the mitochondria via the carnitine acyltransferase controls the oxidation of fatty acids. Although the effects of starvation (37), thyroid conditions (38), diabetes (39) and clofibrate feeding (40) on CPT activity have been examined, no other data regarding the effects of dietary conditions on the activity of CPT have been published.

The marked increase we observed in liver CPT activity from fish oil-fed animals with all of the different fatty acyl substrates may be directly related to an effect of the fish oil diet on the regulation of the enzyme. However, the increased activity may simply be related to an alteration of membrane properties of the mitochondrial membrane as a result of the incorporation of the n-3 PUFA. Brady et al. (41) demonstrated in the Zucker rat that CPT activities respond to alterations in membrane fluidity, i.e., increased fluidity is associated with increased activity. Zammit (42) has suggested that changes in the lipid structure of the inner mitochondrial membrane might alter the interaction between the malonyl-CoA binding protein and CPT. This is supported by the gradual loss in the sensitivity of CPT I to malonyl-CoA observed when the incubation temperature is increased from 0°C to 37°C (43). These data strongly suggest lipid-protein interactions may be important in the control of CPT I, and our data would be consistant with such an interaction.

Decreases in the activities of the three acyl-CoA desaturases have been reported in tumor cells from rat hepatomas with respect to control liver tissue (29). These data indicate a decrease in the de novo synthesis of PUFA and subsequently a decrease in the precursors for prostaglandin synthesis. However, PG concentration in tumor tissues has been found to be increased (14,15). Our data show that feeding fish oil resulted in an increase in the concentration of the n-3 fatty acids in the tumor membranes (Table 4). This could result in a decrease in the ability of the tumor to synthesize prostaglandin (PG) due to the competition of n-3 fatty acids with n-6 fatty acids for the cyclooxygenase (19,44). If tumor growth is correlated with increased amounts of PG, the decrease in tumor growth we noted in the fish oil-fed animals could be then the result of decreased PG synthesis. Furthermore, the slight increase in the Δ^5 and Δ^6 desaturases in tumors from fish oil-fed animals, relative to activities from tumors in corn oil-fed animals, may be an attempt by the tumor to compensate for the lack of PG precursors supplied by the n-3 fatty acids of the fish oil diet by synthesizing more n-6 fatty acids to serve as PG precursors.

Alteration of membrane lipid fatty acid content which would alter membrane fluidity could also have profound effects in the response of tumors to antineoplastic agents by directly modifying an intracellular membrane target site or by affecting the transport of drug to its active site. For example, doxorubicin has been reported to induce modifications in the surface membranes of sarcoma 180 cells (45). Indeed, Tritton and Yee (46) proposed that the antitumor activity of doxorubicin results from its interaction with the tumor cell membranes; thus, dietary alteration of the tumor cell membrane fatty acid composition could be responsible for the enhanced antineoplastic activity observed for doxorubicin when the host was fed fish oil. Alternatively, changing the membrane fluidity through dietary manipulation could alter the transport of doxorubicin and mitomycin c in and out of the tumor cell and/or its organelles. Transport modifications which increase the concentration of antitumor agent at the target site would be expected to increase antitumor activity. Finally, incorporation of the highly polyunsaturated fatty acids from dietary fish oil into the tumor membrane lipids would be expected to increase the susceptibility of the tumor lipids to lipid peroxidation and oxidative stress which could jeopardize membrane integrity, produce toxic aldehyde byproducts, inhibit thiol-containing proteins and cause DNA degradation. Because tumors from fish oil-fed animals would possess high levels of the highly unsaturated n-3 fatty acids, they may be more susceptible to pro-oxidant induced oxidative stress; thus, endogenous lipid peroxidation could be responsible for the slower tumor growth observed in the mice fed fish oil (47). Doxorubicin has been reported to undergo redox cycling with mitochondrial NADH-Coenzyme Q reductase (48) with concomitant production of toxic oxygen species capable of initiating and propagating lipid peroxidation. In fact, Doroshow (49) proposed that oxidative stress was involved in the antineoplastic activity of doxorubicin. Similarly, mitomycin C was reported to undergo bioactivation with subsequent generation of toxic oxygen radicals (50). This, in turn, was correlated to cytotoxicity of aerobically cultured EMT-6 tumor cells. Thus, it is

possible that feeding fish oil predisposes MX-1 human mammary carcinoma to lipid peroxidation and increases its susceptibility to pro-oxidant antitumor agents like doxorubicin and mitomycin C. In addition, the observed increased sensitivity of MX-1 human mammary carcinoma towards these two antitumor agents in the fish oil-fed group may be related to the pro-oxidant activity of these two chemotherapeutic agents. These possible mechanisms of antineoplastic activity require additional experimentation.

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The Common Occurrence of Furan Fatty Acids in Plants

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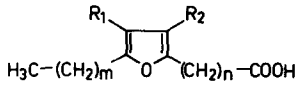
The observation that F-acids (1) occur in rat chow initiated a search for F-acids in human diet. We observed that the amount of F-acids with a pentyl side chain in α-position taken up with a one-day diet correlates well with the amount of excreted degradation products, the pentyl urofuran acids (2), (3) and (4). Therefore it can be concluded that F-acids with a pentyl side chain are not produced in the human body but are introduced through the diet. The origin of F-acids carrying an α-propyl side chain is less clear. The amount of propyl-urofuran acids (2) and (3) excreted in urine was found in one case out of three to be five times higher than the amount of F-acids carrying a propyl group in α-position taken up by the diet. Therefore, it can presently not be excluded that a portion of the propyl F-acids is produced by the body.

F-acids found in human food are mainly introduced into the body by vegetables and fruits. F-acids were found also in birch leaves in considerable amounts, as well as in grasses, dandelion and clover leaves. Thus, we can conclude that F-acids are common constituents of plants. ***Lipids 24,*** **296-298 (1989).**

F-acids (1), first shown by Glass and Schlenk (1,2) to occur in fish, were later found in soft corals (3) and crayfish (4). Recently, Watanabe showed that they are also present in amphibians and reptiles (5). Our research detected F-acids in mammals (6), including man (7). F-acids in plants have been reported only once: Hasma et al. (8) detected an F-acid in *Hevea brasiliensis.*



1

		m	n	R_1	R_2
a:	F_0	4	6	CH_3	CH_3
b:	F_1	2	8	CH_3	CH_3
c:	F_2	4	8	H	CH_3
d:	F_3	4	8	CH_3	CH_3
e:	F_4	2	10	CH_3	CH_3
f:	F_5	4	10	H	CH_3
g:	F_6	4	10	CH_3	CH_3
h:	[a]	3	10	CH_3	CH_3

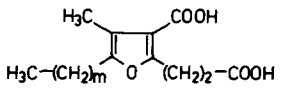
[a]Synthetically produced furan fatty acid.

FORMULA 1

Nevertheless, it was tacitly assumed that F-acids with either 3 or 5 carbons in the aliphatic side chain are produced by animals or man. Biosynthetic experiments with a linoleic acid analog as precursor, in which the side chain had been extended by one carbon atom, were carried out in our laboratory. These experiments failed, because the "labeled" compound was not incorporated into F-acids (9). In the course of these investigations we found that F-acids were introduced into the rats through the diet (9), although previous investigations had shown the absence of F-acids in the rat food (10). This could be explained by the fact that F-acids behave like fatty acids in chromatography and therefore escape detection. If a mixture of fatty acids and F-acids is hydrogenated the resulting tetrahydrofuran acids show a different behavior which allows their separation from fatty acids, even if the latter are present in large quantities (7).

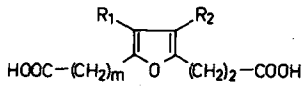
The observation that F-acids are introduced into rats through the diet led us to reexamine whether these compounds may also be part of the human diet. Since it was not known which portion of the diet would contain the F-acids, the whole diet eaten in one day was collected and after extraction an aliquot of the lipids was analyzed for F-acids.

During this experiment we also measured the excretion of the degradation products of F-acids (1), the urofuran acids (2), (3) and (4) (11,12).



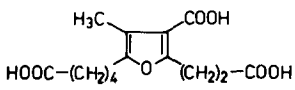
2 m = 2, 4

FORMULA 2



3 m = 2, 4
$R_1 = CH_3$, H
$R_2 = CH_3$, H

FORMULA 3



4

FORMULA 4

RESULTS

Three people each eating a different diet collected for analysis identical quantities of all foods they consumed over the period of the experiment. These food samples were analyzed for lipids as described in the Materials and Methods section of this paper. During the food collection period, urine samples from the test subjects were collected and analyzed for urofuran acids. Each lipid fraction of the dietary samples was hydrolyzed and hydrogenated to tetrahydrofuran acids (7) which, after methylation, can be more easily separated from fatty acids than the corresponding F-acids (7) by thin-layer chromatography (TLC).

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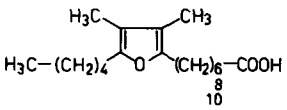
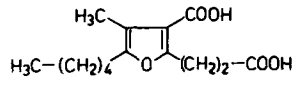
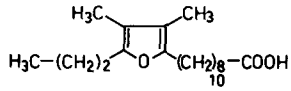
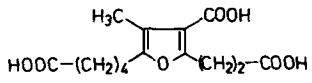
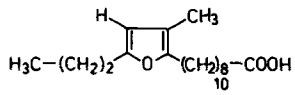
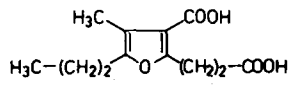
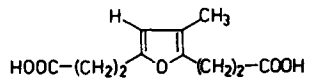
Abbreviations: FID, flame ionization detector; GC, gas chromatography; MS, mass spectrometry; TLC, thin-layer chromatography; WCOT, wall-coated open tubular.

TABLE 1

F-Acids Determined in Food (μmol/day[a])			Urofuran-Acids Expected in the Urine (μmol/day[a])		
	I	1) 7.01 ± 1.64 2) 7.29 ± 2.29 3) 14.21 ± 4.22		II	1) 6.11 ± 0.15 2) 6.07 ± 0.09 3) 9.87 ± 0.11
					and
	IV	1) 0.66 ± 0.17 2) 3.64 ± 0.87 3) 2.32 ± 0.65		III	1) 0.84 ± 0.07 2) 1.02 ± 0.04 3) 1.86 ± 0.11
	VI	1) 4.21 ± 1.01 2) 0.94 ± 0.25 3) 1.81 ± 0.73		V	1) 2.28 ± 0.02 2) 3.71 ± 0.04 3) 3.18 ± 0.03
				VII	1) 3.14 ± 0.13 2) 0.42 ± 0.06 3) 0.84 ± 0.06

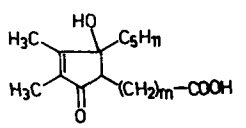
[a]The values were determined by GC. Peak areas of the F-acids were compared with the area of the synthetic F-acid (**1h**) added at an amount of 100 μg as an internal standard. Each sample was lmeasured three times.

The methyl esters of the tetrahydrofuran acids were separated by gas chromatography (GC) and identified by mass spectrometry (MS). Table 1 gives the results of these measurements.

DISCUSSION

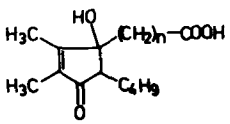
The data in Table 1 show that the amount of F-acids with a pentyl side chain in the α-position introduced into the body through the diet corresponds well with the amount of excreted urofuran acids. The results obtained with α-propyl substituted F-acids do not allow the same clear conclusion to be drawn. Considering the facts that propyl- and pentyl-urofuran acids show a different tendency for adsorption by albumin (13) and that propyl-urofuran acid is excreted in a circadian rhythm while pentyl-urofuran acid does not follow such a rhythm (14), it might be concluded that some of the propyl F-acids are produced in the body.

One source of F-acids may be meat or fish; however, the amounts of F-acids introduced into the body by normal daily fish and meat consumption are low when compared with the excreted amounts of urofuran acids (2), (3) and (4) (11). In looking for other sources of F-acids, we detected these compounds in oranges. Further, F-acids degradation products, the cyclopentenolons (5) and (6) (15), were found in soy oil, but only in low amounts.



5 m = 7, 9

FORMULA 6



6 n = 8, 10

FORMULA 7

These findings, together with Hasma's earlier observation (8) on the occurrence of ab F-acid in the latex of *Hevea brasiliensis*, prompted us to draw the conclusion that F-acids may occur frequently in plants.

Subsequently, a thorough investigation of plant material revealed that F-acids occur in the roots and blades of grasses (*Poaceae spec.*), in clover (*Trifolium pratense*), and also in certain vegetables (e.g. in chive [*Allium sativum*] and cabbages [*Brassica oleracea spec.*]); in potato (*Solanum tuberosum*), wheat (*Triticum aestivum*) and rice (*Oryza sativa*); in some fruits (in lemon [*Citrus limon*], strawberries [*Fragaria spec.*] and orange [*Citrus sinensis osbeck*]); in algae (*Chlorophyta spec.*), in the trunk of birch (*Betula pendula*), and in dandelion (*Taraxacum officinale*). F-acids occur in comparatively high amounts in the green parts of plants (Table 2). Only small amounts (typically 1/100 to 1/1000 of that found in the green parts) occur in the trunks, roots and seeds (Table 2).

We also found the F-acid (**1d**) in mushroom (*Agaricus bisporus*), and traces of the F-acids (**1d**) and (**1g**) in yeast (*Saccharomyces cerevisiae*). In the mushroom the F-acid (**1d**) predominates, while in plants (**1g**) is the main F-acid. In comparison with the amounts found in vegetables (with the exception of potato) and fruits the amounts of (**1g**) found in the blades and leaves of grasses, in dandelion, and in birch are very high. Interestingly, among the land plants and other species so far investigated, F-acids with a propyl side chain occur only in traces. The algae *Chlorophyta spec.* were the only species treated in which high amounts of an F-acid with a propyl side chain was detected (Table 2).

MATERIALS AND METHODS

Gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS). GC was carried out with a

TABLE 2

Occurrence of F-Acids in Plants

		F-acids determined in μg per g of dried plant material[a]				
		(1c)	(1d)	(1e)	(1f)	(1g)
1	Grasses (blade)	<1	50	2	—	189
2	Grasses (root)	—	2	—	—	>1
3	Dandelion (leaf)	<1	2	—	—	82
4	Dandelion (root)	—	—	—	—	<1
5	Dandelion (seed)	—	—	—	—	<1
6	Clover	—	<1	2	<1	18
7	Birch (leaf)	—	6	2	3	183
8	Birch (trunk)	—	<1	—	—	<1
9	Chive	—	—	—	—	16
10	Wheat	—	<1	<1	—	33
11	Rice	—	4	—	—	<1
12	Potato (leaf)	<1	11	4	—	355
13	Potato (fruit)	—	2	—	—	3
14	Cabbage	—	—	—	—	2
15	Orange	2	—	—	—	—
16	Lemon	—	—	—	—	28
17	Strawberry (leaf)	—	—	—	—	6
18	Strawberry (fruit)	—	—	—	—	2
19	Mushroom	—	166	—	—	<1
20	Yeast	—	1	—	—	<1
21	Algae	—	3	145	—	9

[a]The values were determined by comparing the GC peak areas of the identified F-acids with the area of the synthetic F-acid (1h) added to each sample as an internal standard.

Packard model 438S from United Technologies equipped with a flame ionization detector (FID), on a wall-coated open tubular (WCOT)-glass capillary (30 m × 0.3 mm) OV-101 column, temperature programmed from 100°C to 240°C at 2°C min^{-1}. The temperatures of the injector and detector were kept at 270°C and 290°C, respectively. Peak area integration was done by a Shimadzu C-R3A integrator. The carrier gas was hydrogen. The split ratio was 1:10.

GC-MS was performed on a Finnigan MAT 312 GC-MS system with a MAT SS 300 data system. Electron impact mass spectra were recorded with an ionizing energy of 70 eV. The GC column was a 25-m × 0.3-mm i.d. OV-101 WCOT glass capillary column. The carrier gas was helium (2 ml min^{-1}), and the temperature program was the same as used for GC.

Nutrition experiment. Over a period of 5 days, the urine excreted in the 24 hr between 8 a.m. of one day and 8 a.m. of the next day was collected individually from three persons (two male, one female). The urine samples were stored at −20°C.

Quantification of urofuranic acids (2), (3) and (4) in urine. One-ninety sixth (corresponding to 15 min) of a 24-hr urine sample was acidified with conc. HCl, and diluted with water to 20 ml. Ten μg 3-carboxy-4-methyl-5-pentyl-furan-2-acetic acid was added as an internal standard. The extraction of organic compounds and the quantifaication of urofuranic acids (2), (3) and (4) were done as previously described (9), using Chromabond-C_{18} and Chromabond-Si solid phase extraction columns (Macherey & Nagel; D-5160 Düren, Federal Republic of Germany). The amount of urofuranic acids (2) and (3) was calculated by peak area integration, by comparison with the internal standard.

Quantification of F-acids (1) in human food and plants. To each sample 100 μg of (1h) were added as an internal standard. The extraction of the lipids was performed according to the method of Bligh and Dyer (16), and the crude lipid extract was subjected to a "Folch wash" with 0.88% potassium chloride solution (17).

After partition, the chloroform layer was evaporated to dryness under reduced pressure and the residue was saponified with 200 ml 1-N potassium hydroxide solution (methanol/water, 9:1, v/v) under an atmosphere of nitrogen, for 14 hr, which was necessary to hydrolize amid fatty acid bonds, too (18). After cooling and acidification with conc. HCl the potassium chloride was filtered off and 100 ml chloroform and 100 ml water were added. The chloroform layer was separated, the solvent removed under reduced pressure and the residue esterified with ethereal diazomethane. Column chromatography, thin layer chromatography and hydrogenation were done as previously described (7).

The furan fatty acids (1) were quantified as to their tetrahydrofuran methyl esters by peak area integration, using the internal standard (1h) for comparison (7).

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Oxidation of Vitamin E in Red Cell Membranes by Fatty Acids, Hydroperoxides and Selected Oxidants

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Our laboratory previously reported that vitamin E (tocopherol) in human blood platelets was oxidized *in vitro* by various oxidants. This paper shows that diamide, superoxide, hydroperoxides and polyunsaturated fatty acids induce oxidation of tocopherols in red cell membranes. In contrast to platelets, red cell membrane tocopherol was oxidized by hydrogen peroxide and tertiary butyl hydroperoxide. Alpha tocopherolquinone was one of the products of oxidation. Among the fatty acids, the *cis* polyunsaturated acids were the most potent oxidizing agents with monounsaturated and *trans* compounds relatively ineffective. The oxidation is not a detergent effect of the fatty acids since neither the detergents Brij and Lubrol, when present in concentrations under 0.5 mM, nor sodium arachidate (1.25 mM), could oxidize the membrane tocopherol. When red cell membrane samples were incubated with 0.5 mM arachidonate, 47 ± 11% (S.D.) of the tocopherol lost was converted to tocopherolquinone. Unlike arachidonate, oxidants such as diamide, hydrogen peroxide and tertiary butylhydroperoxide are unable to oxidize all of the membrane tocopherol and produce less tocopherolquinone from oxidation (10–15%) under the experimental conditions of this study. Linoleic acid hydroperoxide is a much more potent oxidant and produces less quinone than arachidonate. The mechanisms of tocopherol oxidations induced by the various compounds seem to be different since the yields of quinone during oxidation vary with the nature of the oxidant. Tocopherol is consumed by oxidation as it protects the membrane from oxidant damage induced by compounds such as unsaturated fatty acids and hydroperoxides.
***Lipids* 24, 299–304 (1989).**

The literature contains reports of many experimental investigations demonstrating that tocopherols (T) are very potent lipid antioxidants. Most of these studies have focused on the reactions of T in chemical media and the conversion of T to tocopherolquinone (TQ) (Fig. 1) has been shown to occur under various conditions. For example, Fukuzawa and Gebicki have shown that α-T present in micelles and liposomes is oxidized to TQ by hydroxyl and superoxide free radicals (1). Alpha chromanoxyl radical is an intermediate in this reaction (2). The alpha chromanoxyl radical can be further converted to the quinone and other oxidation products or be regenerated to the original vitamin E through the action of vitamin C (2–4).

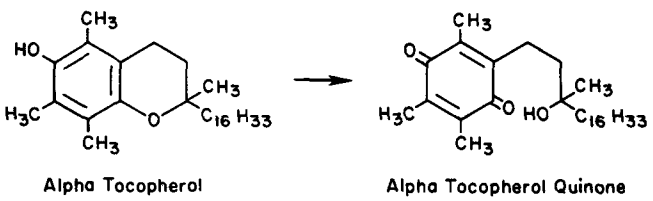

FIG. 1. Oxidation of alpha tocopherol to alpha tocopherolquinone.

The oxidation of α-T to TQ is of considerable significance in understanding the mechanism of action of the T compounds as biological antioxidants. Recently we have observed that T in human platelets can be oxidized in vitro to TQ by various oxidants (5). This report shows that a very similar (but not identical) oxidation of T takes place when human red cell membranes are challenged *in vitro* with unsaturated fatty acids such as arachidonate or other oxidants like peroxides, fatty acid hydroperoxide, diamide or superoxide.

MATERIALS AND METHODS

Human subjects. Human male subjects 25–55 years of age were selected for the study. These healthy volunteers fasted for 12 hr prior to drawing of venous blood from the arm in the morning. None of the subjects were on any weight-reducing or other dietary regimens or were taking high doses of vitamins or minerals.

Chemicals. All chemicals used were of reagent grade purity from standard sources. Solvents used for chromatography were HPLC grade from Burdick and Jackson Laboratories, Inc., Muskegon, MI 49442. Other special chemicals used were purchased from the following sources: α-T, γ-T and TQ from Kodak Laboratory Chemicals, Rochester, NY 14650; tertiarybutylhydroperoxide from Sigma Chemical Company, St. Louis, MO 63178; hydrogen peroxide from Fisher Scientific, Springfield, NJ 07081. Absolute ethanol was obtained from Midwest Solvents Company, Pekin, IL 61555 and was redistilled prior to use. Sodium salts of the fatty acids were obtained from Nuchek Prep Inc., Elsian, MN 56028. The detergents, Brij and Lubrol, were obtained as specially purified 10% aqueous solutions from Pierce Chemical Company, Rockford, IL 61105. Hydroperoxy-linoleic acid (13S Hydroperoxy) was obtained from Oxford Biomedical Research Inc., Oxford, MI 48051.

Isolation of red blood cell membranes and protocol for incubations. Blood was obtained in heparinized vacutainer tubes from human subjects who had fasted overnight. The red blood cell membranes were isolated by a procedure described by Burton et al. (6) which is a minor modification of the original procedure of Dodge et al. (7). Even though no other special precautions were taken the membranes obtained appeared white. The red cells were lyzed and the membranes isolated by centrifugation and washed three times with successively lower concentrations of phosphate buffer (6). The *in vitro* incubation of red cell membranes was done at room temperature (20–22°C) for 120 min in 50 mM potassium phosphate

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Abbreviations: BHT, butylated hydroxytoluene; diamide, diazine dicarboxylic acid *bis*(N,N-dimethylamide); T, tocopherols; TQ, tocopherolquinone.

buffer at pH 7.4. All incubations were performed at a final membrane protein concentration of 300 μg/ml. The total reaction volume ranged from 1 to 2 ml. The sodium salts of fatty acid or other substances were added to start the incubation. The sodium salts of unsaturated fatty acids were always kept under argon after the vial was broken and were used for only a week after opening. The salts were dissolved in water which was exhaustively deaerated wth argon. The tubes were mixed briefly on occasion during the incubations. At the end of incubation, tubes were centrifuged at 27000 g for 12 min, supernatants were removed and the pellets assayed for α-T and TQ.

Biochemical assays. All experiments were repeated on a separate day using blood samples from a different donor. Data from typical experiments are reported. Concentration of total protein was determined by the Lowry technique as modified by Markwell et al. (8).

Liquid chromatography of tocopherols. The liquid chromatographic method for determination of tocopherols was a modification of our published reports (9,10). Briefly, 2 ml ethanol containing 0.025% butylated hydroxytoluene (BHT) and 0.1 ml of 30% ascorbic acid were pipetted into the tubes containing samples for T analysis. The mixture was saponified at 60°C for 30 min after the addition of 1 ml of 10% potassium hydroxide solution. The tubes were cooled and 2 ml of water was added followed by 2 ml of hexane containing 0.025% BHT. T was extracted into the hexane phase by vortexing for one min. Hexane phase was separated out, dried with anhydrous sodium sulfate and analyzed by either normal phase (Zorbax Sil column; mobile phase—1% methanol in hexane) or reverse phase (Zorbax C18; mobile phase—methanol:water, 98:2) liquid chromatography. The tocopherols were detected by their native fluorescence (excitation 295 nm, emission 340 nm).

Liquid chromatographic conditions for analysis of TQ. The details of the procedure for determination of TQ in membrane samples have been reported (11). Briefly, the samples for TQ analysis were mixed with 0.1 ml of 30% ascorbic acid and 2 ml of distilled ethanol containing 0.05% BHT. One ml of 10% potassium hydroxide was added, and all tubes were saponified for 30 min at 55°C. The mixture was cooled and extracted with 2 ml of hexane. Known volumes of the hexane layer were pipetted into tubes and the solvent evaporated off under nitrogen using an N-evap. The residue was redissolved in 0.2 ml of the mobile phase (methanol/water, 98:2) and used for reverse phase chromatography of TQ. The TQ was detected by UV absorption of the compound at 265 nm.

Superoxide production. The xanthine-xanthine oxidase system was used for the production of superoxide as recommended by Fridovich (12). The incubation was done at room temperature (20–22°C) under the following conditions: pH 7.8, xanthine 50 μM, 100 μM EDTA, 50 mM potassium phosphate, xanthine oxidase 0.1 μM. After incubation the red cell membranes were pelleted and analyzed for T and TQ by liquid chromatography.

RESULTS

The influence of the structure of fatty acid upon oxidation of α-T in red cell membrane was studied first. The membrane samples were incubated with fatty acids of different chain lengths and unsaturation with the final concentration being 0.5 mM. The results show that only polyunsaturated fatty acids are able to cause measurable oxidation of α-T (Table 1). The data also demonstrate that linoleate with *cis* double bonds is much more potent than *trans* linoelaidate. Since it is possible that α-T could have been released into the medium when the red cell membranes were incubated with the fatty acid, samples of the incubation medium were collected after centrifugation. Analysis of these samples failed to show measurable levels of α-T in the incubation medium.

Proteins such as serum albumin which bind fatty acids are known to be present in many tissues and physiological fluids. Therefore, the effect of addition of albumin to the incubation medium prior to addition of arachidonate was studied. Oxidation of T in the red cell membrane proceeded until the final concentration of albumin reached 0.1%, at which point oxidation was inhibited. When the final concentration of albumin was 0.3% or above, the oxidation of T was completely inhibited, suggesting that the oxidation is induced by the unbound fatty acids.

Metal ions and their chelates are potent agents affecting redox equilibrium under a variety of biochemical conditions. However, addition of ethylenediaminetetraacetic acid or desferrioxamine methanesulfonate to the incubation medium before addition of arachidonate did not block oxidation of T. This suggests that metal ions or their chelates are not involved in the oxidation of T in red cell membranes by arachidonate.

Chemical changes produced in biological matrices by fatty acids (especially when they are present in high concentrations) could be ascribed to the so-called detergent properties of the fatty acids. It is difficult to rule out such detergent effects. An experiment was conducted where the red cell membranes were incubated with the detergents Brij and Lubrol. Since detergents can solubilize membranes, T concentrations in both membrane pellet and supernatant were determined. (It is interesting to note that when membrane samples were incubated with fatty acids, no T could be detected in the incubation medium.) The results given in Table 2 show that Brij does not alter T concentrations in the membrane even up to a final concentration of 5 mM. Lubrol will oxidze membrane T when the final concentration is near 1 mM. The

TABLE 1

Oxidation of α-Tocopherol in Human Red Cell Membranes Induced by Selected Fatty Acids

Fatty acid added	α-Tocopherol remaining after oxidation (μg)	α-Tocopherol oxidized (μg) (calculated)	% original α-tocopherol oxdized
None	0.253 ± 0.002[a]	0	0
Oleate	0.246 ± 0.003	0.007	3
Linoleate	0.185 ± 0.013	0.068	27
Arachidonate	0.080 ± 0.004	0.173	68
Linoelaidate	0.236 ± 0.003	0.017	7
Arachidate	0.247 ± 0.004	0.006	2

[a]Mean ± S.D. for three separate experiments

Red cell membrane samples were isolated from the venous blood obtained from one adult human male volunteer who fasted overnight. Each incubation tube containing 600 μg of membrane protein was incubated at room temperature (21–23°C) for 2 hr as described under Methods. All fatty acids were 0.5 mM in final solution.

TABLE 2

Effect of Incubating Human Red Cell Membranes with Detergents upon Alpha Tocopherol Concentration in the Membrane

Detergent	Final concentration of detergent	Amount of alpha tocopherol (μg)		
		In membrane pellet	In supernatant	Total (calculated)
None	0	0.099 ± 0.004[a]	0	0.099
Brij	5 mM	0.091 ± 0.003	0.016 ± 0.003	0.107
	0.5 mM	0.098 ± 0.008	0.008 ± 0.003	0.106
Lubrol	1 mM	0.048 ± 0.003	0.021 ± 0.001	0.069
	0.5 mM	0.085 ± 0.003	0.008 ± 0.005	0.093

[a]Mean ± S.D. for three determinations.

Red cell membrane samples were isolated from the blood of one human volunteer. Each incubation tube contained 300 μg of membrane protein and was incubated under conditions given in Methods (also see legend of Table 1) after the addition of the required amount of Brij or Lubrol.

TABLE 3

Yield of Alpha Tocopherolquinone From the Oxidation of Alpha Tocopherol in Human Red Cell Membranes Upon Incubation with Arachidonate

Human donor	Total membrane protein (mg)	Alpha tocopherol originally present in membrane samples (nmol)	Alpha tocopherol oxidized (nmol)	Alpha tocopherol quinone produced (nmol)	Alpha tocoperol quinone produced as % of alpha tocopherol oxidized
A	750	0.432 ± 0.013[a]	0.275 ± 0.018	0.114 ± 0.011	42
B	600	0.423 ± 0.003	0.175 ± 0.007	0.063 ± 0.003	36
C	600	0.574 ± 0.005	0.409 ± 0.016	0.252 ± 0.015	62
D	600	0.586 ± 0.004	0.399 ± 0.009	0.188 ± 0.012	47
					47 ± 11 (S.D.)

[a]Mean ± S.D. for three incubation tubes.

Fasting blood was obtained from four separate human male volunteers, and red cell membranes were isolated. Each incubation tube contained 600–750 μg of total protein and was incubated at room temperature (20–21°C) with 0.5 mM arachidonate. Alpha tocopherol and quinone in the membrane were determined by liquid chromatography.

lack of oxidation of membrane T by 5 mM Brij, 0.5 mM Lubrol as well as saturated fatty acids (1.25 mM arachidate) suggests that T oxidation is not due to the detergent-like property of fatty acids.

The yield of quinone during oxidation of membrane T by arachidonate was then investigated by processing the membrane pellets for quantitative determination of both T and TQ. The results given in Table 3 show that the yield of TQ from T is quite variable in red cell membrane samples from different human subjects and that TQ accounts for an average of 47 ± 11% (S.D.) of the total T oxidized. It is quite likely that other oxidation products of T such as dimeric molecules are also formed during the in vitro incubations.

Many chemical compounds are known to oxidize components within the red blood cell and its membrane. Peroxides form one chemical class of such oxidants. In one set of experiments, red cell membrane samples were incubated with different concentrations of hydrogen peroxide or tertiary butyl hydroperoxide. The results in Table 4 show that substantial oxidation of T is induced by both peroxides. In contrast to arachidonate which was capable of oxidizing nearly the entire amount of tocopherols in the membrane, peroxides did not oxidize all of the membrane T even when they were present in final concentrations of 10 mM. In a separate experiment it was also found that an average of 11.3% of the T oxidized was converted to TQ during oxidations with hydrogen peroxide.

Another oxidant of the peroxide class which is of particular interest is fatty acid hydroperoxide. A comparison between the oxidations induced by arachidonate and linoleic acid hydroperoxide (13S hydroperoxy) is shown in Table 5. Linoleic acid hydroperoxide is much more potent as an oxidizing agent than arachidonate. The mechanism of oxidations of red cell membrane T by arachidonate and linoleic acid hydroperoxide appear to be different since the yield of TQ is much less with the hydroperoxide-induced oxidations (Table 5). In this respect linoleic acid hydroperoxide resembles other peroxides like hydrogen peroxide.

Diamide has been studied in great detail as an oxidant. The results of incubating red cell membranes with varying concentrations of this compound are given in Table 6. Like the other oxidants, diamide also oxidizes T in membranes. Unlike arachidonate, this compound did not completely oxidize T in the membrane sample even when present in high concentrations of up to 10 mM. Furthermore,

TABLE 4

Effect of Addition of Hydrogen Peroxide and Tertbutyl Hydroperoxide to the Incubation Medium Upon Alpha Tocopherol Concentrations in Human Red Cell Membranes

Peroxide used	Final peroxide concentrations (mM)	Alpha tocopherol in membrane after incubation (μg)	Alpha tocopherol oxidized	
			In μg	As % of original alpha tocopherol
Hydrogen peroxide	0	0.128 ± 0.002[a]		
	0.05	0.118 ± 0.008	0.010	7.8
	0.10	0.104 ± 0.004	0.014	10.9
	0.50	0.083 ± 0.004	0.045	35.2
	1.0	0.076 ± 0.006	0.052	40.6
Tertbutyl hydroperoxide	0	0.160 ± 0.005		
	0.1	0.094 ± 0.005	0.066	41.3
	1.0	0.069 ± 0.002	0.091	57.0

[a]Mean ± S.D. for separate incubation tubes.

Red cell membranes were isolated from the fasting blood of two separate human donors. Each incubation tube contained 300 μg of membrane protein. After incubation alpha tocopherol concentrations in the red cell membrane pellets (obtained after centrifugation of the incubation tube) were determined by liquid chromatography.

TABLE 5

Comparison of Oxidation of Tocopherol in Human Red Cell Membranes Induced by Arachidonate and Linoleic Acid Hydroperoxide

Oxidizing agent	Concentration	Alpha tocopherol remaining in the membrane (nmol)	Alpha tocopherol oxidized (nmol)	Tocopherolquinone produced (nmol)	Yield of tocopherol-quinone (%)[c]
None	—	0.525 ± 0.011[a]		—[b]	
Arachidonate	500 μM	0.275 ± 0.010	0.250	0.131 ± 0.007	52.4
Linoleic acid hydroperoxide	10 μM	0.171 ± 0.009	0.354	0.013 ± 0.003	3.6

[a]Mean ± S.D.

[b]The control membrane sample contained 0.008 ± 0.001 nmol of tocopherolquinone. This blank was subtracted from the experimental tocopherolquinone values for the membranes treated with arachidonate or linoleic acid hydroperoxide.

[c]The yield of tocopherolquinone is expressed as percent of alpha tocopherol oxidized.

Red cell membranes were obtained from one human volunteer. Samples containing 450 μg protein were incubated as described under Materials and Methods. Alpha tocopherol and tocopherolquinone were determined by liquid chromatography.

TABLE 6

Oxidation of Alpha Tocopherol and Production of Alpha Tocopherolquinone During Incubation of Human Red Cell Membranes With Diamide

Concentrations of diamide in the medium	Amount of alpha tocopherol in membrane after incubation (nmol)	Amount of alpha tocopherol oxidized (nmol)	Amount of alpha tocopherol quinone produced (nmol)	Alpha tocopherol quinone produced as % of alpha tocopherol oxidized
0	0.718 ± 0.018[a]	0	0	0
2 mM	0.471 ± 0.005	0.247	0.030 ± 0.003	12.2
5 mM	0.386 ± 0.008	0.332	0.034 ± 0.002	10.2
10 mM	0.349 ± 0.006	0.369	0.047 ± 0.04	12.7

[a]Mean ± S.D. for three incubation tubes.

Red blood cell membranes were isolated from the blood of one human volunteer. Enough diamide was added to each incubation tube to reach final concentrations noted in the first column. The individual tubes contained 750 μg of total protein. After incubation, concentrations of alpha tocopherol and quinone in the membrane pellets were determined by liquid chromatography.

TABLE 7

Alpha Tocopherol Oxidation in Human Red Blood Cell Membranes Induced in vitro by Superoxide

	Amount of alpha tocopherol in membrane after oxidation (μg)	Alpha tocopherol oxidized (μg)	Alpha tocopherol oxidized as % of original
Control	0.163 ± 0.003[a]	0	0
Arachidonate (1 mM)	0.034 ± 0.003	0.129	79.1
Xanthine + xanthine oxidase	0.141 ± 0.001	0.022	13.5
Xanthine + xanthine oxidase + superoxide dismutase	0.162 ± 0.001	0	0

[a]Mean ± S.D. for three separate incubation tubes.

Blood was obtained from one human volunteer after an overnight fast and red cell membranes isolated. Each tube containing 514 μg of protein was incubated after adding the different agents. Alpha tocopherol remaining in the membrane was determined by liquid chromatography. These incubations were conducted at a pH of 7.8 in 50 mM phosphate buffer.

TQ accounted for only 10–13% of the total T eliminated. The corresponding figures for the yield of TQ during oxidations induced by arachidonate, hydrogen peroxide and linoleic acid hydroperoxide wre 47% (Table 3), 11.3% (see above), and 3.6% (Table 5), respectively.

Oxidative damage produced by superoxide in biochemical systems has been studied in great detail. Using the xanthine-xanthine oxidase couple for generation of superoxide in situ, an experiment was performed to see the effect of superoxide on T in red cell membrane preparations. The incubation conditions, including a pH of 7.8, were selected to optimize the steady production of superoxide. The results in Table 7 show that superoxide generation in the medium causes oxidation of T in the membrane. TQ could be detected chromatographically as a product of oxidation even though it was difficult to quantitate the small quantity of TQ produced. The low yield of TQ was expected because the T oxidized was only 14% of the total T, and only a fraction of the T oxidized is expected to be converted to the TQ. The blocking of superoxide-induced oxidation of T in membrane by addition of superoxide dismutase to the incubation medium prior to addition of xanthine + xanthine oxidase confirms the finding that superoxide is the active agent causing oxidation of T in membrane.

DISCUSSION

Concentration of the T oxidation product TQ is quite low in normal mammalian tissue or membrane samples according to our analysis as well as the report of Bieri and Tolliver (13). One of the major reasons for this lack of detectable levels of oxidation products from T could be regeneration of T by other antioxidant substances such as ascorbic acid. In 1968, Tappel proposed that vitamin C can regenerate vitamin E from the tocopheroxyl radical (3). Investigations have also been conducted to determine whether vitamin C can have a sparing effect on the utilization of vitamin E. After reviewing several studies, many of which were done in pure chemical media, McCay (14) concluded that the data strongly suggest that vitamin C can regenerate vitamin E even though it is not possible to rule out unequivocally other alternative explanations of all the experimental data.

The experiments reported in this paper clearly demonstrate that oxidants which vary widely in chemical structure such as unsaturated fatty acids, diamide, peroxides such as hydrogen peroxide, tertiary butyl hydroperoxide and hydroperoxy linoleic acid, and superoxide oxidize T present in red cell membrane under the *in vitro* conditions used. As expected, TQ is one of the products of oxidation. Since the oxidation is expected to involve free radicals and might also result in the intermediate formation of tocopheroxyl radical, it is very likely that addition products derived from the tocopheroxyl radical are also being formed. In this study only one oxidation product, TQ, was followed. As described earlier (see Results) oxidation of T induced by arachidonate yields much more TQ than the oxidations by linoleic acid hydroperoxide, hydrogen peroxide, tertiary butyl hydroperoxide or diamide (47% vs less than 13%).

Hydrogen peroxide, tertiary butyl hydroperoxide and diamide oxidized T in red cell membranes (Tables 4 and 6). Vanderpas and Vertongen have reported that when erythrocytes from neonates and adults were incubated with 0.75 to 5.0 mM concentrations of hydrogen peroxide, T in erythrocytes was oxidized and TQ was produced (15). They also demonstrated a similar oxidation of T in red cell ghosts (15). It is important to note that oxidants like tertiary butyl hydroperoxide and diamide are also known to oxidize sulfhydryl groups (16).

In contrast to the polyunsaturated fatty acids, diamide, hydrogen peroxide and tertiary butyl hydroperoxide did not oxidize all of the membrane tocopherol (see Results). It is possible that all of the membrane tocopherol could have been oxidized by the latter three compounds (diamide, hydrogen peroxide and tertiary butyl hydroperoxide) if the incubation time and/or the concentrations of the oxidants had been greatly increased. Furthermore, a labile oxidation product produced by the three compounds may have been reduced back to α-T during saponification in the presence of ascorbic acid. Nevertheless, under the experimental conditions employed, the unsaturated fatty acids did behave differently from diamide, hydrogen peroxide and tertiary butyl hydroperoxide. This observation, as well as the differences in the yield of TQ during the oxidation (see above), suggests that the mechanism of oxidation of membrane T by

polyunsaturated fatty acids is different from that of the other three compounds.

The chemical structure of the fatty acid strongly influences its ability to induce oxidation of membrane T (Table 1). The *cis* polyunsaturates are the most potent fatty acids. Experiments with human platelets also gave very similar results (5). Several factors may contribute to the high oxidizing power of polyunsaturates. The susceptibility of unsaturated fatty acids to undergo oxidation may be an important factor. The size and shape of unsaturated fatty acid molecules could make the polyunsaturates more accessible to the membrane than the rigid, elongated saturated fatty acids. The differences in the critical micellar concentrations between the various fatty acids also may play a role. Recent studies suggest that the unbound fractions of free fatty acids are first associated with the outer layer of the red cell membrane before being translocated across the membrane (17). Therefore, the physical factors controlling the interactions of fatty acids with membranes cited earlier are crucial in understanding the oxidation of membrane tocopherol induced by unsaturated fatty acids. It is also interesting that α-T has been shown to protect the calcium ATPase of the sarcoplasmic reticulum against destabilization during thermal denaturation which was enhanced by unsaturated fatty acids (18). Since the authors did not monitor T concentrations, it is not known whether any T was oxidized during this protective action which they ascribed to stabilization of membranes by T.

Peroxides have been considered to be initiators of oxidation by a number of investigators. The data presented in Table 5 show that linoleic acid hydroperoxide is a very potent oxidizing agent causing oxidation of membrane T. Furthermore, many reports have appeared in the literature suggesting that enzyme activities (especially those involved in the biosynthesis of eicosanoids) are affected by minute traces of peroxides. The work of Lands' group (19) has established that fatty acyl hydroperoxides stimulate cyclooxygenase activity. Hydroperoxyeicosatetraenoic acid was found to stimulate 5-lipoxygenase by Rouser and Samuelsson (20). Conversely, antioxidants inhibited cyclooxygenase activity (19) and α-T inhibited 5-lipoxygenase activity (21). The redox state of membranes which could be modulated by membrane T concentration would therefore be expected to be controlled by changes in concentrations of peroxide intermediates. The converse may also be true. Therefore, as a potent antioxidant, T may be capable of playing a key role in stabilizing the redox state of membranes as well as in controlling the concentration of critical metabolic intermediates such as fatty acyl hydroperoxides.

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Modulation of Eicosanoid Production and Cell-mediated Cytotoxicity by Dietary α-Linolenic Acid in BALB/c Mice[1]

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The effects of dietary α-linolenic acid (18:3n-3) on fatty acid composition, eicosanoid production, and cell-mediated cytotoxic activity of immune cells before and after challenge with virus or poly I-C from BALB/c mice were studied. Weanling BALB/c mice were fed purified diets containing either 10%-by-weight corn oil or linseed oil providing a ratio of 18:3n-3 to 18:2n-6 of 1/32 or 2/1, respectively, for 6–10 weeks. Fatty acid analysis of splenocyte phospholipids showed an appreciable increase in the percentage of n-3, and a decrease in n-6, fatty acids in splenocytes from mice fed the linseed oil diet. Splenocyte prostaglandin E and peritoneal exudate cell leukotriene C production was significantly lower in the linseed oil-fed mice. In general, cell-mediated cytotoxic activity was similar for immune cells from linseed oil and corn oil-fed mice. However, 6 days after the viral challenge, splenocyte cell-mediated cytotoxic activity was significantly higher in linseed oil mice. This higher activity was associated with nonspecific cytotoxicity rather than that of viral-specific cytotoxic T-lymphocytes. Cell yields from the spleen and peritoneum were frequently significantly higher in linseed oil mice. Interactions between dietary 18:3n-3, eicosanoid production, and immune cell proliferation and/or migration are discussed. In summary, feeding mice a diet rich in 18:3n-3 elevates immune cell n-3 fatty acid content, reduces eicosanoid synthesis and, to a limited extent, enhances the cell-mediated cytotoxic response to a viral challenge.
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Lipid modulation of cell-mediated cytotoxic (CMC) activity has been demonstrated by several investigators. Gill and Clark (1) demonstrated that the incorporation of unsaturated fatty acids increased, whereas saturated fatty acids decreased, CMC activity of cytotoxic T lymphocytes (CTL). When Rice *et al.* (2) examined natural killer cell (NK) activity, they found differential effects of unsaturated fatty acid incorporation, with oleic acid (18:1n-9) increasing, and gamma-linolenic acid (18:3n-6) decreasing cytotoxic activity. The addition of exogenous, free 20:4n-6 (3) or 20:5n-3 (4) has also been shown to inhibit NK activity in a dose-dependent manner. Fewer studies have involved the effect of dietary fat. The addition of fat to low-fat diets decreases lymphocyte-mediated cytolytic activity (5,6). Polyunsaturated fats are more effective at decreasing CMC activity than saturated fats (5).

Prostaglandin (PG) E_2 has been shown to suppress the cytotoxic activity of NK (7), macrophages (8) and CTL (9). Feeding a diet rich in 18:3n-3 reduces the prostaglandin synthesizing capacity of various immunologically important tissues, including the liver, spleen, thymus (10), peripheral blood lymphocytes (11) and peritoneal macrophages (12). This occurs because of reduced levels of 20:4n-6 in the tissues, and elevated n-3 fatty acids, particularly 20:5n-3 and 22:6n-3, which directly compete for the enzymes in the eicosanoid synthesizing pathways. Magrum and Johnston (13) demonstrated that arginase activity of rat peritoneal macrophages could be altered by dietary 18:3n-3. It has been proposed that arginase activity is involved in macrophage-mediated cytolytic and cytostatic functions against tumor cells (14). Therefore, it was of interest to examine the possible influence of dietary 18:3n-3 on CMC activity of various immune cells.

MATERIALS AND METHODS

Animals and diets. Weanling BALB/c mice (Harlan Industries, Indianapolis, IN) were used in all studies. Animals were housed in polypropylene cages (4–5) per cage) with Beta-chip hardwood bedding (Northeastern Products Corp., Warrensburg, NY). A diurnal light cycle of 12 hours was maintained. Upon receipt, mice were fed one of two purified diets and provided with tap water *ad libitum.* Fresh diet was provided every other day with any remaining diet being discarded. The diets were formulated according to AIN-76 guidelines (15). Diets differed only in the source of fat, one containing 10%-by-weight corn oil (CO) and the other, 10% linseed oil (LO), providing ratios of 18:3n-3 to 18:2n-6 of 1:32 and 2:1, respectively. The composition of the diets is shown in Table 1. Autoxidation of diets was prevented by the addition of a synthetic antioxidant (0.01% butylated hydroxytoluene) to the oils upon receipt.

Collection of immune cells. Peritoneal exudate cells (PEC) were collected by flushing the peritoneum with 10 ml, followed by 7 ml of ice-cold phosphate-buffered saline (PBS), pH 7.4. Cell suspensions were pelleted (450 × g, 10 min at 4°C), then resuspended in 1 ml of complete Eagle's Minimal Essential Medium (cMEM) which contains nonessential amino acids, 25 mM HEPES (Irving Scientific, Santa Ana, CA), supplemented with 0.292 g/l L-glutamine, 2.2 g/l sodium bicharbonate, 1% antibiotic-antimycotic solution (Gibco Laboratories, Grand Island, NY) and kept on ice.

Single cell suspensions of splenocytes were made by forcing each spleen through a tissue sieve (Bellco Glass Inc., Vineland, NJ) equipped with an 80-mesh stainless steel screen. Using a 10-ml syringe without a needle, cell clumps were dispersed by several gentle washings through the sieve. RBC and dead cells were removed by centrifugation of the spleen cell suspension over

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Abbreviations: CMC, cell-mediated cytotoxic; cMEM, complete Eagle's Minimal Essential Medium; CO, corn oil; CTL, cytotoxic T lymphocytes; LO, linseed oil; LT, leukotriene; NK, natural killer cell; PBS, phosphate-buffered saline; PC, splenocyte choline phosphoglyceride; PE, ethanolamine phosphoglyceride; PEC, peritoneal exudate cells; PG, prostaglandin; CRD, completely randomized design; LSD, Fischer's Least Significant Difference; SAS, Statistical Analysis System; PFU, plaque-forming units.

TABLE 1

Composition of Purified Diets

Ingredients	% by weight (g/100 g) CO	LO
Corn oil[a,c]	*10.0*	—
Linseed oil[b,c]	—	*10.0*
Casein[d]	20.0	20.0
D,L-methionine[e]	0.3	0.3
Cornstarch[f]	30.5	30.5
Dextrose[g]	29.6	29.6
Fiber (α-cellulose)[h]	5.0	5.0
Mineral mix (AIN-76)[i]	3.5	3.5
Vitamin mix (AIN-76)[i]	1.0	1.0
Choline chloride[e]	0.1	0.1

[a] Fatty acid composition (% of total fatty acids): 16:0, 10.3%; 18:0, 1.5%; 18:1n-9, 26%; 18:2n-6, 60.5%; 18:3n-3, 1.2%. Courtesy of Best Foods, CPC International, Inc., Englewoods Cliffs, NJ.

[b] Fatty acid composition (% of total fatty acids): 16:0, 5.7%; 18:0, 3.1%; 18:1n-9, 20.5%; 18:2n-6, 17.9%; 18:3n-3, 49.7%. Expeller oil fraction courtesy of Cargill, Inc., Minneapolis, MN.

[c] Both oils were supplemented with 0.01% butylated hydroxytoluene and 0.735% dl-α-tocopherol acetate.

[d] Shamrock Brand, Erie Casein Co., Erie, IL.

[e] Sigma Chemical Co., St. Louis, MO.

[f] A.E. Staley Co., Decatur, IL.

[g] Staleydex 333, A.E. Staley Co., Decatur, IL.

[h] Solka-Floc, James River Corp., Berlin, NH.

[i] ICN Nutritional Biochemicals, Cleveland, OH.

Lymphocyte Separation Medium, density 1.077–1.080 at 20°C, as described by the manufacturer (Litton Bionetics Inc., Charlestown, SC). Cells at the interface were collected, washed twice and resuspended in 2 ml cMEM.

Splenocytes and PEC were enumerated using a Coulter Counter, Model ZBI (Coulter Electronics, Inc., Hialeah, FL). Cell viability was determined, using the trypan blue exclusion test and cell viability was generally greater than 95%. Cell suspensions were diluted to a final concentration of 1×10^7 cells/ml, or pelleted and stored (−80°C) for lipid analysis.

Fatty acid analysis of splenocyte phosphoglycerides. Splenocytes previously collected were resuspended in 3 ml ice-cold 0.1 mM EDTA. Lipids were extracted with 18 ml chloroform and methanol (2:1, v/v) containing 0.005% BHT. The organic phase containing the lipid extract was collected and reduced in volume under N_2.

Splenocyte phosphoglycerides were separated by two-dimensional thin layer chromatography (16). Phospholipids were identified by comparison to chromatograms of authentic standards (Supelco, Inc., Bellefonte, PA and Applied Science Laboratories, State College, PA). Methyl esters of fatty acids were prepared by transmethylation using 4% H_2SO_4 in methanol. Fatty acid methyl esters of splenocyte choline (PC) and ethanolamine (PE) phosphoglycerides were identified using a Packard gas-liquid chromatograph, Model 428 (Packard Instrument, Co., Downers Grove, IL) with a 180 × 0.4 cm glass column packed with 10% SP 2330 on 100/120 Chromosorb W AW 1-1851 (Supelco, Inc., Bellefonte, PA) operated isothermally at 190°C. Results, expressed as percentage of total fatty acids, were determined using a Hewlett-Packard 3380A integrator (Sunnyvale, CA).

Determination of PG synthesizing capacity of immune cells. One-half ml of immune cells, isolated as previously described, was added to one-half ml of serum-free MEM containing 10 μg/ml (19 μM) calcium ionophore, A23187 (Sigma Chemical Co., St. Louis, MO). After 1 hr at 37°C, the incubation was terminated by the addition of 0.025 ml of an aspirin solution (100 mg/ml of dimethylsulfoxide). Cell-free medium was collected and stored at −80°C. Prostaglandins were determined by radioimmunoassay using rabbit antiprostaglandin E-BSA serum (Miles Laboratories, Elkhart, IN). This antiserum is reported by the manufacturer to have minimal cross-reactivity (less than 3%) with PGA_1, A_2, F_1, B_1 and B_2, 10% with F_2 and 53% cross reactivity with PGE_1. Cross reactivity with PGE_3 was undetermined. No attempt was made to correct for cross reactivity, therefore results are expressed as picograms of PGE/ml culture medium.

Determination of leukotriene (LT) production by PEC. For LT production, half of the mice on each diet treatment received intraperitoneal (i.p.) injections of brewer's thioglycollate (Difco Laboratories, Detroit, MI), three days prior to PEC collection. One ml of PEC (5×10^6 cells) was cultured in the presence of calcium ionophore at 5 μg/ml for 20 min at 37°C. Cell-free medium was collected immediately by pelleting cell suspensions for 15 sec at 12,000 × *g*. Samples were stored at −80°C, until LT determinations could be conducted. Because LTC_4 is the predominant LT synthesized by mouse peritoneal macrophages, it was determined by radioimmunoassay using a ^{3}H-LTC_4 kit from New England Nuclear (Boston, MA). The antiserum is reported by the manufacturer to have minimal cross-reactivity (<0.006%) with LTB_4, various PG, and free 18:2n-6 and 20:4n-6. Cross-reactivity with LTD_4 and LTE_4 was 55.3% and 2.3%, respectively, whereas cross-reactivity with LTC_5 was not reported. Results are expressed as nanograms of LTC/ml of culture medium.

Cell-mediated cytotoxicity studies. The effect of feeding a diet rich in 18:3n-3 on the CMC activity of cells isolated from the spleen and peritoneal cavity of BALB/c mice with or without an immunochallenge was studied.

Study 1. Spontaneous CMC was determined on 48 male mice which had been fed either the CO or LO diets for 6–7 weeks postweaning. Eight mice per diet-group were sacrificed in each of 3 experiments. Immune cells from the spleen and peritoneum were collected as previously described. Splenocytes from each mouse were tested for NK activity using the YAC-1 tumor cell line in a 4-hr Cr-51 release assay, or for natural cytotoxic cell (NC) activity by using the SV-T2 cell line in a 16-hr Cr-51 release assay. Peritoneal exudate cells (PEC) were pooled from two mice from the same diet treatment and tested for CMC activity, using YAC-1 as targets in a 16-hr Cr-51 release assay.

Study 2. Forty male mice fed CO or LO diets for 8–10 weeks postweaning were used to assess CMC 3 days postviral challenge. Mice were injected i.p. with 5×10^6 PFU of vaccinia virus in 0.5 ml of cMEM. Experiments were designed such that NK and NC activity in the spleen, and CMC against SV-T2 by PEC could be measured in each of the 4 mice per diet treatment per experiment.

Study 3. Twenty-four female mice fed a CO or LO diet for 8–10 weeks postweaning were used to determine CMC 6 days postviral challenge. Antigen-specific CMC (i.e., cytotoxic T lymphocyte activity) was demonstrated by

infecting the syngeneic fibroblast cell line (3T3) with vaccinia virus, just prior to using these cells as targets in a 16-hr Cr-51 release assay. Noninfected 3T3 and the simian virus 40-transformed cell line (SV-T2) were used as control targets in parallel assays to assess nonspecific CMC activity.

Study 4. The CMC response to a poly I-C (synthetic double-stranded RNA) immunochallenge was determined for 60 mice fed the CO and LO diets for 8–10 weeks. In each of the first two experiments, CMC activity of 8 mice per diet was tested 24 hr after mice received an i.p. injection of either 100 μg of poly I-C (P-L Biochemicals, Inc., Milwaukee, WI) in 0.5 ml of sterile saline or a sham injection (0.5 ml sterile saline). In the third experiment, mice were tested for splenic NK activity 2 and 6 days after poly I-C administration, and 2 days after sham injections.

Study 5. CMC activity 3, 6, and 9 days after an immunochallenge with two suboptimal doses of vaccinia virus in 160 female mice fed a corn oil (CO)- or linseed oil (LO)-containing diet for 6–10 weeks postweaning was determined. For each experiment 6 mice per diet received i.p. injections of vaccinia virus. Half were injected with 10^4 PFU of virus and the other half with 10^5 PFU. Immune cells were collected after either 3, 6 or 9 days postpinjection (p.i.) as previously described. Splenocytes from each mouse were tested for NK, NC and CTL activity. Cytotoxic activity of peritoneal exudate cells against SV-T2 target cells was also determined.

Cultures and virus. All cell lines were purchased from American Type Culture Collections (Rockville, MD) and maintained *in vitro* in cMEM supplemented with 10% Nu-serum, a serum substitute (Collaborative Research Inc., Lexington, MA). The virus used in this study was the WR strain of vaccinia which was originally obtained from Dr. W.A.F. Tompkins, University of Illinois (Urbana, IL). Virus was grown in VERO (African green monkey kidney) cells and titered to 1×10^7 PFU on VERO monolayers as described by Rawls *et al.* (17). Virus was dispensed in 1 ml aliquots and stored at −80°C.

Cell-mediated cytotoxicity assays. CMC activity by PEC and splenocytes was determined as described by Tompkins *et al.* (18) using a modified Cr-51 release method as originally described by Brunner *et al.* (19). In order to distinguish between NK and NC cell activity in each spleen, aliquots of splenocyte cell suspensions were tested for cytotoxic activity against two different tumor cell lines. NK activity was determined using the NK-sensitive mouse lymphoma cell line YAC-1 (TIB 160) in a 4 hr Cr-51 release assay. Assessment of NC activity in the spleen and CMC activity by peritoneal exudate cells (PEC) was conducted using the virally-transformed fibroblast cell line, SV-T2 (CCL 163.1) in a 16-hr Cr-51 release assay.

The syngeneic fibroblast cell line 3T3 (CCL 163) was used in a 16-hr Cr-51 release assay to assess vaccinia virus-specific CMC activity of splenocytes. Viral infection of target cells with vaccinia was carried out in the wells during a preincubation period, in which adherence, Cr-51 uptake and expression of viral antigens occurred (20). Viral infection of targets did not alter spontaneous release of Cr-51.

Harvesting assays. At the end of the incubation period, 75 μl of cell-free medium was removed from each well, mixed with 0.6 ml of an aqueous scintillation fluid and counted in a Beckman 2800 Liquid Scintillation Counter. Spontaneous release was determined in control wells to which 0.2 ml of cMEM with 10% Nu-serum was added to targets prior to the incubation period. Maximum release was determined by 3 freeze-thaw cycles of half of the control wells. All systems were performed in triplicate. Results of cytotoxicity assays were expressed as % specific Cr-51 released and determined as follows: [(DPMs in test wells − DPMs in spontaneous release wells)/(DPMs in maximum release wells − DPMs in spontaneous release wells)] × 100. In the text and tables, percentage specific Cr-51 released is referred to as % cytotoxicity.

Statistical analysis. Data were analyzed using Statistical Analysis System (SAS). Computer programs PROC ANOVA (for balanced data) and PROC GLM (for unbalanced data) were utilized (21). Differences between diet treatment groups and virus dosage levels were tested with single degree of freedom comparisons for statistical significance by F-test. A completely randomized design (CRD) was used for the poly I-C and the time course studies, in which the treatments were arranged as a $2 \times 2 \times 3$ factorial. In the poly I-C study diet treatment (i.e., CO vs LO), immunochallenge (i.e., sham vs poly I-C) and time post-challenge (1, 2 or 6 days) were factors A, B and C, respectively. In the time course study (Study 5) factor A was diet treatment (i.e., CO vs LO fed mice) and factor B was virus dosage (i.e., 10^4 vs 10^5 PFU) and factor C was days postchallenge mice were sacrificed (i.e., 3, 6 and 9 days). Comparisons over time and within diet treatment groups were made using Fischer's Least Significant Difference (LSD).

RESULTS

Fatty acid composition. There was a significant decrease in all n-6 fatty acids, except 18:2n-6, and an increase in all n-3 fatty acids of splenocyte phospholipids when mice were fed the α-linolenic acid-rich LO diet compared to the linoleic acid-rich CO diet. In PC, the percentage of n-3 fatty acids increased from 3.3 to 15.2% and in PE, from 7.6 to 30.0%. The n-6/n-3 ratios in these phospholipids changed from 12.1 to 1.8 in PC and 7.3 to 1.0 in PE in the CO vs LO mice, respectively.

There were marked reductions in the 20:4n-6 content of both phospholipid classes when mice were fed the LO diet compared with the CO diet. In PC, 20:4n-6 content was 64% lower (20.7% vs 7.5%), whereas in PE there was a 62% reduction (31.7 vs 11.9%) for CO vs LO mice, respectively. The content of 20:5n-3 increased significantly ($P < 0.01$) upon LO feeding and represented 5.4% of the total fatty acids in PC compared with 1.3% when CO was fed. In PE the difference in 20:5n-3 content was more marked than those seen in PC (8.3% compared with 0.9% for LO vs CO, respectively). Although 18:2n-6 content tended to be higher in mice fed LO compared with CO, these differences were not statistically significant in either PC or PE phosphoglyceride. Interestingly, the opposite trend was seen when 18:2n-6 content of splenocyte total lipids was analyzed.

Eicosanoid production. Eight weeks after the initiation of diet treatments, PGE synthesis by splenocytes was 70% lower in LO-fed compared with CO-fed mice (Fig. 1). The PG synthesizing capacity of PEC were similarly effected by dietary 18:3n-3 (data not shown). Feeding mice

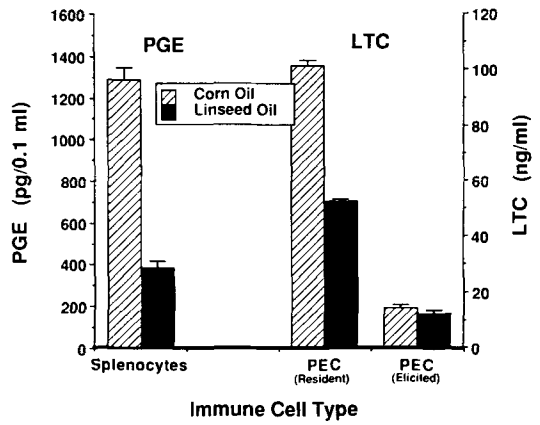

FIG. 1. Effect of high dietary 18:3n-3 intake on PGE and LTC production by immune cells from the spleen and peritoneum. Female BALB/c mice were fed diets containing 10%-by-weight corn oil (low in 18:3n-3) or linseed oil (high in 18:3n-3) for 8–10 weeks prior to the collection and testing of immune cells. Splenocytes and PEC were collected and stimulated to produce eicosanoids as described in the text. Data represents the means + S.E.M. for 4–8 samples per dietary treatment.

an 18:3n-3-rich diet for 8 weeks significantly decreased LTC_4 production by PEC. Resident PEC from CO-fed mice produced nearly twice as much LTC_4 as those from LO mice (Fig. 1). Thioglycollate-elicited PEC produced significantly less LTC compared with resident PEC and the apparent inhibition of LTC_4 production by LO feeding was only 14% in the elicited cells.

Body and spleen weight and immune cell yield. In general, there was no significant effect of dietary n-3 fatty acid intake on body weight nor spleen weight, however, spleen weights were significantly increased ($p < 0.0001$) by both viral and poly I-C challenges (data not shown).

Cell yield (i.e., the number of cells obtained) from the spleen and the peritoneum were significantly influenced by dietary 18:3n-3 intake, as well as by viral and poly I-C challenges. Splenocyte yields from virally-challenged LO-fed mice were consistently greater by 10–20% than the mice fed CO. These differences were statistically significant ($p < 0.05$) when data from Studies 2 and 3 were pooled. On average, the number of splenocytes recovered from poly I-C challenged LO-fed mice was 23% greater than that recovered from CO-fed mice. In the time course study (Study 5) splenocyte yield was significantly influenced by diet treatments ($p < 0.005$), virus dosage ($p < 0.05$) and time post-challenge ($p < 0.001$), with no significant interactions between these factors. Splenocyte yield from LO-fed mice was on the average 24% higher than that from CO-fed mice. For example, mice challenged with 10^4 PFU showed splenocyte yields of 22.6 ± 2.4 vs 24.3 ± 2.7, 24.9 ± 2.1 vs 31.0 ± 2.7, 28.8 ± 2.5 vs 35.8 ± 3.8 ($\times 10^6$ cells) at 3, 6 and 9 days postchallenge for CO vs LO-fed mice, respectively. Those mice challenged with 10^5 PFU showed a similar trend (data not shown).

The influence of diet treatment on PEC yield was variable. In unchallenged mice, LO feeding enhanced (Study 1) and had no effect (Study 4) on the PEC yield compared with CO feeding. PEC yield was significantly lower ($p < 0.05$) in LO vs CO mice at 6 days p.i. of 10^5 PFU (10.9 ± 1 vs $14.7 \pm 1.3 \times 10^6$ cells for LO vs CO mice, respectively). The reduction in PEC after poly I-C injections was significantly greater for LO-fed compared with CO-fed mice, however later recovery was not effected by the diets.

CMC activity. Summarized in Table 2 is the CMC activity in the spleen and peritoneum of unchallenged and virally challenged mice fed CO and LO diets. CMC activity by splenocytes and PEC was greatly elevated 3 days after the viral challenge compared with unchallenged mice. Both CO and LO-fed mice expressed similar levels of CMC activity at these times. CMC activity by splenocytes and PEC was also significantly stimulated by poly I-C injections (Study 3). However, again there were no significant differences between the responses of CO and LO-fed mice (data not shown).

Summarized in Figure 2 are the results of three experiments, where the CMC activity in the spleen was assessed 6 days after viral challenge in mice fed a CO or LO diet (Study 4). CMC activity against vaccinia virus-infected 3T3 cells was significantly higher ($p < 0.005$) in LO compared with CO-fed mice. Background cytotoxicity against noninfected 3T3 and SV-T2 cells was considerably lower compared with vaccina virus-infected 3T3 cells. CMC activity against these other targets was significantly higher by splenocytes from LO-fed compared with CO-fed mice.

Shown in Figures 3 and 4 are some of the results from Study 5, where CMC activity was assessed over time after two suboptimal doses of vaccinia virus challenge. NK activity (Fig. 3) was highest 3 days postchallenge, and declined significantly through days 6 and 9 p.i., whereas a similar response pattern was observed for NC activity in the spleen (data not shown). Activity in LO-fed mice appeared to decline more slowly than in CO-fed mice, with the difference in NK and NC activity being most pronounced on day 6 postchallenge. CMC activity by PEC

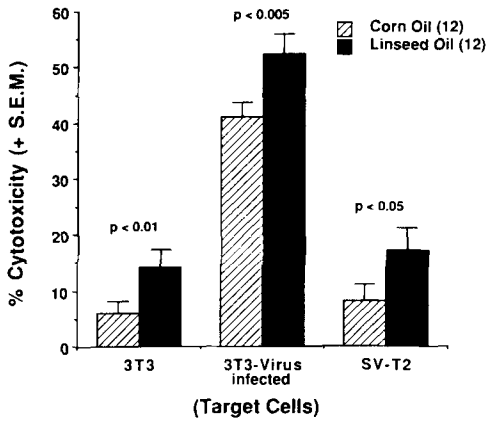

FIG. 2. Effect of high dietary 18:3n-3 intake on splenocyte anti-viral CMC activity, 6 days after a viral challenge. Female BALB/c mice were fed diets containing 10%-by-weight corn oil (low in 18:3n-3) or linseed oil (high in 18:3n-3) for 8–10 weeks prior to receiving i.p. injections of 10^6 PFU of vaccinia virus. In three separate experiments, 6 days postviral challenge, splenocytes from 4 mice per diet treatment were isolated and tested for CMC activity in a 16 hr Cr-51 release assay as described in the Methods section. A syngeneic fibroblast cell line (3T3) was infected with vaccinia virus and used as target cells in the CMC assay, whereas noninfected 3T3 and SV-T2, a virally-transformed cell line, served as controls. Effector-to-target cell ratios were 50:1 in all experiments.

TABLE 2

Cell-Mediated Cytotoxic Activity of BALB/c Mice Fed a Corn Oil (CO) or Linseed Oil (LO)-Containing Diet[a]

Dietary fat	E:T[b]	N[c]	No challenge		With challenge[d]	
			CO	LO	CO	LO
Splenocyte NK	100:1	12	5.0 ± 0.8[e]	5.4 ± 1.7	37.6 ± 5	35.7 ± 5
	50:1	20(16)	2.6 ± 0.4	3.2 ± 0.4	27.6 ± 5	23.2 ± 5
	25:1	16	nd[f]	nd	14.6 ± 3	14.2 ± 3
Splenocyte NC	100:1	4(12)	7.2 ± 1.4	4.6 ± 1.5	27.9 ± 5	27.9 ± 5
	50:1	4(12)	3.4 ± 2.0	2.0 ± 2.4	14.8 ± 5	16.7 ± 5
	25:1	12	nd	nd	5.9 ± 5	7.0 ± 5
Peritoneal exudate cells	100:1	8	nd	nd	57.1 ± 5	62.8 ± 6
	50:1	8(12)	12.4 ± 1.1	12.6 ± 1.5	46.6 ± 14	45.9 ± 7
	25:1	12	nd	nd	32.7 ± 10	37.7 ± 11

[a]Mice were fed diets for 8–10 weeks postweaning prior to testing CMC activity.
[b]Effector-to-target cell ratio.
[c]Sample number per dietary treatment (number in parentheses is equal to "N" in the challenge studies when different from nonchallenged).
[d]Three days after i.p. injection of ca. 10^6 PFU of vaccinia virus.
[e]% Cytotoxicity expressed as mean ± S.E.M.
[f]Not determined.

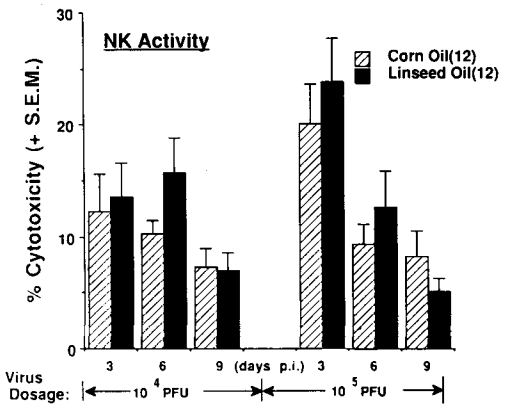

FIG. 3. Effect of high dietary 18:3n-3 intake on NK activity at various times after a viral challenge. Female BALB/c mice were fed diets containing 10%-by-weight corn oil (low in 18:3n-3) or linseed oil (high in 18:3n-3) for 8–10 weeks prior to receiving i.p. injections of 10^4 or 10^5 PFU of vaccinia virus. For each experiment, splenocytes were isolated from three mice per level of viral challenge, per diet treatment and tested for CMC activity either 3, 6 or 9 days postchallenge. NK activity was determined using YAC-1 lymphoma cells as targets in a 4 hr Cr-51 release assay as described in the Methods section. An effector-to-target cell ratio of 50:1 was used in all experiments. Results from 3–4 experiments per day are presented. Spontaneous release of the Cr-51 label averaged 8.6 ± 0.6% in the NK assays.

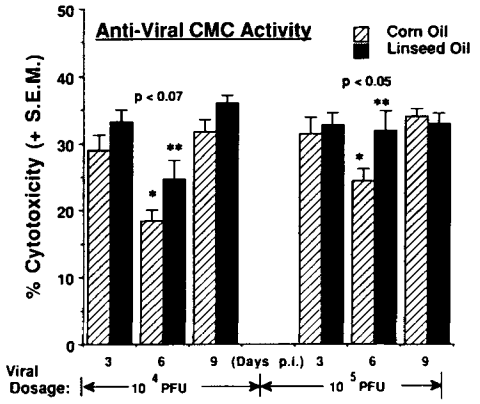

FIG. 4. Effect of high dietary 18:3n-3 intake on antiviral CMC activity at various times after a viral challenge. The same protocol outlined for Fig. 2 was followed, only target cells used to assess CMC activity differed. Antiviral CMC activity was determined using 3T3 cells, which had been infected with vaccinia virus 5–7 hours prior to use, in a 16 hr Cr-51 release assay. Spontaneous release from these cells averaged 1.6 ± 0.3%/hr. Noninfected 3T3 cells served as controls and CMC activity against these cells ranged from 1–8% (data not shown).

PEC remained elevated throughout the study period with mice fed CO and LO diets expressing similar activities (36.4 ± 4.5 vs 42.6 ± 3.9, 37.2 ± 5.1 vs 35.9 ± 4.8, 32.9 ± 5.3 vs 35.9 ± 6.5 for CO vs LO mice at 3, 6, and 9 days postchallenge with 10^5 virus, respectively). At the lower viral dose (i.e., 10^4 PFU), activity appeared to be declining by day 9 postchallenge in both the CO-fed and LO-fed mice (data not shown).

Figure 4 shows the CMC activity of splenocytes against vaccinia virus-infected 3T3 cells. Cytotoxic activity against the noninfected cells was low and ranged 2–8%. CMC activity against vaccinia virus-infected targets (3T3) was significantly greater ($p < 0.05$) on day 6 in LO vs CO-fed mice. At the lower viral dose, CMC activity was high 3 day p.i., declined on day 6, then rose to its highest level on day 9. The CMC activity was consistently higher in mice fed LO compared with CO diets. These differences between the diet groups were significant ($p < 0.005$) for days 6 and 9 together, and nearly so, individually ($p < 0.07$).

DISCUSSION

Feeding mice a diet rich in 18:3n-3 dramatically altered membrane fatty acid profiles and PG synthesizing capacity of immune cells. Similar observations have been made

and reported for various rat tissues (22-24). In the mouse, suppression of 20:4n-6 and the elevation of 20:5n-3 levels in splenocyte phospholipid fractions exceeded those achieved in rats (10), which may in part reflect the relatively greater activity of liver desaturase enzymes in mice compared with rats (25,26).

The magnitude of the reduction in LT biosynthesis by feeding an 18:3n-3 rich diet depended on the prior conditioning of the PEC. Resident PEC from LO-fed mice produce less than 50% the LTC_4 compared with CO-fed mice, whereas there was only a 14% difference in thioglycollate-elicited PEC. The observation that resident PEC produced significantly greater quantities of LT than elicited PEC, is in agreement with Humes *et al.* (27). They showed that PEC isolated after an acute bacterial infection, produced significantly less PGE_2, PGI_2 and LTC_4 compared with resident PEC. Tripp *et al.* (28) later demonstrated that the alteration in eicosanoid production was due to the migration of monocytes into the peritoneum.

Our studies demonstrate that feeding mice a diet rich in 18:3n-3 can significantly enhanced CMC activity. The effects on CMC were less dramatic than the changes in fatty acid composition, PGE and LT biosynthesis and were observed under limited circumstances. Dietary influences over CMC activity were only seen at certain times after a specific immunochallenge. Enhancement was not observed in unchallenged mice. Others have reported that alterations in endogenous PG production did not significantly alter "natural" NK activity in the spleen (29). Voth *et al.* (30) demonstrated that relatively large doses (200 μg) of the PG synthesis inhibitor, indomethacin, were required to significantly enhance CMC activity of NK or peritoneal macrophages in the absence of an immunochallange. In fact, the levels of indomethacin that were effective (200-400 μg) were very near levels that proved to be lethal (800 μg). This drug-induced enhancement in CMC activity is transient, peaking 3 days p.i., and lasts a total of 6 days. Weaker PG synthesis inhibitors were less effective, as were lower levels of indomethacin. Thus, our dietary data supports the concept that PG do not play an important role regulating basal CMC activity.

In our studies, higher NK/NC activity in the LO-fed mice accounted for some, but not all, of the higher CMC activity against vaccinia virus-infected targets at the later time points (i.e., 6 and 9 days postchallenge). This does imply that PG do act as natural feedback inhibitors of "stimulated" CMC activity, as suggested by Tracey and Adkinson (29). We believe that the reduced PGE synthesizing capacity of LO-fed mice, resulted in a slower or less complete shut-down of the nonspecific phase of the antiviral response compared with the CO-fed mice. We observed that vaccinia virus-specific CMC activity also tended to be higher in LO-fed mice. This is in agreement with the reported inhibitory action of PG on CTL generation (9,35). Lowering the concentration of the viral challenge by 10- and 100-fold, did not accentuate the diet-induced differences in the CMC response. Instead, at the lower viral concentration, stimulation of splenic NK activity was marginal. At both concentrations, the induction of antiviral CMC activity was delayed, compared with our earlier studies and the reports of others (36,37).

Two different immunochallenges were used to stimulate CMC activity above basal levels: live vaccinia virus and a synthetic double-stranded RNA (poly I-C). Although the CMC response to vaccinia virus is well defined, it is slow to develop and involves complex interactions between numerous cell types (31). Poly I-C has been shown to quickly (i.e., within hours) stimulate CMC activity by peritoneal macrophages (32) and spleen NK cells (33). We hoped that this alternate immunochallenge would avoid some of the possible confounding variables in our studies using a live virus. Both challenges share a common pathway for the stimulation of the immune system, that is, an elevation in interferon release and production. That others (34) have shown that PG do not affect interferon production may explain why we observed no differences between CO and LO-fed mice in the early stages of the CMC response to vaccinia virus or poly I-C.

Cell yields from the spleen of immunochallenged LO-fed mice were consistently greater than from CO-fed mice. This is consistent with evidence suggesting that PGE suppresses lymphocyte proliferation, and that reductions in PGE production results in enhanced T-cell proliferation (38). To the contrary, previous findings in our laboratory suggest that mitogen-induced lympho-proliferation is not regulated by PGE (39). An alternative explanation may be that dietary 18:3n-3 influenced the recruitment or migration of immune cells. Although LTB_4 production was not measured, it is probable that like LTC_4, it was also reduced in LO-fed mice. Because LTB_4 is a powerful chemotactic eicosanoid, such a reduction could have altered cell migration to or from the peritoneum and spleen. Koga *et al.* (40) showed that inhibition of PG synthesis by indomethacin resulted in an increase in T-lymphocytes in the spleen. This effect was dependent on the thymus, and appears to be the result of an elevated migration of lymphocytes from the thymus to the spleen (41).

PG also appear to modulate cell movement through the peritoneum. Voth *et al.* (30) recovered 2-fold greater numbers of PEC from indomethacin-treated mice. The 32% greater PEC recovered from the unchallenged LO-fed compared with CO-fed mice may also have been a result of the reduced PGE_2 synthesizing capacity of the LO mice. Moore *et al.* (42) demonstrated that endogenous PGE_2 inhibit macrophage proliferation and proposed that PGE_2 is the natural feedback regulator of this process.

Although the Cr-51 release assay is the most commonly used method for assessing CMC activity, it, like most other in vitro assays, has shortcomings (43). One of those is that the effector cell concentrations across treatment groups are equalized to keep E:T ratios the same. Therefore, the present results do not completely reflect the effect that dietary 18:3n-3 might have on disease resistance as it relates to *in vivo* CMC activity in the whole animal. We have shown that the number of effector cells as well as their relative activity appears to be enhanced by dietary 18:3n-3. This suggests the possibility that even greater differences in disease resistance may exist between CO-fed and LO-fed mice. Further studies using various viral, bacterial and parasitic challenges will help determine if this is the case.

ACKNOWLEDGMENTS

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Synergistic Enhancement of the Antiproliferative Activity of *cis*-Diamminedichloroplatinum(II) by the Ether Lipid Analogue BM41440, an Inhibitor of Protein Kinase C

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The new phospholipid analogue 3-hexadecylmercapto-2-methoxy-methyl-propyl-1-phosphocholine inhibits the phospholipid-calcium-dependent protein kinase, partially purified from Walker carcinoma cells with a K_i value of 0.56 μM. The compound inhibits the phorbol ester stimulated phosphorylation of the ribosomal protein S6 indicating that the depression of Ca^{2+}-phospholipid-dependent protein kinase by the alkyl phospholipid also occurs in intact cells. The dose effect curve for the inhibition of cell proliferation by 3-hexadecylmercapto-2-methoxy-methyl-propyl-1-phosphocholine in Walker cells exhibits a close correlation to the dose effect curve for the depression of Ca^{2+}-phospholipid-dependent protein kinase activity. Although alternative mechanisms cannot be excluded, the data suggest that the growth inhibitory activity of 3-hexadecylmercapto-2-methoxy-methyl-propyl-1-phosphocholine correlates with the inhibition of Ca^{2+}-phospholipid-dependent protein kinase. The antiproliferative activity of 3-hexadecylmercapto-2-methoxy-methyl-propyl-1-phosphocholine is synergistically enhanced by *cis*-diamminedichloroplatinum(II).

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Alkyl-lysophospholipids (ALP), a new class of antitumor drugs, represent synthetic 1-*O*-alkyl analogues of 2-lysophosphatidylcholine. ALP have been reported to inhibit the growth of a broad variety of malignant cells in culture (1-4). ALP also prevent tumor growth or inhibit metastases *in vivo* (5, 6). In contrast to the effect on malignant cells, human embryonic fibroblasts (2) and bone marrow cells (7) were found to be less affected by doses of ALP that are toxic to malignant cells.

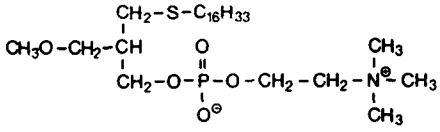
The sensitivity of malignant cells to ALP has been discussed as resulting from the inability to metabolize the drug due to the lack of 1-*O*-alkyl cleaving enzymes (8). But, despite efforts by several groups, the mechanism underlying the cytotoxic activity of ALP has not yet been elucidated (9).

Ca^{2+}-phospholipid-dependent protein kinase (protein kinase C) represents an essential element in signal transduction of a variety of growth factors and mitogens (10). It has been demonstrated, that the ALP 1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine and lysosphingolipids inhibit protein kinase C activity (11, 12) and this effect has been discussed as the possible mechanism that is responsible for the antitumor activity of these agents.

Recently the new alkyllysophospholipid analogue BM41440 has been synthesized which differs from the alkyllysophospholipids available so far (Fig. 1): The long chain alkyl group is linked by a thioether bond and the β-hydroxyl group of the glycerol has been substituted by a methoxymethyl moiety.

BM41440 is one of the most potent cytotoxic phospholipid analogues (13-15). Compared with the *O*-alkylglycerophospholipids studied so far, this molecule shows little resemblance to biological lyso-or alkylphospholipids and should, therefore, exhibit a different behavior in biological systems. Indeed, it has been demonstrated that the introduction of the thioether bond drastically reduces the platelet aggregating (PAF-like) activity, compared with corresponding *O*-alkyl derivatives (16). This is explained by the low affinity of the thioether compound to the PAF receptor, if compared with *O*-alkyl derivatives (17). The substitution of glycerol by a 2-alkyl propanol should have additional biological consequences. The enzymatic formation of alkylacyl- or dialkyl glycerols has been shown to contribute to the biological effects of alkylphospholipids (18). It is conceivable, therefore, that the lack of a glycerol moiety in BM41440 affects its biological properties. Thus, the effects of the new alkylphospholipid BM41440 on protein kinase C may differ from those of the *O*-alkylglycerophospholipids investigated so far. Furthermore, it is still unclear whether the inhibition of protein kinase C which has been observed with alkylphospholipids is related to the growth inhibitory effect of these compounds. It is shown here that BM41440 is a particularly potent inhibitor of protein kinase C and that the dose effect curves for the depression of protein kinase C and the inhibition of cellular replication are superimposable, suggesting a close correlation between these two parameters.

In a preceding publication it has been demonstrated that inhibitors of protein kinase C synergistically en-



BM41440

3-Hexadecyl-mercapto-2-methoxymethyl-propyl-1-phosphocholine

FIG. 1. Structure of BM41440.

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Abbreviations: ALP, alkyl-lysophospholipid; BM41440, 3-hexadecylmercapto-2-methoxy-methyl-propyl-1-phosphocholine; *cis*-DDP, *cis*-diamminedichloroplatinum(II); MOPS, 3-N-morpholino propanesulfonic acid; protein kinase C, Ca^{2+}-phospholipid-dependent protein kinase; TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate; Tris-HCl, Tris(hydroxymethyl)-aminomethane; CI, combination index; DMEM, Dulbecco's modified minimal essential medium.

hance the antiproliferative effect of *cis*-platinum (19). It seemed interesting, therefore, to investigate whether BM41440 exhibits a similar behavior in combination with platinum complexes.

MATERIALS AND METHODS

Chemicals. BM41440, horse serum and Dulbecco's modified minimal essential medium (DMEM) were from Boehringer Mannheim (Mannheim, FRG) and *cis*-diamminedichloroplatinum(II) (*cis*-DDP) was donated by Homburg Pharma (Frankfurt, FRG). [γ^{32}P]-ATP (10 Ci/mmol) and ^{32}P-orthophosphate were from the Radiochemical Centre (Amersham, UK). GF/F-filters and DEAE-52 cellulose were from Whatman (Clifton, NJ). Leupeptin, aprotinin, 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA), β-glycerophosphate, histone H1 (type III S), 3-N-morpholino propanesulfonic acid (MOPS) L-α-phosphatidyl-L-serine and 1,2-*sn*-diolein were obtained from Sigma (Munich, FRG). Tris(hydroxymethyl)-aminomethane (Tris-HCl), EGTA and Triton X-100 were purchased from Serva (Heidelberg, FRG).

Cell culture and evaluation of combined drug effects. Walker rat carcinoma cells were kindly donated by Dr. J.J. Roberts, Institute of Cancer Research, Royal Cancer Hospital, Sutton, Surrey, UK, and grown in suspension at 36.8°C in DMEM supplemented with 10% horse serum and buffered with 25 mM MOPS (pH 7.35 at 20°C). Dose-response curves were established with BM41440 by addition of the drug dissolved in 20mM Tris-HCl, pH 7.4, to Walker cells (10^5 cells/ml). After incubation for 48 hours, cells were counted by an electronic counter (Coulter-Electronics, Luton, UK). Cellular multiplication (M) was calculated by $M = T_t\text{-}T_o / C_t\text{-}C_o \times 100$, where C are untreated controls, T are drug treated cells, o and t equal the number of cells at time O and t (48 hrs), respectively. Evaluation of the effects of drug combinations were performed as described by Chou and Talalay (20, 21) employing data from at least 3 different experiments. Computation was performed employing the computer program (Apple II) by J. Chou and T.-Ch. Chou, *Dose effect analysis with microcomputers* (Elsevier Biosoftware, Cambridge, UK).

Protein kinase C. Protein kinase C was purified 31-fold from Walker cells with a yield of ca. 80% employing a DEAE-52 cellulose chromatography of cell extracts as described by Kreutter et al. (22). Protein kinase C activity was determined according to Regazzi et al. (23) by measuring [^{32}P]orthophosphate incorporation into histone H1. Briefly, the reaction mixture (125 μl) contained 0.5 μCi [γ^{32}P]-ATP, 40 mM Tris-HCl, pH 7.4, 1 mM $CaCl_2$, 700 μM EGTA, 50 μg histone and different concentrations of phosphatidylserine, diolein and BM41440, as described in the corresponding figure legend and ca. 1 μg/20 μl of enzyme protein. Assays were run for 10 minutes at 32°C. The reaction was stopped by addition of 1 ml 20% trichloroacetic acid. The precipitated proteins were collected on GF/F filters and radioactivity counted in a liquid scintillation spectrometer.

Phosphorylation of ribosomal protein S6. Cells were grown in DMEM with 0.5% horse serum for 15 hr. After 1 hr incubation in phosphate free medium [^{32}P]orthophosphate (4 μCi/ml) TPA, (0.5 μM) or BM41440 (20 μM) plus TPA (0.5 μM) was added. After 1.5 hr of incubation, cells were lysed in 50 mM Tris-HCl, pH 7.4, 25 mM KCl, 5 mM $MgCl_2$, 0.33 M saccharose, 1% Triton X-100, 1 mM phenylmethanesulfonylfluoride, 20 μg/ml leupeptin, 2 μg/ml aprotinin and 80 mM β-glycerophosphate. After 10 min at ice temperature the lysate was centrifuged for 3 hours at 100 000 × *g*. The pellet was analyzed by gel electrophoresis in a 15% polyacrylamide gel (24). Ribosomal protein S6 was identified after blotting to nitrocellulose by employing anti-S6 antiserum (kindly donated by Prof. G. Stoeffler, Innsbruck). Cell viability was checked by the trypan blue exclusion assay. Incubation with BM41440 for 1.5 hr did not reduce the number of living cells.

RESULTS

The inhibition by BM41440 of protein kinase C from Walker rat carcinoma cells is shown in Fig. 2. The inhibitory effect is inversely related to the concentration of lipid cofactors (phosphatidylserine/diacylglycerol) in the reaction mixture (Fig. 3). A Lineweaver Burk plot of the data, reveals a competitive type of inhibition in respect to lipid with a K_i of 0.56 μM. Inhibition of protein kinase C by BM41440 in cell-free extracts does not necessarily indicate that this compound reduces the enzyme activity in intact cells. Inhibition of partially purified protein kinase C depends on the concentration of phospholipids (Fig. 3). Fifteen μg/ml of the phosphatidylserine in the assay was sufficient to eliminate the inhibitory effect of BM41440. Considering the fact that a substantial fraction of protein kinase C is membrane bound (25), and that the membrane associated enzyme may represent the biologically active form (25), it may be argued that protein kinase C is hardly affected by BM41440 in intact cells. In order to analyze the inhibition by BM41440

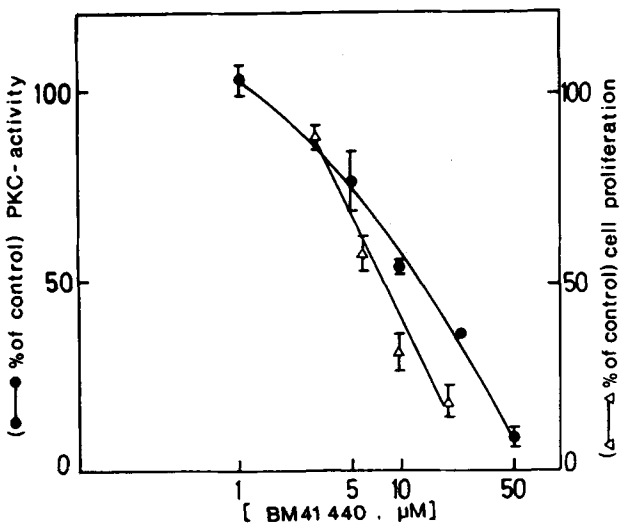

FIG. 2. Effects of BM41440 on cellular replication and protein kinase C. Protein kinase C was determined as described in the "Methods" section in presence of 2.0 μg/ml phosphatidylserine and 0.2 μg/ml diolein. 100% kinase activity represents 23 pmol phosphate/min. Data represent means ± SEM. Cellular replication, △; protein kinase C, ●.

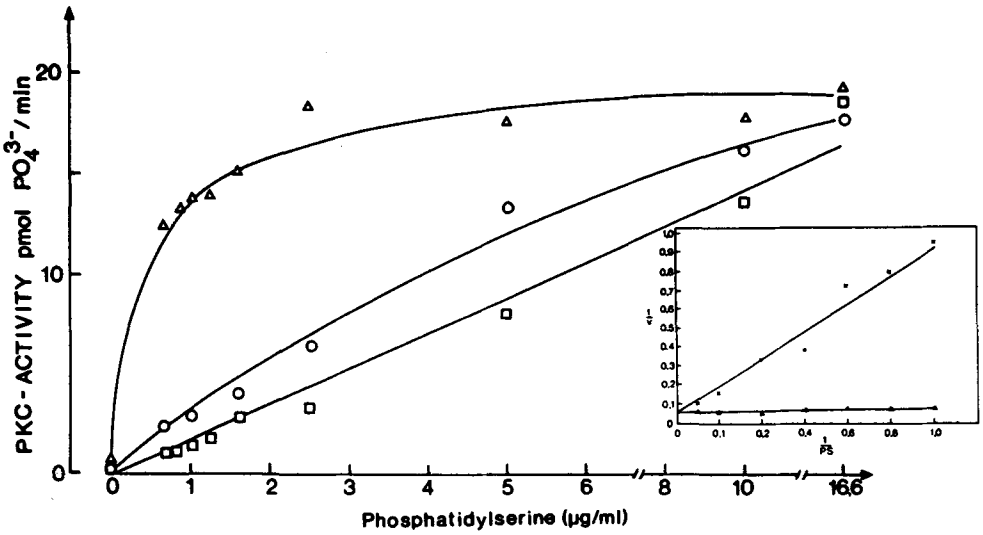

FIG. 3. Inhibition of protein kinase C by BM41440 as a function of the phosphatidylserine concentration. Protein kinase C was determined as described in the "Methods" section. The assays contained 1 µg/ml diacylglycerol and various amounts of phosphatidylserine as indicated. Each point represents the mean from three experiments. Standard deviation was < 5%. Control, △; BM41440 10 µM, ○; BM41440 20 µM, □.

of protein kinase C *in vivo*, we followed the phosphorylation of the ribosomal protein S6. Phosphorylation of S6 is catalyzed by a S6-kinase which in turn has been shown to be under control of protein kinase C (26). As protein kinase C is activated by phorbol esters, like TPA, a depression of the phorbol ester stimulated phosphorylation of S6 should be an indirect measure of intracellular protein kinase C activity. Figure 4 demonstrates the activation of the phosphorylation of the ribosomal protein S6 by TPA and an inhibition by 20 µM BM41440. The results suggest that protein kinase C is sensitive to BM41440 in intact cells.

Considering the essential role of protein kinase C in growth regulation (10) it seemed possible that the depression of protein kinase C by BM41440 is correlated to the growth inhibitory effect of this compound. In order to analyze whether an inhibition of protein kinase C is correlated to the reduction of cellular prolif-

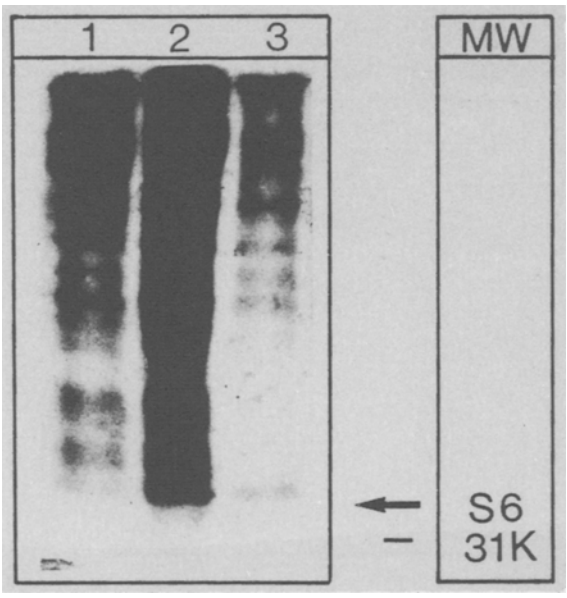

FIG. 4. Inhibition of the phosphorylation of ribosomal protein S6 by BM41440 in intact Walker cells. Ribosomal protein S6 was identified employing molecular weight markers and anti-S6 antiserum. Lane 1, control; lane 2, + TPA (0.5 µM); lane 3, TPA (0.5 µM) + BM41440 (20 µM).

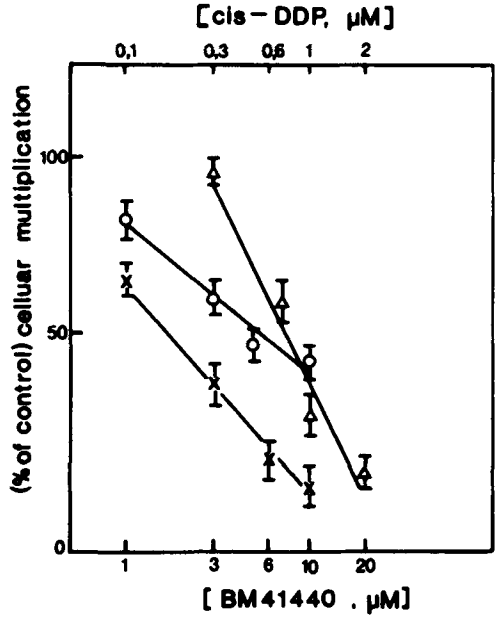

FIG. 5A. Antiproliferative effect of BM41440, *cis*-DDP and a BM41440/*cis*-DDP mixture on Walker carcinoma cells in culture. Cells were grown in presence of the drug for 48 hrs. Cell multiplication was determined as described under "Methods." BM41440, △; *cis*-DDP, ○; BM41440/*cis*-DDP (M ratio 10:1), ×; bars indicate SEM.

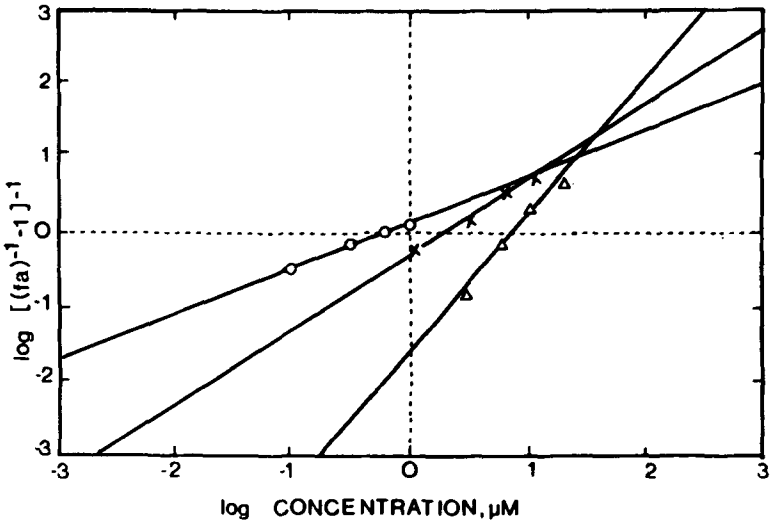

FIG. 5B. Calculated "Median Effect Plots" employing the data from Fig. 5A. Computations were performed according to Chou and Talalay (18,19). The abscissa presents: log *cis*-DDP, ○; log BM41440 + *cis*-DDP, × or log BM41440, △. Inhibition of cell multiplication is expressed as fraction affected (f_a); f_a 0.9 indicates 90% inhibition compared with controls.

eration, the dose dependent inhibition of both parameters were determined. As can be seen in Figure 2, concentrations of BM41440 which inhibit protein kinase C reduce cell proliferation to about the same extent, strongly suggesting the close correlation between these two parameters.

BM41440 enhances the antiproliferative activity of *cis*-DDP (Fig. 5A). The data from Figure 5A have been used to produce "median effect plots" (Fig. 5B) according to Chou and Talalay (20, 21). Computed regression coefficients of the linearized dose-effect curves proved to be > 0.98, indicating that the data fulfill the criteria for computation of the "combination index" (CI) according to Chou and Talalay (20, 21). This combination index has been shown to be extremely useful for quantitative evaluation of drug and inhibitory combinations. As outlined by Chou and Talalay (21), the combination index yields values of CI = 1, if summation is indicated, CI > 1, in case of antagonistic activities, and CI < 1, in case of synergism. In Figure 5C the combination indices for a mixture of BM41440 with *cis*-DDP are plotted with respect to the extent of inhibition of cell multiplication. It is evident that both drugs act synergistically (CI < 1).

DISCUSSION

O-Alkylglycerophospholipids have been shown to inhibit protein kinase C and this effect has been discussed as a possible mechanism responsible for the antitumor activity of these compounds (11, 27, 28). The new alkylphospholipid BM41440 differs from the substances studied so far. It contains a thioether instead of an *O*-ether and the glycerol is replaced by a 2-methoxymethyl-propanol.

The compound seems interesting as its exhibits strong antitumor activity against various *in vitro* and *in vivo* tumor systems (13-15). In view of the major differences between BM41440, biological phospholipids and phospholipid analogues investigated so far, it seemed interesting to determine whether BM41440 affects protein kinase C and, if this is the fact, whether a depression of the enzyme could account for the growth inhibitory effect of the molecule. The data presented here demonstrate that BM41440 inhibits protein kinase C in cell free extracts. The mechanism of inhibition appears similar to that suggested for the effect of the *O*-alkylglycerophospholipids, which are thought to compete with the phospholipid binding site of the enzyme (11). The K_i-value for BM41440 corresponds to K_i values reported for other alkylphospholipids. Helfman et al. (11) report an inhibition of protein kinase C from

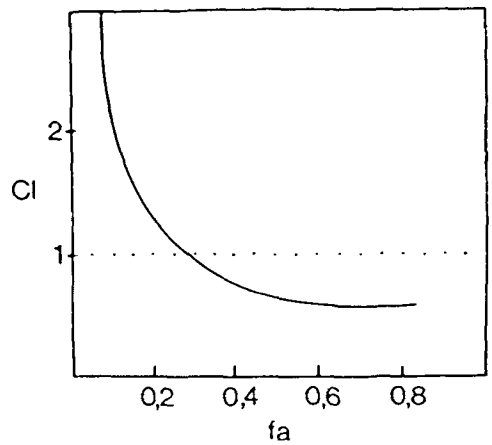

FIG. 5C. Computed plot of the combination index (CI) with respect of inhibition of cell multiplication by *cis*-DDP and BM41440 (molar ratio 1:10). Inhibition of cell multiplication is expressed as fraction affected (f_a); f_a 0.9 indicates 90% inhibition compared with controls. Computation was performed as described by Chou and Talalay (19) employing data from chart 5b. For definition of combination index see text.

human myelocytic leukemia cells by 1-octadecyl-2-methyl-*sn*-glycero-3-phosphocholine with a K_i of 6.3 μM.

The phosphorylation of the ribosomal protein S6 was used as an indirect marker for the intracellular protein kinase C activity. Our data demonstrate that submaximal inhibitory concentrations of BM41440 reduce the phosphorylation of S6 in intact cells. These results suggest that BM41440 at therapeutic concentrations affects protein kinase C in intact cells.

The dose effect plot for a protein kinase inhibition by BM41440 closely resembles the dose effect curve of the growth inhibitory action of this phospholipid analogue. Although it can be questioned whether the conditions in the cell-free extract permits conclusions concerning the behavior of the enzyme in intact cells, the data are in agreement with the assumption that protein kinase C is the major target of a alkylphospholipid—although additional mechanisms cannot be excluded.

Various inhibitors of protein kinase C have been shown to enhance the antiproliferative activity of *cis*-DDP (19). The data demonstrate that BM41440 shares this property. The protein kinase C inhibitors which have been studied with respect to their behavior in combination with *cis*-DDP comprise a variety of chemically distinct compounds with very different mechanisms of action and include quercetin, tamoxifen, phorbol esters, staurosporine and alkylphospholipids. None of these drugs is specific for protein kinase C. But, the common denominator of these chemically very-different compounds is their inhibitory effect on protein kinase C. It is likely, therefore, that it is indeed the ability of these agents to depress protein kinase C which is responsible for their enhancement of *cis*-DDP activity. By which mechanism inhibitors of protein kinase C potentiate the antiproliferative activity of cis-DDP is still obscure. It should be noticed, however, that *cis*-platinum complexes exert a series of membrane effects which seem to be correlated to the sensitivity against *cis*-platinum and which may contribute to the cytotoxic activity of these drugs (29—31). There is no direct inhibition by *cis*-DDP of protein kinase C (data not shown).

An enhancement of the antiproliferative activity of *cis*-DDP by ether lipid analogues has recently also been described by Noseda et al. (32). In contrast to our findings demonstrating a synergistic potentation of *cis*-DDP activity by BM41440, the compounds investigated by Noseda et al. (32) increase the growth inhibitory effect of *cis*-DDP via an additivity phenomenon. Whether this discrepancy is explained by the chemical differences of the compounds used or whether it is due to biological differences between the cell types studied remains to be investigated.

It seems unlikely that the synergistic enhancement of the antiproliferative activity of BM41440 by *cis*-DDP is specific for platinum complexes because similar effects have been obtained employing combinations of various protein kinase C-inhibitors with nitrogen mustard (19). It should be emphasized, however, that nitrogen mustard, as well as other alkylating agents, cause a variety of membrane lesions at therapeutic concentrations (33–36) which may be related to the potentiation of the antitumor activity by protein kinase C-inhibitors.

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Environmental Temperature and Metabolism of the Molecular Species of Phosphatidylcholine in the Tissues of the Rainbow Trout

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The phospholipid composition and the fatty acid pattern of phosphatidylcholine from major tissues of the rainbow trout acclimated to 11° and 21°C were analyzed. A significant difference in the level of 20:5ω3 fatty acid between gill, kidney, intestine and liver phosphatidylcholine is observed in both groups of animals. These differences correlate with a modification of the molecular species distribution in the phosphatidylcholine in these organs. In vivo incorporation of ^{32}P into phospholipids of gills, kidney, intestine, liver and muscle was determined in trout acclimated to 11° and 21°C. Temperature acclimation specifically modify the relative specific radioactivity of the monoene and hexaene molecular species of phosphatidylcholine in gills, intestine, kidney and liver. It can be concluded from these results, that the metabolism of the diacylglycerol moiety and the coupled fatty acids of phosphatidylcholine differs with the tissue and the temperature. It is suggested that different associated mechanisms specific for each organ determine the fatty acid composition of phosphatidylcholine required for its physiological functions at each temperature.
Lipids 24, 318-324 (1989).

In fish, phospholipids have a characteristic and complex fatty acid pattern with large amounts of highly unsaturated fatty acids (1). It is often assumed that this unsaturation of the diacylglycerol moiety of phospholipids in these animals is related in a direct manner to the cell membrane fluidity (2,3). In fact, a wide variety of poikilotherms possess the ability to alter the fatty acid composition of their phospholipids in response to a change in environmental temperature (2,4,5). The adjustment of the types of fatty acid esterified in phospholipids of the membranes probably involves various regulatory mechanisms and may occur at several points in the lipid metabolic pathway (3). However, these adaptation mechanisms can differ among tissues of a single species, and additional studies are needed to understand the metabolic alterations that occur during homeoviscous adaptation (6).

The metabolic reactions involved in the changes in fatty acid composition of phospholipids in poikilothermic organisms acclimated to extreme temperature have not been completely elucidated. Recently, a temperature-controlled metabolic heterogeneity in the molecular species of different phospholipids has been observed in crabs (7), trout (8,9) and goldfish (10), suggesting that a possible adaptative mechanism may occur at this level.

Phospholipids could be synthesized by several different routes: de novo synthesis from the 1,2-diacylglycerols (11); reacylation of lysophospholipids (12); stepwise methylation of phosphatidylethanolamine to form phosphatidylcholine (13); decarboxylation of phosphatidylserine to give phosphatidylethanolamine (14); and base exchange reactions (14). However, these different pathways have not the same activity in the major organs of fish and evaluating their respective roles requires additional studies in these species.

The present work was undertaken to determine, in the different tissues of the rainbow trout (*Salmo gaidnerii*), the influences of the environmental temperature on the biosynthesis of the major molecular species of phosphatidylcholine. We have investigated by ^{32}P labeling, whether phospholipid biosynthesis is temperature-dependent in all tissues and whether the temperature of the adaptation medium alters, similarly in all organs, the concentration and renewal of the molecular species of phosphatidylcholine.

MATERIALS AND METHODS

Rainbow trout (weighing 150-180 g) were purchased from a local supplier and kept in highly aerated running water at 11° and 21°C for at least six weeks. The animals were fed daily with SARB (Decines, France) commercial trout food. Before use, the animals were starved 24 hr. Each trout received 300 μCi of $^{32}PO_4Na_2H$ (10 mCi/ml, Amersham, Buckinghamshire, UK) in isotonic solution by intraperitoneal injection. Water radioactivity was measured for each trout 3 hr after ^{32}P injection to estimate the leakage of radioactive material from the point of injection, and animals with a loss of less than 10% of the injected dose were used. Injected trout were killed 12, 24 and 48 hr after the initial injection and tissue lipids were extracted by a modification of Folch's method (15) as previously described (9,16). The washed organic phase was evaporated to dryness in a rotary evaporator, and the lipid extract stored in a minimum volume of 2:1 (v/v) benzene/methanol at −70°C.

Phospholipids were separated by two dimensional thin-layer chromatography on 10 × 10 cm silica gel 60 precoated plates (Merck, Darmstadt, Germany) using solvents previously described (17). The spots on the chromatogram were visualized with the phosphorus-specific spray reagent of Dittmer and Lester (18). Spots were scraped off into counting vials and 4 ml of water/ethanol (1:1, v/v) and 10 ml of Picofluor (Packard, Downers Grove, IL) were successively added (19). After mixing, each vial was counted. Counting efficiency was

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Abbreviations: DPG, diphosphatidylglycerol; LPC, lysophosphatidylcholine; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SPH, sphingomyelin; UI, unsaturation index.

determined by the external standard channels ratio method. Under these conditions, 95–98% of the radioactivity in each spot was recovered (20).

The phosphatidylcholines were purified by one-dimensional thin-layer chromatography on 0.5-mm thin layers silica gel H (Macherey-Nagel, Düren, Germany) plates with chloroform/methanol/acetic acid/water (60:30:7:3, v/v) as developing solvent (21). After localization with primuline spray reagent under UV light (22), the phospholipids were eluted with a mixture of chloroform-methanol-acetic acid-water (50:39:1:10, v/v) and washed as described by Arvidson (23). The purity of the isolated phosphatidylcholine was controlled by analytical two-dimensional thin layer chromatography on silica gel 60 precoated chromatoplates (Merck, Darmstadt, Germany) according to Portoukalian et al. (17).

Molecular species of the purified phosphatidylcholines were fractionated on precoated silica gel 60 plates impregnated with $AgNO_3$ by dipping for 30 min in 100 ml of 50% (w/v) Ag NO_3 in water. The plates were dried for 10 min at 115°C, stored in the dark and activated for 8 min at 170°C immediately before use. A maximal amount of 2 mg of phosphatidylcholine was applied to each plate, which was developed in chloroform/methanol/water (60:40:7.5, v/v) (24). The 4 major separated fractions stained with phosphorus-specific spray were either scraped off into scintillation vials for liquid scintillation counting or eluted and washed as described by Arvidson (23). Determination of lipid phosphorus was made on each eluate by Bartlett's method (25) as modified by Portoukalian et al. (17). The fatty acid composition was analyzed by gas chromatography after methanolysis of each eluate by the procedure previously described (26). All analyses were carried out isothermally at 190°C using a gas liquid chromatograph (Packard, Downers Grove, IL) with a 10% SP 2330 on 100/120 chromosorb W AW column of 9 ft 1/16 in. S.S.

RESULTS

Phospholipid composition of the tissues of rainbow trout acclimated at 11° and 21°C. The phospholipid content of the different tissues differ widely from 2.2% of wet tissue in liver to 0.6% in muscle (Table 1). Increased acclimation temperature induced a significant decrease of phospholipid content in gills and kidney.

The phospholipids present the same general pattern in the liver, gills, kidney, muscle and intestine, but differences exist in the composition, which is mostly quantitative. As shown in Table 1, there is a significantly higher percentage of phosphatidylcholine (PC) in liver and muscle as compared with intestine, kidney and gills. This observation is in agreement with the data obtained by one-dimensional TLC of lipids extracted from liver and gills of trout by Hazel (27,28). The highest ratio of PC/sphingomyelin (SPH), resulting from a lower percentage of SPH is observed in the muscle (PC/SPH = 21.6 at 11°C and 27.5 at 21°C) and the lowest in intestine where the ratio decreases to 5.9 at 11°C and 6.8 at 21°C. The ratios phosphatidylethanolamine (PE)/PC and PS/phosphatidylinositol (PI) were higher in the three organs implicated with osmoregulatory processes. The highest proportion of diphosphatidylglycerol (DPG) exists in the lipids from liver. The greatest amount of lysophosphatidylcholine (LPC) appears to be in intestine and gills, two organs in contact with the environmental medium. 11° or 21°C-Acclimation temperature have no particular effect on this composition.

Fatty acid composition of phosphatidylcholines from the tissues of rainbow trout acclimated at 11°C and 21°C (Table 2). The fatty acid pattern of phosphatidylcholines extracted from the tissues of 11° and 21°C-acclimated trouts is summarized in Table 2. Examination of their fatty acid compositions revealed that palmitic (16:0), oleic (18:1) and docosahexaenoic (22:6ω3) acids are the major fatty acids in all tissues and represent 70–75% of the total fatty acids esterified in PC. A similar pattern for PC fatty acids has been observed in the liver by Hazel (27) and in the gills by Sellner and Hazel (29). Muscle PC appears as the most unsaturated as shown by its unsaturation index (UI). The higher proportion of 20:4ω6 was observed in gills, kidney and intestine, but the sum of ω6 fatty acids remains approximately the same in all tissues. In contrast, the sum of ω3 varies largely between the different organs, the highest being in liver and muscle. An important point is that the temperature of acclimata-

TABLE 1

Effect of Acclimatation Temperature on the Phospholipid Composition of the Rainbow Trout Tissues

	Gills		Kidney		Liver		Muscle		Intestine	
	11°C	21°C	11°C	21°C	11°C	21°C	11°C	21°C	11°C	21°C
Plip[a]	11.4±0.2	10.5±0.2[b]	20.0±0.6	18.1±0.5[b]	22.4±0.5	21.4±1.0	6.6±0.2	6.4±0.2	14.0±0.8	16.4±0.2
LPC°	1.8±0.6	1.3±0.2	0.6±0.2	0.5±0.1	0.7±0.3	0.8±0.3	tr	tr	2.2±0.5	2.7±0.4
SPH	5.8±0.9	5.4±0.5	7.7±0.9	7.6±0.6	5.2±0.7	5.0±0.3	3.0±0.6	2.4±0.4	9.0±0.3	7.9±0.3[b]
PC	57.8±1.9	58.9±1.4	55.0±0.7	54.7±1.7	61.0±1.8	60.9±1.2	64.8±1.4	65.9±1.0	53.2±1.3	54.0±1.2
PS	4.5±0.9	4.2±0.6	5.9±0.7	5.0±0.4	3.1±0.9	3.6±0.5	3.5±0.7	2.6±0.1	5.6±0.8	6.5±0.6
PI	3.8±0.6	3.4±0.5	4.0±0.7	4.4±0.7	5.4±0.7	4.4±0.4	4.0±0.4	4.9±0.5	5.7±0.6	5.9±0.9
PE	22.9±1.0	23.8±0.9	23.7±0.8	24.4±1.3	19.4±1.2	20.1±0.6	21.7±0.7	21.0±0.5	21.3±1.0	20.0±1.0
DPG	3.0±0.4	2.5±0.5	2.9±0.5	3.0±0.6	4.9±0.7	4.9±0.9	2.3±0.7	2.6±0.6	2.4±0.5	2.6±0.7
AP	0.4±0.2	0.5±0.3	0.3±0.1	0.4±0.2	0.3±0.1	0.3±0.1	0.7±0.3	0.6±0.2	0.5±0.2	0.5±0.2

[a]Plip. expressed the phospholipid content of each tissue in mg. of phospholipid per g of wet tissues.
[b]Values statistically different ($p<0.01$) between 11° and 21°C trout with the student t test.
°All values of phospholipid composition are expressed as the % of total P recovered from the chromatogram and are the means ± SEM of 12 animals.

TABLE 2.

Fatty Acid Composition of Phosphatidylcholines From the Tissues of Rainbow Trout Acclimated at 11°C and 21°C

	Gills		Kidney		Liver		Muscle		Intestine	
Fatty Acid	11°C	21°C	11°C	21°C	11°C	21°C	11°C	21°C	11°C	21°C
16:0	29.4±0.8	30.7±0.8	31.6±1.5	33.0±0.8	30.9±0.1	33.0±0.1[a]	33.7±1.2	35.1±1.7	29.8±1.9	29.8±1.9
16:1	0.6±0.1	0.8±0.2	1.2±0.2	1.3±0.1	tr	tr	tr	tr	tr	tr
18:0	6.5±0.4	6.8±0.3	3.5±0.2	3.9±0.2	8.2±0.5	7.3±0.8	3.0±0.5	2.9±0.1	3.5±0.1	3.7±0.2
18:1	31.6±0.5	31.6±1.4	23.0±1.5	25.3±0.5	15.9±0.4	20.3±1.0[a]	12.4±0.8	11.5±0.5	27.9±0.5	28.0±0.9
18:2ω6	5.0±0.2	5.3±0.6	5.8±0.4	5.0±0.6	6.3±0.4	5.9±0.1	6.4±0.5	7.0±0.6	5.3±0.4	5.4±0.1
18:3ω3	0.1	0.1	0.5±0.1	0.4±0.1	0.4±0.1	0.1	1.2±0.1	1.3±0.1	tr	tr
20:1	0.6±0.1	0.7±0.1	0.5±0.1	0.5±0.1	0.6±0.1	1.3±0.2	0.3	0.4±0.1	0.9±0.1	0.9±0.1
20:2ω6	0.5±0.1	0.5±01	0.6±0.1	0.8±0.1	0.8±0.1	1.6±0.2[b]	0.4±0.1	0.5±0.1	0.9±0.3	1.4±0.1
20:3ω3	0.8±0.1	0.9±01	1.1±0.2	1.2±0.1	1.4±0.2	1.3±0.1	1.2±0.1	1.0±0.1	1.2±0.1	1.7±0.1[b]
20:4ω6	3.5±0.5	3.1±0.1	3.1±0.2	2.6±0.1	2.5±0.3	1.8±0.3	2.6±0.3	2.9±0.3	3.4±0.2	3.0±0.1
20:5ω3	5.2±0.3	3.6±0.2[a]	7.3±0.1	5.4±0.4[a]	5.8±0.8	2.3±0.3[a]	7.7±1.0	9.7±1.2	6.0±0.4	3.8±0.4[a]
22:3	0.2	0.1	1.1±0.1	1.3±0.1	ND	tr	ND	ND	tr	tr
22:5ω6	0.2	0.4	0.4	0.3	tr	0.9±0.1	0.5±0.2	0.5±0.2	0.2	0.5±0.1
22:5ω3	0.5±0.1	0.6±0.1	0.2	0.5±0.1	0.5±0.1	0.8±0.1	tr	tr	0.9±0.1	0.8±0.2
22:6ω3	15.5±2.0	14.7±2.2	20.1±1.5	18.6±0.9	26.8±1.0	23.6±2.8	30.5±1.9	26.8±1.1	20.2±0.6	21.3±0.3
UI	183±6	171±5	218±8	200±6	238±4	209±5[a]	268±9	257±6	215±4	212±3

Values are the means ± SEM.
[a]$p<0.01$.
tr, Traces, ND not detected.
UI, the unsaturation index, is the sum of the percentage multiplied by the number of double bonds for each fatty acid in the mixture.

tion does not seem to affect greatly the major fatty acid pattern of PC in all tissues. Only a significant decrease of eicosapentaenoic acid (20:5ω3) is seen in gills, kidney, liver and intestine of 21°C-acclimated trout. In contrast, in muscle, there is an increase in the percentage of this fatty acid. On the basis of these analyses, it is only possible to differentiate the two kinds of tissues by considering the ratio, 20:5ω3/20:4ω6, which decreases significantly in liver from 2.3±0.2 at 11°C to 1.3±0.1 at 21°C. A similar variation is observed in gills (from 1.5±0.1 to 1.2±0.1), kidney (from 2.4±0.2 to 2.1±0.1) and intestine (from 1.8±0.2 to 1.3±0.1). In muscle, the ratio increases from 3.0±0.1 to 3.3±0.2 at 21°C.

In vivo incorporation of $^{32}PO_4HNa_2$ into phospholipids of rainbow trout acclimated at 11° and 21°C. To determine whether the differences in the composition of tissue phospholipids in 11°- and 21°C-acclimated trout result from variations in their synthesis, the kinetics of in vivo incorporation of radioactive phosphate was investigated. Figure 1 shows the total amount of radioactivity accumulated in the tissue lipids of *Salmo gaidnerii* 12, 24 and 48 hr after injection of the radiolabel. During the 48-hr period after the ^{32}P injection, the level of radioactivity in total lipids increases continuously (see Fig. 1). The slowest uptake appears in the muscle and the fastest in liver. Increasing acclimation temperature results in an increased rate of ^{32}P incorporation in gills, kidney and liver lipids. In muscle, no significant difference between 11° and 21°C is observed.

In all tissues, the major part of the radioactivity is concentrated in three lipid species, 60-80% in the PC, 15-30% in the PE and 5-30% in the PI (Table 3). The remainder is distributed between the other constituents, chiefly in PS (1-4%), SPH (1-3%), DPG (0.5-1.5%) and LPC (0.5-1.5%). The distribution of the radioactivity into the different constituents of the phospholipid pool remains approximately the same during the 48-hr period of incubation in the liver (see Table 3). In contrast, in gills, kidney, muscle and intestine, the percentage of radioactivity incorporated into PC, PE and PI varies largely with time and adaptation temperature. The percentage of radioactivity declined for PI in these tissues at both temperatures; whereas, the radioactivity of PC increased at 11° and remained stable at 21°C. For PE, the percentage decreased in the kidney and intestine at 11° and in muscle at 21°C, and remained stable in gills and muscle at 11°C. At 21°C, the percentage of radioactivity in PE increases in gills, kidney and muscle.

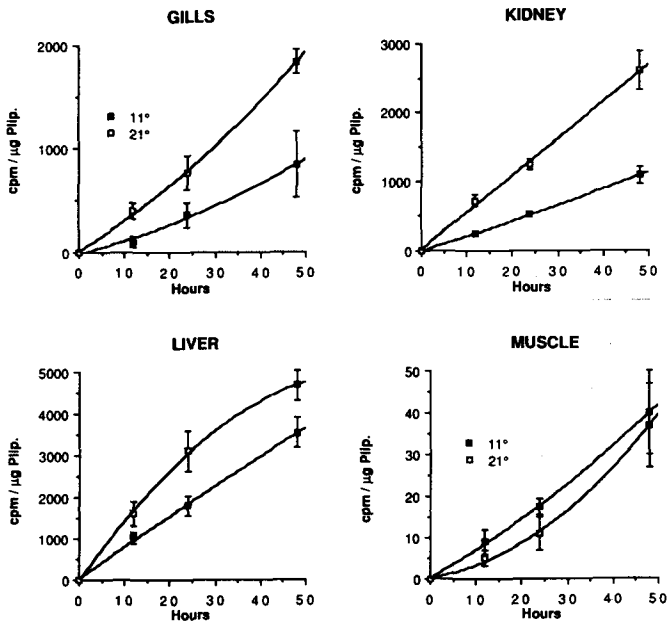

FIG. 1. Incorporation of $^{32}PO_4HNa_2$ into total phospholipids of rainbow trout acclimated at 11° and 21°C.

TABLE 3

Distribution of the Radioactivity into the Major Phospholipids of Tissues From 11°C- and 21°C-Acclimated Rainbow Trout

	11°C			21°C		
Hours	PC	PI	PE	PC	PI	PE
			Gills			
12	46.4±2.9[a]	29.2±3.7	14.6±2.1	55.4±2.0[b]	18.2±0.2[b]	18.7±2.2
24	59.6±7.1	18.3±4.5	14.9±1.7	58.1±1.2	14.3±0.2	20.0±1.5
48	65.8±0.2	11.4±0.5	15.8±1.1	54.9±2.7[c]	9.0±0.7[b]	26.3±2.2[c]
			Kidney			
12	45.2±4.5	16.7±3.4	32.4±0.1	59.7±2.5[b]	12.8±0.9	22.8±1.3[c]
24	55.1±1.0	14.9±2.6	23.7±3.0	57.2±1.2	12.1±0.7	25.2±1.9
48	63.4±2.6	9.3±1.1	21.2±0.8	54.3±0.4[c]	8.4±1.1	28.5±0.1[c]
			Liver			
12	74.7±3.1	6.6±1.4	14.7±1.0	78.2±0.1	5.2±0.1	13.5±0.1
24	71.6±1.0	6.1±0.4	18.1±0.6	76.2±0.1[c]	4.9±0.1[b]	14.3±0.1[c]
48	70.6±1.4	5.5±0.7	18.9±0.3	75.2±1.5	5.2±0.2	14.4±1.8[b]
			Muscle			
12	46.4±4.3	17.7±1.7	14.9±1.4	64.6±0.3[c]	15.4±0.9	11.1±0.7
24	67.1±5.3	8.9±2.3	12.0±1.4	66.4±1.8	14.4±1.2	12.1±1.4
48	69.7±1.7	6.9±1.2	12.7±0.4	68.3±1.7	7.4±0.1	15.1±0.3
			Intestine			
12	42.6±1.0	13.9±0.6	36.1±0.2	45.6±1.2	14.4±1.2	34.5±3.0
24	52.8±0.2	10.6±0.1	31.1±0.1	52.8±0.5	9.4±0.9	32.6±1.9
48	51.8±0.8	9.3±1.6	30.8±0.8	50.5±0.4	9.2±0.8	32.8±0.1[b]

[a]Results are expressed as dpm % of the total dpm recovered from the chromatogram and were the means ± SEM of 6 measures.
[b]$p<0.05$, value statistically different between 11 and 21°C.
[c]$p<0.01$; value statistically different between 1 and 21°C.

As can be seen in Table 4, the highest specific radioactivities of PC and PE are observed in liver and are 2- to 3-fold more than those in the gills, kidney and intestine at both temperatures. Increasing the temperature multiplies by 1.5-2 the specific radioactivity of these two phospholipids in these last tissues. PC and PE have the lower specific activity in muscle in which phospholipids have a slow labeling. However, in intestine, in contrast with the other tissues, PE has a higher specific radioactivity than PC.

The results presented above give only an imperfect picture of the extent of the changes due to temperature influence, as PC consists of molecular species with different degrees of unsaturation. For this reason, we have examined the molecular species of PC synthetized in the both groups of trout.

Distribution of radiolabel among the different molecular species of PC of trout acclimated at 11° and 21°C. After ^{32}P injection, PC from tissues from 11° and 21°C-acclimated trout have been resolved into fractions of different degrees of unsaturation by chromatography on silica gel plates impregnated with $AgNO_3$. Trout PC were routinely separated into four fractions stained by phosphorus reagent on the chromatogram, but in some cases, the fourth fraction was separated into two bands. As their fatty acid compositions were relatively close, we used these as a single fraction. Each fraction separated on the chromatogram was arbitrarily named according to its unsaturated fatty acid content.

Figure 2 shows the fatty acid compositions of the molecular species isolated by $AgNO_3$ thin-layer chromatography from PC. The percentage of C_{18}, C_{20} and C_{22} fatty acid in the major molecular species of PC extracted from the different tissues showed striking variations corresponding to the differences in fatty acid composition of the initial phospholipid (see Table 2). However, no statistically significant differences were observed in relative proportions of fatty acids in the similar molecular species of PC isolated from the same tissues from either 11°- or 21°C-acclimated trout. In fraction 4, separated in some cases as two bands on the chromatogram, differences were observed in the two separated bands, but were not statistically significant.

From the data on the percentage distribution of P between the subfractions of PC in Table 5, it is seen that the monoenoic and hexaenoic fractions together account for 70-80% of total PC phosphorus. In liver PC, there is 2-fold more hexaenoic fraction than in gills, and this difference corresponds to the higher

TABLE 4

Average of Specific Activities of Phosphatidylcholine and Phosphatidylethanolamine During the 48-hr Experimental Period

	Phosphatidylcholine		Phosphatidylethanolamine	
	11°C	21°C	11°C	21°C
Gills	469[a]	947	293	995
Kidney	623	1444	626	1705
Liver	2486	3921	1967	2220
Muscle	23	18	13	11
Intestine	538	682	870	1205

[a]Specific radioactivity was expressed as dpm/μg of Plip. Values are means of 12-, 24- and 48-hr specific activities of PC or PE.

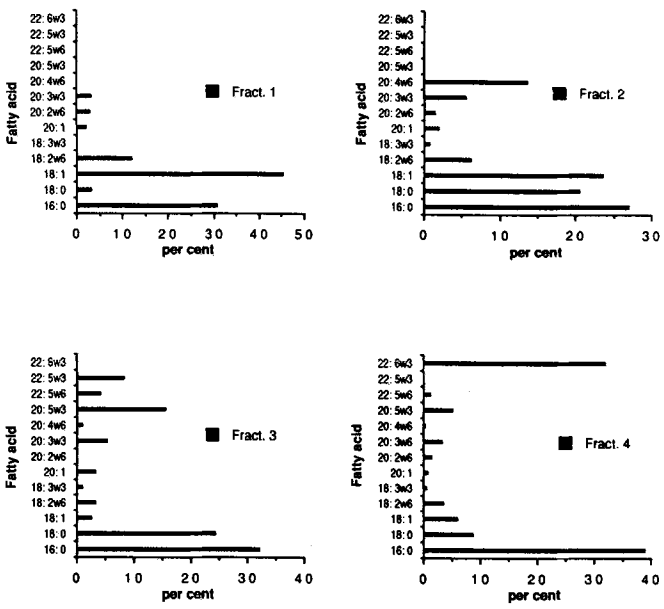

FIG. 2. Fatty acid compositions of the molecular species isolated by $AgNO_3$ thin-layer chromatography from phosphatidylcholine of gills.

content of 22:6ω3. By increasing acclimation temperature, a higher percentage of the more saturated fraction is observed in liver (+92%), gills (+16%) and kidney (+17%). These increases were concomitant with decreases of hexaenoic fraction.

To compare the labeling of each constituent of the PC between animals living at 11° and 21°C, we have calculated their relative specific radioactivities (sp. act. of the component/sp. act. of total lipids) which are given in Table 6. These ratio values which are independent of the rate of ^{32}P incorporation into the PC facilitate the comparison of the renewal of the species studied. Significant differences in renewal of the most saturated (monoene) and the unsaturated (tetraene and hexaene) fractions between 11° and 21°C trout were observed in gills, kidney, intestine and liver (Table 6). At 21°C, the monoene and tetraene fractions have a higher renewal compared with 11°C in gills, kidney and intestine. In contrast, these fractions have a lower renewal in liver at 21°C. The most unsaturated fraction (hexaene), has a lower renewal at 21°C in gills and intestine, but a higher one in liver. A higher ratio (sp. act. of monoene fraction/sp. act. of hexaene fraction) of 12.6±1.6 in the liver as compared, respectively, with 2.7±0.3, 2.6±0.6 and 3.9±0.9 in the gills, kidney and intestine was observed in 21°C-acclimated trout. However, at 11°C, this ratio has the higher value (13.4±0.9) in intestine as compared with 4.8±0.5, 3.9±0.7 and 4.5±0.8, respectively, in gills, kidney and liver.

DISCUSSION

Phosphatidylcholine, one of the most abundant of commonly occurring phospholipids in trout tissues (see Table 1), has been studied during acclimatation of the trout at two different temperatures.

At 11°C, our results show that the organs involved in osmoregulation (gills, kidney and intestine) differ greatly from the liver and muscle. The PC of these organs presents the lowest percentage of saturated fatty acids and ω3 fatty acids, the highest percentage of arachidonic acid (20:4ω6) and the lowest ratio, Σ ω3/Σ ω6. Also, the most abundant molecular species of these PC is monoene, whereas in the liver it is hexaene.

Increasing the acclimation temperature from 11° to 21°C results in the following changes in the PC: increase in saturated fatty acids, except the intestine; decrease in 22:5ω3 and 22:6ω3 acids, and in ratio 20:5ω3/20:4ω6; and decrease in ratio, Σω3/Σω6, except in the kidney.

This results in an increase in the monoenoic molecular species and a decrease in the hexaene. The tetraene molecular species remains stable; whereas, there is a significant decrease in the pentaenes in the gills and the liver.

The increase in the temperature, also accelerates the incorporation of ^{32}P into the tissue lipids, with the exception of the muscle. There is an increase of radiolabel incorporated into the most saturated (monoene) species of PC at 21°C in the osmoregulatory organs and a decrease in the liver. It is similar for the tetraene,

TABLE 5

Effect of the Temperature on the Distribution of the Molecular Species in PC of the Trout Tissues

		Molecular species			
	Temp (°C)	Monoenes	Tetraenes	Pentaenes	Hexaenes
Gills	11°	49,5±1.8[a]	11,6±1,2	12,2±0,3	26,9±0,3
	21°	57,2±0,7[b]	10,6±0,7	10,9±0,2[b]	21,4±1,7[c]
Kidney	11°	45,1±1,2	10,0±2,1	10,1±0,1	34,9±3,3
	21°	52,7±1,9[c]	9,2±0,7	10,8±1,2	27,3±0,6
Liver	11°	22,4±1,2	7,6±0,4	12,8±0,1	57,3±1,3
	21°	43,1±3,9[d]	7,7±0,7	8,1±1,0[d]	41,1±3,7[d]
Intestine	11°	45,2±0,1	13,6±1,0	11,4±1,3	29,9±0,3
	21°	45,5±0,2	13,2±1,7	10,2±0,3	31,2±1,3

[a]Results are expressed as the percentage of total phosphorus recovered on the TLC plate. Values [mean ± SEM (n = 6)] with a superscript are significantly different by Student t test between the 11°C and 21°C trout.
[b]$p>0.02$, [c]$p<0.05$, [d]$p<0.01$.

TABLE 6

Relative Specific Activities[a] of Molecular Species of Phosphatidylcholines

		Monoenes	Tetraenes	Pentaenes	Hexaenes
Gills	11°C	0.45±0.4	0.80±0.07	0.90±0.02	2.14±0.08
	21°C	0.68±0.06[b]	1.04±0.05[c]	0.87±0.05	1.84±0.07[c]
Kidney	11°C	0.46±0.03	0.68±0.10	1.04±0.04	1.77±0.11
	21°C	0.66±0.05[c]	0.78±0.04	1.04±0.10	1.74±0.13
Liver	11°C	0.31±0.04	0.63±0.07	0.70±0.06	1.38±0.04
	21°C	0.16±0.03[c]	0.50±0.04	0.76±0.10	2.02±0.03[b]
Intestine	11°C	0.18±0.01	0.61±0.01	0.99±0.03	2.42±0.04
	21°C	0.49±0.08[c]	0.70±0.02[c]	0.85±0.18	1.91±0.12[b]
		Distribution in dpm %			
			Fract. 1+2	Fract. 3	Fract. 4
Muscle	11°C		20.1±2.7	6.1±0.9	73.8±2.1
	21°C		28.5±8.0	6.1±1.0	66.3±7.3

[a]Relative specific activity was the ratio of the specific radioactivity of a molecular species to the specific radioactivity of total PC. Muscle results are expressed only as the distribution of ^{22}P among PC of different degrees of unsaturation.
[b]$p<0.01$; [c]$p<0.05$.

but the hexaene is renewed more slowly in the gills and in the intestine and quicker in the liver.

From these results, it can be concluded that the metabolism of the diacylglycerol moiety and the coupled fatty acids of PC differs with the tissue and the temperature. They can be modified selectively. This determines for each organ a fatty acid composition of PC required for physiological functions at different temperatures.

This restructuration of the PC in function of the temperature is probably the result of an overall rearrangement, conjunction of : direct influence of the temperature on the speed of synthesis or degradation of these molecules within each organ (10,30-34); influence of the concentration of different substrates and in particular the relative ratio of the different constituents of the fatty acyl-CoA pool in each organ (35-37); activation or inactivation in certain organs of accessory pathways of PC synthesis, such as acylation of LPC (38-41), decarboxylation of PS to PE (42, 43), methylation of PE (44-46) or base exchange (41); vesiculation processes of cell membranes more or less important depending on the organ (47) and provoking the exportation of certain molecular species of PC present in the lipid layer of the membrane (46).

These modifications, in the composition and distribution of the molecular species of each phospholipid or their turnover, will have an effect on the physical properties of each membrane bilayer (48).

However, this compensation against the temperature requires an external source of certain essential elements, such as the ω3 and ω6 polyunsaturated fatty acid (49-53). This varies with the animal studied and these diverse requirements can be explained by differences in the activity of Δ9, Δ6 and Δ5 desaturases (54-56). The activities of these desaturases vary differently with the temperature (57, 58) and so the metabolism of certain molecular species of PC will be dependent with the evolution of the latter. However, the process is probably more complex, certain fatty acids (20:4 ω6 and 20:5 ω3) are transformed into prostaglandins in osmoregulatory organs (59-60). Thus, the increase of specific radioactivity of the tetraene fraction in these organs and decrease in the liver could be due to the exchange of constitutive fatty acids between these different organs. An inverse phenomenon is observed with the hexaene fraction. Acting as a salvage pathway, this process could deliver to the organs which need them the essential fatty acids to function. Such an exchange process has been described in the crab, *Carcinus maenas* (46), and in the eel, *Anguilla anguilla* (unpublished observations).

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Sterol Metabolism in the Nematode *Panagrellus redivivus*

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Panagrellus redivivus **was propagated in media containing three structurally different sterols: 7-dehydrocholesterol, campesterol or stigmastanol. Nematodes propagated with 7-dehydrocholesterol contained mostly lathosterol and 7-dehydrocholesterol. Nematodes propagated with campesterol contained mostly cholesterol and cholestanol. Nematodes propagated with stigmastanol contained mostly cholestanol. The sterol ester fraction was enriched with 4α-methylsterols and contained the same sterols as the free sterol fraction except for nematodes propagated with 7-dehydrocholesterol, where no dietary sterol was found in the ester fraction.** ***P. redivivus*** **is capable of reducing the Δ^5-bond, C-24 dealkylation and methylating the sterol nucleus at C-4.**
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Nematodes lack the ability to synthesize sterols *de novo* and therefore possess a nutritional requirement for exogenous sterols (1—4). Nematodes, however, do possess the ability to metabolize dietary sterols (5—14). Although the sterol content and composition of many nematodes is known, the difficulty in propagating plant-parasitic nematodes axenically has limited studies to comparisons of sterol composition of the host and the parasite (15—21). Extensive studies on sterol metabolism in nematodes, therefore, have utilized the free-living nematode *Caenorhabditis elegans* as an axenically grown model organism (6—10, 12). *C. elegans* is capable of C—24 dealkylation but generally produces 7-dehydrocholesterol (cholesta-5,7-dien-3β-ol), a sterol not yet detected in parasitic nematodes (6—10, 12).

When fed Δ^5-sterols, the free-living nematode *Panagrellus redivivus* produces cholesterol (cholest-5-en-3β-ol) and cholestanol (5α-cholestan-3β-ol) as its principal sterols (13). As sterols with a Δ^5-bond or a saturated nucleus are more characteristic of plant-parasitic nematodes (18, 20, 22), we suggested that *P. redivivus* may be a more suitable model organism for investigating sterol metabolism representative of these nematodes (13). Therefore, it was of interest to extend our previous study on sterol metabolism in *P. redivivus* to include sterols with differing side chains and nuclear modifications.

EXPERIMENTAL

Nematode cultures. Panagrellus redivivus was propagated axenically at 22°C in a semi-defined aqueous medium containing 30 mg/ml yeast extract, 30 mg/ml soy peptone, 10 mg/ml casein hydrolyzate and 10 mg/ml dextrose (23). All ingredients except the soy peptone were extracted (3×) with chloroform/methanol (2:1, v/v) to remove any exogenous sterols. A filter-sterilized stock solution of dichloromethane-extracted hemoglobin (0.5 mg/ml in the final medium) was added after autoclaving of the basal medium. The dietary sterols were solubilized in aqueous Tween 80, filter-sterilized and similarly added at a final concentration of 10 μg/ml. The nematodes were acclimated through two subcultures in the appropriate medium prior to introduction into mass culture.

Dietary sterols. The dietary sterols contained no impurities by thin layer chromatography (TLC). The stigmastanol (24α-ethyl-5α-cholestan-3β-ol) and 7-dehydrocholesterol (cholesta-5,7-dien-3β-ol) contained no detectable impurities (<0.05%) by capillary gas liquid chromatography (GC). The campesterol (24α-methylcholest-5-en-3β-ol) contained approximately 4% sitosterol (24α-ethylcholest-5-en-3β-ol) by capillary GC.

Sterol isolation. Replicate cultures of living nematodes were isolated from late logarithmic phase cultures by centrifugation and sucrose flotation (24). Total lipids were isolated from lyophilized nematodes by homogenization (4×) with chloroform/methanol (2:1, v/v) in a Ten Broeck tissue grinder. The neutral lipids were separated by column chromatography through silica gel 60 (E. Merck, Darmstadt, FRG) by eluting with chloroform. The neutral lipids were then fractionated on a 15-g silica gel 60 column (22 mm i.d.) by eluting with: 1) 100 ml hexane; 2) 50 ml hexane/benzene (1:1, v/v); 3) 50 ml hexane/benzene (2:8, v/v); 4) 50 ml benzene; 5) 50 ml chloroform; 6) 50 ml chloroform; 7) 50 ml ether, and 8) 100 ml methanol. Fractions 1—3 (steryl esters) and 6—7 (free sterols) were saponified for 4 hr in 4% KOH in methanol at 65°C.

The sterols were isolated from the saponification products on a 7-g silica gel 60 column (1.0 cm i.d.) by eluting with 20 ml each of 0, 10, 12, 14, 16, 17, 20, 30 and 100% ether graded into hexane. The 4-methylsterols eluted in the 16 and 17% ether fractions and the 4-desmethylsterols eluted in the 20 and 30% ether fractions. The sterols were acetylated with pyridine/acetic anhydride (2:1, v/v) for 16 hr at ambient temperature. The steryl acetates were fractionated on a 5-g 20% $AgNO_3$-Unisil column (1.0 cm i.d) by eluting with 20 ml each of 0, 2, 3, 4, 5, 6, 7 and 100% ether graded into hexane. All chromatographic columns except the 20% $AgNO_3$-Unisil columns were monitored by thin-layer chromatography (TLC) on Anasil H TLC plates (Analabs, North Haven, CT) developed in hexane/ether/acetic acid (8:2:0.1, v/v/v) and visualized by acid charring.

Instrumentation. GC of the sterols and steryl acetates was performed isothermally on a glass column (2mm i.d. × 2 m) packed with 2% OV-17 and a J&W DB-1 fused silica capillary column (0.25 mm i.d. × 15 m, 0.25 μm film) in a Varian model 3700 gas chromatograph equipped with a flame ionization detector (FID). Retention times and areas were recorded on a Shimadzu C-R1B Chromatopac data processor. GC/mass spec-

Abbreviations: FID, flame ionization detector; GC, gas chromatography; MS, mass spectroscopy; RRT, relative retention times; TLC, thin-layer chromatography.

TABLE 1

Lipid and Sterol Content of *Panagrellus redivivus*

Dietary sterol	Total lipid[a] % dry wt	Total sterol[b] % dry wt	Esterified sterol[b] % total sterol
Campesterol	18.5	0.04	2.4
Stigmastanol	17.2	0.03	12.1
7-Dehydrocholesterol	17.7	0.05	2.5

[a]Determined gravimetrically.
[b]Determined by OV-17 GC quantitation.

troscopy of the steryl acetates was performed at 70 eV on a Finnigan model 4510 equipped with a J&W DB-1 fused silica capillary column (0.32 mm i.d. × 30 m, 0.25 μm film) and interfaced with an Incos Data System. Ultraviolet spectroscopy of the steryl acetates (when applicable) was performed with a Perkin-Elmer 559 spectrophotometer scanning between 190 and 400 nm.

RESULTS

The total lipid content on a dry weight basis was similar when *Panagrellus redivivus* was propagated with any of the three dietary sterols (Table 1). The total sterol content ranged from 0.03 to 0.05% of the nematode dry weight. The steryl esters comprised 2.4 and 2.5% of the total sterol when propagated with campesterol and 7-dehydrocholesterol (respectively); however, elevated levels of esterified sterols (12.1%) were present when *P. redivivus* was propagated with stigmastanol.

The isolated sterols and steryl acetates possessed GC relative retention times (RRT) to cholesterol and cholesteryl acetate (Table 2), respectively, identical to authentic standards and were in agreement with previously published values (6—10, 13, 14, 25). The 4α-methylsteryl acetates exhibited the characteristic shift in RRTs previously reported (6). The mass spectra and UV spectra of the steryl acetates were identical to authentic standards and were in agreement with previously published values (6—10, 13, 14, 25). The cholesta-7,9(11)-dien-3β-ol was identified by the mass spectrum and UV spectrum of the acetate. This sterol possessed a mass spectrum for the acetate with ions at m/z (relative intensity): 426 (M^+, 100), 411 (22), 366 (19), 351 (37), 313 (30), 286 (19), 271 (11), 259 (31), 253 (38), with other prominent ions at 211 (62), 199 (33), 183 (21), 171 (22), 157 (31), 145 (50), 131 (42), 118 (30), 105 (43), 81 (40), 69 (40), 55 (50). The molecular ion at m/z 426 indicated a diunsaturated sterol. The fragments for loss of side chain (m/z 313) and loss of side chain and acetic acid (m/z 253) indicated that both double bonds were in the nucleus. The prominent molecular ion (m/z 426) for the acetate indicated the absence of a C-5 double bond. The UV spectrum exhibited λ_{MAX}^{EtOH} nm: 233, 243, 253 characteristic of a 7,9(11)-ring system (25). This sterol was therefore identified as cholesta-7,9(11)-dien-3β-ol.

Exclusive of dietary sterol, the steryl ester fractions contained the same sterols as the free sterol fractions and were enriched with 4α-methylsterols (Table 3). When *P. redivivus* was propagated with 7-dehydrocholesterol as the dietary sterol, the major recovered free sterol was lathosterol (46.8%) with lesser amounts of dietary sterol (31.0%). Cholesta-5,7,9(11)-trien-3β-ol comprised only 5.3% of the free sterol fraction but was the major sterol in the sterol ester fraction (51.8%).

P. redivivus metabolized greater than 70% of the campesterol taken up from the medium; however, less than 57% of the isolated free sterols were dealkylated at C-24 (Table 3). Exclusive of dietary sterol, the major isolated free sterols were cholesterol (36.1%) and cholestanol (15.1%) with lesser amounts of campestanol (9.0%). Substantial quantities of the unusual 24-methyl-23-dehydrocholesterol were also present as previously reported (14).

When *P. redivivus* was propagated with stigmastanol, 93.1% of the isolated free sterol was dealkylated at C-24 (Table 3). The major isolated free sterols were cholestanol (79.9%) and 4α-methylcholestanol (7.4%). Only 1.8% of the free sterol possessed nuclear unsaturation, whereas higher levels (8.9%) of unsaturated sterols were detected in the steryl ester fraction.

TABLE 2

Gas Chromatographic Relative Retention Times of Sterols and Steryl Acetates Isolated From *Panagrellus redivivus*

Sterol	RRT[a] DB-1	RRT[a] OV-17
Cholesterol	1.00	1.00
Cholestanol	1.02	1.02
Lathosterol	1.12	1.17
Campesterol	1.30	1.32
Campestanol	1.32	1.34
24-Methyl-23-dehydrocholesterol	1.26	1.36
Sitosterol	1.60	1.64
Stigmastanol	1.63	1.66
Desmosterol	1.08	1.20
Cholest-24-enol	1.11	1.26
7-Dehydrocholesterol	1.11	1.17
Cholesta-5,7,9(11)-trienol	0.98	1.06
Cholesta-7,9(11)-dienol	1.05	1.16
4α-Methylcholestanol	1.20 (1.17)	1.15 (1.13)
4α-Methylcholest-7-enol	1.31 (1.27)	1.35 (1.32)
4α-Methylcholest-8(14)-enol	1.17 (1.14)	1.16 (1.13)
4α,24α-Dimethylcholestanol	1.53 (1.50)	1.52 (1.48)

[a]Relative retention times of sterols to cholesterol. If not indicated, relative retention times of the steryl acetates to cholesteryl acetate are the same as those for the sterol to cholesterol.
[b]Numbers in parentheses are relative retention times of steryl acetates relative to cholesteryl acetate.

TABLE 3

Relative Percentages of Free Sterol (FS) and Esterified Sterol (ES) Recovered From *Panagrellus redivivus* Grown on Various Dietary Sterols

	Dietary sterol							
	7-Dehydro-cholesterol		Campesterol		Stigmastanol		Sitosterol[a]	Cholesterol[a]
Isolated sterol	FS	ES	FS	ES	FS	ES	Total sterol	Total sterol
Cholesterol	3.1	—	36.1	49.7	0.6	8.2	61.7	69.8
Cholestanol	1.6	2.6	15.1	8.7	79.9	73.0	20.3	26.6
Lathosterol	46.8	30.4	0.6	6.2	1.2	0.7	0.4	0.4
7-Dehydrocholesterol	31.0	—	—	—	—	—	—	—
Cholesta-5,7,9(11)-trienol	5.3	51.8	—	—	—	—	—	—
Cholesta-7,9(11)-dienol	5.2	2.7	—	—	—	—	—	—
Campesterol	—	—	29.8	16.7	—	—	—	—
Campestanol	—	—	9.0	5.1	—	—	—	—
24-Methyl-23-dehydrocholesterol	—	—	4.1	2.3	—	—	—	—
Sitosterol	—	—	—	—	—	—	6.7	—
Stigmastanol	—	—	—	—	6.9	1.2	0.1	—
Desmosterol	—	—	3.1	2.4	—	—	6.1	—
Cholest-24-enol	—	—	—	—	4.0	2.2	—	—
4α-methylcholestanol	—	—	1.9	2.1	7.4	14.7	4.2	3.2
4α-methylcholest-7-enol	0.2	3.2	—	—	—	—	—	—
4α-methylcholest-8(14)-enol	0.7	8.8	—	—	—	—	—	—
4α,24α-dimethylcholestanol	—	—	0.3	6.8	—	—	—	—
Others	6.1	—	—	—	—	—	0.5	—

[a]Previously reported (9).

DISCUSSION

The total lipid and sterol content are consistent with previous reports for *P. redivivus* and other species of free-living nematodes (6—9, 12). However, elevated levels of esterified sterols (12.1%) occurred when the nematodes were propagated with stigmastanol. The reason for the elevated levels of steryl esters when propagated with a sterol with a saturated nucleus is currently unknown. Previous reports with *Caenorhabditis elegans* indicate that the levels of steryl esters are depressed from the usual 13% to 21% level to 7.3% when propagated with stigmastanol (9). However, when *C. elegans* was propagated with cholestanol, 19.1% of the sterols were found in the steryl ester fraction (12). This might be indicative of the variability of the steryl ester fraction with respect to a storage function or might be peculiar to the metabolism of sterols with a saturated nucleus in these nematodes.

P. redivivus has the ability to dealkylate the 24-alkyl group of phytosterols, reduce a Δ^5-bond and introduce a methyl at C-4 of the sterol nucleus (Table 3). Dealkylation of the 24-alkyl group appears to be independent of nuclear unsaturation as both sitosterol and stigmastanol are dealkylated to greater than 80%. The presence of sterol metabolites possessing the Δ^{24}-bond when *P. redivivus* was propagated with 24-alkylsterols suggests that dealkylation proceeds via 24-alkylsterol → 24-alkenesterol → 24-desalkyl-24-dehydrosterol as demonstrated in *C. elegans* and *Turbatrix aceti* (7–11, 13, 14). As in *C. elegans, P. redivivus* is less efficient in removing a 24-methyl group than a 24-ethyl group, as 43.2% of the free sterols still possessed a methyl group at C-24 when the nematodes were propagated with campesterol as the dietary sterol. *C. elegans* was demonstrated to exhibit a preference for demethylating 24β-methylsterols as only 43% of the dietary campesterol (24 α-epimer) was dealkylated but 67% of the dietary 22-dihydrobrassicasterol (24 β-epimer) was dealkylated (9). Whether this preference is shared by *P. redivivus* is unknown. The presence of the unusual 24-methyl-23-dehydrocholesterol in *P. redivivus* and *C. elegans* suggests that an alternative minor demethylation pathway may also exist in these nematodes as previously reported (14).

P. redivivus reduces the Δ^5-bond in the sterol nucleus, as evidenced by the production of substantial quantities of cholestanol when the nematodes were propagated with campesterol, sitosterol or cholesterol and by the production of lathosterol when propagated with 7-dehydrocholesterol (Table 3). The production of 4α-methylcholest-8(14)-enol in 7-dehydrocholesterol-fed *P. redivivus* suggests that isomerization from the Δ^7- to the $\Delta^{8(14)}$-bond also occurs as in other nematodes (6—13). An isomerization mechanism is supported by the lack of $\Delta^{8(14)}$-sterols when *P. redivivus* is propagated with any of the Δ^5-sterols. *P. redivivus* might reduce the Δ^7-bond to some extent, as small quantities of cholesterol and cholestanol are detected when the nematodes are propagated with 7-dehydrocholesterol. However, the lack of 4α-methylcholestanol, the preferred 4-methylsterol in *P. redivivus*, suggests that this does not occur. It appears more likely that the cholesterol is a contaminant concentrated from the medium and the cholestanol is a metabolite of this contaminant.

Whether *P. redivivus* is capable of introducing a double bond into the sterol nucleus is unclear. The small quantities of lathosterol detected when the nematodes were propagated with the Δ^5-sterols and with the Δ^0-sterol would indicate that Δ^7-unsaturation might occur to some extent. The introduction of a Δ^7-double bond has been demonstrated in the free-living nema-

todes *Caenorhabditis elegans* (6—10, 12) and *Turbatrix aceti* (8, 13). This is not limited to nematodes, as some marine invertebrates (26—28) and certain insects (29—31) share this ability. However, the isolation of consistently low levels of Δ^7-sterols when *P. redivivus* was propagated with any of the Δ^5-sterols or the Δ^0-sterol indicates that the lathosterol might also be concentrated from the medium. Trace quantities (<15 μg/l) of ergosterol (24β-methylcholesta-5,7,22-trien-3β-ol) from the yeast extract have been detected when some media preparations have been extracted (unpublished data). The low levels of lathosterol might represent the concentration and metabolism of this trace media contaminant. Investigations with radiolabeled dietary sterols should resolve this question.

Of particular interest is the 4α-methylation pathway, which is unique to nematodes. The 4α-methylsterols of *P. redivivus* consist entirely of 4α-methylstanols except when propagated with 7-dehydrocholesterol. In the latter case, the Δ^5-double bond is still reduced indicating that lathosterol may be a better substrate for the 4α-methylation enzymes than 7-dehydrocholesterol. In campesterol-fed nematodes, the 4α-methylation enzymes were not hindered by the presence of the 24-methyl group as the majority of the 4α-methylsterols in the steryl ester fraction were still alkylated in the side chain. In contrast, a 24-ethyl substituent apparently abolishes any affinity for binding between the sterol substrate and the nuclear methylating enzymes.

P. redivivus is similar to several economically important genera of plant-parasitic nematodes in producing substantial quantities of saturated sterols from unsaturated dietary sterols (32). The Δ^5 and Δ^0-sterols may be better suited for reproduction and development in these nematodes. Whether all plant-parasitic nematodes are also capable of C-24 dealkylation and 4α-methylation is unknown and warrants further investigation. The nutritional requirement of *P. redivivus* for exogenous sterol, the production of substantial quantities of saturated sterols and the ability to disrupt nematode growth and reproduction by inhibitors of nematode sterol metabolism make *P. redivivus* a good model organism for development of compounds that may disrupt the nematode life cycle.

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Quantification, Characterization and Fatty Acid Composition of Lysophosphatidic Acid in Different Rat Tissues

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The amount and composition of lysophosphatidate present in different rat tissues have been estimated by an internal standard method in which a synthetic unnatural isomer (1-heptadecanoyl-*rac*-glycerol-3-phosphate) was added to the total lipid extracts, and the fatty acid composition of purified lysophosphatidate was determined. Lipids from tissues were extracted under acidic conditions, and the lysophosphatidate was purified by solvent partitions followed by thin-layer chromatography in multiple solvent systems. The purified lipid was shown to be 1-acyl-*sn*-glycerol-3-phosphate by chromatographic and chemical analysis, by its resistance to hydrolysis when treated with phospholipase A_2 and also by its complete conversion to 1-acyl-*sn*-glycerol when treated with alkaline phosphatase. The fatty acid consituents of this lipid were determined by gas-liquid chromatography of the derived methyl esters. The concentrations (nmol/g of tissue) of lysophosphatidate in various tissues were: 86.2 ± 4.2 in brain, 60.3 ± 6.3 in liver, 46.4 ± 6.5 in kidney, 30.6 ± 5.0 in testis, 22.3 in heart and 19.3 in lung. Mostly (80%) saturated fatty acids were found to be present in this lyso lipid. A significantly high level of stearic acid was present in this lipid from all the tissues (50–60% in liver, kidney, brain and testis, and about 40% in heart and lung) compared to palmitic acid (10–15% in liver, kidney and brain and 25–30% in testis, heart and lung). The fatty acid compositions of phosphatidic acid, the putative product of lysophosphatidate acylation, from different tissues were also determined and palmitate was found to be the major saturated fatty acid. These results suggest that tissue lysophosphatidic acid is not only formed by *de novo* biosynthesis but is also generated via the breakdown of phospholipids such as phosphoinositides.
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Lysophosphatidic acid (lysoPA) is a key intermediate for the biosynthesis of glycerolipids (1–3). This lipid is formed in three different subcellular compartments, either via direct acylation of glycerol-3-phosphate (G-3-P) in mitochondria and microsomes or via acylation of dihydroxyacetone phosphate (DHAP) in peroxisomes followed by reduction (2–4). This lyso lipid may also originate from the degradation of other lipids (5). The steady state concentrations of this important phospholipid in different animal tissues and its fatty acid compositions have not yet been reported in the literature. This is probably because during standard lipid extraction method (6), at neutral pH, this acidic lyso lipid is lost with the aqueous wash and therefore escapes detection (7). We recently developed a combined acid extraction and solvent partition method to isolate acyl DHAP from guinea pig liver and found that lysophosphatidate is extracted and partitioned in the same manner (8). After purification by TLC and analysis it was found that guinea pig liver contained fairly high level of lysoPA (140 nmol/g) and stearic acid was the major fatty acid component (66%) of this lipid. We also estimated lysoPA in three different rat tissues by enzymatically measuring the *sn*-glycerol-3-phosphate released after alkaline methanolysis of partially purified lipid extracts and found it to be relatively high (50–90 nmol/g) (8). However, in that work the loss incurred during purification of lysoPA was not considered and whether the lysoPA in rat tissues was also enriched in stearic acid had not been determined. Therefore, the present method of analysis of lysoPA in different tissues was developed where an internal standard was used to account for the loss of lysoPA during its extensive purification and for the quantitative determination and the fatty acid composition of this polar lipid. These results are reported here.

MATERIALS

Gas-liquid chromatographic (GLC) reference mixture of saturated fatty acid methyl esters of different carbon chains was purchased from Nu Chek Prep, Inc. (Elysian, Minnesota) and that of polyunsaturated fatty acids (PUFA, No. 2, animal source) was from Supelco, Inc. (Bellefonte, Pennsylvania) and used for the identification of unknown saturated and unsaturated fatty acids. n-Heptadecanoic acid was from Aldrich Chemical Co. (Milwaukee, Wisconsin); the methyl ester of this compound showed a single peak on GLC chromatogram. Authentic lipids including lysoPA, phosphatidic acid (PA), 1- and 2-palmitoyl-*sn*-glycerol, phospholipase A_2 from *Naja naja* venom and alkaline phosphatase from *E. coli* were obtained from Sigma Chemical Co. (St. Louis, Missouri). 1-Heptadecanoyl DHAP was prepared from n-heptadecanoic acid and glycolic acid as described before (9). 1-Heptadecanoyl-*rac*-G-3-P, which was used as an internal standard during the present studies for estimation of lysoPA, was made by reducing 1-heptadecanoyl DHAP by $NaBH_4$ in ethanol at pH 7.5 for 2 hr at 37°C as described before (8). This synthesized material gave one spot on TLC in different solvent systems and was found to contain only heptadecanoic acid as checked by GLC of the derived methyl ester. The fatty acid to phosphate ratio was 1:1. The phosphorus concentration of this material and other lipids was determined by the method of Ames and Dubin (10). Adult Sprague-Dawley male rats (200–250 g) were purchased from Harlan Sprague-Dawley, Inc. (Indianapolis, Indiana) and fed standard diet (Purina Chow) and water ad libitum. Other materials were the same as described previously (8,9).

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METHODS

Extraction of lysoPA from tissues. Total lipids from liver, kidney, brain, testis, heart and lung of adult rats (150–200 g) were extracted by an acidic solvent extraction method (8). The rats were anesthetized with diethyl ether, the tissues were removed and immediately (within 15 sec) frozen in an isopentane/dry ice bath and, if necessary, stored at −70°C. The frozen tissues were weighed and quickly ground into small pieces with a pre-chilled (−20°C) mortar and pestle and immediately homogenized in chloroform/methanol/2M H_3PO_4 (1:1:0.05). During homogenization of the tissues, 25 nmol of synthetic 1-heptadecanoyl-*rac*-G-3-P per g of tissue was added to the homogenates. The lysoPA was separated from most other tissue lipids by partitioning the lipids between two phases, first at pH 4.4 where lysoPA was partitioned into the upper aqueous layer and then at pH 1.5 when lysoPA is extracted back into the lower chloroform layer by the methods previously described (8).

Purification of lysoPA. This partially purified lysoPA was further purified to homogeneity by using three consecutive thin layer chromatographic systems. The lipid extract was put as a band on a preparative TLC plate (E. Merck, Silica Gel-60, 0.5 mm thick) along with an authentic standard lysoPA spot side-by-side and the plate was developed in the first solvent system, chloroform/methanol/acetic acid/5% sodium bisulfite (100:40:12:4). The lipid bands were visualized under UV light after spraying the plate with Primuline (11). The lysoPA band was located by comparing its mobility to that of standard lysoPA (Rf = 0.33) and the content of this band was recovered by scraping it out and extracting the scrapings three times with chloroform/methanol (1:1) containing HCl (0.1M). The combined extract was washed with half volume of water to remove the acid. The lower layer containing the lysoPA was dried by blowing nitrogen, and the dried lipid was subjected to a second TLC using a basic solvent system i.e. chloroform/methanol/8 M NH_4OH (60:40:5). The lysoPA (Rf = 0.07) was recovered from the TLC plate as described above and then finally purified by TLC by using another solvent system of chloroform/methanol/acetic acid/acetone/water (100:30:20:40:10) (12). The lysoPA (Rf = 0.46) was extracted out from the TLC spot as described above and used for analysis.

Hydrolysis by alkaline phosphatase. About 50 μg of standard lysoPA or rat liver lysoPA (purified without the addition of the internal standard) dispersed in Tris buffer (0.15M, pH 8.5) by sonication was incubated with *E. coli* alkaline phosphatase (35 μg, 1.5 U) in a final volume of 0.5 ml. The mixture was incubated at 37°C for 2 hr. The product was extracted using an acidic Bligh and Dyer extraction method (13). When necessary, the progress of the reaction was studied by taking out an aliquot of the reaction mixture, extracting the lipids under acidic conditions (8) and separating them on TLC using chloroform/methanol/acetic acid/water (100:40:12:4). In this system the product monoacyl glycerol has an Rf of 0.88 and the Rf of lysoPA is 0.42.

Separation of monoacylglycerols. The positional isomers of monoacyl glycerol(1-*sn* and 2-*sn*) were separated from each other by TLC on silica gel containing boric acid (14). The boric acid impregnation was done by immersing the plates (5 × 20 cm Merck Silica gel-60) in methanolic boric acid (12% w/v) for 30 min. The plates were air dried for 15 min, activated at 110°C for 30 min and used immediately. By this procedure, the TLC plate was found to contain boric acid about 10% of the weight of the adsorbent. The monoacylglycerols were separated from each other by using a solvent system of chloroform/acetone (90:10). The respective Rf values for 1-palmitoyl and 2-palmitoyl-*sn*-glycerol were 0.12 and 0.20.

Extraction and purification of PA. The lower, washed chloroform layer which was saved during the lipid partitioning process (8) was concentrated under a stream of N_2. From this total lipid extract PA was isolated by two-dimensional TLC on a preparative plate (0.5 mm thick E. Merck Silica gel 60) using the solvent mixture of chloroform/methanol/8 M HN_4OH (17:7:1) in the first dimension followed by chloroform/methanol/acetic acid/water (80:40:7.4:1.2) in the second dimension essentially as described by Pumphrey (15). Purified PA migrated as a single spot having Rf of 0.04 in the first and 0.55 in the second solvent.

Treatment with phospholipase A_2. To 100 μg of PA or lysoPA dissolved in 0.5 ml of diethyl ether/methanol (98:2), 50 μl of a solution of lyophilized *Naja naja* venom (1 mg/0.5 ml of 0.3 M Tris-HCl, pH 7.5 containing 20 mM $CaCl_2$) was added. The mixture was vortexed vigorously for 30 sec and then incubated at room temperature for 3–4 hr with gentle magnetic stirring under N_2 essentially as described by Wells and Hanahan (16). After the reaction, most of the ether and methanol were evaporated off under N_2 and the product was dissolved in 2 ml of chloroform/methanol (1:1), followed by the addition of 0.8 ml of water containing 0.1M HCl. The lower chloroform layer was washed with chloroform/methanol/water (1:12:12). An aliquot of the lipid extract was put on TLC plate and developed with chloroform/methanol/acetic acid/water (100:40:12:4) for identification of the products by comparing the Rf values of standard compounds applied side by side. The Rf values of PA, lysoPA and free fatty acid in this solvent system were 0.77, 0.42 and 0.98, respectively.

Preparation and analysis of fatty acid methyl esters from lysoPA and PA. Fatty acid methyl esters were prepared from these lipids by alkaline methanolysis (17) with modification. In a screw-capped tube, a portion of lipid solution (~ 15 μg of lipid phosphorus) was dried down under N_2. One ml of 0.2 M NaOH in methanol and 2.0 ml of chloroform were added. The reaction mixture was mixed well and left at room temperature. After one hr of incubation, 0.75 ml of 0.35 N acetic acid was added, mixed well, and the phases were separated by centrifugation. Upper layer was removed and the lower layer was washed with 2 ml of 0.9% NaCl/methanol mixture (1:1). The methyl esters present in the final chloroform layer were purified by TLC using a solvent system of n-hexane/ether/acetic acid (50:50:2). The purified ester (Rf = 0.67) was extracted from TLC powder by three extractions with diethyl ether. After removing the ether by blowing N_2, the sample was dissolved in a small volume of n-hexane

and analyzed in a Hewlett-Packard gas chromatograph, Model No. 5710A, equipped with flame ionization detector and an electronic integrator (Spectra Physics). The stationary phase was 15% Silar 10C supported on Gas Chrom R in a glass-lined stainless steel analytical column (183 cm × 0.32 cm) and the temperature programming of 150-220°C was used for the GLC run. The components of methyl esters were identified by comparing the retention times with those of standards.

Estimation of lysoPA in tissues. The amount of lysoPA present in different tissues was evaluated from the relative amount of 17:0 in the fatty acid mixtures derived from the lipid. Because the endogenous 17:0 content of lysoPA isolated from different tissues was found to be very low (<1% of total fatty acids), it was assumed that all the 17:0 present in the final GLC analysis of the fatty acid composition of lysoPA was from the heptadecanoyl GP added (25 nmol/g tissue) during the lipid extraction process. This and the assumption that the proportional loss of this internal standard during purification is the same as the other molecular species of lysoPA were used to calculate the lysoPA content of different tissues. For example, nmol lysoPA/g tissue = [(100 − wt % 17:0)/wt % 17:0] × 25. The amount of each individual fatty acid present in lysoPA can also be calculated this way.

All other methods were the same as described previously (8,9). Lipids were stored, when necessary, at −70°C under N_2.

RESULTS

Isolation and characterization of lysoPA. The lysoPA purified by the above method migrated as a single spot with the mobility same as standard lysoPA in two other TLC systems: (a) chloroform/methanol/2-propanol/0.25% aqueous potassium chloride/ethyl acetate (30:9:25:6:18) (18) (Rf = 0.15) and (b) chloroform/methanol/acetic acid/water (100:40:12:4) (Rf = 0.42). When analyzed (8), the phosphorus/*sn*-glycerol-3-P/fatty acid molar ratio in different lysoPA tissue samples was found to be 1.0:1.0:1.0. From these criteria the isolated lysoPA was judged to be pure. The recovery of lysoPA from each TLC purification was 80-90% as measured by using radioactive lysoPA (8). The overall recovery of lysoPA from the crude lipid extract varies from 50-60% as indicated by the recovery of the internal standard (17:0) by GLC.

The lysoPA isolated from liver and kidney seemed to be 1-acyl isomer since it was not hydrolyzed by phospholipase A_2 as indicated by the absence of free fatty acid as a product when analyzed on the TLC plate and the total phosphorus content of lysoPA before and after the enzymatic treatment was the same. This is confirmed by hydrolyzing the lysoPA by alkaline phosphatase and analyzing the product. The lysoPA from liver, like the standard lysoPA, was completely hydrolyzed in 2 hr when treated with alkaline phosphatase as described in Methods. The resulting product, i.e., monoacyl glycerol, when chromatographed on 10% boric acid impregnated silica gel plate migrated as a single spot with authentic 1-palmitoyl-*sn*-glycerol. There was no spot corresponding to 2-palmitoyl-*sn*-glycerol.

TABLE 1

Lysophosphatidate Content of Different Rat Tissues[a]

Tissue	LPA (nmol/g of tissue) ± S.D.
Liver	60.3±6.3 (n=4)
Kidney	46.4±6.5 (n=4)
Brain	86.2±4.2 (n=3)
Testis	30.6±5.0 (n=3)
Heart	19.5, 25.1[a]
Lung	17.0, 21.6[a]

[a]The contents of LPA in different tissues of rat were estimated by GLC analysis of the fatty acid methyl esters including the internal standard methyl heptadecanoate (17:0) as described in the text.

n, Number of experiment; a, results of two experiments.

Tissue distribution of lysoPA. As shown in Table 1, the contents of lysoPA in different tissues of rat ranged from 20-90 nmol/g of tissue. Brain contained the highest level of this lipid (78-92 nmol/g of tissue) compared to other organs (20-60 nmol/g of tissue). Very little variation was observed in the lysoPA content of corresponding tissues from different animals. This is probably because of the use of adult animals of the same age group and using identical conditions to harvest the tissues. The amount of lysoPA present, however, is sufficient to isolate it from a small amount (0.5 g) of tissues and accurately analyze its fatty acid composition.

The presence of alkali stable lysoPA, i.e., lysoPA which may contain ether bond (instead of ester bond) was investigated by using rat kidney lysoPA. The phosphorus concentration before and after alkaline methanolysis of lysoPA was determined. The results indicated that about 10% of kidney lysoPA was alkali stable. The nature of this alkali stable lipid, however, was not investigated further. This is most probably alkyl G-3-P as any alkenyl G-3-P will be degraded by the repeated acidic extractions used to purify the lysoPA.

Fatty acid compositions of lysoPA from different tissues. Table 2 presents the fatty acid compositions of lysoPA from six different tissues of rat. Basically,

TABLE 2

Fatty Acid Compositions of LPA in Different Tissues of Rat[a]

Fatty acid	Composition in wt %					
	Liver	Kidney	Brain	Testis	Heart	Lung
14:0	9.5	5.2	0.5	tr	0.6	tr
16:0	16.0	13.6	9.9	31.5	26.1	28.4
18:0	50.6	59.9	51.3	56.6	35.6	42.6
18:1(n-9)	17.3	10.3	31.6	9.2	25.4	20.8
18:2(n-6)	5.2	6.9	tr	1.6	6.2	7.1
18:3(n-3)	—	—	5.0	—	1.5	1.0
20:4(n-6)	1.4	2.1	1.5	0.9	tr	tr

[a]The methyl esters were prepared from the purified LPA of different tissues containing 1-O-heptadecanoyl-rac-G-3-P as internal standard and analyzed by GLC as described in the text. The wt % of each fatty acid was calculated from the known amount of the internal standard (17:0). The data are the averages (ranges ± 3%) of three experiments.

tr, Trace, less than 0.1 %.

16:0, 18:0 and 18:1 were the three major components of lysoPA from all tissues. As can be seen (Table 2), 18:0 was the major fatty acid present in the lysoPA, e.g., more than half (50–60%) of total fatty acids were composed of 18:0 in the lysoPA isolated from liver, kidney, brain and testis. The ratio of 18:0/16:0 is also very high (3 to 5) in most of the tissues (Table 2). The fatty acid compositions of lysoPA from all these tissues were found to be very similar to guinea pig liver lysoPA which also contained very high amounts of stearic acid (65.9%) (8).

Purification and fatty acid composition of PA from different tissues. We also analyzed the fatty acid compositions of PA, the putative biosynthetic product from lysoPA, isolated from different tissues. Although the fatty acid compositions of rat liver and brain PA have been reported previously (19, 20), no information is available regarding the composition of PA of other organs. PA was isolated by two dimensional TLC using the same solvent systems as described by Pumphrey (15). An excellent and reproducible separation of this lipid from all other lipids was achieved; however, instead of the double spots for PA reported by Pumphrey (15), a single spot was found. This difference is probably because under the acidic lipid extraction used here, all the tissue phosphatidates were converted to the free acid form which migrated as a single spot on the TLC plate.

The amounts of PA present in various organs of rat, human and other animals have been reported by other workers (21—23). The fatty acid compositions of PA of different tissues of rat are presented in Table 3. It should be noted that, except in the case of brain and heart, the levels of 16:0 in the PA of different tissues were much higher than that of 18:0. For example, the ratio of 16:0/18:0 in brain was 0.53, which is lower than that in liver (1.48), kidney (1.25), testis (2.62) lung (1.31) and heart (0.91) (Table 3). The values of this ratio as calculated from literature reports are 2.2 (19) and 0.87 (20) for rat liver and brain, respectively. Among the mono- and polyunsaturated fatty acids, 18:1, 18:2 and 20:4 were the major components; however, in brain, a significant amount of 18:3 (6.0%) was also present.

Possmayer et al. have shown that rat liver PA contained 51% of 16:0 and 33% of 18:0 at the *sn*-1 position of glycerol (19). We determined the positional distribution of fatty acids in rat kidney PA by stereospecific hydrolysis with phospholipase A_2 and then analyzing the fatty acid composition of the resulting lysoPA. The results showed that 16:0 is present at a relatively higher amount (53%) than 18:0 (24%) at the *sn*-1 position of rat kidney PA.

TABLE 3

Fatty Acid Compositions of PA in Different Tissues of Rat[a]

Fatty acid	Composition in wt %					
	Liver	Kidney	Brain	Testis	Heart	Lung
14:0	1.8	3.8	tr	0.3	0.2	0.8
16:0	23.2	24.4	10.7	38.0	18.5	23.7
16:1(n-9)	4.0	3.7	0.8	2.2	2.1	3.5
18:iso	1.0	0.2	0.5	0.2	—	0.5
18:0	15.6	19.6	20.1	14.5	20.3	18.0
18:1(n-9)	20.1	12.2	33.0	17.5	14.6	15.7
18:2(n-6)	21.7	8.8	3.1	4.3	20.3	14.3
20:0	—	2.2	2.0	3.8	3.0	2.6
18:3(n-3)	1.5	0.2	6.0	—	—	1.6
18:4(n-3)	—	—	0.8	—	0.2	tr
20:2(n-6)	0.3	0.2	0.8	tr	0.4	0.5
20:3(n-6)	0.4	2.4	2.8	1.6	1.5	1.7
20:4(n-6)	6.3	10.0	9.7	9.5	6.6	6.6
20:5(n-3)	0.4	—	0.6	—	0.3	0.6
22:4(n-6)	—	0.9	2.8(?)	—	tr	0.9
22:5(n-3)	—	—	—	3.7(?)	—	0.7
22:6(n-3)	—	—	0.3	—	2.2	0.9

[a]PA from different tissues were isolated by two dimensional TLC and the total fatty acid contents were analyzed by GLC (see text). The data are the averages (ranges ± 5%) of two experiments.

tr, Trace, less than 0.1 %.

DISCUSSION

The use of the internal standard, 1-heptadecanoyl glycerol-3-P, was advantageous for the simultaneous quantification of the amount and fatty acid composition of the lipid. Since, like any other internal standard method a quantitative recovery of lysoPA was not necessary, the estimation was more easily done than any other method. This method should have a general applicability for the accurate analysis of any tissue lipid as the standards containing unnatural fatty acids can be easily synthesized by chemical or enzymatic methods.

As described above, lysoPA isolated from different tissues was found to be pure by chromatographic and chemical analysis. The lysoPA of rat liver was characterized as 1-acyl-*sn*-glycerol-3-P by the following criteria: (a) After alkaline methanolysis of the lipid, the products, i.e., fatty acid methyl esters and *sn*-glycerol-3-P formed were in 1:1 molar proportion. (b) Hydrolysis by alkaline phosphatase yielded 1-acyl-*sn*-glycerol as the sole lipid product. (c) The lipid is not hydrolyzed by phospholipase A_2. Van Deenen and de Haas (24) have shown that lysophosphatides (2-acyl) are hydrolyzed by snake venom phospholipase A_2.

Though the exact structure of lysoPA of organs other than liver was not determined, the similarity in the chromatographic migration rate with rat liver lysoPA, formation of 1:1 molar proportion of fatty acid and *sn*-glycerol-3-P after alkaline hydrolysis and the presence of mainly saturated fatty acids in the purified lysoPA (Table 2) indicate that this lipid in all tissues is predominantly 1-acyl-*sn*-glycerol-3-P.

The tissue concentrations of lysoPA, as reported here, are relatively high (20–90 nmol/g of tissue) compared to similar lipid biosynthetic intermediates such as acyl DHAP (2–10 nmol/g of tissue) (8) or CDP diacylglycerol (9–15 nmol/g of tissue) (25) but are lower than the corresponding amounts of phosphatidic acid present in these tissues (150–200 nmol/g of tissue) (21, 22). LysoPA is biosynthesized in three different subcellular compartments, i.e., mitochondria, peroxisomes and endoplasmic reticulum, but only in endoplasmic reticulum it is enzymatically acylated to PA (2, 3). This may account for the relatively high steady-state concentration of lysoPA in different tissues because the mitochondrial and peroxisomal-derived lysoPA must be transported to the endoplasmic reticulum for lipid biosynthesis. Also, from the high stearate content of

lysoPA (Table 2), it seems that a large fraction of tissue lysoPA originates from sources other than via the biosynthetic routes. This is because biosynthetically-derived lysoPA should have mostly 16:0, as both glycerophosphate acyltransferase and dihydroxyacetone phosphate acyltransferase have been shown to preferentially utilize 16:0 rather than 18:0 for the acylation of GP and DHAP, respectively (26-28). The predominance of 16:0 at the *sn*-1 position of PA as shown here and by other workers (19) also indicates that biosynthetic lysoPA should contain a relatively higher amount of 16:0 than 18:0. Therefore, our results suggest that a major fraction of lysoPA is probably formed via catabolic breakdown of lipids. One such possibility is the formation of this lipid from phosphoinositides which have mostly 18:0 at the *sn*-1 position (20, 29, 30). Lapetina and co-workers have shown that by the consecutive actions of phospholipase C, diacylglycerol kinase and PA-specific phospholipase A_2, the phosphoinositides are converted to lysoPA (5, 31). Therefore, it is possible that a portion of the tissue lysoPA originates from such receptor mediated breakdown of membrane lipids (32, 33). Further experiments such as the effects of chronic stimulation of phosphoinositide-linked receptor on the concentration and composition of lysoPA in cells would be useful to establish the source and function of this polar lipid in cellular metabolism.

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Dietary Saturated Fat Level Alters the Competition Between α-Linolenic and Linoleic Acid

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Male weanling rats were fed semi-synthetic diets high in saturated fat (beef tallow) vs high in linoleic acid (safflower oil) with or without high levels of α-linolenic acid (linseed oil) for a period of 28 days. The effect of feeding these diets on cholesterol content and fatty acid composition of serum and liver lipids was examined. Feeding linseed oil with beef tallow or safflower oil had no significant effect on serum levels of cholesterol. Serum cholesterol concentration was higher in animals fed the safflower oil diet than in animals fed the beef tallow diet without linseed oil. Feeding linseed oil lowered the cholesterol content in liver tissue for all dietary treatments tested. Consumption of linseed oil reduced the arachidonic acid content with concomitant increase in linoleic acid in serum and liver lipid fractions only when fed in combination with beef tallow, but not when fed with safflower oil. Similarly, ω3 fatty acids (18:3ω3, 20:5ω3, 22:5ω3, 22:6ω3) replaced ω6 fatty acids (20:4ω6, 22:4ω6, 22:5ω6) in serum and liver lipid fractions to a greater extent when linseed oil was fed with beef tallow than with safflower oil. The results suggest that the dietary ratio of linoleic acid to saturated fatty acids or of 18:3ω3 to 18:2ω6 may be important to determine the cholesterol and arachidonic acid lowering effect of dietary α-linolenic acid.
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Hypocholesterolemic effects of polyunsaturated fatty acids have been thought to be mediated via a redistribution of cholesterol from blood to the tissue pools (1-8). In a recent study, we demonstrated that this redistribution may not be true for all types of polyunsaturated fatty acids but may be restricted to ω6 fatty acids (9). Omega-3 fatty acid from linseed oil (i.e., 18:3ω3) decreases cholesterol content of plasma without affecting the liver cholesterol concentration (9). Omega-3 fatty acids of marine origin such as eicosapentaenoic acid (20:5ω3) and docosahexaenoic acid (22:6ω3) are more effective than ω6 fatty acids in reducing serum or plasma levels of cholesterol (10-13). In terms of platelet aggregation, polyunsaturated fatty acids of the ω6 family present in vegetable oils have pro-aggregatory thrombotic effects (14-16) while the ω3 polyunsaturated fatty acids of seafoods increase bleeding time. On this basis, we postulated that 18:3ω3 may have an intermediate place for prevention of atherosclerosis and thrombosis between ω6 fatty acids and C20 and C22 ω3 fatty acids (20:5ω3 and 22:6ω3) of marine oils (9).

The present study examined the effects of feeding 18:3ω3 to rats in the presence of either saturated fatty acids or linoleic acid on the cholesterol content and fatty acid composition of serum and liver lipids. The results suggest that the dietary ratio of 18:3ω3/18:2ω6 or of linoleic acid to saturated fatty acids may be important in determining the cholesterol and 20:4ω6 lowering effect of dietary 18:3ω3.

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MATERIALS AND METHODS

Animals and diets. Male Sprague-Dawley rats weighing 30-40 g were maintained on Wayne rat chow (Allied Mills Inc., Chicago, IL) for three days before feeding the experimental diets. The semipurified diets fed (20) contained 200 g fat/kg diet from one of the following sources: 90% w/w hydrogenated beef tallow plus 10% w/w safflower oil (beef tallow diet); 100% w/w safflower oil (safflower oil diet); 70% w/w hydrogenated beef tallow plus 25% w/w linseed oil plus 5% safflower oil (beef tallow and linseed oil diet); 75% w/w safflower oil plus 25% w/w linseed oil (safflower oil and linseed oil diet). The fatty acid composition of the diets is indicated (Table 1). The beef tallow diet contained mainly saturated fatty acids (86.3% w/w of total fatty acids) and enough linoleic acid (9.5% w/w) to prevent essential fatty acid deficiency. The safflower oil diet was enriched with linoleic acid (70.5% w/w). Both the beef tallow and safflower oil diets with linseed oil provided similar amounts of α-linolenic acid (ca. 18% w/w) but differed in linoleic acid to saturated fatty acid ratios (0.16 and 5.85, respectively). Animals were housed individually in hanging stainless steel cages in a well ventilated room maintained at 22°C on a 12/12 hr light/dark cycle. All diets were prepared weekly and stored at −20°C. Food and water were provided ad libitum. Body weights were monitored once a week throughout the feeding trial.

Lipid analysis. After four weeks of feeding experimental diets, the rats were killed by decapitation, between 8 and 10 a.m. Livers were quickly excised and rinsed in ice-cold saline. Blood samples were also collected for separation of serum by centrifugation. Total lipids from serum and liver samples were extracted with chloroform/methanol (2:1, v/v) (21) containing 0.005% (w/v) butylated hydroxytoluene. Total, free and esterified cholesterol contents in aliquots of serum and liver lipid extracts were determined enzymatically (22,23). Total phospholipids, triacylglycerol and cholesterol esters were separated by thin layer chromatography on Silica Gel G plates (Analtech, 250 μm, 20 × 20 cm) using a solvent system comprised of petroleum ether/diethyl ether/acetic acid (80:20:1, v/v/v) (24). Phospholipids were methylated with 14% (w/w) BF_3-methanol reagent at 100°C for one hr (25). Triacylglycerol and cholesterol ester fractions were saponified with 0.5 M methanolic potassium hydroxide by heating at 85°C for 90 min, followed by methylation with 14% (w/w) BF_3-methanol reagent (25). Fatty acid methyl

TABLE 1

Fatty Acid Composition of the Experimental Diets (wt %)

Diet fat	Tallow alone	Tallow plus linseed oil	Safflower oil alone	Safflower oil[a] plus linseed oil
14:0	4.2	2.7	0.3	0.1
16:0	28.9	20.4	7.6	6.2
16:1ω7	0.5	0.4	0.1	0.1
17:0	2.1	1.4	0.1	0.1
18:0	51.2	38.2	5.8	3.4
18:1ω9	3.2	7.4	14.6	15.2
18:1ω7	0.2	0.3	0.2	0.1
18:2ω6	9.5	10.2	70.5	56.7
18:3ω3	0.1	18.8	0.6	17.9
U.I.[b]	23.2	84.9	157.7	182.5
Saturated	86.3	62.7	13.8	9.7
Monounsaturated	4.1	8.1	14.9	15.4
ω6	9.5	10.2	70.5	56.7
ω3	0.1	18.8	0.6	17.9
18:2ω6/SFA ratio	0.11	0.16	5.11	5.85
18:3ω3/18:2ω6 ratio	0.01	1.84	0.01	0.31

[a]Fats were added to the basal diet in the proportions explained in Materials and Methods. The basal diet contained the following components (g/kg diet): casein, 270; starch, 200; glucose, 207; nonnutritive cellulose, 50; vitamin mix, 10; mineral mix, 50; choline, 2.75; L-methionine, 2.5, and inositol, 6.25, as described elsewhere (20).

[b]U.I., Unsaturation Index; Σ [(a)(b)], where a is the percentage of each unsaturated fatty acid and b is the number of double bonds for that particular fatty acid.

esters were extracted and analyzed by automated capillary gas chromatography (Varian Model 6000 equipped with a flame ionization detector) (26). Authentic standard mixtures of fatty acid methyl esters were injected to identify fatty acid methyl esters. Peak areas and weight percent fatty acid composition were computed by a chromatography data system (Varian Model DS 604).

Statistical analyses. The data are expressed as mean ± standard deviation. The effect of diet fat treatment was determined by analysis of variance procedures. The comparison between treatments was made using Newman-Keuls multiple range test (27).

RESULTS

Body weights, liver weights and food consumption. All animals appeared healthy after four weeks of feeding. The dietary fat treatment had no significant effect on body weight, liver weight or liver weight to body weight ratio in these animals. Rats fed the different dietary regimens consumed a similar amount of food (data not shown).

Cholesterol content of rat serum and liver lipids. The total cholesterol content of serum in rats fed the safflower oil diet was significantly higher than those fed the beef tallow diet, while addition of linseed oil to these diets had no significant effect on serum cholesterol levels (Fig. 1). Neither free nor esterified cholesterol content of serum was affected by the dietary fat composition.

In liver tissue, the level of cholesterol was significantly higher in animals fed safflower oil than in those fed the beef tallow diet (Fig. 1). Addition of linseed oil either to the beef tallow or safflower oil diet lowered the cholesterol content of liver. The increase in liver cholesterol concentration following feeding of the safflower oil diet was detected in both free and esterified cholesterol content. The decrease in cholesterol level after consumption of diets containing linseed oil was associated with the esterified cholesterol content and not with the nonesterified cholesterol content (Fig. 1).

Fatty acid composition of rat serum and liver phospholipids. Feeding rats the safflower oil diet increased the 18:2ω6 and 20:4ω6 content of serum and

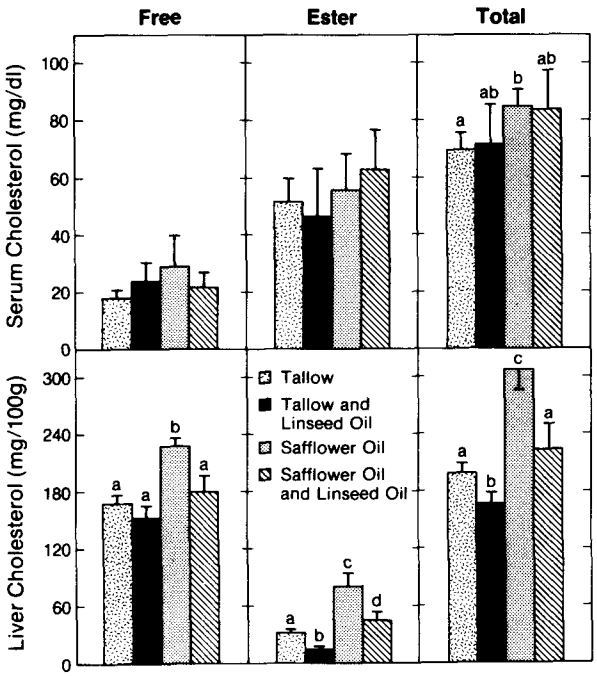

FIG. 1. Effect of dietary α-linolenic acid on cholesterol content of rat serum and liver. Values are the mean ± standard deviation of 5 rats (n = 5) in each dietary group. Values without a common superscript are significantly different ($p < 0.05$).

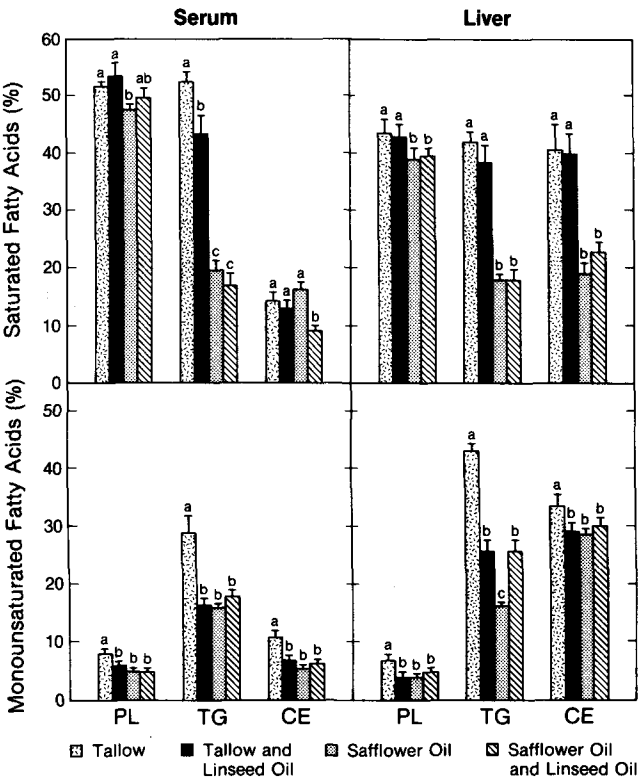

FIG. 2. Effect of dietary α-linolenic acid on saturated and monounsaturated fatty acid content of rat serum and liver lipid fractions. PL, phospholipid; TG, triacylglycerol; CE, cholesteryl ester. Values are the mean ± standard deviation of 5 rats (n = 5) in each dietary group. Values without a common superscript are significantly different ($p < 0.05$).

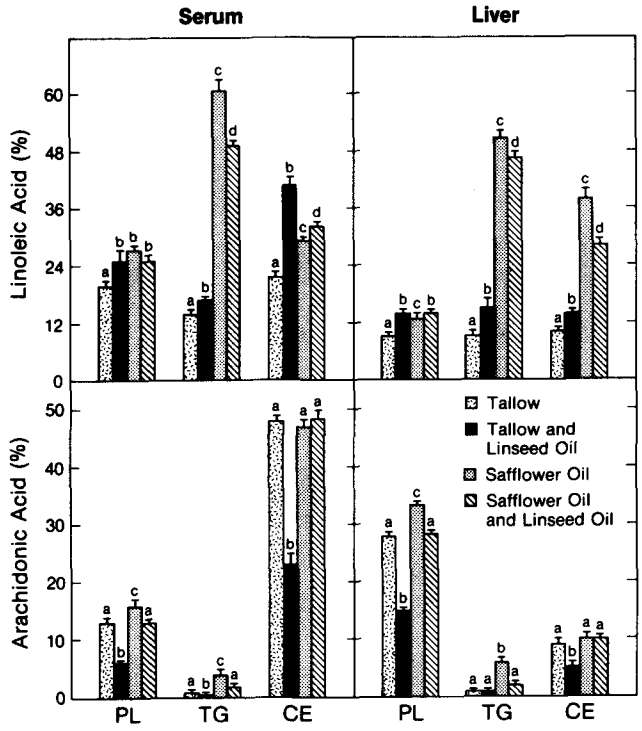

FIG. 3. Effect of dietary α-linolenic acid on linoleic and arachidonic acid content of rat serum and liver lipid fractions. PL, phospholipid; TG, triacylglycerol; CE, cholesteryl ester. Values are the mean ± standard deviation of 5 rats (n = 5) in each dietary group. Values without a common superscript are significantly different ($p < 0.5$).

liver phospholipids with an accompanied decrease in saturated (16:0), monounsaturated (18:1ω9) and 22:6ω3 content (Fig. 4). Consumption of 18:3ω3 with saturated fatty acids reduced the 20:4ω6 content of both serum (55%) and liver (45%) phospholipids followed by a concomitant increase of 18:2ω6 content compared with that observed for animals fed the diet high in saturated fatty acids without 18:3ω3. These changes in 18:2ω6 and 20:4ω6 with the beef tallow and linseed oil diet were also followed by decreases in 22:4ω6 and 22:5ω6 and increases in 20:5ω3 and 22:5ω3 contents of serum and liver phospholipids. Liver phospholipid 22:6ω3 content increased but serum phospholipid 22:6ω3 levels were not affected (Figs. 2-4). Animals fed the linseed oil with safflower oil diet exhibited amounts of 20:4ω6 in serum and liver phospholipids similar to those in animals fed the beef tallow diet. The safflower oil and linseed oil diet also failed to elevate phospholipid 20:5ω3 and 22:6ω3 levels (Figs. 2-4).

Fatty acid composition of serum and liver triacylglycerols. The fatty acid composition found in the serum triacylglycerol fraction resembled that of the dietary fats fed. Animals fed the beef tallow diet exhibited high levels of saturated (52.8%) and monounsaturated (28.7%) fatty acids; those fed the safflower oil diet had elevated levels of 18:2ω6 (60.7%); and linseed oil fed animals had higher levels of 18:3ω3 (17.1 and 10.6%) in serum triacylglycerols (Figs. 2-4). Feeding 18:3ω3 in combination with saturated fatty acids (beef tallow and linseed oil diet) increased the level of 18:3ω3, 20:5ω3, 22:5ω3 and 22:6ω3 at the expense of ω6 fatty acids (20:4, 22:4 and 22:5) in both serum and liver triglycerides (Figs. 2-4). Changes in fatty acid composition observed after feeding the safflower oil and linseed oil diet were similar but of smaller magnitude than those changes observed after feeding the beef tallow and linseed oil diet (Figs. 2-4).

Fatty acid composition of serum and liver cholesteryl esters. The cholesterol ester fraction of serum lipids exhibited the largest change in 18:2ω6, 20:4ω6 and 20:5ω3 content following consumption of the beef tallow and linseed oil diet. Serum levels of 18:2ω6 in the cholesteryl ester fraction almost doubled, and 20:4ω6 content decreased to less than half in rats fed the beef tallow and linseed oil diet compared to animals fed the beef tallow diet (Fig. 3). There was a 10-fold increase in 20:5ω3 content and a small increase in 22:5ω3 and 22:6ω3 following the feeding of linseed oil in the saturated fat diet. Feeding 18:3ω3 in combination with 18:2ω6 failed to produce change in 20:4ω6, 20:5ω3,

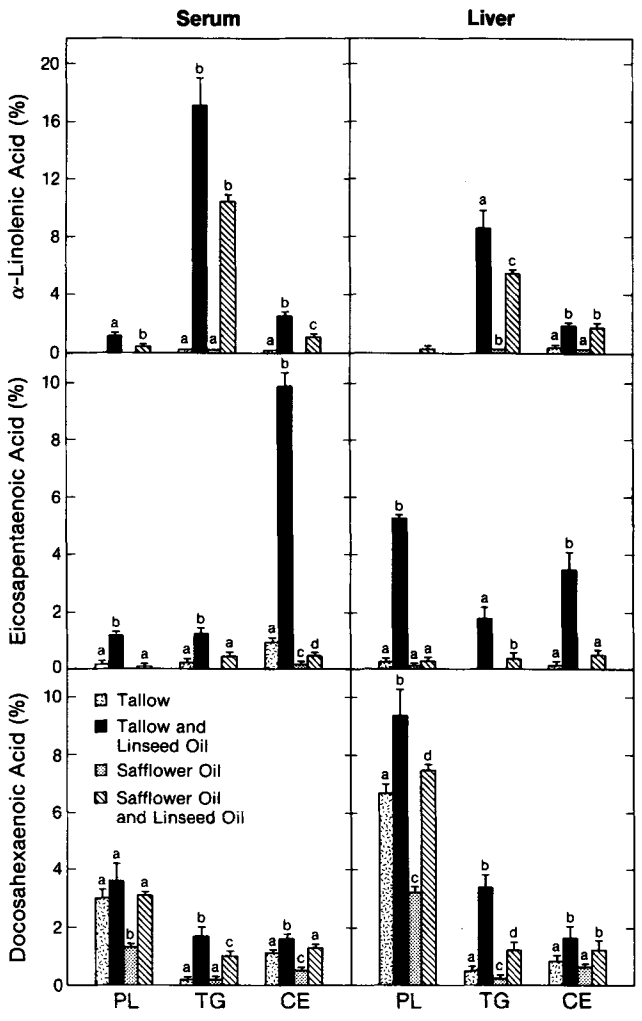

FIG. 4. Effect of dietary α-linolenic acid on α-linolenic, eicosapentaenoic and docosahexaenoic acid content of rat serum and liver lipid fractions. PL, phospholipid; TG, triacylglycerol; CE, cholesteryl ester. Values are the mean ± standard deviation of 5 rats (n = 5) in each dietary group. Values without a common superscript are significantly different ($p < 0.05$).

22:5ω3 and 22:6ω3 levels in the cholesteryl ester fraction of rat serum (Figs. 3 and 4). The pattern of changes in fatty acid composition of liver cholesteryl esters was similar to but of smaller magnitude than that found in the serum (Figs. 2–4).

DISCUSSION

Increased attention has been given in the past few years to the question of the potential health benefits of ω3 of polyunsaturated fatty acids (28–30). While ω3 fatty acids (20:5ω3 and/or 22:6ω3) of marine origin decrease cholesterol and triacylglycerol levels in normal (10–13) and hyperlipidemic subjects (31–32) and have beneficial effects against cardiovascular diseases (28–30), controversy exists concerning the usefulness of 18:3ω3 as a potential therapeutic agent (33–37). The effects of dietary 18:3ω3 on lipid metabolism in animals (16, 38–40) and humans (34,36,37) have been examined by various workers, but the importance of the dietary ratio of linoleic acid to saturated fatty acids has not been considered. In the present study, we compared the effect of feeding 18:3ω3 in combination with saturated fatty acids or 18:2ω6 on the cholesterol content and fatty acid composition of serum and liver lipids. It is important to stress that the effects observed in this study are specific to the dietary 18:3ω3 level and not due to increase in the overall unsaturation of the diet, as the beef tallow and linseed oil diet was found to be most effective in modifying cholesterol and fatty acid profiles despite having a lower unsaturated (U.I. 84.9) fatty acid composition than the safflower oil diet with (U.I. 182.5) or without (U.I. 157.7) addition of linseed oil (Table 1).

Feeding the safflower oil diet for 28 days elevated serum cholesterol levels (Fig. 1). Previously, other workers have reported either no change (8,41) or a decrease (42,43) in cholesterol concentration in serum after feeding diets enriched with 18:2ω6. The reason for this discrepancy is not clear but may be due to differences in the length of the feeding period, level of 18:2ω6 and species of animals used. In liver tissue, the cholesterol content was elevated by feeding the safflower oil diet and suppressed by feeding the beef tallow and linseed oil diet. Both free and esterified cholesterol content were increased by feeding the safflower oil diet. The increase in cholesteryl ester content is most likely due to increased acylcoenzyme A:cholesterol acyltransferase activity in liver microsomes after consumption of the 18:2ω6 enriched diet (44,45). The increase in free cholesterol may be due to increased capacity of hepatic membranes to accommodate more cholesterol following feeding of 18:2ω6 enriched diets (46).

The fact that high dietary 18:3ω3 levels suppressed the cholesterol level in liver merits further investigation. 18:3ω3 is a precursor for the formation of 20:5ω3 and 22:6ω3. Recently, it has been demonstrated that consumption of 20:5ω3 and 22:6ω3 accelerates the flow of cholesterol toward bile formation and secretion (10). Therefore, decrease in free cholesterol content following the feeding of the diets containing linseed oil may be due to increased utilization of cholesterol for bile synthesis in the liver. These observations are supported by the fatty acid compositional data of liver lipid fractions which indicate accumulation of metabolites of 18:3ω3 (20:5ω3, 22:5ω3 and 22:6ω3) after feeding diets high in 18:3ω3 in the presence of saturated fatty acids. Thus, the importance of feeding 18:3ω3 in increasing cholesterol content in the bile needs to be explored.

Dietary linseed oil suppresses the 20:4ω6 content of serum and liver lipid fractions only when fed with saturated fatty acids and not with 18:2ω6. Previous reports have shown that 18:3ω3 competes with 18:2ω6 for Δ^6-desaturation and chain elongation (47,48). The present study demonstrates that this competition is apparent only when the dietary 18:2ω6 content is low. The optimum ratio of 18:2ω6 to saturated fatty acids required for competition of 18:3ω3 with 18:2ω6 for Δ^6-desaturation at enzyme or molecular level remains to be determined. Some work suggests that dietary 18:3ω3 does not inhibit conversion of 18:2ω6 to 20:4ω6 when the 18:3ω3 content of the diet is < 15% (38). In

the present study both diets containing linseed oil provide ca. 18% of the diet fat as 18:3ω3. Feeding diets containing safflower oil and linseed oil did not affect the 20:4ω6 content of serum and liver lipid fractions even though the 18:3ω3 content of this diet is more than that reported to be required to reduce 20:4ω6 levels (38). On the other hand, when a similar amount of 18:3ω3 was fed in combination with saturated fatty acids (beef tallow and linseed oil diet), 20:4ω6 levels were reduced with a concomitant increase in 18:2ω6 concentration indicating impairment of desaturation and chain elongation of 18:2ω6. Thus, 18:3ω3 competes with 18:2ω6 for desaturation at Δ^6-desaturase or molecular level, but this process appears to be dependent on the 18:2ω6 to saturated fatty acid or 18:3ω3 to 18:2ω6 ratios of the diet. These observations are supported further by a recent study which, using cultured glioma cells, demonstrates that a balance of saturated and unsaturated fatty acids is important to enzymes responsible for synthesis of crucial membrane components and substrates for eicosanoid formation (20:4ω6, 20:5ω3 and 22:6ω3) (49). The identification of this ideal balance for the activity of the Δ^6-desaturase in hepatic and intestinal tissue remains to be established.

Another critical question is to what extent 18:3ω3 can be converted to 20:5ω3 and 22:6ω3 in humans and animals. Existing evidence suggests that both animals and humans possess limited ability to convert 18:3ω3 to 20:5ω3 and 22:6ω3, whereas 18:2ω6 is rapidly desaturated and elongated to 20:4ω6 (33,34,50). Therefore, in the studies of Dyerberg (33) and Sanders and Rasahanai (34), 18:3ω3 supplementation failed to produce substantial increases in 20:5ω3 and 22:6ω3 content of plasma lipid fractions. The present study suggests that conversion of 18:3ω3 to 20:5ω3 and 22:6ω3 can be accelerated if the rest of the fat in the diet is saturated fat. Dietary α-linolenic acid was associated with the accumulation of 20:5ω3 to a greater extent in serum and liver lipid fractions when fed in combination with beef tallow than with safflower oil. The possibility that 18:3ω3 is poorly absorbed when 18:2ω6 content of the diet is high (51), e.g., after consumption of the safflower oil and linseed oil diet, cannot be ruled out. However, this seems unlikely as dietary 18:3ω3 has been shown to be absorbed to the same extent regardless of the quality of the rest of the dietary fat (Thomson et al., unpublished data).

Controversy exists about the usefulness of 18:3ω3 in platelet aggregation and atherosclerosis (50) and revolves around the rate of desaturation and elongation of 18:3ω3 in man. Although it may be inappropriate to interpret the animal data in humans, the present study suggests that conversion of 18:3ω3 to 20:5ω3 and inhibition of the conversion of 18:2ω6 to 20:4ω6, both steps considered as inhibitory for platelet aggregation and atherosclerosis, can be maximized by partial replacement of dietary 18:2ω6 by saturated fat. In this regard, high dietary levels of 18:3ω3 also appear to be hypocholesterolemic, decreasing liver cholesterol levels regardless of the dietary fat fed. These observations could provide a useful basis for formulating human clinical trials to examine the efficacy of 18:3ω3 on platelet aggregation and cholesterol metabolism.

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The Essential Fatty Acid Requirement for Azoxymethane-Induced Intestinal Carcinogenesis in Rats

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The essential fatty acid requirement for the development of intestinal carcinogenesis was determined and compared to the overall essential fatty acid status of the animals as measured by the triene/tetraene ratio in the plasma, liver and colon. To induce tumors, male Sprague-Dawley rats were given two weekly injections (20 mg/kg body wt) of azoxymethane. Two weeks after the last injection, the rats were divided into groups of 25 and given one of six diets containing various levels of essential fatty acids (as linoleate). The diets contained 5% total fat and were prepared by mixing safflower oil (high essential fatty acids, beef fat (low essential fatty acids), and medium chain triglyceride oil (no essential fatty acids). One group of rats was fed a 20% beef fat diet. The range of essential fatty acids was from <0.03% to 1.28% (w/w). Twenty-six weeks after the first azoxymethane injection, the animals were killed and intestinal tumor incidence and multiplicty were determined. Samples of plasma, liver and colon were also taken for measurement of the triene/tetraene ratio by gas chromatography.

Large bowel tumor incidence showed a dependence on the essential fatty acid content of the diet. The results were as follows: (percent essential fatty acids: percent tumor incidence) Group A (1.28: 72.4), Group B (0.60: 73.3), Group C (0.11: 55.2), Group D (0.08: 39.3), Group E (<0.03: 37.9) and Group F, which was fed 20% beef fat, (0.34: 88.5). These data suggest the essential fatty acid requirement for colon tumorigenesis is much lower than values previously reported for tumorigenesis in the breast and pancreas. The plasma and liver triene/tetraene ratios showed clear-cut essential fatty acid deficiency (ratio >0.4) in Groups D and E, although no clinical symptoms were evident. In all dietary groups, the triene/tetraene ratio in the colon was lower than 0.3. In addition, in the colon, the percentage of fatty acids present as 20 carbon polyunsaturated fatty acids was lower than in the plasma and liver. These data suggest the colon possesses low levels of the fatty acid desaturase and elongase needed for conversion of linoleate to 20 carbon fatty acids, and therefore, that the colonic requirement for essential fatty acids may be low. Furthermore, in the absence of other clinical symptoms, the reduced tumorigenesis observed in the groups fed low essential fatty acids suggests the essential fatty acid requirement of tumor tissue may be higher than that of normal colon mucosa.
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Intestinal carcinogenesis has been shown to be strongly influenced by environmental factors. The environmental factor with the greatest impact appears to be the diet, and in particular the fat content of the diet. A majority, but not all, studies of intestinal carcinogenesis have shown that a high-fat diet enhances tumorigenesis (1, 2). For most fats, the enhancement of tumorigenesis has been shown to occur during the post-initiation phase (3,4). Recent investigations of the relationship between dietary fat and tumorigenesis have concentrated on the fatty acid composition of different fats. Reddy and Maeura have shown that high-fat diets containing predominantly unsaturated sources of fat enhance tumorigenesis more than diets containing fairly highly saturated fats (5). Another group of investigators demonstrated greater tumorigenesis in rats fed 5% linoleate as a fat source than in rats fed 0.5% linoleate and 4.5% stearate (6). Thus, at both high- and low-fat levels, it appears that unsaturated fats promote tumorigenesis more than saturated fats.

The effect of polyunsaturated fatty acids (PUFA) on intestinal tumorigenesis is similar to that reported in several other organs in which dietary fat acts as a promoter of carcinogenesis. There is evidence from these studies that linoleate is required for the development of cancer (7). In particular, studies in the breast and pancreas have demonstrated the influence of PUFA in tumor promotion (8, 9). Furthermore, in these organs, the specific level of essential fatty acid (EFA) necessary for maximal tumorigenesis has been defined (10, 11). To our knowledge, a similar titration of the PUFA requirement for colon tumorigenesis has not been reported.

Essential polyunsaturated fatty acids play numerous roles in cellular physiology. One major metabolic fate is to serve as precursors for the biosynthesis of arachidonic acid with subsequent conversion to prostaglandins and lipoxygenase products (12). These fatty acid oxygenation products have been shown to play important roles in many cellular processes. The involvement of these compounds in tumorigenesis is supported by the fact that inhibitors of prostaglandin biosynthesis as well as lipoxygenase inhibitors reduce the development of tumors in animal models (13-16).

The present investigation was undertaken to determine the influence of EFA status on intestinal tumorigenesis. The overall EFA status of the animals was determined by evaluating the plasma and liver triene to tetraene ratios. The intestinal EFA requirement was also assessed and compared to that necessary for maintaining the general health of the animal.

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Abbreviations: ANOVA, analysis of variance; AOM, azoxymethane; BF, beef fat; EFA, essential fatty acids; FID, flame ionization detection; GC, gas chromatography; MCT, medium chain triglyceride; NRC, National Research Council; PUFA, polyunsaturated fatty acids; SO, safflower oil; 20:4, 5,8,11,14-eicosatetraenoate; 20:3ω-9, 5,8,11-eicosatrienoate.

The relationship of these criteria to the development of azoxymethane-induced intestinal tumors was examined. The results of this study provide information with respect to the enhancement of intestinal tumorigenesis by dietary fat.

EXPERIMENTAL

Azoxymethane (AOM, CAS: 25843-45-2) was purchased from Ash-Stevens, Inc. (Detroit, MI) and was prepared as an aqueous solution for injection. Dextrose was obtained from a local bakery supply house. Beef fat was donated by Belmont Packing Co. (Detroit, MI) and was rendered in the laboratory prior to use in preparation of the diets. Medium chain triglyceride oil (MCT) was donated by Capital City Products Corp. (Columbus, OH). All other dietary components were obtained from Dyets, Inc. (Bethlehem, PA). Solvents for lipid extraction were purchased from a local laboratory supply company and were of the highest purity obtainable.

Animals and diets. Male Sprague-Dawley rats (240) weighing 100–125 g were obtained from Charles-River breeding Laboratories Inc., Portage, MI. Animals were housed individually in hanging wire cages and maintained under conditions of controlled lighting (12 hr/day), temperature and humidity. Diets and water were provided *ad lib.* Diets were prepared fresh weekly and stored at 4°C prior to dispensing to the animals. Body weights were recorded weekly, and food consumption was determined monthly.

Upon arrival, rats were fed a diet containing 5% corn oil. After one week acclimation, 180 rats received sc injections of AOM (20 mg/kg body wt) once a week for two weeks. Noncarcinogen controls (60 rats) received injections of sterile water. One week after the last injection the rats were placed into one of six dietary groups. Each group contained 30 carcinogen-treated and 10 control rats. Five of the groups were fed diets containing 5% total fat, and one group was fed a diet containing 20% beef fat. The 5% fat diets were designed to provide different levels of essential fatty acids and were prepared by mixing either safflower oil (SO), beef fat (BF), or MCT oil. These three fats were chosen because they contain high (SO), low (BF), or no (MCT) essential fatty acids. From previous studies using 5% beef fat (0.1% EFA) it was suspected the EFA requirement for colon tumorigenesis may be low. Therefore, two groups of rats were fed diets containing high proportions of MCT oil to make the diets significantly EFA deficient. Samples of each diet were analyzed to determine precisely the level of dietary linoleic acid given to each group of animals. The individual dietary groups are presented in Table 1. The composition of the diets (wt %) was as follows: mineral mix 3.6, vitamin mix 2.0, D,L-methionine 0.2, cellulose 5.0, casein 20.0 (16). In addition, the 5% fat diets contained 5% fat, 45.2% dextrose and 19.0% cornstarch and had a caloric density of 3.818 kcal/g, while the 20% fat diet contained 20% fat, 40.2% dextrose and 9.0% cornstarch, and had a caloric density of 4.568 kcal/g.

Twenty six weeks after the first carcinogen injection, all rats were killed and necropsies performed. Tissues were examined grossly for tumors; the intestine was slit lengthwise from stomach to anus and rinsed free of debris; the number, size and location of all intestinal tumors was recorded. Only those tumors grossly observable (>1.0 mm) were included for analysis. Extensive experience with this animal model has shown that at 26 weeks after the initiation of carcinogen treatment, the vast majority of induced tumors are detectable upon gross examination. Ten representative large intestinal tumors from each dietary group were taken for histological analysis. The tumors were removed, fixed in phosphate buffered formalin, embedded in paraffin and stained with hematoxylin and eosin then examined microscopically. The data were analyzed statistically by Chi-square (tumor incidence) and analysis of variance (ANOVA) (tumor frequency).

Lipid analysis. At the time of sacrifice, samples of plasma, liver and colon were collected for analysis of lipid composition to determine EFA status. The tissue samples were extracted with $CHCl_3$/methanol (2:1), transesterified, methylated and subjected to analysis by gas chromatography (GC) utilizing flame ionization detection (FID) and electronic integration to quantitate individual fatty acids. The fatty acids were identified by co-elution with authentic standards. The ratio of 5,8,11-eicosatrienoate (20:3ω-9) to 5,8,11,14-eicosatetraenoate (20:4) was determined to obtain a quantitative assessment of the EFA status of animals consuming a particular diet. The results of the lipid analyses were evaluated statistically by ANOVA.

RESULTS

Body weights and food consumption. In the carcinogen-treated animals, there were no significant differences in mean body weight between any of the dietary groups at any point in the experiment. In general, the carcinogen-treated animals weighed less than animals in the re-

TABLE 1

Dietary Groups to Determine Effect of Linoleic Acid on Intestinal Tumorigenesis

Group	Dietary fat source (wt %)	Actual 18:2 (wt %)[a]
A	1% Beef fat + 4% safflower oil	1.28
B	3.75% Beef fat + 1.25% safflower oil	0.6
C	5% Beef fat	0.11
D	2.5% Beef fat + 2.5% MCT oil[b]	0.08
E	0.5% Beef fat + 4.5% MCT oil	<0.03
	20% Be	0.34

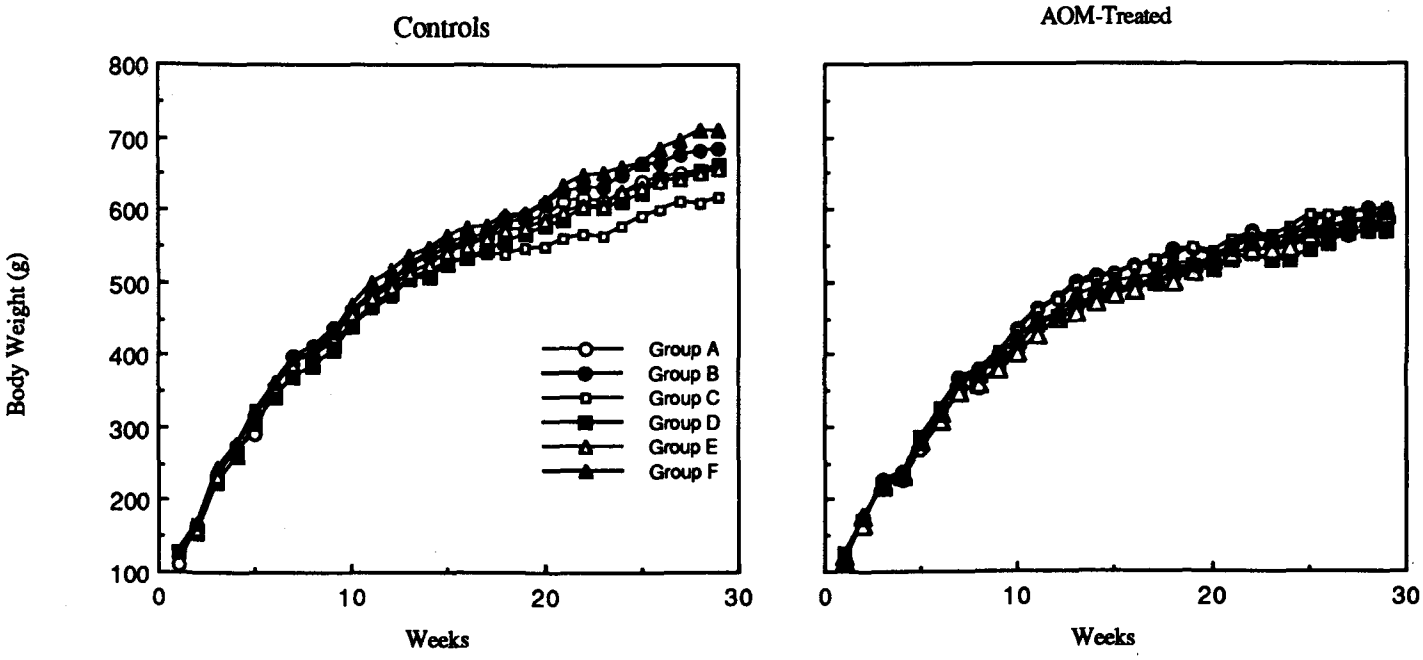

FIG. 1. Mean body weights of animals in the tumorigenesis study. There were no statistically significant differences in the mean body weights between any of the dietary groups.

spective control group. The mean body weights of control animals were also similar, although toward the end of the experiment, the rats fed 20% beef fat tended to weigh more than those fed 5% beef fat, which is consistent with previous observations (3). These data are presented in Figure 1.

The food consumption of rats consuming the 5% fat diets was similar for all the various diets. Rats fed 20% fat ate less food than those fed 5% fat and, therefore, maintained similar body weights. The greatest difference was observed between rats fed 20% beef fat and those fed 5% beef fat where the rats fed high fat ate an average of 20.9% less food over the course of the experiment. While reduced food consumption in animals fed high-fat diets has been reported by others, in our previous experience, the divergence in food consumption did not occur until the fat content of the diet was 30% or more. The vitamin and mineral mixes used in preparation of the diets in this experiment contained two times the National Research Council (NRC) requirement for maintenance of laboratory rats (17). Therefore, the slightly reduced food consumption in the animals fed high fat was not great enough to lead to nutrient deficiency. The caloric intake of rats fed the different diets is shown in Figure 2. For the first two months of the experiment, rats fed the 20% fat diet had a slightly higher caloric consumption than the rats fed the 5% fat diets. However, in the third through sixth months the caloric intake was similar in all dietary groups.

Tumor incidence and multiplicty. The incidence (percent of animals with tumors), number of tumors per tumor-bearing animal, and tumor frequency (number of tumors per rat) of intestinal tumors in the AOM treated animals are shown in Table 2. There were no tumors in the animals that did not receive AOM injections. For the initial recording of tumor data, the small and large intestines were divided into proximal and distal halves. However, for presentation, the numbers were combined since there were no differences in the proximal and distal halves that were not evident in the combined data. Ten representative large intestinal tumors from each group, five larger than 0.5 cm and five smaller, were examined histologically. All lesions, regardless of size, were adenocarcinomas with varying degrees of differentiation and invasion of the intestinal wall. These characteristics were not significantly different between the various groups. The production of 100% adenocarcinoma with carcinogen treatment pro-

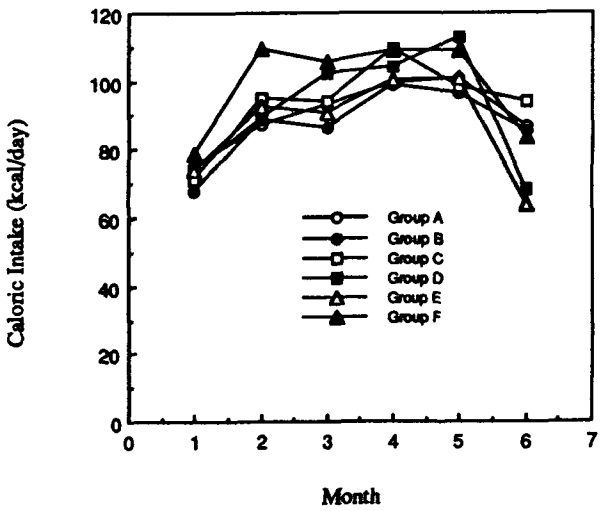

FIG. 2. Daily caloric intake of rats in the tumorigenesis study. The data presented represent the mean daily intake per rat. For data collection, the food consumption of five animals in each group was measured over a three-day period.

TABLE 2

Intestinal Tumor Incidence and Multiplicity

Group (no. of animals)	% linoleate	Incidence, %		Mean no. tumors/ tumor-bearing rat		Tumor frequency: tumors/rat	
		Small	Large	Small	Large	Small	Large
A(29)	1.28	72.4	72.4	2.0±1.1	2.0±0.9	1.5±1.3	1.5±1.2
B(30)	0.60	76.7	73.3	3.0±1.5[a]	2.1±1.4	2.3±1.8	1.5±1.5
C(29)	0.11	79.3	55.2[c]	2.9±1.4[a]	2.3±1.3	2.3±1.7	1.2±1.5
D(28)	0.08	82.1	39.3[a,b,c]	1.9±1.2[b,c]	1.8±1.2	1.5±1.3	0.7±1.2[c]
E(29)	<0.03	82.8	37.9[a,b,c]	2.1±1.0[b,d,c]	1.7±0.9	1.7±1.2	0.7±1.0[c]
F(26)	0.34	76.9	88.5	3.2±2.5[a]	3.3±3.3[a,b,e,f]	2.4±2.6[a]	2.9±3.3[a,b,d,e,f]

[a]P<0.05 to P<0.005 compared to Group A.
[b]P<0.05 to P<0.005 compared to Group B.
[c]P<0.05 to P<0.0005 compared to Group F.
[d]P<0.05 to P<0.005 compared to Group C.
[e]P<0.05 to P<0.005 compared to Group D.
[f]P<0.05 to P<0.005 compared to Group E.

tocol used in the present study is consistent with previous studies utilizing this animal model (3, 16).

Small intestinal tumors developed almost exclusively in the proximal half. In fact, out of a total of 333 small bowel tumors in all the groups, only 4 (1.2%) were found in the distal segment. In the large bowel, the distribution of tumors, both incidence and multiplicity, was nearly the same between the proximal and distal halves. The only notable difference was found in the rats consuming 20% beef fat where the incidence in the distal half (73.3%) was slightly higher than in the proximal segment (53.8%).

Tumor metastasis occurred in only two of the carcinogen-treated rats, one in group A and one in D. The frequency of ear tumors was also low with a total of only eight tumors observed in all the carcinogen-treated rats. There was no clear correlation between metastasis or ear tumor formation and either the linoleate or total fat content of the diet.

The incidence of large bowel tumors showed a dependence on the linoleate content of the diet. The NRC recommendation for EFA content in the diet of rats is 0.6% (17). The diet fed to Groups A and B met or exceeded this recommendation. The diet fed to Groups C (0.11%), D (0.08%) and E (<0.03%) were below this level, and animals in these groups showed a reduced tumor incidence. The lowest tumor incidence was observed in groups of rats fed less than 0.1% linoleic acid. It is noteworthy that rats consuming the 20% beef fat diet (0.34% linoleate) had less than the recommended EFA level (0.6%) but had the highest incidence of tumors of any group in the study. It is also interesting that the tumor incidence did not increase as the EFA content of the diet was doubled from 0.6% (Group B) to 1.28% (Group A).

On the other hand, in the large intestine, the number of tumors per tumor-bearing animal did not reflect the dietary EFA content, although it did reflect the total fat content of the diet. Thus, a statistically significant increase in tumors per tumor-bearing animal was noted in the 20% beef fat group (Group F) compared to those groups fed 5% fat (Groups A, B, D and E) with the exception of Group C (5% BF, 0.11% linoleate). Since the incidence of tumors varied, the tumor frequency (average number of tumors per rat) of the individual dietary groups showed the same trend as the tumor incidence. Moreover, the frequency of large intestinal tumors in the group fed 20% beef fat (Group F) was greater than that in the groups fed the 5% fat diets, regardless of the linoleate content. The difference was statistically significant in all cases.

Tumor yield in the small intestine was distinctly different than that in the large intestine. The incidence of small intestinal tumors was generally equal to or greater than that in the large intestine and independent of the EFA content of the diet. Thus, rats consuming the lowest level of EFA (<0.03%) had essentially the same tumor incidence as those consuming the highest level of EFA (1.28%). The number of tumors per tumor-bearing animal was highest in groups B, C and F compared to the other three groups. However, this did not correlate with the linoleate content of the diet since Group A, which was fed the highest linoleate level, had a relatively low number of tumors per tumor-bearing animal.

Quantitative EFA status of animals. In an attempt to provide a quantitative assessment of the EFA status of the animals in this study, samples of plasma, liver and colon were taken at the termination of the study. The lipids in these samples were extracted, methylated and subjected to analysis by gas chromatography. The relative amounts of the major fatty acids were determined, and particular attention was paid to the amounts of 20:3ω-9 (triene) and 20:4 (tetraene). The ratio of these two fatty acids (triene/tetraene) is frequently used as a measure of the EFA status of an animal with a ratio, in the plasma, of greater than 0.4 usually taken as an indication of EFA deficiency (18).

The results of analysis of the different diets are shown in Figure 3. The various diets provided a good spectrum of EFA levels (as linoleate) with the highest level of 1.28% linoleate in Group A and the lowest linoleate content of <0.03% in Group E. The difference in measured linoleate content was statistically significant between all dietary groups. The level of linolenate provided by the diets was negligible in all groups. In addition to the fatty acids shown in Figure 3, the diets in Groups D and E also contained significant quantities of octanoate (8:0) and decanoate (10:0) which were supplied by the MCT oil. These two

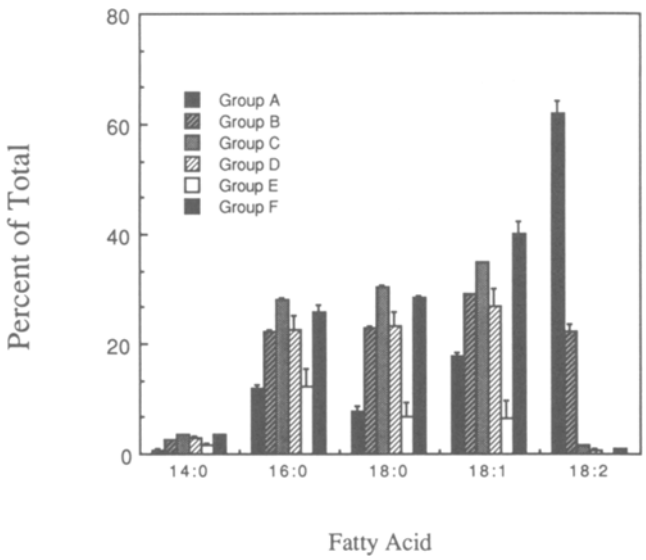

FIG. 3. Major fatty acid composition of the diets in the tumorigenesis study. Fatty acids are presented as percent of total fatty acids. The diets fed to Groups D and E also contain significant quantities of 10:0 and 12:0 derived from the MCT oil. Data presented are the mean ± SD.

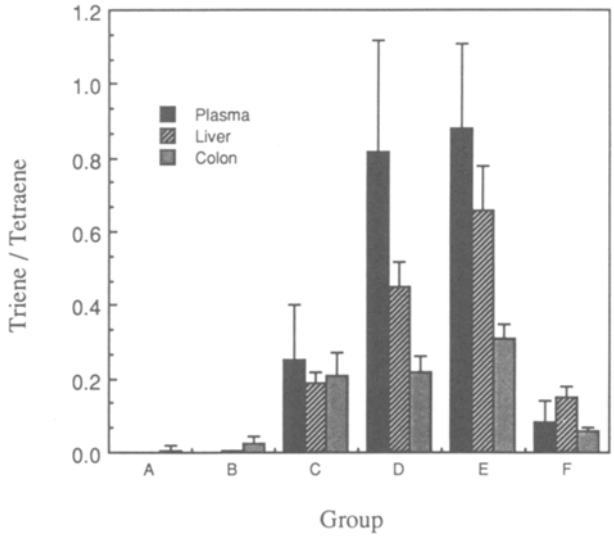

FIG. 4. Triene/tetraene ratios of rats in the various dietary groups. Where no column is shown, the level of triene detected was too low to allow calculation of a ratio. Due to technical problems, the lipid content in Group B was not determined. Data presented are the mean ± SD for 5 animals in each group.

fatty acids were present in only trace amounts in the other dietary groups.

The triene/tetraene ratios of the plasma, liver and colon from animals in the various groups is shown in Figure 4. There was little 20:3ω-9 in the plasma of animals fed 1.28 or 0.6% linoleate in the diet, thus the triene/tetraene ratio in these animals is very low. In contrast, animals in groups D and E were clearly EFA deficient as evidenced by plasma ratio of 0.82 and 0.88, respectively. On the other hand, Groups C and F had detectable plasma levels of 20:3ω-9 although the triene/tetraene ratios obtained were well below those indicative of deficiency. In the plasma, the difference between the triene/tetraene ratio in the different groups is statistically significant with the exception of Groups C versus F and Groups D versus E.

The liver triene/tetraene ratios showed a trend similar to those in the plasma although they were generally lower. As before, only Groups D and E had ratios high enough to indicate EFA deficiency. For all dietary groups, the difference in the liver triene/tetraene ratio is statistically significant with the exception of Group D versus F. None of the animals, in any dietary group, showed visible clinical symptoms of EFA deficiency.

The fatty acid content of the colon was significantly different than that of the plasma and liver as shown in Figure 5. In the colon, the total amount of the 20 carbon fatty acids, 20:4 and 20:3ω-9, was small in all dietary groups. For example, in group A, arachidonic acid represented 19.5% of the fatty acids in the plasma and 13.7% in the liver. In contrast, the percentage of 20:4 in the colon was only 3.1% of the total fatty acids. Similarly, low levels of 20:3ω-9 were also found. This observation of a low level of 20 carbon fatty acids in the colon was seen in all the dietary groups. Within any given dietary group, the difference between the 20:4 content in the colon compared to the plasma and liver was statistically significant.

In addition to a low level of 20 carbon fatty acids in the colon, the triene/tetraene ratio was also low. Group E, which received the lowest level of dietary linoleate (<0.03%), had the highest ratio of 0.31. Thus, while data from the plasma and liver suggested a general state of EFA deficiency, the colon did not give a similar indication.

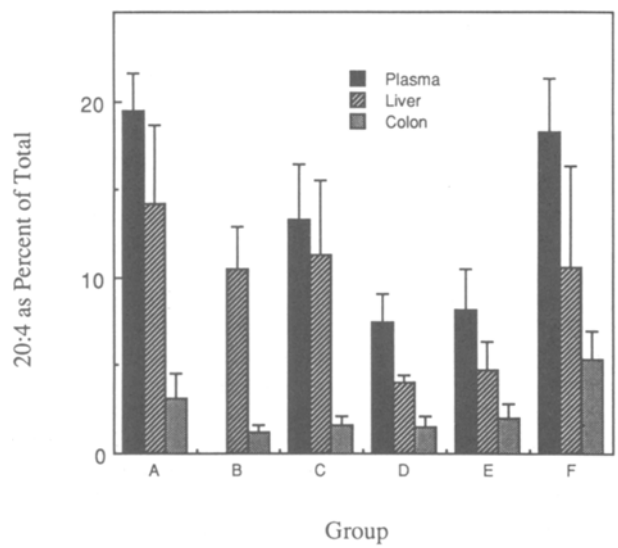

FIG. 5. Content of arachidonic acid (20:4) in the plasma, liver and colon of rats in the various dietary groups. Data are the amount of 20:4 as a percent of total major fatty acids. Due to technical problems, the plasma content of 20:4 in Group B was not determined. Mean ± SD for 5 animals in each group.

DISCUSSION

In the present experiment the effect, on intestinal tumorigenesis, of the EFA content of 5% fat diets and the overall EFA status of rats fed the diets was studied. The EFA status of the animals was assessed by measurement of the triene/tetraene ratios in plasma, liver and colon. Large bowel tumorigenesis was shown to be dependent upon the EFA content of the diet; however, this dependence did not become apparent until the dietary EFA content was much lower than the amount considered optimal for overall animal health. The EFA requirement for general health of laboratory rats is 0.6% of the diet (17). In the present experiment, large bowel tumor incidence was only slightly depressed in animals fed 0.1% EFA compared to those fed 0.6% (55.6% incidence vs 73.3% incidence). On the other hand, an increase in EFA content from 0.6% to 1.28% was not associated with enhanced tumorigenesis. Furthermore, in rats fed 20% beef fat which provided 0.34% EFA, the yield of tumors was greater than that in animals fed 0.6% EFA. These results suggest the EFA requirement for tumorigenesis in the large bowel is much lower than that for overall health and that the upper limit of the EFA requirement is between 0.1% to 0.34%.

It has been shown previously that the response of various organs to EFA deficiency is different (19). This suggests the EFA requirement for maintenance of normal function also differs. For example, under conditions of EFA deprivation, the polyunsaturated fatty acid content of certain liver phospholipids has been shown to decrease rapidly while those in the heart and kidney are maintained at near normal levels for longer periods of time. Based on these observations, plus the results of the current study, it is reasonable to suggest that the EFA requirement for the promotion of tumorigenesis may also differ between organs. In fact, the finding that tumorigenesis in the small bowel appeared to be independent of EFA status, whereas colon tumorigenesis showed the opposite response, may be relevant to this point.

The finding that large bowel carcinogenesis is reduced when EFA levels fall below 0.6%, but does not increase with levels of EFA greater than 0.5%, is in direct contrast to results obtained in other organ systems responsive to dietary fat. For example, the EFA requirement for optimal development of breast tumors has been estimated to be 4.4% (10). Similarly, high values of the EFA requirement have also been reported in studies of the enhancement of pancreatic carcinogenesis by dietary fat (11). Thus, an important distinction can be made between tumorigenesis in the colon compared to these other organs. Specifically, the EFA requirement for optimal tumorigenesis in the breast and pancreas is in excess of that required for good nutrition, whereas in the colon the optimal EFA requirement is less than the required amount.

It appears that the enhancement of cancer by high-fat diets is composed of two factors; the first is dependent on EFA and their metabolism, while the second is the result of other effects of fat. Thus, once the EFA requirement is met, the non-EFA dependent factors could make an additional contribution to carcinogenesis. There is evidence from studies of mammary carcinogenesis that this situation exists (20). A major difference between studies of EFA and carcinogenesis in the other organs and the present experiment is that the other studies utilized a high-fat diet whereas this experiment was conducted primarily with 5% fat diets. Thus, in the current study, the contribution of non-EFA dependent fat effects would be small.

Along these same lines, the observation of a higher tumor yield in rats fed 20% beef fat compared to those fed 5% fat is important. This observation suggests the enhancement of colon tumorigenesis by beef fat is not due solely to a difference in EFA content. While some of the enhancement may be due to this factor, it is clear that an increase in EFA from 0.34% (20% beef fat) to 1.28% (1% BF + 4% SO) did not enhance tumorigenesis. In fact, the rats fed the lower level of EFA, but higher total fat content, actually had the highest tumor yield. Thus, the enhancement of colon tumorigenesis by high beef fat diets must include non-EFA dependent components. These non-EFA components may include biliary steroids, although it has been suggested recently that oxidation products of unsaturated fatty acids, including those of oleic acid, may be a contributing factor (21, 22).

It has been suggested the enhancement of tumorigenesis by high dietary fat is due to a difference in caloric consumption and not to any direct effect of fat itself. In the present experiment this does not appear to be the case as there are clear differences in the tumor yield among rats fed only 5% fat diets, for example, Group A as opposed to Group E. Thus the alteration in tumorigenesis observed must be due to the composition of the dietary fat and not the caloric content of the diet. Furthermore, with respect to the high-fat versus low-fat diets, the rats fed the high-fat diets had a similar caloric intake, throughout most of the experiment, to rats fed 5% fat diets, again suggesting a fat-specific as opposed to a caloric effect. In support of this, the data of Reddy and Maeura demonstrate a difference in tumorigenesis due to fat composition, not caloric content (5).

The determination of the triene/tetraene ratios in the animals in this study provided some interesting results. A plasma triene/tetraene ratio of greater than 0.4 is usually considered indicative of EFA deficiency (18). The ratio in the plasma in the two groups of rats fed the lowest level of EFA (Groups D, 0.08%, and E, <0.03%) clearly indicates EFA deficiency. There were no overt clinical signs of deficiency such as scaly tails, weight loss, poor coat development or hematuria, although it is known that elevated triene/tetraene ratios will precede the clinical manifestation of deficiency. From the data in this experiment, it is apparent that tumorigenesis is affected even though other symptoms of deficiency do not develop. Since the disease process is affected while the target tissue does not indicate EFA deficiency, the possibility exists that the biochemical definition of EFA deficiency is different for different organs. For example, an organ such as the colon may be functionally EFA deficient at a triene/tetraene ratio much lower than the accepted plasma value of 0.4. Alternatively, as discussed below, it is possible the EFA requirement of normal colon mucosa

is different than that of colon tumors. Thus, while the normal colon was unaffected, the tumor tissue may have been EFA deficient and its growth depressed.

In the present experiment, the colonic content of 20:4 was found to be a much lower percentage of total fatty acids than in either the plasma or liver. Similarly, the level of 20:3ω-9 in the colon was also low. These two fatty acids are biosynthesized from precurosr fatty acids by the action of delta-5 and delta-6 desaturases and a fatty acid elongase (12). While we did not measure the amount of elongase and desaturase activity, the data from this experiment suggest that, in the colon, the activity of these enzymes is low. Low levels of these enzyme activities have been reported previously in rat and guinea pig skin, which is similar to the colon in the need for constant cell turnover during epithelial renewal (23). There are two interpretations to this observation. One possibility is that, since the colon cannot efficiently elongate EFA to eicosanoid precursors, there is a stringent requirement for dietary EFA. The other possibility is that low levels of biosynthesis reflect a decreased requirement of the tissue for 20 carbon PUFAs. Data from the present experiment suggest the latter possibility is operative since colon tumorigenesis was not affected until the EFA level was much lower than the required daily amount.

It is generally believed that the major role of EFA is to provide precursors for the biosynthesis of prostaglandins and other eicosanoids (12). How, then, is it possible to reconcile the finding of a low colon requirement for EFA with the observations of inhibition of colon tumor growth by inhibitors of prostaglandin biosynthesis such as piroxicam and indomethacin (14-16)? One possible explanation is that as colonic tissue undergoes the transformation from normal mucosa to maligant tissue, the essential fatty acid requirement also changes. This suggestion is consistent with the observation that indomethacin is an effective inhibitor when administered late in the carcinogenic process and actually exerts a chemotherapeutic action on colon tumor growth (24-26). The suggestion that a change in the EFA requirement occurs during cell transformation, if confirmed, may prove useful in the chemoprevention and treatment of colon cancer.

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METHODS

On-Line Hydrogenation in GC-MS Analysis of Cyclic Fatty Acid Monomers Isolated from Heated Linseed Oil[1]

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A simple on-line hydrogenation method in GC-MS analysis of unsaturated fatty acid esters is described. Using hydrogen as carrier gas, hydrogenation takes place in a capillary reactor connected to the outlet of the analytical column in the oven of the gas chromatograph. The reactor is a fused silica tube (60 cm × 0.32 mm i.d.) coated with palladium acetylacetonate. Selective hydrogenation of olefinic bonds is achieved after a normal chromatographic run. Structural information (carbon-skeleton, double bond equivalents) can thus be deduced, and structural correlations between the saturated and unsaturated components can be obtained. Structures of cyclic fatty acid esters isolated from heated linseed oil were elucidated using this simple method which was found very useful for structural investigations on unsaturated compounds by GC-MS.

Lipids 24, 347-350 (1989).

Separation and spectral characterization of the methyl esters of unsaturated cyclic fatty acid monomers (CFAM) isolated from heated vegetable oils were described recently (1,2). However, many structural problems remained unsolved because the mass spectra of the unsaturated compounds of the mixtures were ambiguous. Generally, the mass spectra of saturated species are interpreted more readily than those of related unsaturated compounds. For example, distinction of the carbon-skeleton types is much easier (ring size, nature of the substituents).

The easiest and most commonly used method to obtain saturated components is by catalytic hydrogenation of the unsaturated mixture before analysis. This was done very recently on CFAM mixtures isolated from sunflower and linseed oils. The carbon-skeletons of the major constituents were elucidated, and the double bond equivalents were determined (1,3).

However, this kind of data is useful only if the correlations between the saturated and their corresponding unsaturated analogues can be carried out easily. Correlations become difficult if various unsaturated species are converted into the same saturated products. Positional and geometrical isomers, for example, apparently rather abundant in the CFAM mixtures (2), will give the same hydrogenated product; therefore, only one peak will be detected by gas chromatography. Consequently, it was impossible to determine correlations between the unsaturated and saturated CFAM mixture obtained after total hydrogenation.

Another method of hydrogenation would be to obtain selective hydrogenation of all the unsaturated species after chromatographic separation. An on-line technique (4,5), just before mass spectrometry, may be used. We now describe an on-line hydrogenation method for use in GC-MS analyses of methyl esters of CFAM isolated from a heated linseed oil. This method proved easy and useful in structural studies.

EXPERIMENTAL

Cyclic fatty acid monomers were isolated from linseed oil heated at 275°C under nitrogen and esterified according to published procedure (1).

Gas chromatography-mass spectrometry (GC-MS). All GC-MS analyses were performed with a Nermag R 10-10 C mass spectrometer directly coupled to a Girdel 31 gas chromatograph. The analytical column used throughout the study was a fused silica capillary column (30 m × 0.25 mm i.d.) coated with DB Wax (J and W Scientific, 0.5 μm film thickness).

Splitless injections were used and oven temperatures were programmed from 60 to 180°C/min at 10°C/min, then to 220°C at 3°C/min and held at 220°C until completion of the analyses. Hydrogen was used as the carrier gas with a linear velocity of 53 cm/s at room temperature. Electron impact mass spectra were generated at 70 eV with a source temperature of 150°C, the instrument scanning from 42 to 310 amu on an 0.8 s cycle.

Hydrogenation capillary reactor. A deactivated fused silica capillary column (5 m × 0.32 mm i.d.) was statically coated with a 0.5% methylene chloride solution of palladium acetylacetonate (Fluka). After drying under a stream of nitrogen, 50 cm of both ends of the column were discarded. A 60-cm piece of the capillary was inserted with zero-dead volume butt connectors (Supelco Inc., Bellefonte, PA) between the analytical column and a deactivated fused silica capillary column (3 m × 0.25 mm i.d.) directly connected, through the heated interface, to the ion source of the mass spectrometer.

Palladium metal was precipitated in a stream of hydrogen carrier gas by heating to the normally-applied column temperature (220°C).

RESULTS AND DISCUSSION

A recent structural study on unsaturated CFAM methyl esters isolated from heated linseed oil revealed that the CFAM fraction was a mixture of cyclohexenyl and cyclopentyl and/or cyclopentenyl derivatives (2). After total hydrogenation of the CFAM fraction, another study (3) partially confirmed an earlier one (6) while extending our knowledge of the MS fragmentation of alkylcyclopentyl esters. The structures of some of them were definitely established by organic synthesis (7).

[1]Presented in part at the AOCS meeting in May 1988 in Phoenix, AZ.

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Abbreviations: CFAM, cyclic fatty acid monomers; GC, gas chromatography; MS, mass spectroscopy; TIC, total ion currents.

However, in the GC-MS analyses of the hydrogenated CFAM mixture isolated from linseed oil, the chromatograms obtained before and after total hydrogenation were, as expected, completely different (2,3). It was then rather difficult to assign structures to positional isomers of unsaturated CFAM which gave only one product after hydrogenation.

In order to obtain better GC correlations between saturated and unsaturated species, we investigated an on-line hydrogenation method first described by Schomburg et al. (4,5). Schomburg and his coworkers determined important parameters (activity of catalysts, hydrogen flow in the capillary reactor and temperature of hydrogenation) using a double oven chromatograph equipped with a switching device (5). However, a simpler way to operate, compared to the method described by Schomburg et al., would be to connect a capillary catalytic reactor either before or after the analytical column, directly in the oven of the gas chromatograph. Hydrogen would be provided as the carrier gas and reaction temperature would be the oven temperature. The availability of deactivated fused silica capillaries and of various zero-dead volume connectors permitted us to investigate this direct simple arrangement.

For the selective hydrogenation of olefinic double bond, a palladium catalyst was chosen, because platinum catalysts may also hydrogenate other functions, such as ester groups (5).

The capillary reactor was placed in the oven of the GC behind the analytical column and ahead of the mass spectrometer. Connecting the reactor to the inlet of the analytical column would not afford an advantage as compared to classical hydrogenation before GC-MS analysis.

The activity of the catalyst, its selectivity for carbon-carbon double bonds, and adsorption on its surface were tested with mixtures of unsaturated esters. For instance, a mixture of methyl oleate and methyl linoleate (Fig. 1A) gave two peaks of methyl stearate when injected with the Pd reactor connected to the outlet of the column (Fig. 1B). Only slight variations of a few seconds were noted between independent runs. Peak tailing, due to adsorption of the eluates on the surface of the catalyst, was considered acceptable. Complete hydrogenation was obtained when the sample load did not exceed 1 μg/peak.

The unsaturated CFAM mixture described above was therefore submitted to post-column hydrogenation. The total ion currents obtained without and with the Pd

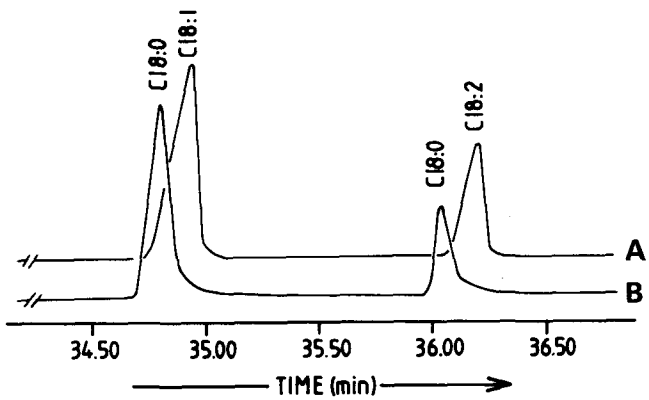

FIG. 1. Total ion currents (TIC) of a mixture of methyl oleate (C18:1) and methyl linoleate (C18:2). (A) Without Pd reactor. (B) With Pd reactor. Column: DB Wax 30 m × 0.25 mm i.d. programmed from 60 to 220°C at 5°C/min. Pd capillary reactor: 60 cm × 0.32 mm i.d. Carrier gas: H_2

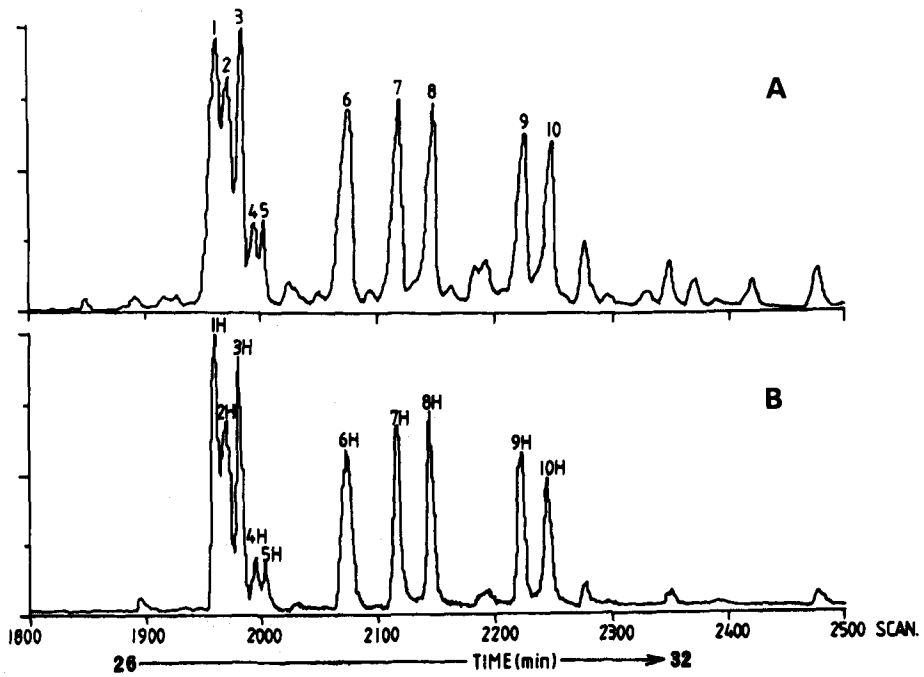

FIG. 2. TIC of CFAM esters isolated from heated linseed oil. (A) Without Pd reactor. (B) With Pd reactor. Column: DB Wax 30 m × 0.25 mm i.d. programmed from 60 to 180°C at 10°C/min, then to 220°C at 3°C/min. Pd capillary reactor: 60 cm × 0.32 mm i.d. Carrier gas: H_2

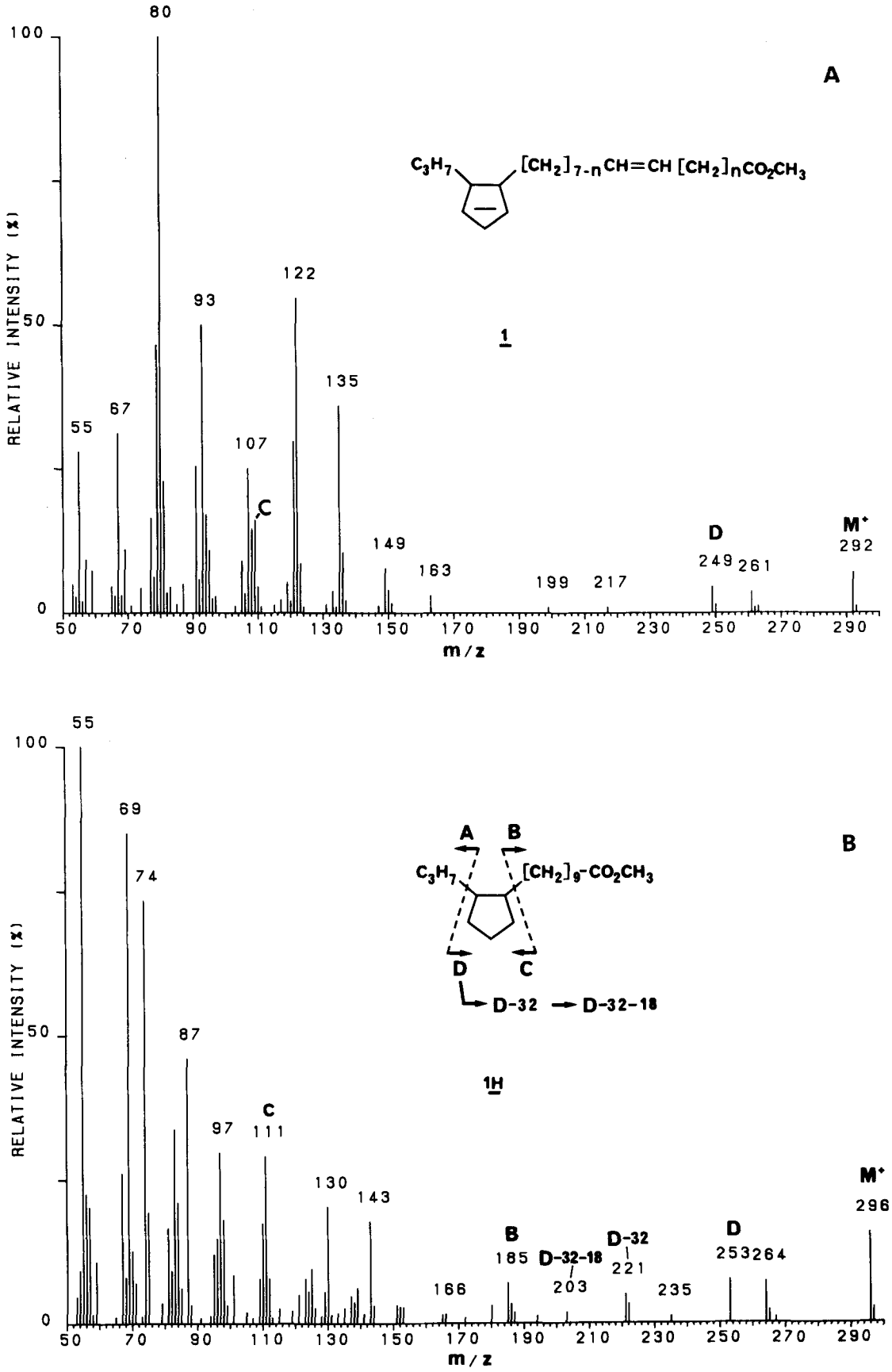

FIG. 3. Mass spectra (70 eV) of compound 1 (n = 0–7, A) and its hydrogenated counterpart 1H (B), with their identification and characteristic fragmentations. Peak numbers are those of Figure 2.

reactor could be superimposed (Fig. 2). Under the same chromatographic conditions, retention times were not altered by inserting the capillary reactor into the system. Only slight tailing of peaks was observed due to adsorption. Mass spectra of the pure hydrogenated species were obtained for each of the major 10 peaks. The carbon skeleton of each of the compounds (peaks 1 to 10) could clearly be established by the information gained through the mass spectra of their hydrogenated counterparts 1H to 10H (Fig. 2). For instance, peak number 1 was identified as a propylcyclopentenyl decenoate, because the mass spectrum of its hydrogenated product 1H (Fig. 3B) was identified as that of methyl (2-propylcyclopentyl) decanoate. The molecular ion was increased by four mass units from 292 to 296 (Fig. 3). Extensive changes in the spectrum occurred in the mass range of 50–150 as a result of hydrogenation. Diagnostic ions (Fig. 3B) B (*m/z*: 185), C (*m/z*: 111), D (*m/z*: 253), D-32 (*m/z*: 221) and D-32-18 (*m/z*: 203) were unambiguously assigned. We also noted ions of low intensity (*m/z*: 267 and 235) and attributed them to fragment ions D_β and D_β-32 resulting from a second cleavage of the alkyl moiety in the β-position. This hypothesis was confirmed by mass spectrometry of some synthesized alkyl-cyclopentyl derivatives (7), and the β-fragmentation of the alkyl chain seems to be characteristic of these alkyl-cyclopentyl esters.

Peak 2 was identified as the *cis*-isomer of 1, peaks 3 to 6 were identified as butylcyclopentenyl nonenoates, differing only by the position of the double bond in the side-chain, and peaks 7 to 10 were confirmed (2) to be isomers of propylcyclohexenyl nonenoates.

Activity and selectivity of the catalyst were tested periodically. Good results were obtained through several days of operation using one 60-cm piece of capillary reactor. The technique was found useful for structural investigations on mono- and polyunsaturated compounds of various origins as they occur in complex mixtures.

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COMMUNICATIONS

The Effect of Dietary Fish Oil on Muscle and Adipose Tissue Lipoprotein Lipase

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The activity of lipoprotein lipase (LPL) in the adipose tissue and skeletal muscle of rats fed glucose- or fructose-based diets containing fish oil, corn oil or tallow was examined. In addition, heart LPL activity was measured in rats fed a glucose-based diet containing either corn oil or fish oil. Adipose tissue LPL activity was unaffected by dietary fat. In both heart and skeletal muscle, LPL activity was higher in rats fed the fish oil diet. These results suggest that increased removal of triglyceride by muscle may contribute to the blood triglyceride lowering effect of dietary fish oil.
Lipids 24, 351-353 (1989).

Dietary omega-3 fatty acids have been shown to lower blood triglyceride levels. This effect has been shown to be due at least in part to decreased hepatic synthesis of triglycerides coupled with decreased VLDL secretion (1-5). However, increased clearance of triglyceride-rich lipoproteins could also contribute to the triglyceride-lowering effect of dietary fish oils. Harris and Connor (6) reported that in subjects who had consumed diets enriched in salmon or corn oil for two weeks, the increase in plasma triglyceride after a single dose of salmon oil was lower than after corn oil. They suggested this could be due to more rapid clearance of the omega-3 containing chylomicrons. However, they subsequently reported that postheparin lipolytic activity was unaffected by fish oil consumption (7). Brockerhoff et al. (8) and Bottino et al. (9) suggested that fish oils were poorly digested and absorbed, which could explain the lower plasma triglyceride responses reported by Harris and Connor (6). Chen et al. (10,11) have shown that the hydrolysis of chylomicron triglyceride enriched with EPA is similar to that of those enriched with oleate using recirculating heart perfusion, and this was confirmed in vivo. In the studies of Chen et al., the rats had been fed rat chow (Purina Rat Chow, Ralston Purina Co., St. Louis, MO). If lipoprotein lipase (LPL) activity and consequently, chylomicron clearance, changes with different dietary fats, it would not have been observed in these studies. Because of the inconclusive nature of these studies with regard to the effect of dietary fish oils on lipoprotein lipase activity, we have studied the activity of lipoprotein lipase in cardiac and skeletal muscle and adipose tissue in animals fed diets rich in saturated or omega-6 or omega-3 fatty acids. In addition, because of the known hypertriglyceridemic effects of dietary fructose, we have also examined the effects of these fats in animals consuming fructose- or glucose-based diets.

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Abbreviations: ANOVA, analysis of variance; FCO, fructose + corn oil; FMaxEPA, fructose + fish oil; FT, fructose + tallow; GCO, glucose + corn oil; GMaxEPA, glucose + fish oil; GT, glucose + tallow; LDL, low density lipoproteins; LPL, lipoprotein lipase; VLDL, very low density lipoproteins.

MATERIALS AND METHODS

Male Sprague-Dawley rats were obtained from Canadian Biobreeding Labs (Montreal). Animals were housed individually in plastic metabolic cages. The animals were maintained at a temperature of 25°C in a light controlled room (lights on 0800-2000). The rats were given 20 g of food per day at 0800. Uneaten food was removed at 1100. Thus, the animals had access to food for only 3 hr per day. Water was available *ad libitum.* Food consumption was measured daily. Six diets were used: fructose + tallow (FT); fructose + corn oil (FCO); fructose + fish oil (FMaxEPA); glucose + tallow (GT); glucose + corn oil (GCO); and glucose + fish oil (GMaxEPA). The diets had the compositions shown in Table 1. No antioxidant other than the vitamin E contained in the vitamin mix was added to the diets, and no attempt was made to equalize the cholesterol content. The diets were made fresh every other day and stored in sealed containers at −70° to minimize oxidation. In each experiment, four rats received each diet. The average initial weight of the animals was 220 ± 8 g (mean ± S.D.) in the fed experiment, 211 ± 12 in the fasted experiment. In a separate experiment, rats were meal fed a glucose based diet containing either corn oil or fish oil as described above. The average weight of all the rats in this experiment was 224 ± 13 g (mean ± S.D.). The average final weight of the fasted rats for each group was GCO, 248 g; GT, 250; GMaxEPA, 252; FCO, 259; FT, 258; FMaxEPA, 242. The average final weight of the fed rats in each group was GCO, 286 g; GT, 287; GMaxEPA, 283; FCO, 277; FT, 273; FMaxEPA, 263. There was no significant difference in initial or final weight among the dietary treatments in any experiment.

Dietary fats were transmethylated according to the procedure of Parkes and Thompson (12). Fatty acid methyl

TABLE 1

Composition of Experimental Diets (g/kg diet)[a]

Component	g
Glucose, or fructose	600
Casein	200
d,l methionine	3
Alpha cellulose	50
Fat (tallow, corn oil or MaxEPA)	100
AIN mineral mix	35
AIN vitamin mix	10
Choline Cl	2

[a]Diet components were obtained from ICN Nutritional Biochemicals except for maize oil, which was obtained locally (Mazola, Canada Starch Co., Montreal) and MaxEPA, a gift of D. B. Hutchinson, R. P. Scherer (Canada), Windsor, Ontario.

esters were quantitated by gas chromatography using a Supelco SP2330 capillary column (0.25 mm × 30 m) in a Perkin Elmer 8310 gas chromatograph. The oven temperature was 180°C. The carrier gas was Helium at a flow rate of 2 ml/min. Fatty acids were identified by retention time in comparison with known standards. The fatty acid composition of the dietary fats is presented in Table 2. Fatty acids which make up less than 1% of the total are not included.

LPL activity was determined in the epididymal fat pads and skeletal muscle of rats which had been fed on the day of the experiment and in rats which had not been fed on the day of the experiment. Tissue samples from fed rats were taken at 10:00, 2 hr after the start of the meal. Fasted samples were also taken at 10:00, that is 23 hr after the end of the previous days meal. In addition, LPL was determined in the hearts of fasted, glucose-fed rats. Fed rats had full stomachs at the time of the experiment. Rats which were not fed had empty stomachs. LPL activity was determined from the rate of release of [^{14}C]-oleate from ^{14}C-labelled triolein emulsion (specific activity = 7.5×10^5 dpm/μmol) according to the method of Nilsson-Ehle et al. (13). Extracts of acetone powders were used for the determination of epididymal fat pad LPL activity. Heart and skeletal muscle LPL were assayed on extracts of acetone powders of heart and the muscles from an entire hindlimb. Heart or muscle was rapidly frozen in liquid nitrogen and ground to a fine powder. A portion of the powder was homogenized in acetone. The acetone powder was extracted with 0.05 M NH_4OH/NH_4Cl buffer pH 8.1, and this extract was used for the assay of LPL activity. Activity was shown to be linear with time and enzyme concentration. LPL activity was calculated as the serum dependent, NaCl inhibitable activity.

Results for adipose tissue and hindquarter were analyzed by two way analysis of variance (ANOVA); heart LPL was analyzed by Student's t test. Where the ANOVA showed significant treatment effects, individual mean differences were determined by Duncan's new multiple range test (14). Effects were considered significant if $p < 0.05$.

Chemicals and biochemicals were obtained from Fisher Scientific Co., Sigma Chemical Co. or Boehringer Mannheim. Diet components were obtained from ICN Nutritional Biochemicals except for maize oil, which was obtained locally (Mazola, Canada Starch Co., Montreal) and MaxEPA, a marine oil which was a gift of D. B. Hutchinson, R.P. Scherer (Canada), Windsor, Ontario. Isotopes were obtained from New England Nuclear (LaChine, Quebec).

RESULTS AND DISCUSSION

Adipose tissue LPL activity was unaffected by either dietary fat or carbohydrate (Table 3), although activity was reduced by fasting as previously reported (15). There was no effect of diet composition on the weight of the epididymal fat pads in either the fed or fasted rats ($p > 0.05$ for main effects and interaction by ANOVA). The fatty acid composition of the diet has been shown to alter adipose tissue LPL activity although with varying results. In male Wistar rats fed a 30% fat diet of which 11 vs 2.5% were n-3 polyunsaturated fatty acids, LPL activity was lower in those fed 11% PUFA (16). In contrast, rats fed 20% corn oil vs lard had higher LPL (17). Similarly, male guinea pigs fed diets rich in CO had higher LPL activity than those fed tallow (18). Haug and Høstmark (19) reported that rats fed fish oil or a 50:50 mixture of fish oil and coconut oil had lower activity of epididymal fat pad LPL and hepatic lipase compared to those fed coconut oil. Nadeau et al. (20) reported that LPL was lower in rats fed corn oil than lard.

The activities of both heart and skeletal muscle LPL were greater in rats fed fish oil compared to corn oil (Tables 4 and 5). Skeletal muscle LPL activity was increased by starvation as previously reported for both heart and skeletal muscle LPL (21,22). Our finding of a modest increase in heart LPL is inconsistent with the findings of Chen et al. (10) that the clearance of oleate and EPA enriched chylomicrons by nonworking perfused rat hearts was the same. However, the hearts were from rats which had been consuming Purina Rat Chow, and it is probable that the differences we observed in LPL activity require adaptation to the dietary fats. Further, the

TABLE 2

Fatty Acid Composition of the Dietary Fats[a]

Fatty acid	Tallow	Corn oil	MaxEPA
14:0	2.3	0.1	7.0
16:0	29.3	10.3	17.3
16:1(n-7)	3.0	—	8.7
18:0	19.8	1.4	2.4
18:1(n-9)	36.9	25.5	14.9
18:2(n-6)	1.6	59.9	1.6
18:3(n-3)	—	—	2.4
18:4(n-3)	—	—	3.4
20:1	—	—	2.2
20:4(n-6)	—	—	1.1
20:5(n-3)	—	—	17.7
22:1	—	—	3.1
22:5(n-3)	—	—	2.1
22:6(n-3)	—	—	10.1
Unidentified	7.1	2.8	6.0
Σ saturates	51.4	11.8	26.7
Σ mono	36.9	25.5	28.9
Σ n-6	1.6	59.9	2.7
Σ n-3	—	—	35.7

[a]Fatty acid analysis is described in Materials and Methods. Fatty acids making up less than 1 % of the total are not included.

TABLE 3

Lipoprotein Lipase Activity in the Epididymal Fat Pads of Fed or Fasted Rats Fed Diets Containing Corn Oil, Tallow or MaxEPA[a]

	Corn oil	Tallow	MaxEPA
Fasted			
Glucose	2.7 ± 0.1	2.9 ± 1.0	3.8 ± 1.0
Fructose	3.2 ± 0.7	2.5 ± 0.6	2.6 ± 0.9
Fed			
Glucose	5.1 ± 1.7	6.1 ± 1.6	6.4 ± 0.8
Fructose	4.8 ± 0.5	5.4 ± 0.9	5.6 ± 2.0

[a]Carbohydrate was either glucose or fructose. Activities (total units/pr of epididymal fat pads) are presented as mean ± S.D. (n = 4).

TABLE 4

Lipoprotein Lipase Activity in the Hindlimb of Fed or Fasted Rats Fed Diets Containing Corn Oil, Tallow or MaxEPA[a]

	Corn oil	Tallow	MaxEPA
Fasted			
Glucose	44.2 ± 6.4[a]	49.0 ± 10.4[a]	80.1 ± 16.6[b]
Fructose	41.4 ± 8.5[a]	68.2 ± 11.5[b]	111.6 ± 42.7[c]
Fed			
Glucose	19.6 ± 3.8[a]	36.0 ± 11.4[b]	58.0 ± 13.1[c]
Fructose	27.7 ± 3.4[a,b]	35.1 ± 9.6[b]	45.0 ± 10.2[b,c]

[a]Carbohydrate was either glucose or fructose. Activities (mU/g of tissue) are presented as mean ± S.D. (n = 4).

In each dietary state (fed or fasted), values with a different superscript are significantly different ($p < 0.05$).

TABLE 5

Lipoprotein Lipase Activity in the Heart of Fasted Rats Fed a Glucose Based Diet Containing Either Corn Oil or MaxEPA

	mU/g (mean ± S.D.)
Corn oil (n = 10)	430 ± 73
MaxEPA (n = 11)	511 ± 71[a]

[a]Significantly different ($p < 0.05$).

chylomicrons prepared by Chen et al. were collected by lymph cannulation, washed and then used in the perfusions. It is possible that the modest difference in LPL activity we report is insufficient to alter the rate of clearance by perfused heart. Subsequent work by Chen et al. (11) showed no differences in the rate of clearance *in vivo*, but again in rats fed Purina Rat Chow so there was no opportunity for adaptation of LPL to the different dietary fats.

Huff et al. have reported increased conversion of VLDL apo B to LDL in miniature pigs fed fish oil compared to corn oil (23). However, the fish oil reduced both post-heparin lipoprotein and hepatic triglyceride lipase. In humans consuming fish oil, there was no difference in post-heparin lipolytic activity compared to subjects consuming vegetable oils (7). We don't feel that these results are inconsistent with our finding of increased muscle LPL in fish oil fed rats. Post-heparin plasma LPL activity represents both muscle and adipose tissue activity. We found no differences in adipose tissue LPL due to dietary fat. In post heparin plasma, the difference in heart and skeletal muscle activities could be masked by the contribution of adipose tissue activity to the total measured.

In addition to the differences in the omega-3 fatty acid content, the dietary fats used in this study also differed in their content of oleic and linoleic acids. The tallow and corn oil have very different content of linoleic acid but rats consuming these fats had similar activity of muscle LPL, so it does not seem that differences in this fatty acid contribute to the observed effects of diet on muscle LPL activity. The case of oleic acid is not as clear, but if the increased activity of LPL were due to the reduction in the oleic acid content of the diet one might expect there to be a concentration dependence of the effect. That is, the activity of muscle LPL should be intermediate for the corn oil diet. In fact, the activity of muscle LPL in rats fed the tallow diet is generally between the other two. If the classes of fatty acids as given at the bottom of Table 2 are examined, the predominant difference between the MaxEPA diet which resulted in the highest muscle LPL activity and the other two diets is the omega-3 fatty acid content. We believe that it is the omega-3 fatty acid content of the fish oil diet which is responsible for the higher activity of muscle LPL activity.

We believe that increased heart and skeletal muscle LPL activity could contribute to the lower plasma triglyceride in fish oil fed animals and humans. It might also direct more of the omega-3 enriched chylomicrons toward muscle for oxidation of the n-3 fatty acids. Increased oxidation of omega-3 fatty acids by muscle could at least partially explain the low quantities of n-3 fatty acids in the adipose tissue of rats fed omega-3 enriched diets (24).

ACKNOWLEDGMENTS

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Association of the Intestinal Brush-Border Membrane Phospholipase A_2 and Lysophospholipase Activities (Phospholipase B) with a Stalked Membrane Protein

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We have attempted to determine the size and membrane orientation of a recently described rat jejunal brush-border protein possessing phospholipase A_2 and lysophospholipase activities (phospholipase B) (Pind, S. and Kuksis, A. [1988] *Biochim. Biophys. Acta 938,* 211-221). The phospholipase A_2 and lysophospholipase activities were renatured following nonreducing sodium dodecyl sulphate polyacrylamide gel electrophoresis of the total membrane proteins and were shown to migrate as a component of a protein band having a relative molecular mass of 170 kDa. This band accounted for approximately 1% of the total Coomassie Blue staining proteins. Phospholipase B was also shown to be solubilized from the membranes, in an active form, by a proteolytic digestion with papain. Papain solubilization resulted in a loss of the hydrophobic properties observed for the intact phospholipase. These results suggest that the active site of the phospholipase projects from the luminal surface of the membrane vesicles. In support of this, phospholipase activity towards exogenous, detergent-solubilized phosphatidylcholine was demonstrated under conditions in which the membranes remained intact. We conclude that the phospholipase B has the characteristics of a stalked, brush-border membrane protein and may be considered as another digestive enzyme anchored in this membrane. *Lipids 24,* 357-362 (1989).

The intestinal brush-border membrane is characterized by the presence of several stalked membrane proteins that project from the membrane surface into the intestinal lumen (1-4). These proteins, often dimeric in structure, are hydrolytic enzymes capable of splitting peptide, glycoside or ester bonds, thereby converting complex macromolecules into lower-molecular-weight components prior to their absorption from the gut. They are stalked proteins in that most of their mass, including their active sites, is not in direct contact with the membrane; anchorage to the membrane is accomplished via small hydrophobic segments, generally comprising only 3-6% of the total protein, and which are connected to the main protein mass by polypeptide stalks. These enzymes are intrinsic membrane proteins in that detergents are necessary for their solubilization. However, many can also be released from the membrane by protease digestion (usually with papain or elastase) of the stalk region. Perhaps the best characterized of these proteins is the sucrase-isomaltase complex (4) which comprises approximately 10% of the total brush-border membrane protein and has been recently cloned (5).

We have previously shown that purified brush-border membrane vesicles from rat jejuna contain membrane-bound phospholipase A_2 and lysophospholipase activities (6,7). The A_2 activity is Ca^{2+}-independent, maximally active at pH 8-11, and specifically stimulated by bile-salt detergents. The lysophospholipase activity is also Ca^{2+}-independent, but is only minimally activated by bile salts. Based on competition experiments, we proposed that a single active site was responsible for both activities (i.e., phospholipase B) (7). It has been suggested (8,9) that a phospholipase in the brush-border membrane may play a role in digestion of dietary phospholipids. In order to further characterize this protein, we have attempted to identify the size and location of the enzyme within the brush border. This study shows that both enzymatic activities are associated with a protein having a relative molecular mass of ca. 170 kDa which is located on the luminal surface of the membrane. This phospholipase B conforms with the general model of a stalked membrane protein and may, therefore, be considered as another digestive enzyme associated with this membrane. An abstract has appeared (10).

MATERIALS AND METHODS

Materials. Papain (from *Papaya carica*) and Triton X-114 (purified for membrane research) were obtained from Boehringer Mannheim Canada Ltd. (Dorval, Que.). Phenyl-Sepharose CL-4B and Sephacryl S-300 were purchased from Pharmacia (Canada) Ltd. (Dorval). Aquacide II was from Calbiochem-Behring Corp. (San Diego, CA). Lysopalmitoylphosphatidylcholine, L-1-[palmitoyl-1-^{14}C] (55 mCi/mmol), was obtained from New England Nuclear/Dupont Canada Inc. (Lachine, Que.). All other materials were as previously described (6,7) or were of reagent grade or better quality.

Brush-border membrane preparation. Brush-border membrane vesicles were purified 25-30-fold from rat jejunal scrapings as described (6). Membranes were suspended, at a concentration of 1-2 mg protein/ml, in 50 mM mannitol/2 mM Hepes (pH 7.1) and stored at −30°C. All experiments were done with samples stored for less than one week.

Phospholipase assays. Phospholipase A_2 and lysophospholipase activities were assayed as previously described (7) using egg yolk phosphatidylcholine mixed with tracer amounts of 1-palmitoyl-2-[1-^{14}C]oleoyl-*sn*-glycerol-3-phosphorylcholine, and using *sn*-1-acyl-lysophosphatidylcholine mixed with tracer quantities of 1-[1-^{14}C]-palmitoyl-*sn*-glycerol-3-phosphorylcholine, respectively. The labeled phosphatidylcholine was diluted to ca. 130 cpm/nmol. Unlabeled *sn*-1-acyl-lysophosphatidylcholine

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Abbreviations: BCA, bicinchoninic acid; CHAPS, 3-[(cholamidopropyl)-dimethylammonio]-1-propanesulphonate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate (disodium); Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; PBS, phosphate buffered saline; SDS, sodium dodecyl sulphate; Tris, 2-amino-2-hydroxymethylpropane-1,3-diol.

was prepared from egg yolk phosphatidylcholine (7) and mixed with the palmitate-labeled material to yield ca. 130 cpm/nmol. All assays were done in the presence of 1% CHAPS detergent. The phospholipase activity of intact brush-border membrane vesicles was also measured. For this purpose membranes (300 μg protein) were incubated in 7 mM taurocholate/1 mM labeled phosphatidylcholine mixed micelles (1 ml final volume in 20 mM Hepes, pH 7.2), prepared as described by Child and Kuksis (11). Following 15 min at 37°C the membranes were separated from the aqueous solution by centrifugation at 15,000 × *g* for 30 min in an Eppendorf microcentrifuge (Brinkman Instruments Ltd., Toronto). The resulting supernatant was removed and free fatty acids were extracted and quantitated (7), whereas the pellet was resuspended and assayed for protein content.

Renaturation of phospholipase activities. Renaturation of proteins separated by SDS-polyacrylamide gel electrophoresis was accomplished essentially as described by Hager and Burgess (12), with the exception that reducing agents were not used in any of the steps and the dilution buffer was modified to contain 0.5% CHAPS. Briefly, aliquots of the membrane vesicles, corresponding to 200 μg total protein, were centrifuged at 15,000 × *g* for 30 min. The resulting pellets were resuspended in 100 μl of nonreducing Laemmli sample buffer (13) containing 10 μg soybean trypsin inhibitor as a low-molecular-weight protective agent, and heated at 95°C for 5 min. Samples were loaded into 8-mm wide lanes and electrophoresed down a 7.5% polyacrylamide slab gel at 8 mA constant current (13). When the dye front reached the bottom of the gel (16–18 hr) the gel was removed and cut lengthwise into two. One-half was stained with Coomassie Blue R-250 (6), whereas the other half was visualized with ice-cold 0.25 M KCl. Individual gel lanes were isolated and cut into 3- to 10-mm pieces which were placed into 13 × 100 mm borosilicate glass tubes, rinsed with H_2O and crushed in 1.5 ml of the SDS-containing elution buffer (−DTT). After 6 hr of elution at room temperature the buffer was removed, a further 1.0 ml was added to the gel pieces, and elution was allowed to continue overnight. The next morning the second gel eluate was combined with the first and the eluted proteins were recovered by acetone precipitation and centrifugation. The acetone precipitate was allowed to dry for 10 min and then dissolved in 40 μl of 6 M guanidine-HCl in the modified dilution buffer (+0.5% CHAPS, −DDT) and allowed to stand at room temperature for 20 min. Following this, 2.0 ml of the modified dilution buffer was added and the samples were left to renature for 2 hr at room temperature. Aliquots (0.2 ml) of each sample were then assayed for phospholipase A_2 and lysophospholipase activities.

Papain digestion. Papain solubilization of brush-border membrane proteins was accomplished using the methods described by Sigrist *et al.* (14). Commercial papain was activated by diluting 100 μl (1 mg protein) of the crystalline suspension with 2.9 ml of 50 mM KH_2PO_4/1 mM EDTA (pH 6.5) containing 5 mM freshly added cysteine hydrochloride, Buffer I, and allowing the mixture to stand for 30 min at room temperature. Prior to digestion, membrane vesicles were pelleted by centrifugation at 15,000 × *g* for 30 min in the microcentrifuge and were resuspended at a concentration of ca. 1 mg protein/ml in Buffer I. Seven volumes of the membranes were mixed with 2 volumes of the activated papain and incubated at 37°C for various time periods. Following incubation, 1 volume of 0.1 M iodoacetamide was added to stop the digestion and the solubilized proteins were separated from the remaining membranes by centrifugation, as previously described.

Gel-filtration chromatography. The solubilized fraction arising from papain digestion of the brush-border membranes from 6 rats was separated from the residual membranes by centrifugation at 27,000 × *g* for 30 min (Sorvall, SS34). The volume of this fraction (20 ml) was reduced to 3–4 ml by placing it in a dialysis bag and surrounding the bag with dry Aquacide II. The concentrated fraction was dialyzed overnight against 500 volumes of PBS (2.7 mM KCl, 1.5 mM KH_2PO_4, 137 mM NaCl, 8 mM Na_2HPO_4, pH 7.4) and then loaded onto a column of Sephacryl S-300 (1.5 × 102 cm) which had been packed and equilibrated in the PBS. The column was eluted with PBS, under gravity flow, at a rate of ca. 10 ml/hr.

Hydrophobic-interaction chromatography. Proteins were solubilized from brush-border membranes by papain digestion, as indicated, or with 2% CHAPS as previously described (7). Aliquots (2 ml, 1.1–1.5 mg protein) of the supernatants obtained following centrifugation were placed into dialysis bags and dialyzed against 500 volumes of 25 mM Tris-HCl (pH 7.8). As a control, one aliquot of the papain solubilized proteins was mixed with CHAPS (final concentration 2%) prior to dialysis. Following dialysis for 1.5 days, the samples were passed over 1-ml columns of phenyl-Sepharose (packed in Pasteur pipets) which had been previously equilibrated in the 25 mM Tris-HCl buffer. Unbound proteins were eluted by washing with a further 5 ml of 25 mM Tris-HCl and then bound proteins were eluted with 10 ml of 1% CHAPS in the same buffer.

Other methods. Brush-border membrane proteins solubilized following extraction with 1% (w/v) Triton X-114 and centrifugaton were subjected to phase separation as described by Bordier (15). Proteins were assayed using the BCA protein assay reagent as previously described (7). Gels to be silver stained were fixed in 40% ethanol–10% acetic acid and rinsed in 10% ethanol (16), before equilibrating overnight in distilled water and staining as described by Morrissey (17), beginning at his step four. The staining reaction was stopped using acetic acid (16). Sucrase was assyed as previously described (6).

RESULTS

Preliminary trials of the protein renaturation procedures described by Hager and Burgess (12) revealed that the brush-border phospholipase activity could be recovered if reducing agents were omitted. Exposing SDS or guanidine-HCl denatured brush-border membrane proteins to 1 mM DTT resulted in a complete absence of activity following renaturation. This effect was specific for the denatured protein as 1 mM DTT caused only a slight (10%) inhibition of phospholipase activity when the native enzyme was assayed in the presence of this reducing agent. Figure 1 shows the region of the gel from which phospholipase A_2 and lysophospholipase activities were recovered following electrophoresis. The two activities comigrated exactly on the gel and also appeared to comigrate with a protein band having a relative molecular

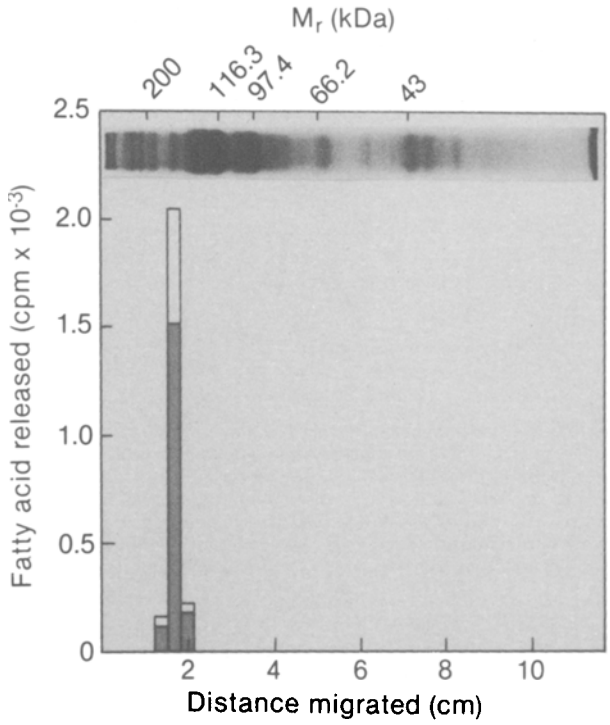

FIG. 1. Location of brush-border membrane phospholipase activity following SDS-polyacrylamide gel electrophoresis. 7.5% gels were run under nonreducing conditions and the separated proteins were eluted, renatured and assayed as described in Materials and Methods. The graph shows the distance migrated by phospholipase A_2 (open bars) and lysophospholipase (shaded bars) activities. Shown at the top of the figure is a corresponding Coomassie Blue-stained lane, aligned with the distance markers on the bottom. The mobilities of molecular weight standards are shown along the top edge of the figure. In this experiment 38% of the total phospholipase activity loaded onto the gel was recovered in the active fractions.

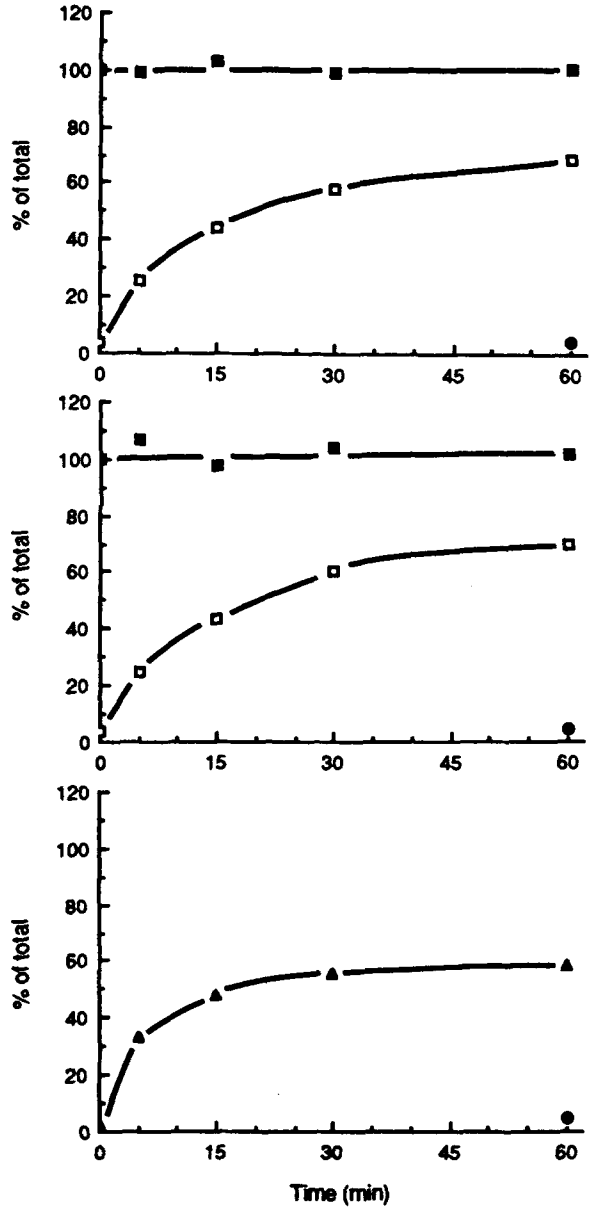

FIG. 2. Solubilization of phospholipase activity and protein by papain digestion of brush-border membrane vesicles. Papain incubation and centrifugal separation of the solubilized and residual membrane components were done as described in Materials and Methods. Phospholipase activities in the soluble and membrane bound fractions and the protein content of the membranes were measured as described in Materials and Methods. The % of total activity remaining (soluble + membrane bound, closed squares) and the % of activity solubilized (open squares) is shown for phospholipase A_2 (top) and lysophospholipase (middle), following papain digestion for 0–60 min. The bottom graph shows the % of total protein solubilized during the same incubations. The closed circle in each graph shows the % of activity (protein) that was solubilized during an incubation in the absence of papain.

mass of 170 kDa. Based on Coomassie Blue staining intensities, this band accounts for ca. 1% of the total brush-border membrane proteins. Although this band appears well separated from most of the others, this analysis does not reveal how many protein components overlap at this position. Our recent results indicate that three distinct proteins migrate with this 170 kDa band (Pind, S. and Kuksis, A., unpublished results). The total enzymatic activities recovered following elution and renaturation consistently corresponded to 25–50% of those loaded onto the gel.

Figure 2 shows that phospholipase A_2 and lysophospholipase activities were solubilized in an identical manner from the brush-border membrane by digestion with papain. Papain was essential for this solubilization as less than 5% of the activities or total protein were released during a 60-min incubation in the absence of this protease. Release of total protein and phospholipase activities was initially rapid but leveled off to a plateau value by 20 (protein) or 30 min (activities). Recovery of the total phospholipase activities (soluble + membrane bound) remained constant throughout the incubations, indicating that the protease was not affecting the enzyme's active site. A papain digestion of 60 min resulted in the solubilization of ca. 70% of both enzyme activities and ca. 60% of the total membrane protein. A 60-min digestion also solubilized 97% of the total sucrase activity. Several previous studies have shown that virtually all of the brush border sucrase activity and from 30–60% of the total membrane proteins are solubilized during papain digestion (18–20), in agreement with our results.

Figure 3 shows that the papain digestion did effect a specific solubilization of the membrane proteins, with

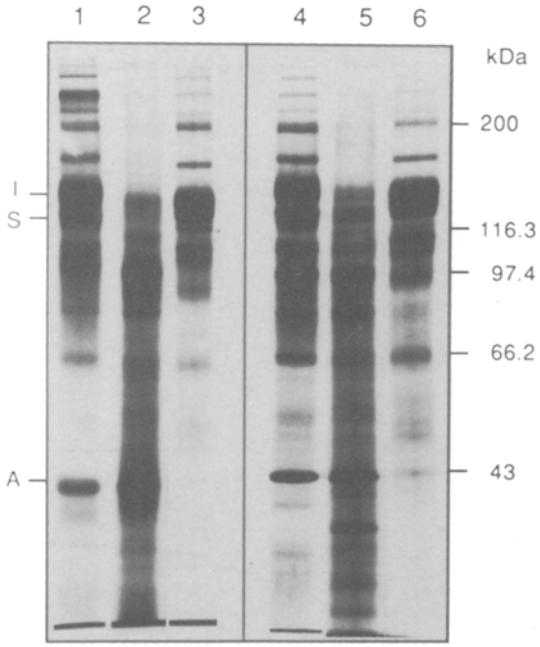

FIG. 3. SDS-polyacrylamide gel analysis of the various fractions resulting from papain digestion. Protein from the intact brush-border membranes (lanes 1 and 4) and the residual membrane (lanes 2 and 5) or the soluble fraction (lanes 3 and 6) obtained following a 60-min papain digestion were electrophoresed under nonreducing (lanes 1-3) or reducing (lanes 4-6) conditions on 7.5% polyacrylamide gels. Bands were visualized by silver staining. The mobilities of molecular weight standards are shown along the righthand margin. I, isomaltase-rich band; S, sucrase-rich band; A, actin-rich band.

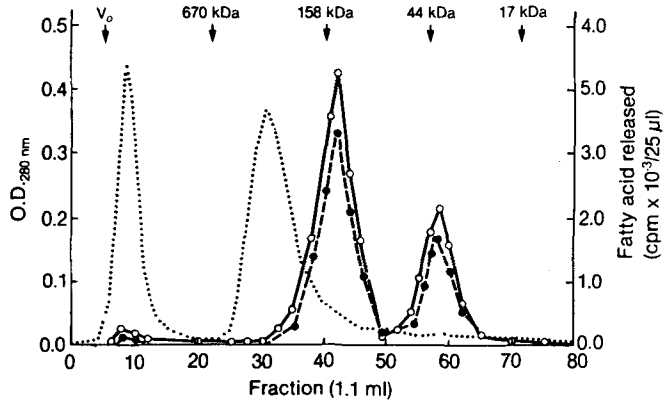

FIG. 4. Gel-filtration profile of the proteins solubilized by a 60-min papain digestion. The solubilized sample was prepared and applied to the Sephacryl S-300 column as described in Materials and Methods. A 55-ml fraction was collected before beginning the 1.1 ml fractions. The elution position of gel-filtration standard proteins (Bio-Rad) are shown along the top edge of the figure. ·····, O.D. 280 nm; O—O, phospholipase A_2 activity; ●—●, lysophospholipase activity.

some bands being retained by the residual membrane (lanes 2 and 5) and others appearing in the soluble fraction (lanes 3 and 6). For example, the band composed primarily of actin, which originates from the interior of the vesicles (3), was not digested with papain and was recovered almost exclusively in the membrane pellet, whereas the isomaltase- and sucrase-rich bands were largely transferred to the supernatant fraction. These bands were identified by their size, the prominence of the respective proteins in the brush-border membrane (1-4), by comparison with published figures (21,22), and with the knowledge that sucrase activity was solubilized by papain. That an intact actin band was observed following papain digestion suggests that the vesicles remained sealed during digestion, in agreement with previous results (20,21). Although the bands were sharper under reducing conditions (lanes 4-6), the protein profiles appeared similar under reducing and nonreducing conditions. The protein profile of the residual membrane fraction is similar to that published by Klip *et al.* (21,22), following papain digestion of rabbit brush-border membranes. These results indicate that the active site of the phospholipase is present on the luminal surface of the vesicles and that a portion of the enzyme containing the active site can be released from the membrane by papain digestion.

Figure 3 also shows that papain digestion solubilized a protein band having an apparent molecular mass of 160-165 kDa, which was similar in abundance to the 170 kDa band in the total membrane fraction. It is not possible, as yet, to definitely assign the solubilized phospholipase as a component of this band. Renaturation experiments were inconclusive as very little total activity was recovered. Gel-filtration chromatography of the proteins released by a 60-min papain digestion showed that the active site of the solubilized phospholipase was associated with two major fragments, having approximate molecular weights of 120 and 35 kDa (Fig. 4). Phospholipase and lysophospholipase activities were found in similar proportions in both of these fragments, again indicating that one active site is responsible for both activities. Following a 2-hr papain digestion, the relative proportion of the 35 kDa fragment increased, suggesting that it was produced from the larger fragment by a further proteolytic step. This result shows that the phospholipase has more than one susceptible site for protease attack during the papain digestion. SDS-polyacrylamide gel analysis of the 120 kDa region of the elution profile in Figure 4 showed that a 160-165 kDa band, among others, was found in this region (not shown).

Papain digestion was shown to reduce the hydrophobicity of the phospholipase by two different criteria. First, more than 75% of the activity in a sample of CHAPS-solubilized and dialyzed brush-border membrane proteins became bound to a hydrophobic column of phenyl-Sepharose during a buffer wash and was eluted only after 1% CHAPS was added to the eluant. In contrast, more than 95% of the phospholipase activity passed through the column during the buffer wash following papain solubilization of the proteins (+/− added CHAPS). Second, during Triton X-114 phase partitioning of the intact membrane proteins, the phospholipase partioned exclusively (>98%) with the detergent phase, whereas following papain digestion, the phospholipase remained (>98%) in the aqueous phase. In both of the above procedures, phospholipase A_2 and lysophospholipase activities were distributed identically. These results indicate that the intact phospholipase possesses a significant hydrophobic character that is lost upon papain solubilization.

To investigate the ability of intact brush-border membranes to hydrolyze exogenous phospholipids, the vesicles were incubated with mixed taurocholate-phosphatidylcholine micelles. These conditions have previously been shown to be nondestructive towards brush border (11) and red blood cell (23) membranes. Furthermore, taurocholate displayed the mildest membrane-solubilizing effect towards the brush border of a number of different surfactants analyzed (7,24). Under the test conditions, the membranes were recovered intact and the incubation resulted in significant hydrolysis of phosphatidylcholine. The specific activity of the phospholipase A_2 hydrolysis was calculated to be ca. 50 nmol/mg protein per min, which is about 40% of that observed under maximal conditions (7). More than 80% of the total protein was recovered in the membrane pellet following incubation.

DISCUSSION

The results presented in this report substantiate and extend our earlier claim (7) that a single brush-border membrane protein possesses both phospholipase A_2 and lysophospholipase activities. The activities were found at the same position and renatured to the same extent following SDS-polyacrylamide gel electrophoresis, were solubilized in an identical fashion by papain digestion, possessed similar amphipathic properties either before or after papain digestion, and coeluted during gel-filtration chromatography. In other studies, we have shown that the phospholipase is retained by a column of Concanavalin A-Sepharose, suggesting that it is a glycoprotein (Pind, S. and Kuksis, A., unpublished data). Our present results further indicate that this protein migrates on nonreducing SDS-polyacylamide gels with an apparent molecular mass of 170 kDa. This size is significantly larger than that of most other intra- or extracellular phospholipases studied to date (25–28), but should be taken as an estimate as many glycoproteins behave anomalously during electrophoresis (29). However, it is also noted that a size of 170 kDa is similar to that determined for several of the other brush-border membrane hydrolases (1,2).

Papain solubilization of phospholipase B activity, combined with the knowledge that papain attacks only the outer, luminal surface of brush-border membrane vesicles (18–20), indicates that the active site projects into the intestinal lumen. It also shows that this phospholipase shares structural similarities with the other brush-border hydrolases and is likely to be a stalked membrane protein. Papain digestion removed the hydrophobic character of the phospholipase, presumably by releasing the nonpolar part which interacts with the membrane bilayer. This hydrophobic part also caused the intact protein to associate with the detergent phase in the Triton X-114 partitioning assay. Tiruppathi *et al.* (30) have shown that many of the brush-border membrane enzymes (alkaline phosphatase, leucine aminopeptidase, γ-glutamyl transpeptidase and Ca^{2+}-Mg^{2+} ATPase) partition into the detergent phase, while several others (glucoamylase, sucrase-isomaltase, trehalase and lactase) remain in the aqueous phase. The phospholipase appears to be most similar to leucine aminopeptidase (aminopeptidase N) and γ-glutamyl transpeptidase in that these enzymes have sufficient hydrophobic character to partition into the Triton detergent phase and are also solubilized from the membrane by papain digestion (1,2).

Although most of the membrane hydrolases are normally resistant to proteolytic digestion following papain solubilization (1,2), it is expected that prolonged digestion would lead to multiple cleavages of a brush-border enzyme, provided that the appropriate susceptible sites were accessible. For example, porcine enterokinase is solubilized from duodenal brush-border membranes with papain, but rapidly loses activity due to further degradation of the solubilized fragment by this protease (31). In addition, aminopeptidase N from porcine intestine (32,33) or kidney (32,34) is converted by a trypsin treatment from a single subunit into two smaller subunits. It appears that the phospholipase has at least two papain susceptible regions; cleavage at one site (the stalk region) releases a large active fragment from the membrane, whereas cleavage at a second site produces a much smaller active fragment. Identifying the location of these cleavages and their resulting products will require more sensitive methods than those presently applied, including an analysis of the action of papain on the purified phospholipase.

Only one other intestinal phospholipase has been studied in detail. A low molecular weight, Ca^{2+}-dependent phospholipase A_2 with a substrate preference for phosphatidylglycerol has been described (35) and purified (36) from pig ileum. This enzyme has subsequently been localized in the Paneth cells and was postulated to be of importance in the digestion of plant and bacterial phospholipids (37). This enzyme is, thus, quite different from the phospholipase B described in the present report.

Although the physiological importance of a brush-border phospholipase B has yet to be determined, this enzyme may assist pancreatic phospholipase A_2 in the digestion of dietary or biliary phospholipids. We have previously shown that the phospholipase can attack endogenous, membrane phosphatidylethanolamine *in vitro* (6) and that the detergent-solubilized enzyme can attack exogenous, detergent-solubilized phospholipids (7). We have now shown that brush-border vesicles can hydroyze exogenous phospholipids in mixed bile-salt micelles, under conditions in which the membrane remains intact. This is consistent with the papain digestion results, predicting a localization of the active site on the luminal surface of the membrane. Numerous studies in animals and humans have shown that luminal phospholipids are hydrolyzed to *sn*-1-acyl-lysophospholipids and free fatty acids prior to their absorption into the enterocyte (38–43). Several of these experiments demonstrated that significant amounts of the resulting lysophospholipids were further hydrolyzed (38–42). Le Kim and Betzing (42) have estimated, on the basis of a test meal in rats, that up to 50% of the phosphatidylcholine was completely hydrolyzed to free fatty acids and glycerophosphocholine or its hydrolysis products. The brush-border phospholipase B has the ability to remove both acyl groups from luminal phospholipids. We have shown that the lysophospholipase activity predominates in the absence of detergent, whereas in the presence of bile salts, the normal luminal detergents, diacyl- and lysophospholipids compete for hydrolysis (7). At any given instant, phospholipase activity would then be determined by the bile-salt concentration and the relative proportions of diacyl- and lysophospholipids which reach the luminal surface of the

brush-border membrane. This membrane is, thus, well equipped to digest luminal lipids, as it also contains enzymes that hydrolyze glycosylceramides (44), sphingomyelin (45) and ceramides (45). The recognition that two enzyme activities are associated with a single, 170 kDa protein ought to facilitate purification of this phospholipase to homogeneity.

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1-*O*-Alk-1′-enyl-2-acyl and 1-*O*-Alkyl-2-acyl Glycerophospholipids in White Muscle of Bonito *Euthynnus pelamis* (Linnaeus)

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The existence of ether-linked phospholipids, including 1-*O*-alk-1′-enyl-2-acyl and 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholines and ethanolamines, in bonito *Euthynnus pelamis* (Linnaeus) white muscle, was investigated by gas chromatography and gas chromatography–mass spectrometry. Chemical ionization (*iso*-butane) mass spectrometry of trimethylsilyl ethers derived from the corresponding ether-linked glycerophospholipids proved effective not only for determining molecular weights but also for structural identification based on the ions $[M - R]^+$, $[M - RO]^+$ and $[M + 1]^+$. 1-*O*-Alk-1′-enyl-2-acyl-*sn*-glycero-3-phosphocholine and ethanolamine accounted for 3.0–6.0% and 3.6–7.6% of the total glycerophospholipids, respectively. 1-*O*-Alkyl-2-acyl-*sn*-glycero-3-phosphocholine and ethanolamine were also determined for one fish and accounted for 1.4% and 0.6% of the total glycerophospholipids, respectively. The predominant long chains in the *sn*-1 position of the glycerol moieties were 16:0, 18:0 and 18:1 in the case of the alkenylacyl and alkylacyl components. Fatty acid distribution of individual glycerophospholipids was also determined.
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Ether linked phospholipids, including 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine and ethanolamine and 1-*O*-alk-1′-enyl-2-acyl-*sn*-glycero-3-phosphocholine and ethanolamine (plasmalogens), occur in various species of marine organisms (1–3). Lewis reported on the alkyl chain composition of total phospholipids of anchovy, shrimp and squid (4). Marine worms (5), abalone (6), sponges (7) and starfish (8) also contain a significant amount of plasmalogen. The gills of crustaceans such as crayfish and lobster, and the body lipids of certain species of crab, mussel, oyster and scallop are rich sources of plasmalogen (9–12). Goldfish brain (13) and garfish olfactory nervous systems (14) are well known to contain alk-1′-enylacyl glycerophospholipids as choline and ethanolamine analogues of the better-known diacyl glycerophospholipids, although their biological significance is not well understood (15). There is little information on ether-linked phospholipids in fish muscle; the presence of plasmalogen was confirmed in South African pilchard flesh by a positive Schiff reaction (16). The occurrence of plasmalogen has also been reported in carp muscle mitochondria (17), rock trout, saury, sardine, codfish (18) and dogfish (19).

In the present study, ether-linked phospholipids of fish muscle were investigated and found to be present in bonito white muscle at a relatively high level. Identification of 1-*O*-alkylacyl and 1-*O*-alk-1′-enylacyl glycerophospholipids of bonito were carried out by gas liquid chromatography (GLC) combined with chemical ionization (CI) mass spectrometry (MS) of the corresponding trimethylsilyl (TMS) ether derivatives.

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Abbreviations: CI, chemical ionization; CPG, choline glycerophospholipid; DMA, dimethylacetal; EI, electron impact; EPG, ethanolamine glycerophospholipid; GLC, gas-liquid chromatography; LCA, long chain aldehyde; LPC, lyso choline glycerophospholipid; LPE, lysoethanolamine glycerophospholipid; MS, mass spectrometry; O, origin; PUFA, polyunsaturated fatty acids; SF, solvent front; TLC, thin-layer chromatography; TMS, trimethylsilyl.

MATERIALS AND METHODS

Standard materials. Bovine heart ethanolamine plasmalogen and 1-palmityl glyceryl ether were purchased from Serdary Research Laboratories (London, Ontario, Canada).

Lipid extraction and fractionation. White muscle lipid of bonito, *Euthynnus pelamis* (Linnaeus), was extracted with chloroform/methanol according to the Bligh and Dyer procedure (20). The phospholipid fraction was separated from other lipids by column chromatography on Bio-Beads S-X2, using benzene as eluent (21).

Quantitation of phospholipid species. Phospholipid classes were separated from each other by two-dimensional thin-layer chromatography (TLC) on precoated Silica Gel G plates (0.25 mm thickness, Merck Japan Ltd., Tokyo) using the solvent system chloroform/methanol/water/acetic acid (65:35:2:1, v/v/v/v) in the first direction and chloroform/methanol/water (60:30:8, v/v/v) in the second direction. The spots corresponding to phospholipids were visualized by exposing the plates to iodine vapor and were each scraped off. The phospholipids were extracted from the silica gel with chloroform/methanol (2:1, v/v). Phosphorus content was determined spectrophotometrically according to the method of Bartlett (22). The ratios of 1-*O*-alkyl-2-acyl vs 1,2-diacyl compounds were estimated by measuring total fatty acids, using 13:0 methyl ester as internal standard (23).

Mild acid hydrolysis and Iatroscan thin-layer chromatography. An aliquot of a chloroform solution of the phospholipid class was evaporated to dryness in a round-bottomed flask, which was then placed in a glass tank saturated with hydrochloric acid fumes for 5 min at room temperature (24). After removal of hydrochloric acid by flushing with a nitrogen stream, the acid-hydrolyzed phospholipid in the flask was subjected to Iatroscan TLC on Chromarods S-II (Dia-Iatron Co., Tokyo, Japan) using a double development technique in the same direction with acetone first to move neutral lipids and then a mixture of chloroform/methanol/acetic acid/water to resolve polar lipids (25).

Lipid derivatization. Phospholipids were reduced with Vitride reagent (Tokyo Kasei Co., Ltd., Tokyo, Japan) to the corresponding 1-alk-1′-enyl or 1-alkyl *sn*-glycerols (26). 1-*O*-Alkyl and 1-*O*-alk-1′-enyl glycerols were separated from each other by TLC on Silica Gel G (diethyl ether/ammonia, 100:0.25, v/v) and extracted from the silica gel with chloroform/methanol (1:2, v/v). Treatment with a mixture of hexamethyldisilazane and trimethylchlorosilane in dry pyridine produced the TMS ether derivatives (27).

Dimethylacetal (DMA) derivatives of long chain aldehydes, which were liberated from the corresponding

alkenylacyl phospholipids by the mild acid hydrolysis mentioned above, were prepared by heating the aldehydes under reflux with 5% methanolic hydrogen chloride (28).

Determination of 1-O-alkyl-2-acyl, 1-O-alkenyl-2-acyl, and 1,2-diacyl chain distribution. The individual phospholipid classes were analyzed according to Mueller *et al.* (29). Our preliminary examination showed that iodine vapor used for visualizing the spots on the TLC plate could cause decomposition of the alkenylacyl phospholipids. Therefore, 2′,7′-dichlorofluorescein in ethanol was used for detection of the samples prepared for the analysis of fatty chain distribution. The purified choline and ethanolamine glycerophospholipid fractions were treated with phospholipase C (Boehringer Mannheim GmbH-Yamanouchi, Tokyo, Japan) to yield diradyl glycerols (30). The products were then acetylated (31) to the corresponding 1-*O*-alkyl-2-acyl-3-acetylglycerol, 1-*O*-alk-1′-enyl-2-acyl-3-acetylglycerol and 1,2-diacyl-3-acetylglycerol. These three acetylglycerols thus obtained were separated from each other by preparative TLC on Silica Gel G using the solvent system of petroleum ether/diethyl ether/acetic acid (90:10:1, v/v/v). The purified acetyl glycerols were saponified individually with 1 N KOH in ethanol at 85°C for one hr. Unsaponifiable matter (1-*O*-alkylglycerol or 1-*O*-alk-1′-enylglycerol) was extracted by *n*-hexane and converted to the corresponding TMS ether derivatives as mentioned above to analyze the aliphatic chain distribution in the *sn*-1 position of the glycerol moiety. The fatty acid compositions in the *sn*-1 and *sn*-2 positions of 1,2-diacyl glycerophospholipid and in the *sn*-2 position of ether-linked glycerophospholipids were analyzed as their methyl esters by GLC as mentioned below.

Gas liquid chromatography and gas chromatography-mass spectrometry (GC-MS). TMS ether derivatives of 1-*O*-alkylglycerol and 1-*O*-alk-1′-enylglycerol were analyzed by GC using a Shimadzu GC 12A instrument (Kyoto, Japan) equipped with a Supelcowax-10 fused silica open tubular capillary column (30 m × 0.25 mm i.d., Supelco Japan Ltd., Tokyo) and a Shimadzu CLH 702 split injector. The injector and the column were held at 250 and 210°C, respectively, the split ratio was 1:60, and helium was used as carrier gas at a constant inlet pressure of 1.2 Kg/cm^2.

Analysis of fatty acid methyl esters was performed on a Shimadzu GC 8APF gas chromatograph with a Chromatopack CR6A data processor. The conditions were similar to those of the TMS ether derivatives analysis, except that the column temperature was at 195°C.

Mass spectrometric analysis of the TMS ether derivatives was carried out on a Shimadzu GC-MS 9020 DF mass spectrometer fitted with a CI source to which an outlet of Supelcowax-10 column (30 m × 0.25 mm i.d.) was connected directly. The column and a Shimadzu movable on-column injector were kept isothermally at 220 and 300°C, respectively. Data acquisitional processing was carried out by an on-line Shimadzu GC-MS PAC 1100 data system. The sample, which was separated by GLC, was ionized in the CI mode by adding *iso*-butane as a reagent gas into 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.

Electron impact (EI) mass spectra of DMA derivatives of long chain aldehydes were obtained by a Shimadzu QP 1000 quadrupole mass spectrometer with a Supelcowax-10 column (30 m × 0.25 mm i.d.), under the conditions of 70 eV electron beam energy, 3 KV acceleration energy and a source temperature of 250°C.

RESULTS AND DISCUSSION

Iatroscan TLC-flame ionization detection analyses of acid-labile phospholipid. Separations of bonito white muscle phospholipids before and after mild acid hydrolysis are compared in Figure 1. The phospholipid before acid hydrolysis showed four well-resolved peaks corresponding to choline and ethanolamine glycerophospholipids (abbreviated as CPG and EPG respectively, in Fig. 1) and lysoglycerophospholipids (LPC and LPE in the figure); the peak ascribed to LPC was larger than that of CPG and the peak ascribed to LPE was smaller than that of EPG, as had been reported previously (32,33). After mild acid hydrolysis, the peaks of LPC and LPE were large, compared with those of CPG and EPG, respectively, and a moderate peak was found in the highest moving band on the Chromarod S-II. The highest peak in Rf was consistent with that of long chain aldehydes. These changes clearly indicate that the EPG and CPG in the phospholipids were hydrolyzed with hydrochloric acid into LPE, LPC and LCA, respectively. It is well accepted that an ether bond in the alk-1′-enyl group is acid-labile, and that phospholipids having such chemical bonds are readily hydrolyzed into lysophospholipids and LCA under mild acidic condition such as saturated hydrochloric acid fumes (8). Therefore, the LPE and LPC that were produced on mild acid hydrolysis seemed to indicate the existence of alk-1-enyl ether bonds in the precursor lipid classes, EPG and CPG, of bonito white muscle phospholipids. These observations correspond well with the results of Foot and Clandinin, in which they separated those hydrolysis products of acid-labile phospholipids by Iatroscan TLC-flame ionization detection (FID) on Chromarod S-II (34).

Identification of ether-linked glycerophospholipids. Gas chromatograms of 1-*O*-alk-1′-enyl-2,3-TMS ether glycerols

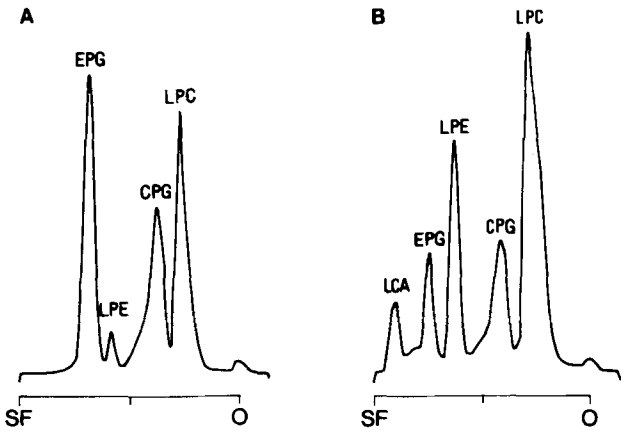
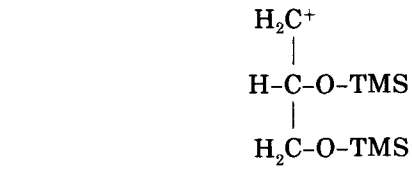
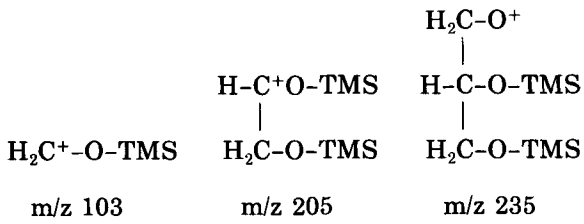


FIG. 1. Iatroscan TLC-FID chromatograms of the phospholipids of bonito white muscle before (A) and after (B) mild acidic hydrolysis. EPG, ethanolamine glycerophospholipid; LPE, lysoethanolamine glycerophospholipid; CPG, choline glycerophospholipid; LPC, lyso choline glycerophospholipid; LCA, long chain aldehyde; O, origin; SF, solvent front.

derived from a commercially available bovine heart ethanolamine plasmalogen (authentic), a laboratory-prepared bovine heart phospholipid, and a bonito white muscle phospholipid are shown in Figure 2 (A, B and C, respectively). Wood and Snyder reported that TMS ether derivatives of alkyl glycerol were successfully separated by GLC on a polar liquid phase column such as diethyleneglycol succinate polyester (35). Therefore, a Supelcowax-10 column, similar to a DEGS column in polarity of liquid phase, was used in the present study. In all samples, well resolved peaks were obtained on chromatograms: 6, 13 and 13 peaks in the authentic plasmalogen standard, bovine heart and bonito white muscle phospholipids, respectively. The CI spectrum of the most prominent peak of the authentic plasmalogen standard (No. 8 in Fig. 2A) was determined and is illustrated in Figure 3A. The spectrum showed a base peak at m/z 219 which indicates formation of the following ion by cleavage of alk-1′-enyl group from the glycerol moiety:



The relatively intense fragment peaks at m/z 103, m/z 205 and m/z 235 in Figure 3A strongly suggest that the following fragments, from the glycerol backbones in the TMS ether derivatives, are produced:



The peak at m/z 459 was considered attributable to $[M + 1]^+$, indicating the TMS ether derivative to have

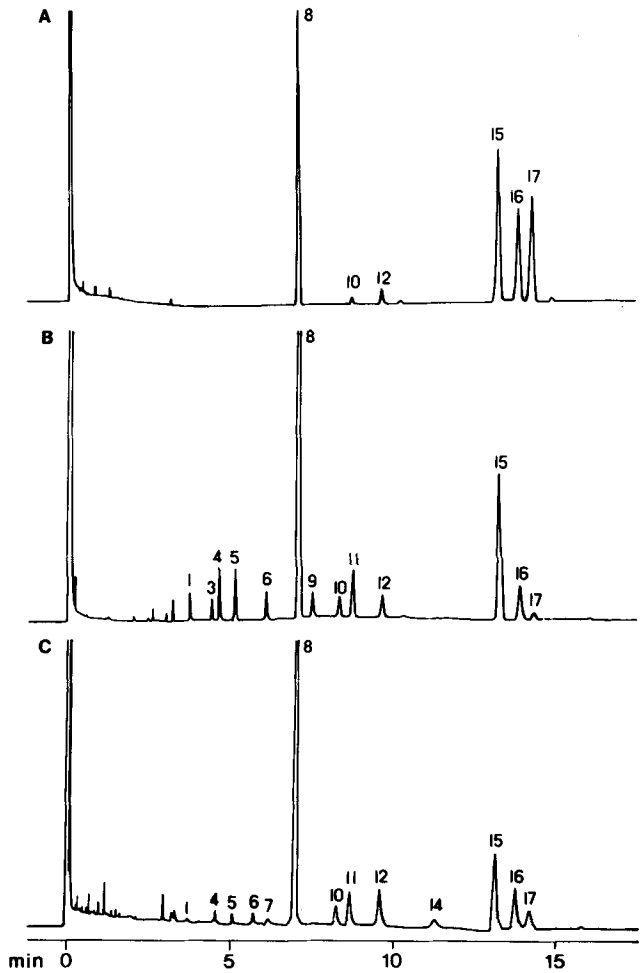


FIG. 2. GC analyses of the 2,3-TMS ether of 1-*O*-alk-1′-enyl-glycerols from (A) bovine heart muscle ethanolamine plasmalogen (authentic), (B) bovine heart muscle phospholipids (prepared in this laboratory) and (C) bonito white muscle phospholipids. See the text for peak components. Supelcowax-10 FQ column, 25 m × 0.25 mm i.d. and operated at 210°C, with helium as carrier gas.

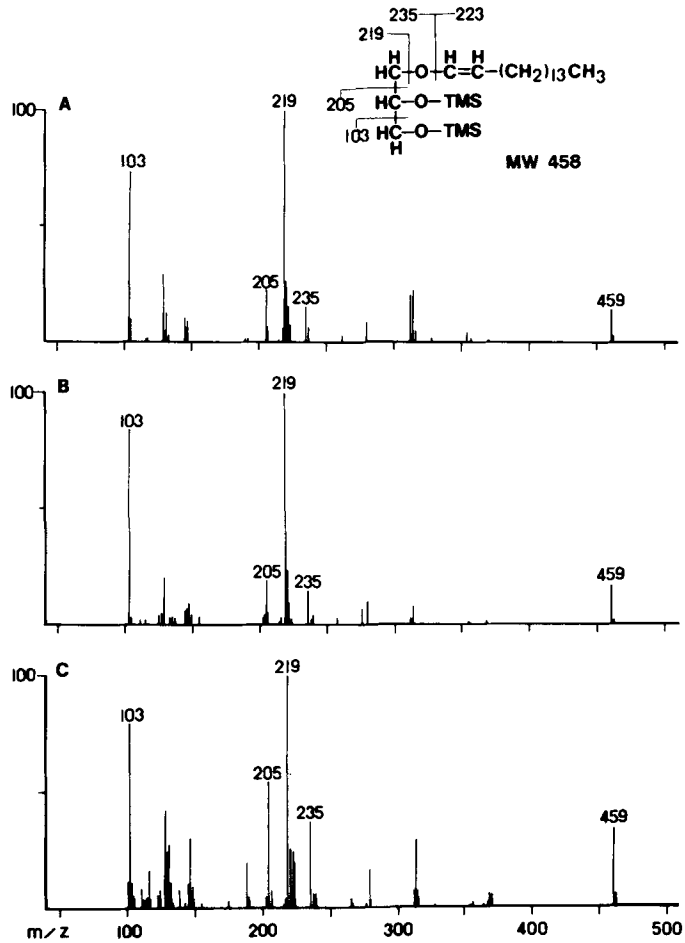


FIG. 3. CI (*iso*-butane) mass spectra of the predominant 2,3-TMS ethers of 1-*O*-alk-1′-enyl-glycerols derived from (A) bovine heart muscle ethanolamine plasmalogen (authentic), (B) bovine heart muscle phospholipids (prepared in this laboratory) and (C) bonito white muscle phospholipids. 200 eV electron beam energy, 200 μA emission current, 3 KV accelerating voltage, and source temperature of 250°C.

a molecular weight of 458. The characteristic fragmentation shown in Figure 3A definitely suggests that, in the TMS ether derivative, a hexadecenyl ether group is linked to the glycerol moiety.

Each TMS ether derivative prepared from the bovine heart phospholipid (Fig. 2B) and the bonito white muscle phospholipid (Fig. 2C) presented similar chromatographic patterns, although the former lacked peaks No. 7 and No. 14, while the latter lacked peaks No. 3 and No. 9. Of those peaks on the chromatograms, retention times (Rt) of the peaks of No. 8, 10, 12, 15, 16 and 17 were consistent with those on the chromatogram of the authentic plasmalogen standard (Fig. 2A). The CI mass spectra of three peaks numbered 8 closely corresponded to one another, as shown in Figure 3 (A, B and C).

The mass spectra in Figure 3 (A, B and C), however, did not show any specific fragment ions which offered information concerning a location of the double bond on the hexadecenyl ether group. In order to clarify this point, the alkenyl group on the glycerophospholipid was completely hydrolyzed to the corresponding LCA under mild acid conditions (8), and the chain structure of the DMA derivatives of the LCA thus obtained was explored by EI-MS.

Figures 4A and 4B show the gas chromatogram of the DMA derivatives of the LCA prepared from the bonito phospholipid by mild acid hydrolysis and the EI mass spectrum of peak No. 8 of Figure 4A, respectively. The spectrum yielded characteristic ions of $[M - 31]^+$ (due to the loss of methoxy group from the parent ion) and of m/z 75 (due to $[CH(OCH_3)_2]^+$) which usually appear in EI spectra of DMA derivatives (36). From these results, it is obvious that the predominant LCA has a structure of $CH_3(CH_2)_{14}CHO$, which is liberated with acid hydrolysis from the 1-*O*-alk-1′enyl group of plasmalogen prepared from the bonito phospholipid. In addition to this, the difference in mass number units between molecular ion and m/z 235 ion, i.e. $(M^+ - 235)$, is proposed to reflect the molecular weight of the alk-1′-enyl group; in the case of 1-*O*-alk-1′-enyl-2,3-TMS ether glycerol (Fig. 3A), mass number unit $(M^+ - 235)$, i.e. 223 in mass number unit, corresponds to the molecular weight of the ion $[CH{=}CH(CH_2)_{13}CH_3]^+$ (refer to Fig. 3A). On the basis of these results, it is concluded that 1-*O*-alk-1′-enyl glycerophospholipids certainly exist in bonito white muscle. The other components corresponding to the peaks shown in Figures 2A, 2B and 2C were identified in the same manner as follows: 1, 14:0; 3, *iso*-15:0; 4, *anteiso*-15:0; 5, 15:0, 6, *iso*-16:0; 7, *anteiso*-16:0; 9, 16:1; 10, *iso*-17:0; 11, *anteiso*-17:0; 12, 17:0; 14, *iso*-18:0; 15, 18:0; 16, 18:1; 17, 18:1 (abbreviated as carbon number:number of double bonds in the alkenyl group). The peaks numbered 2 and 13 are omitted on the chromatogram, because the alkyl components having the corresponding carbon number and number of double bonds were detected as a result of GLC analysis, as will be mentioned below (Fig. 5B).

Figure 5 compares gas chromatograms of 1-*O*-alkyl-2,3-TMS ether derivatives prepared from an authentic hexadecyl ether glycerol (A) and of bonito white muscle (B). The retention time of the most prominent peak in the chromatogram of the bonito phospholipid was consistent with that of the authentic hexadecyl ether glycerol (Figs. 5A and 5B). The CI mass spectra of these peaks are illustrated in Figure 6. The TMS ether derivatives from the bonito sample yielded m/z 461 ion (base peak) as $[M + 1]^+$, and m/z 205, m/z 219 and m/z 103 ions occurred from their glycerol backbone similarly in the case

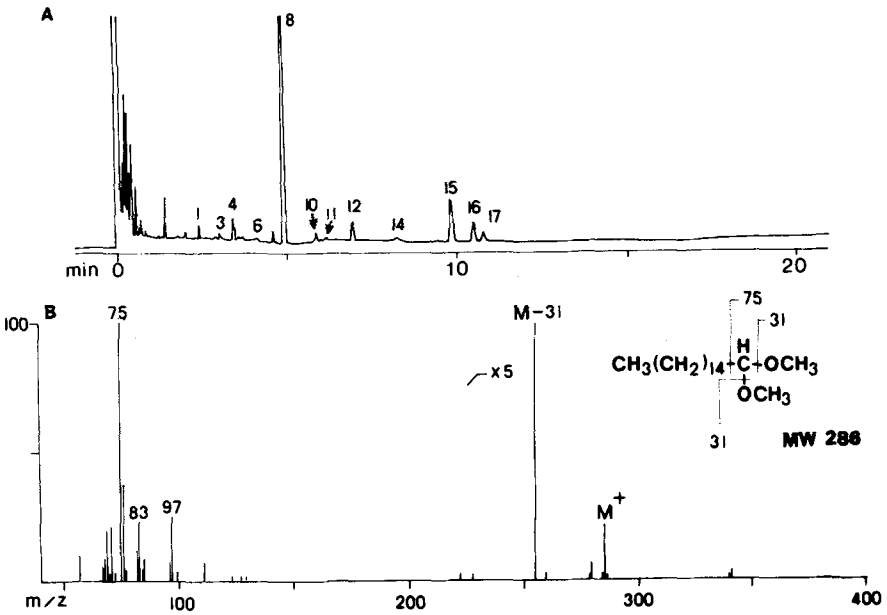


FIG. 4. GC analysis of dimethylacetal derivatives of long chain aldehydes liberated from 1-*O*-alk-1′-enyl-2-acyl glycerophospholipid of bonito white muscle by mild acid hydrolysis (A) and a typical EI mass spectrum of peak No. 8 (B). 1, 14:0; 3, *iso*-15:0; 4, *anteiso*-15:0; 6, *iso*-16:0; 10, *iso*-17:0; 11, *anteiso*-17:0; 12, 17:0; 14, *iso*-18:0; 15, 18:0; 16, 18:1; 17, 18:1. The components of No. 16 and No. 17 are positional isomers. GLC conditions are similar to those of Fig. 2, but column temperature was 195°C.

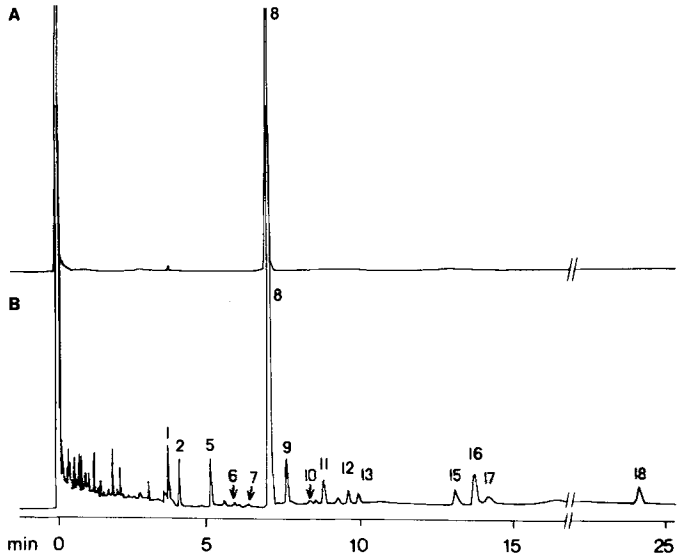


FIG. 5. GLC analyses of the 2,3-TMS ethers of 1-*O*-alkyl-glycerols derived from (A) authentic 1-*O*-hexadecyl glycerol and (B) bonito white muscle phospholipids. The analysis was performed under the conditions given in Fig. 2.

of 1-*O*-alk-1′-enyl-2,3-TMS ether derivatives, which have already been mentioned, although the 1-*O*-alkyl-2,3-TMS ether glycerol derivative did not yield a fragment ion of m/z 235. This fragmentation pattern in CI-MS of the sample prepared from bonito white muscle accorded well with that of authentic hexadecyl ether glycerol which is the component of peak No. 8 in Figure 5A. Similarly, the following results were obtained from the peak components shown in Figure 5B: 1, 14:0; 2, 14:1; 5, 15:0; 6, *iso*-16:0; 7, *anteiso*-16:0; 9, 16:1; 10, *iso*-17:0; 11, *anteiso*-17:0; 12, 17:0; 13, 17:1; 15, 18:0; 16, 18:1; 17, 18:1; 18, 20:0. The peaks numbered 16 and 17 were separated from each other as positional isomers.

Content of ether-linked glycerophospholipids. Table 1 lists the phospholipid composition of bonito white muscle. The total amount of alkylacyl glycerophospholipid and diacyl glycerophospholipid was determined in the fish samples numbered 1 to 5. Only in fish sample No. 6 were the choline and ethanolamine glycerophospholipids isolated separately from other phospholipids by column chromatography on silicic acid (37), and the proportional ratio of 1-*O*-alkyl-2-acyl glycerophospholipid and 1,2-diacyl glycerophospholipid was determined.

In the case of the choline glycerophospholipid, the alk-1′-enylacyl analogue accounted for 3.0–6.0% of the total phospholipid (5.7–11.2% of choline glycerophospholipids). The level of alkylacyl analogue was very low, only 2.4% of choline glycerophospholipids in fish sample No. 6. The most prominent component of the phospholipids seemed to be diacyl, accounting for 91.4% of choline glycerophospholipids of fish sample No. 6. In the case of ethanolamine glycerophospholipids of bonito white muscle, the amount of alk-1′-enylacyl analogue showed considerable variation, accounting for 3.6–7.6% of the

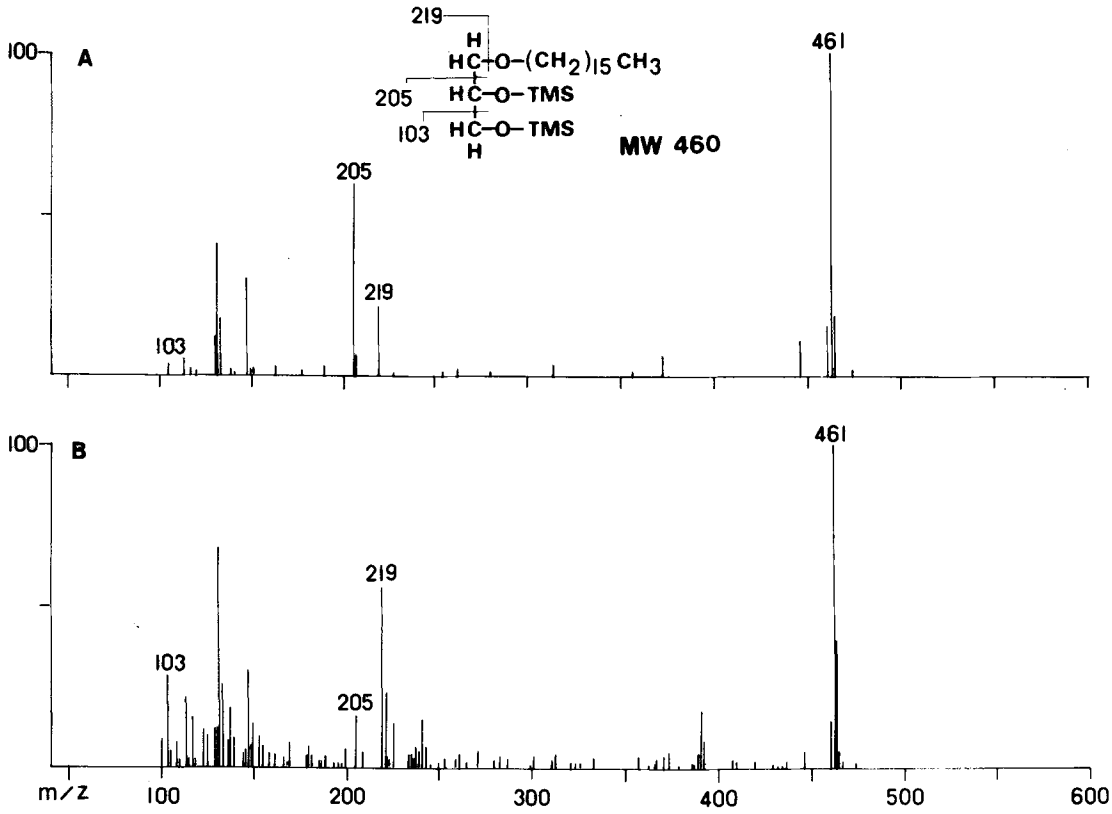


FIG. 6. CI (*iso*-butane) mass spectra of the 2,3-TMS ethers of 1-*O*-alkyl-glycerols derived from (A) authentic 1-*O*-hexadecyl glycerol and (B) bonito white muscle phospholipids. GC–MS conditions are as given in Fig. 3.

TABLE 1

Glycerophospholipid Compositions of Bonito White Muscle (as % of Recovered Phosphorus)

Lipid class	Fish sample 1	2	3	4	5	6
Choline glycerophospholipids	53.2	52.2	46.7	52.1	53.4	57.3
Alk-1'-enylacyl	5.9	3.1	3.1	3.0	6.0	3.5
Alkylacyl + diacyl	47.3	49.1	43.6	49.1	47.4	52.4[a]
Alkylacyl[b]	—	—	—	—	—	1.4[b]
Ethanolamine glycerophospholipids	17.8	24.5	22.3	21.4	25.0	23.4
Alk-1'-enylacyl	3.6	7.1	7.2	5.1	7.6	4.1
Alkylacyl + diacyl	14.2	17.4	15.1	16.3	17.4	18.7[a]
Alkylacyl[b]	—	—	—	—	—	0.6[b]
Serine glycerophospholipids	4.0	4.5	4.8	5.1	2.5	3.9
Inositol glycerophospholipids	3.8	4.4	5.0	3.6	4.4	1.3
Sphingomyelin	6.3	4.6	5.7	6.0	4.6	4.1
Lyso choline glycerophospholipids	14.2	8.7	11.8	11.4	7.5	6.8
Other	0.5	1.1	3.5	0.5	2.5	3.0

[a]The proportional ratios of alkylacyl and diacyl choline and ethanolamine glycerophospholipid were determined by GLC (see text). Value is the percentage of diacyl analogue in fish No. 6.

[b]Value is the percentage of alkylacyl analogue in fish No. 6.

TABLE 2

Fatty Chain Distribution of Choline Glycerophospholipids[a]

Fatty chain[b]	Class / Position	Diacyl *sn*-1 + *sn*-2	1-*O*-Alkyl-2-acyl *sn*-1	1-*O*-Alkyl-2-acyl *sn*-2	1-*O*-Alk-1'-enyl-2-acyl *sn*-1	1-*O*-Alk-1'-enyl-2-acyl *sn*-2
14:0		—	11.47 ± 0.75	1.46 ± 0.12	2.34 ± 0.39	1.42 ± 0.07
14:1		—	7.31 ± 0.57	—	—	—
15:0		—	5.51 ± 0.19	—	1.99 ± 0.10	1.13 ± 0.01
15:1		—	1.59 ± 0.18	—	—	—
16:0		45.81 ± 0.90	55.50 ± 1.16	15.22 ± 0.29	75.35 ± 1.20	6.93 ± 0.16
16:1n-7		1.12 ± 0.20	6.18 ± 0.14	—	—	1.35 ± 0.07
16:2n-6		—	—	—	—	3.00 ± 0.18
17:0 iso		—	1.59 ± 0.31	—	2.28 ± 0.09	—
17:0		—	—	—	2.26 ± 0.10	—
18:0		4.42 ± 0.12	—	4.96 ± 0.02	2.69 ± 0.17	2.75 ± 0.16
18:1n-9		8.57 ± 0.26	3.58 ± 0.16	16.96 ± 0.12	4.01 ± 0.03	27.49 ± 0.31
18:1n-7		1.65 ± 0.09	—	—	1.17 ± 0.17	—
20:0		—	3.37 ± 0.37	—	4.31 ± 0.02	—
20:4n-6		1.72 ± 1.35	—	3.64 ± 0.03	—	7.68 ± 0.16
20:5n-3		6.55 ± 0.24	—	3.85 ± 0.12	—	5.32 ± 0.04
21:5n-3		—	—	—	—	2.89 ± 0.20
22:5n-6		1.14 ± 0.06	—	1.75 ± 0.41	—	1.71 ± 0.37
22:6n-3		24.24 ± 0.95	—	44.59 ± 0.77	—	33.59 ± 1.63
Other[c]		4.78	3.90	7.57	3.60	4.74

[a]The data are presented as the mean ± standard deviation of three separate determinations done on the purified choline glycerophospholipid of fish No. 6.

[b]The position of double bond applies to fatty acyl chains.

[c]Other fatty acid less than 1% each of the total included 16:4n-3, 16:4n-1, 18:2n-9, 18:2n-7, 18:3n-6, 18:4n-1 and 22:5n-3.

total phospholipid (17.5–32.2% of the ethanolamine glycerophospholipids). The alkylacyl analogue was 0.6% of the total phospholipid (2.5% of the ethanolamine glycerophospholipids) as shown in fish sample No. 6 (Table 1). Of other lipid classes, lysocholineglycerophospholipid accumulated at a relatively high level (6.8 to 14.2% of the total phospholipid). These results obtained by phosphorus analysis were also supported by Iatroscan TLC-FID analysis (Fig. 1). In general, the plasmalogen content in ethanolamine glycerophospholipid is higher than that of the choline analogue. This may be a typical example, since plasmalogens in the gill lipid of two species of Pacific crab, *Cancer antennarius* and *Portanus xantusi*, were found to occur at higher levels in the ethanolamine

TABLE 3

Fatty Chain Distribution of Ethanolamine Glycerophospholipids[a]

	Class	Diacyl	1-*O*-Alkyl-2-acyl		1-*O*-Alk-1′-enyl-2-acyl	
Fatty chain[b]	Position	*sn*-1 + *sn*-2	*sn*-1	*sn*-2	*sn*-1	*sn*-2
14:0		—	6.37 ± 0.12	6.75 ± 0.71	—	—
15:0		—	—	3.47 ± 0.16	—	—
15:1		—	1.19 ± 0.04	—	—	—
16:0 iso		—	—	3.19 ± 0.27	—	—
16:0		17.64 ± 1.03	47.82 ± 0.10	40.59 ± 1.45	53.33 ± 0.35	3.65 ± 0.18
16:1n-7		—	5.37 ± 0.42	3.37 ± 0.12	—	—
16:1n-5		—	—	2.47 ± 0.18	—	—
16:2n-6		—	—	—	—	1.32 ± 0.04
17:0 iso		—	—	—	2.38 ± 0.12	—
17:0		1.61 ± 0.05	3.00 ± 0.28	2.55 ± 0.17	4.85 ± 0.24	—
16:4n-3		—	—	1.23 ± 0.14	—	—
18:0 iso		—	2.01 ± 0.32	—	1.87 ± 0.61	—
18:0		34.71 ± 0.27	15.06 ± 0.35	14.21 ± 0.15	19.44 ± 0.31	1.14 ± 0.03
18:1n-9		2.81 ± 0.04	10.84 ± 0.19	5.63 ± 0.03	10.83 ± 0.34	7.09 ± 0.17
18:1n-7		1.75 ± 0.04	6.03 ± 0.02	—	3.95 ± 0.13	—
19:0		1.03 ± 0.01	—	—	—	—
20:0		—	—	—	—	1.61 ± 0.04
20:4n-6		3.18 ± 0.05	—	1.25 ± 0.30	—	5.09 ± 0.05
20:5n-3		2.96 ± 0.03	—	1.08 ± 0.25	—	6.43 ± 0.06
22:5n-6		1.83 ± 0.05	—	—	—	1.13 ± 0.03
22:6n-3		27.94 ± 1.01	—	9.06 ± 0.70	—	63.17 ± 0.29
Other[c]		4.54	2.31	5.15	3.35	9.37

[a]The data are presented as the mean ± standard deviation of three separate determinations done on the purified ethanolamine glycerophospholipid of fish No. 6.

[b]The position of double bond applies to fatty acyl chains.

[c]Other fatty acids less than 1% each of the total included 16:4n-3, 16:4n-1, 18:2n-9, 18:2n-7, 18:3n-6, 18:4n-1 and 22:5n-3.

components (38). Also, bonito white muscle was rich in ethanolamine glycerophospholipid compared with choline analogue.

Fatty chain distributions of choline (CPG) and ethanolamine glycerophospholipids (EPG). The fatty chain distribution of CPG in bonito (fish No. 6 in Table 1) white muscle is shown in Table 2. The prominent fatty acids in the *sn*-1 and *sn*-2 positions of 1,2-diacyl CPG were 16:0, 22:6n-3, 18:1n-9 and 20:5n-3, and the percentages of polyunsaturated fatty acids (PUFA) were quite high. For 1-*O*-alkyl-2-acyl GPC, the percentages of saturated alkyl chains such as 16:0 and 14:0 were higher in the *sn*-1 position; in the *sn*-2 position PUFA were predominant. In 1-*O*-alk-1′-enyl-2-acyl CPG, the sum of monounsaturated and saturated aliphatic chains in the *sn*-1 position amounted to over 80%, while PUFA in the *sn*-2 position accounted for more than 48%.

Table 3 shows the chain distributions of EPG in the white muscle of bonito (fish No. 6 in Table 1). The prominent fatty acids of 1,2-diacyl EPG were similar to those of CPG, except that 18:0 in the former was higher, accounting for 34%. The degrees of unsaturation of the alkyl chain in the *sn*-1 position and of the fatty acyl chain in the *sn*-2 position of 1-*O*-alkyl-2-acyl EPG were lower than those of CPG; more than 66% of the alkyl chains were saturated and 22:6n-3 fatty acid amounted to only 9%. For 1-*O*-alk-1′-enyl-2-acyl EPG, the composition of the alkenyl chain in the *sn*-1 position showed a lower degree of unsaturation, although 22:6n-3 accounted for 63% of the fatty acyl chains in the *sn*-2 position. These observations made for the ether-linked glycerophospholipids of bonito white muscle agreed well with previous data on the ether-linked glycerophospholipids of rabbit alveolar macrophages (39), human platelets (23) and murine mastocytoma (40), except that PUFAs such as 22:6n-3 and 20:5n-3 were prominent in the phospholipid classes of bonito white muscle examined here.

In order to identify ether-linked glycerophospholipids, various analytical techniques, such as two-dimensional TLC (8,41), and GLC of DMA (36) and TMS ether derivatives (35), have so far been applied as conventional methods. More recently, Iatroscan TLC-FID combined with a mild acid hydrolysis procedure (34), GLC (42) and high-performance liquid chromatography (43) of *tert*-BDMS ether derivatives and fast atom bombardment mass spectrometry without any derivatization of the phospholipid (44) have been used successfully. Of these techniques, EI mass spectra of *tert*-BDMS ether derivatives, which are prepared from ether-linked glycerophospholipids, provide information concerning their molecular weights by yielding a $[M - 57]^+$ ion, differing from those of the corresponding TMS ether derivatives (42). On the other hand, the proposed CI-MS technique of TMS ether derivatives, which are prepared from ether-linked glycerophospholipids, seems to be effective not only for determining molecular weights but also for obtaining structural information on ether-linked glycerophospholipids.

In conclusion, the occurrence of novel ether-linked glycerophospholipids has been confirmed in bonito white muscle. Recent interest in the unique physiological and biological properties of 1-*O*-alkyl-2-acetyl glycerophospho-

cholines as the platelet-activating factor (23,40,45-48) also suggests a need for further studies on the molecular species of marine lipids.

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New Phospholipid Fatty Acids from the Caribbean Sponge *Ectyoplasia ferox*

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The phospholipid fatty acids from the Caribbean sponge *Ectyoplasia ferox* were studied. The novel fatty acids 25-methyl-5,9-heptacosadienoic (1) and 26-methyl-5,9-heptacosadienoic (2) were identified in 3.4 and 2.0% abundance, respectively, representing the longest set of Δ5,9 *iso* and *anteiso* acids yet isolated from a marine sponge. The new acid 10,13-dimethyltetradecanoic (3), the unusual acid 15-methyl-11-hexadecenoic (4) and the also novel acid 9-methyl-11-hexadecenoic (5) were also identified in *E. ferox*. The principal sterols isolated from *E. ferox* were 24-ethylcholest-5-en-3β-ol (46%) and 24(R)-methylcholesta-5,22-dien-3β-ol (14%).
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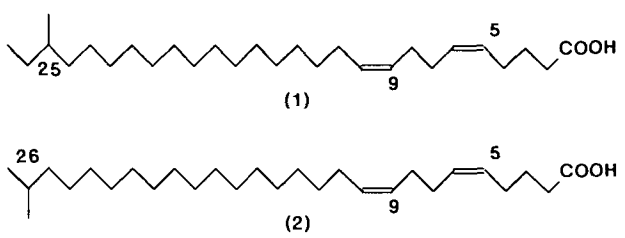
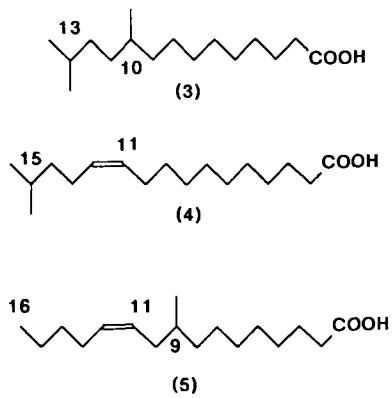
It is now well established that the phospholipids of marine sponges are a rich source of structurally unusual and interesting fatty acids. Common phospholipid fatty acids from marine sponges include 5,9-hexacosadienoic (26:2), which occurs in most known sponges as described originally by Litchfield and coworkers (1), 5,9-heptacosadienoic (27:2) and 5,9-octacosadienoic (28:2). However, there are other very long chain phospholipid fatty acids that have been encountered recently in several species. For example, work by Ayanoglu et al. (2) with the sponge *Petrosia ficiformis* 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. The Australian sponge *Jaspis stellifera* has also been shown to contain the same 27:2 *iso* and *anteiso* acids (3). However, in the sponges analyzed to date, the only reported cases of terminal *iso* and *anteiso* branching are those of the 26:2 and 27:2 series. In our search for similar acids in Caribbean sponges around Puerto Rico we have found that the sponge *Ectyoplasia ferox* contains the 28:2 series of the hitherto undescribed very long chain phospholipid fatty acids 25-methyl-5,9-heptacosadienoic (1) and 26-methyl-5,9-heptacosadienoic (2), which now represents the longest set of Δ5,9 *iso* and *anteiso* phospholipid fatty acids from any marine sponge. We also wish to report in this work the isolation of the new acid 10,13-dimethyltetradecanoic (3), the acid 15-methyl-11-hexadecenoic (4) and the also novel acid 9-methyl-11-hexadecenoic (5). Herein we report the results of our investigation.



SCHEME 1



SCHEME 2

EXPERIMENTAL PROCEDURES

Ectyoplasia ferox was collected May 3, 1988 near La Parguera, Puerto Rico. The sponge was washed in sea water, carefully cleaned of all nonsponge debris and cut into small pieces. Immediate extraction with chloroform/methanol (1:1, v/v) yielded the total lipids. The neutral lipids, glycolipids and phospholipids were separated by column chromatography on ammonium hydroxide-treated silicic acid (100-200 mesh) using the procedure of Privett et al. (4). The phospholipid classes were investigated by preparative thin-layer chromatography (TLC) using silica gel and chloroform-methanol-water (65:25:4, v/v/v) as solvent. The fatty acyl components of the phospholipids were obtained as their methyl esters by reaction of the phospholipids with methanolic hydrogen chloride (5) followed by purification on column chromatography eluting with hexane/ether (9:1, v/v). The resulting methyl esters were analyzed by gas chromatography-mass spectrometry (GC-MS) using a Hewlett Packard 5995 A gas chromatograph-mass spectrometer equipped with a 30-m × 0.32-mm fused silica column coated with SE-54. 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 (1 hr at 100°C) followed by ethereal extraction from the acidified solution and purification by preparative layer chromatography (PLC). Hydrogenations were carried out in 10 ml of absolute methanol and catalytic amounts of platinum oxide (PtO_2). Mass spectral data of key fatty acids for this discussion are presented below.

10,13-Dimethyltetradecanoic acid methyl ester. MS m/z (rel intensity) 270 (M^+, 7), 256 (4), 227 (6), 213 (2), 199 (7),

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Abbreviations: GC-MS, gas chromatography-mass spectrometry; HPLC, high pressure liquid chromatography; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PLC, preparative layer chromatography; PS, phosphatidylserine; TLC, thin-layer chromatography.

185 (3), 171 (4), 157 (4), 143 (27), 129 (13), 101 (7), 87 (62), 74 (100), 57 (57), 55 (37).

10,13-Dimethyltetradecanoic acid pyrrolidide. MS m/z (rel intensity) 309 (M^+, 2), 294 (1.6), 266 (1.5), 252 (2), 238 (1.5), 210 (2), 196 (1.7), 168 (1.4), 154 (1), 140 (2), 126 (25), 113 (100), 70 (12), 55 (20).

9-Methyl-11-hexadecenoic acid pyrrolidide. MS m/z (rel intensity) 321 (M^+, 9), 278 (5), 252 (46), 250 (5), 236 (6), 224 (1), 196 (3), 182 (5), 168 (4), 154 (2), 140 (11), 126 (56), 113 (100), 98 (43), 70 (51), 57 (18).

9-Methylhexadecanoic acid methyl ester. MS m/z (rel intensity) 284 (M^+, 8), 241 (4), 199 (2), 185 (18), 157 (10), 143 (26), 135 (10), 129 (14), 115 (10), 111 (10), 101 (13), 97 (18), 87 (63), 74 (100), 69 (29), 57 (45), 55 (53).

11-Methyloctadecanoic acid methyl ester. MS m/z (rel intensity) 312 (M^+, 12), 269 (8), 213 (7), 199 (5), 185 (9), 181 (3), 163 (5), 157 (8), 143 (36), 129 (16), 125 (8), 111 (13), 101 (12), 97 (21), 87 (76), 83 (23), 74 (100), 69 (29), 57 (52), 55 (54).

11-Methyloctadecanoic acid pyrrolidide. MS m/z (rel intensity) 351 (M^+, 2), 266 (1), 252 (1.1), 224 (1.1), 210 (1), 196 (1), 182 (1), 168 (1.3), 154 (1.4), 140 (1.6), 126 (11), 113 (100), 85 (5), 71 (10), 70 (11), 57 (11), 55 (24).

15-Methyl-11-hexadecenoic acid methyl ester. MS m/z (rel intensity) 282 (M^+, 1), 241 (4), 199 (4), 185 (12), 171 (2), 157 (8), 143 (25), 130 (10), 129 (10), 111 (11), 101 (10), 97 (20), 87 (66), 74 (93), 55 (100).

15-Methyl-11-hexadecenoic acid pyrrolidide. MS m/z (rel intensity) 321 (M^+, 5), 278 (1.7), 252 (1.7), 250 (1.7), 236 (1.7), 224 (1), 210 (1), 196 (1.2), 182 (4), 168 (2), 154 (1.4), 140 (4), 126 (37), 113 (100), 98 (28), 70 (31), 55 (64).

25-Methyl-5,9-heptacosadienoic acid methyl ester. MS m/z (rel intensity) 434 (M^+, 12), 402 (6), 360 (3), 320 (3), 306 (3), 292 (6), 195 (4), 182 (7), 164 (7), 154 (8), 150 (26), 141 (33), 136 (20), 110 (28), 109 (64), 96 (29), 81 (100), 67 (70), 57 (90).

25-Methylheptacosanoic acid methyl ester. MS m/z (rel intensity) 438 (M^+, 22), 395 (3), 207 (8), 199 (4), 143 (24), 129 (10), 111 (12), 97 (22), 87 (77), 85 (19), 83 (27), 75 (54), 74 (100), 71 (34), 69 (34), 57 (82), 55 (66).

25-Methyl-5,9-heptacosadienoic acid pyrrolidide. MS m/z (rel intensity) 473 (M^+, 3), 234 (1), 181 (4), 180 (18), 140 (2), 126 (17), 113 (100), 98 (13), 85 (7), 72 (8), 70 (8), 57 (11), 55 (16).

26-Methyl-5,9-heptacosadienoic acid methyl ester. MS m/z (rel intensity) 434 (M^+, 5), 394 (12), 285 (12), 253 (6), 157 (17), 150 (27), 141 (37), 136 (27), 110 (23), 109 (62), 97 (30), 81 (99), 67 (62), 57 (59).

26-Methylheptacosanoic acid methyl ester. MS m/z (rel intensity) 438 (M^+, 24), 143 (34), 111 (22), 97 (42), 87 (83), 85 (14), 83 (27), 75 (68), 74 (100), 71 (23), 69 (32), 57 (60), 55 (48).

26-Methyl-5,9-heptacosadienoic acid pyrrolidide. MS m/z (rel intensity) 473 (M^+, 4), 181 (3), 180 (13), 126 (16), 113 (100), 98 (9), 85 (8), 72 (6), 70 (8), 57 (6), 55 (13).

RESULTS

The phospholipid fatty acids isolated from *Ectyoplasia ferox* are shown in Table 1. The fatty acid mixture presented a rather unusual series of highly branched-unsaturated acids. One acid that occurred in big amounts (10.6%) was 4,8,12-trimethyltridecanoic (16:0), an acid that has been encountered before in several sponges and was easily characterized by its typical mass spectrum (6). Much to our surprise, we also encountered a second highly branched acid that has not been identified before in any marine sponge, namely the new acid 10,13-dimethyltetradecanoic (3). The multiple methyl substitution in the latter acid was evidenced by a low ECL value of 15.05 for its fatty acid methyl ester but the methyl group positions were determined by synthesizing the corresponding pyrrolidide and measuring its electron impact mass spectrum (Fig. 1). The pyrrolidide spectrum displayed the expected molecular ion peak at m/z 309 with diminished fragments at m/z 280 (carbon 13) and at m/z 224 (carbon 10). Therefore, we have to conclude that the compound in question is the new acid 10,13-dimethyltetradecanoic (3) which occurred in 1.8% abundance in this sponge.

TABLE 1

The Phospholipid Fatty Acids from *Ectyoplasia ferox*

Fatty acid	Abundance (%)
Tetradecanoic (14:0)	1.6
4,8,12-Trimethyltridecanoic (16:0)	10.6
13-Methyltetradecanoic (15:0)	5.0
10,13-Dimethyltetradecanoic (16:0)[a]	1.8
14-Methylpentadecanoic (16:0)	0.6
13-Methylpentadecanoic (16:0)	2.5
Hexadecenoic (16:1)	7.4
Hexadecanoic (16:0)	2.1
9-Methyl-11-hexadecenoic (17:1)[a]	8.0
15-Methyl-11-hexadecenoic (17:1)	1.0
Octadecenoic (18:1)	4.4
Octadecanoic (18:0)	5.1
11-Methyloctadecanoic (19:0)	5.6
17-Methyloctadecanoic (19:0)	0.6
5,8,11,14-Eicosatetraenoic (20:4)	7.7
6,9,12,15-Eicosatetraenoic (20:4)	6.4
Pentacosanoic (25:0)	3.0
24-Methyl-5,9-pentacosadienoic (26:2)	2.7
5,9-Hexacosadienoic (26:2)	5.3
25-Methyl-5,9-hexacosadienoic (27:2)	6.9
5,9-Heptacosadienoic (27:2)	2.4
26-Methyl-5,9-heptacosadienoic (28:2)[a]	2.0
25-Methyl-5,9-heptacosadienoic (28:2)[a]	3.4
5,9-Octacosadienoic (28:2)	2.5

[a]These compounds are unprecedented in nature.

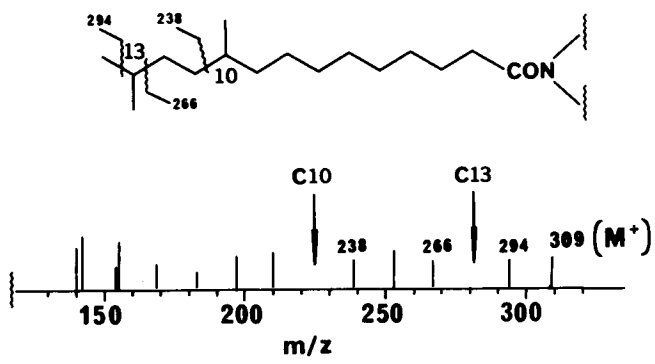

FIG. 1. Partial mass spectrum of 10,13-dimethyltetradecanoic acid pyrrolidide. Spectrum is expanded four times.

Two very similar but rather unusual C17:1 fatty acids were also isolated from *Ectyoplasia ferox*. Critical for the characterization of these acids were their corresponding pyrrolidides. The first acid, which occurred in ca. 8.0% abundance, presented a molecular ion peak at m/z 321 indicating 17 carbons with one unsaturation. The unsaturation was readily determined when it was observed that a difference of 12 amu occurred between fragments at m/z 224 (C_{10}) and m/z 236 (C_{11}) indicating Δ11 desaturation. Methyl branching was also evident since a diminished peak occurred at exactly m/z 210 corresponding to carbon 9 (Fig. 2). Methyl branching at position 9 was also corroborated by the MS spectrum of the corresponding hydrogenated fatty acid methyl ester (ECL 16.42) which essentially displayed a diminished peak at m/z 171 (C_9) concurrent with enhanced C_8 (m/z 157) and C_{10} (m/z 185) peaks. All of our experimental data supports the acid 9-methyl-11-hexadecenoic (5) which, to the best of our knowledge, is unprecedented in nature. The other C17:1 fatty acid (1.0%) turned out to be 15-methyl-11-hexadecenoic (4), a new fatty acid for marine sponges but known in several bacteria (7). The latter acid was again readily characterized by means of mass spectrometry of the corresponding pyrrolidide since it also presented a 12-amu difference between fragments at m/z 224 and m/z 236 supporting Δ11 unsaturation. Hydrogenation of the parent fatty acid methyl ester afforded the known 15-methylhexadecanoic acid (ECL 16.62) which was compared with an authentic sample isolated by us from other sponges.

Two 19:0 branched acids were detected in *E. ferox* in a combined 6% abundance. The first of these acids (ECL 18.39) presented a molecular ion peak, as the methyl ester derivative, of m/z 312. The most important feature of the spectrum was that peaks at m/z 185 and m/z 213 were bigger than normal (as compared with a straight chain acid) and the peak at m/z 199 was diminished. A further study of the pyrrolidide spectrum revealed a diminished peak at exactly m/z 238, thus indicating methyl substitution at position 11. This acid was characterized as the rare 11-methyloctadecanoic, which has been reported before in the demosponge *Aplysina fistularis* (8). The other 19:0 acid (ECL 18.61) was readily characterized as 17-methyloctadecanoic acid by comparison with an authentic sample and GC-MS data.

The most interesting feature in *E. ferox* was the presence of seven very long chain phospholipid fatty acids possessing the typical Δ5,9 unsaturation of "demospongic" acids. The Δ5,9 unsaturation was readily identified since all of these acids afforded the characteristic peak at m/z 180 when the corresponding pyrrolidides were analyzed by GC-MS, a typical peak resulting from allylic cleavage between carbons 7 and 8 of the fatty acid (8). Three of these straight-chain acids were characterized as 5,9-hexacosadienoic (26:2), 5,9-heptacosadienoic (27:2) and 5,9-octacosadienoic (28:2), on the basis of their mass spectra, ECL values of the corresponding hydrogenated acids, and comparison with authentic samples isolated before by us from other sponges. Again, critical for the double bond assignments were the peaks at m/z 81 (base peak) for the methyl esters and m/z 180 for the pyrrolidides. The corresponding *iso* acids of the latter phospholipid acids were also present in the mixture, namely 24-methyl-5,9-pentacosadienoic (*iso*-26:2), 25-methyl-5,9-hexacosadienoic (*iso*-27:2), and 26-methyl-5,9-heptacosadienoic (*iso*-28:2). The first two phospholipid fatty acids (*iso*-26:2 and *iso*-27:2) have been reported before in other sponges (2) but the *iso*-28:2 (2.0% abundance) is, to the best of our knowledge, new in nature. The corresponding saturated *iso*-28:0 (after hydrogenation) presented an ECL value of 27.61. In addition to the *iso*-28:2 acid we also identified, in the mixture, the corresponding *anteiso*-28:2 acid (3.4% abundance), namely 25-methyl-5,9-heptacosadienoic (1), which also has not been reported to occur in nature (Fig. 3). The latter acid, after hydrogenation, presented an ECL value of 27.72, typical of *anteiso* acids (Fig. 4).

The phospholipids from *E. ferox* were analyzed by TLC and they mainly consisted of phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylcholine (PC), phosphatidylinositol (PI) and small amounts of phosphatidylglycerol (PG). Phosphatidylethanolamine and phosphatidylserine were observed in ca. 60% and 18%, respectively, while phosphatidylinositol and phosphatidylcholine were present in ca. 13% and 8%, respectively. The new acids isolated in this work, especially the 26:2-28:2 series, were concentrated in PE, PS, and PI with lesser amounts in PC and PG.

Because sterols are known to coexist with phospholipids in cell membranes, we decided to take a look at the sterols from *E. ferox* in detail to see if unusual branching was present in the sterol mixture. By means of standard isolation procedures and final separation by high pressure liquid chromatography (HPLC) and characterization aided by 300 MHz ^{1}H NMR, MS, and comparison with authentic samples, the following sterols were isolated from *E. ferox:* cholesta-5,22-dien-3β-ol (3% abundance of

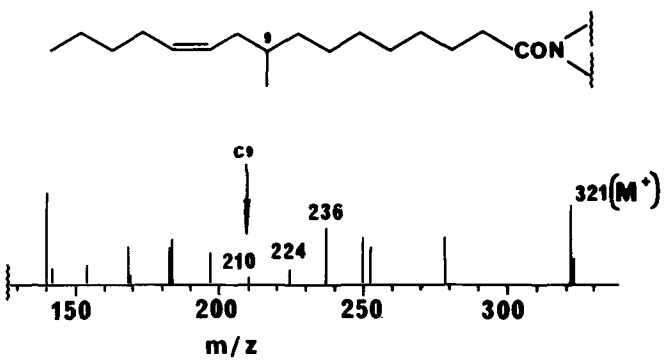

FIG. 2. Partial mass spectrum of 9-methyl-11-hexadecenoic acid pyrrolidide. The spectrum is expanded four times.

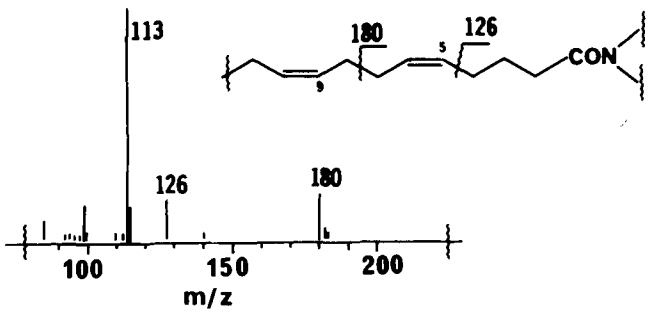

FIG. 3. Partial mass spectrum of 25-methyl-5,9-heptacosadienoic acid pyrrolidide showing the typical fragmentation peak at m/z 180.

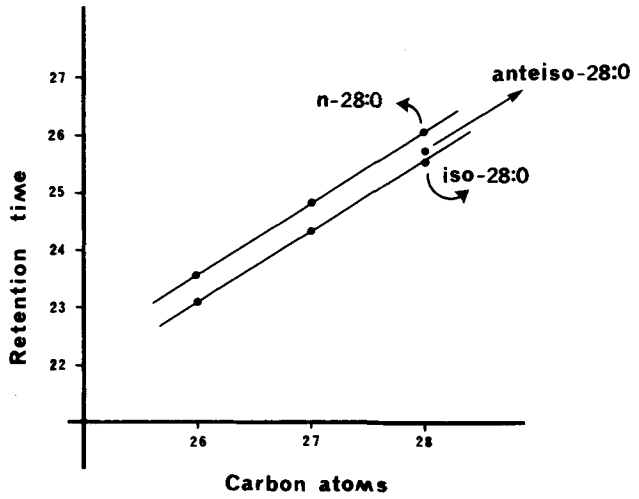

FIG. 4. Plot of retention time (min) vs carbon atoms for the hydrogenated mixture of the very long chain acids from *E. ferox*. Notice the 26:0–28:0 *iso* series as well as the *anteiso*-28:0 compound.

the total sterol mixture), 24(S)-methylcholesta-5,22-dien-3β-ol (8%), 24(R)-methylcholesta-5,22-dien-3β-ol (14%), cholesterol and fucosterol (7%), 24-ethylcholesta-5,22-dien-3β-ol (12%), 24-methylcholest-5-en-3β-ol (10%), and 24-ethylcholest-5-en-3β-ol (46%). Therefore, *E. ferox* presented a common sponge sterol composition.

DISCUSSION

The main thrust of this report, namely the isolation of the very long chain fatty acids 25-methyl-5,9-heptacosadienoic (1) and 26-methyl-5,9-heptacosadienoic (2), adds a new dimension to the series of possible Δ5,9 branched very long chain "demospongic" acids. One previously encountered pair of *iso* and *anteiso* 27:2 acids from the sponge *Jaspis stellifera* have been the subject of various biosynthetic experiments (3) revealing that the branched 27:2 acids are true sponge metabolites originating from their corresponding *iso-anteiso* short-chain analogues by a mechanism of chain elongation and subsequent desaturation. Possibly in *E. ferox* a similar biosynthetic pathway might be operative, but until the necessary biosynthetic experiments are performed we can only speculate at this point.

Of the two branched 17:1 acids isolated in this work, 9-methyl-11-hexadecenoic acid (5) is certainly the most interesting since, to the best of our knowledge, it has not been reported to occur in nature before. The saturated analogue, namely 9-methylhexadecanoic acid, has been reported to occur before in *Staphylococcus aureus* (9). We think that the acid reported here is probably of bacterial origin. The *iso* acid 15-methyl-11-hexadecenoic (4) has been isolated before from several sources including the marine bacterium *Flexibacter polymorphus* (10).

A very interesting finding in *E. ferox* is the presence of the new branched acid 10,13-dimethyltetradecanoic (3). It origin in sponges is certainly worthy of investigation. The final unusual acid isolated in this sponge, namely 11-methyloctadecanoic (19:0), seems to be a two-carbon extension of 9-methylhexadecanoic acid (17:0).

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Effect of pH on the Affinity of Phospholipids for Cholesterol

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The ability of multilamellar vesicles of phosphatidylcholine and phosphatidylethanolamine in aqueous phase to prevent access to cholesterol by a nonpolar solvent was examined. Phosphatidylethanolamine vesicles retained less sterol than phosphatidylcholine vesicles. In mixed vesicles, cholesterol was retained in proportion to the amount of phosphatidylcholine. To alter the charge and hydration of head groups, pH was adjusted from 1.2 to 12.5. Above pH 8, both phosphatidylethanolamine and phosphatidylcholine retained sterol in a 1:1 molar ratio of phospholipid to cholesterol, regardless of acyl side chain composition. Between pH 2.0 and pH 8.0, sterol retention varied with type of head group and side chain. Lipids with 16-carbon saturated side chains retained more sterol than 18-carbon unsaturated or 12-carbon saturated side chains. Between pH 1.1 and 2.0, none of the phosphatidylethanolamines retained sterol, but long chain phosphatidylcholines, saturated or unsaturated, retained sterol in a 1:1 molar ratio of phospholipid to sterol. Short chain phosphatidylethanolamines and phosphatidylcholines retained 0 to 20% at the low- to mid-pH range. Size of multilamellar vesicles, measured by Doppler effect light scattering analysis, had no bearing on sterol retention. Sonication of vesicles, which increases surface curvature, increases the retention of sterol. Fluorescence polarization indicated that cholesterol does not interact with DPPC or DLPC side chains. The observations can be interpreted in terms of space requirements of head groups, including charge repulsion and hydration. Other factors, such as monovalent cation replacement by protons, juxtaposition of charged groups on vesicle surfaces and length and unsaturation of acyl side chains affect the affinity of phospholipids for cholesterol.
Lipids 24, 375–382 (1989).

We have described previously a procedure for quantifying lipid retention by multilamellar vesicles (MLV) of phosphatidylcholine (PC) by extraction with a nonpolar solvent (1). The study showed that MLV of PC without cholesterol retained most of their phospholipid upon extraction with petroleum ether, but if the sterol was present, MLV varied in their retention of sterol or phospholipid. The ability of sterol-containing vesicles to retain lipid upon extraction was related to mobility of acyl side chains. MLV of PC above their transition temperatures (T_c) retained less sterol or phospholipid than PC below their T_c. In the present study, observations on lipid retention were extended to MLV of phosphatidylethanolamine (PE) and mixtures of PC and PE. We found that PE, like PC, retains cholesterol as a function of side chain mobility, but PE vesicles retained less sterol than equivalent PC vesicles. These findings led us to explore some of the reasons why PE vesicles retain less cholesterol than PC vesicles on extraction with a nonpolar solvent.

We based this study on the premise that cholesterol is best protected from extraction when it is sequestered beneath the polar surfaces of phospholipids, i.e., when the phospholipids are in a lamellar phase. Extraction of cholesterol from a phospholipid-sterol complex will occur when the phospholipid assumes a reverse hexagonal phase or when the lamellar phase becomes unstable. Several authors have discussed parameters which control lamellar phase formation. Israelachvili *et al.* (2) proposed that molecular shape is a critical factor. Cylindrical phospholipids, with cross-sectional areas of head groups and of acyl side chains nearly equal, would be most likely to form bilayers, whereas conical lipids, with cross-sectional areas of polar head groups smaller than acyl side chains, would form reverse hexagonal (H_{II}) phases. DeKruijff *et al.* (3) modified the concept of molecular shape and included other factors which may influence the shape occupied by lipid molecules. Hydration of head groups (4,5), intermolecular hydrogen bonding (6–8) and rotation about the long axis of the molecule (9) have been shown to affect molecular shape. We found it useful to consider the space occupied by the molecules, taking into account the influence of like charge repulsion and surface curvature of vesicles. Repulsion of like charge will cause adjacent head groups to occupy more space than predicted by considerations of the shape of single molecules. Organization of phospholipids into vesicles with highly curved surface will have the same effect.

The space requirements of anionic and zwitterionic lipids can be manipulated by varying the pH of the bulk phase surrounding the lipid or by controlling the size, and therefore, the surface curvature, of lamellar vesicles. By these methods, a conical lipid can be made to occupy a cylindrical space without altering its physico-chemical properties, as occurs with the addition of bulky substituent groups (10). Unsaturated phosphatidylethanolamines, which tend to form H_{II} phase structures at ambient temperature and neutral pH (11–13), can be induced to assume a lamellar phase by increasing the pH above the pK_a of the amine group (14,15).

To test the hypothesis that cholesterol is sheltered when entrapped beneath the polar head groups of phospholipids, we varied the pH of the aqueous phase to influence the charge on head groups. At pH 2 or less, both PC and PE are fully protonated and have a net positive charge (16). We expected the effect of a net positive charge to be counterbalanced by the loss of hydration around the phosphate group. PC, because of its large choline group, would be more likely to form a lamellar phase at low pH than PE. At pH 10 and above, PE is deprotonated and has a net negative charge, whereas PC at the same pH is neutral (16). Both PC and PE should form lamellar phases at high pH because the phosphate groups will be hydrated and the bulk provided by hydration will be additive to the space occupied by the choline groups in the former and by the space required by charge

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Abbreviations: DLPC, 1,2-dilauroyl-*sn*-glycero-3-phosphocholine; DLPE, 1,2-dilauroyl-*sn*-glycero-3-phosphoethanolamine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPPE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; PC, phosphocholine; PE, phosphoethanolamine; MLV, multilamellar vesicles.

repulsion in the latter. At extremes of pH, we expected the cholesterol retaining ability of PE to almost equal the ability of PC vesicles to retain the sterol. Between pH 3 and pH 8, intermolecular hydrogen bonding would discourage lamellar formation in PE but not in PC. We also expected that unsaturated PC would approach the capacity of saturated PC's of equivalent side chain length to retain cholesterol at both high and low pH because the space requirements for the head groups would be large in either case. PC or PE with short acyl side chains were not expected to retain cholesterol at any pH because the side chains are too short to accommodate the sterol. All expectations were met, except the last. Short chain phospholipids of both types retained cholesterol at high pH. Suggested reasons for this observation are discussed below.

METHODS

Materials. 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dilauroyl-*sn*-glycero-3-phosphocholine (DLPC) and 1,2-dilauroyl-*sn*-glycero-3-phosphoethanolamine (DLPE) were purchased from Sigma Chemical Co. (St. Louis, MO); 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), from Avanti Polar Lipids (Birmingham, AL); and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE(from Calbiochem (La Jolla, CA). Purities of phospholipids were checked by thin layer chromatography on silica gel G plates, purchased from Applied Science (State College, PA) and developed with chloroform/methanol/water (65:25:4, v/v/v). Phospholipids were visualized with iodine vapors. Cholesterol was recrystallized twice from methanol; 4-[^{14}C]cholesterol was obtained from Amersham (Arlington Heights, IL). All other substances and solvents were reagent grade and were used without further purification.

Preparation of MLV. The procedures for preparing MLV, quantitating cholesterol and phospholipid extracted from vesicles, and measurement of fluorescence polarization have been described (1). At pH 1.2, MLV were prepared in 0.1 N phosphoric acid; at pH 2.0, they were made in 0.1 M potassium dihydrogen phosphate and phosphoric acid; and at pH 4.0, they were prepared in unbuffered 0.9% sodium chloride. For pH ranges between 5.8 and 7.8, 0.1 M potassium phosphate buffers were used. MLV at pH 10.0 were prepared in 0.1 M CAPS (cyclohexylaminopropane sulfonic acid) and sodium hydroxide and for pH 12.5 they were prepared in 0.15 M potassium chloride and sodium hydroxide. The extraction with petroleum ether and with chloroform/methanol (2:1, v/v) was carried out at 22°C and has been described (1). Sonicated vesicles were prepared from dried lipid films in unbuffered 0.9% sodium chloride in an Artek Dismembrator Model 300 (Farmingdale, NY) at 105 W for 10 min in an ice bath. Fluorescence polarization (p), corrected for light scattering, was determined with 1,6-diphenyl-1,3,5-hexatriene as a lipid probe and measured in a Perkin-Elmer Model MFP 44 spectrophotometer (1). The sizes of vesicles of DOPC and DPPE with cholesterol at various pH values were determined by Doppler Effect Laser Light Scattering Analysis (9) on a Coulter DELSA instrument generously made available by Langley-Ford Instruments in Amherst, MA. Light scattering measurements were made with a frequency range of 500 Hz and a frequency shift of 250 Hz. Current was at 6 mA, on time was 2 sec and off time was 0.5 sec.

RESULTS

Cholesterol retention by PE and mixed PC/PE MLV. MLV were prepared in unbuffered 0.15 M NaCl from phospholipid mixtures, with and without equimolar amounts of cholesterol, and extracted three times with petroleum ether. Fluorescence polarization for each sample was determined with diphenylhexatriene as a lipid probe. Figure 1 (A) shows cholesterol retention by vesicles

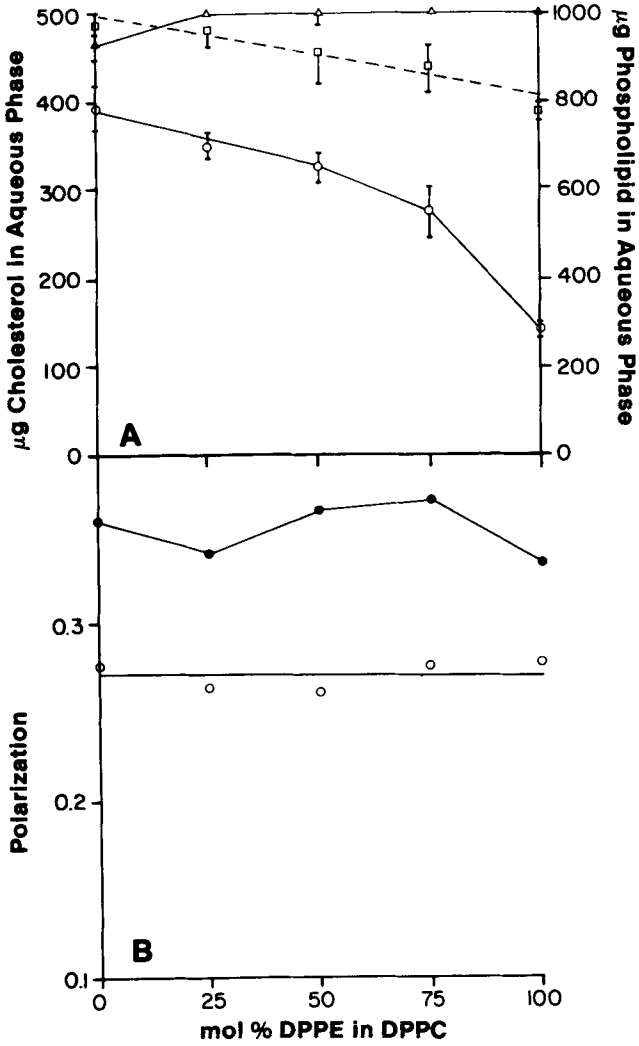

FIG. 1. (A) Cholesterol and phospholipid retention by MLV of DPPC and DPPE, with or without cholesterol, in molar ratio of 1:1 of sterol to phospholipid. Vesicles were prepared as described in Materials and Methods. Sterol was determined by [4-^{14}C]cholesterol remaining in the aqueous phase after petroleum ether extraction. Phospholipid was determined by phosphate assay of dry petroleum ether extracts. Points are the means of 4 to 7 samples; S.D.s are shown by bars. ○, Cholesterol in aqueous phase; □, phospholipid in aqueous phase from vesicles prepared with cholesterol; △, phospholipid in aqueous phase from vesicles without cholesterol. (B) Fluorescence polarization of vesicles prepared as in (A). Each point is the mean of 3 samples; S.D.s are included in the points. ○, DPPC and DPPE prepared with cholesterol; ●, DPPC and DPPE without cholesterol.

of DPPC, DPPE and mixtures of the two at pH 5.8. Extractability of phospholipid in presence and absence of cholesterol was determined. In absence of cholesterol, phospholipid was not extractable from the vesicles but with sterol present, 20% of the lipid was extractable. Cholesterol was selectively removed from vesicles of DPPE and DPPC and their mixtures. The amount of cholesterol retained decreased with increasing DPPE content from 80% with DPPC alone to 30% with DPPE alone. Fluorescence polarization, Figure 1B, was somewhat lower for vesicles with cholesterol than for vesicles without sterol. The failure of DPPE to retain substantial amounts of sterol is not due to mobility of the acyl side chains of the phospholipids because cholesterol retention decreases even though the p value remains fairly constant.

Cholesterol retention and fluorescence polarization for MLV prepared from DLPE and DPPE were measured (Fig. 2). Vesicles containing DLPE or mixtures of DLPE and DPPE retain almost no sterol, whereas DPPE vesicles retain about 30%. The measured pH of the unbuffered medium of these preparations was 3.5-4.0. No phospholipid was extracted from DPPE vesicles, whereas mixed vesicles of DLPE and DPPE lost from 60 to 80% of their lipid. Vesicles of DLPE lost somewhat less phospholipid than vesicles made from DLPE mixed with DPPE. In these preparations, the presence of cholesterol had little influence on the amount of phospholipid extracted. Fluorescence polarization for vesicles with mixed DLPE and DPPE was lower than that of either lipid alone. The presence of cholesterol decreased polarization for all vesicles except those made entirely from DLPE. The fact that fluorescence polarization did not change when cholesterol was present in DLPE vesicles and that no sterol was retained indicates that cholesterol is not incorporated into the vesicles. In this case, as in the case of DPPC and DPPE mixtures described above, cholesterol retention was not related to side chain mobility.

The pattern of cholesterol retention by DOPE/DPPC vesicles is shown in Fig. 3A. In absence of cholesterol, no phospholipid could be extracted from DPPC vesicles

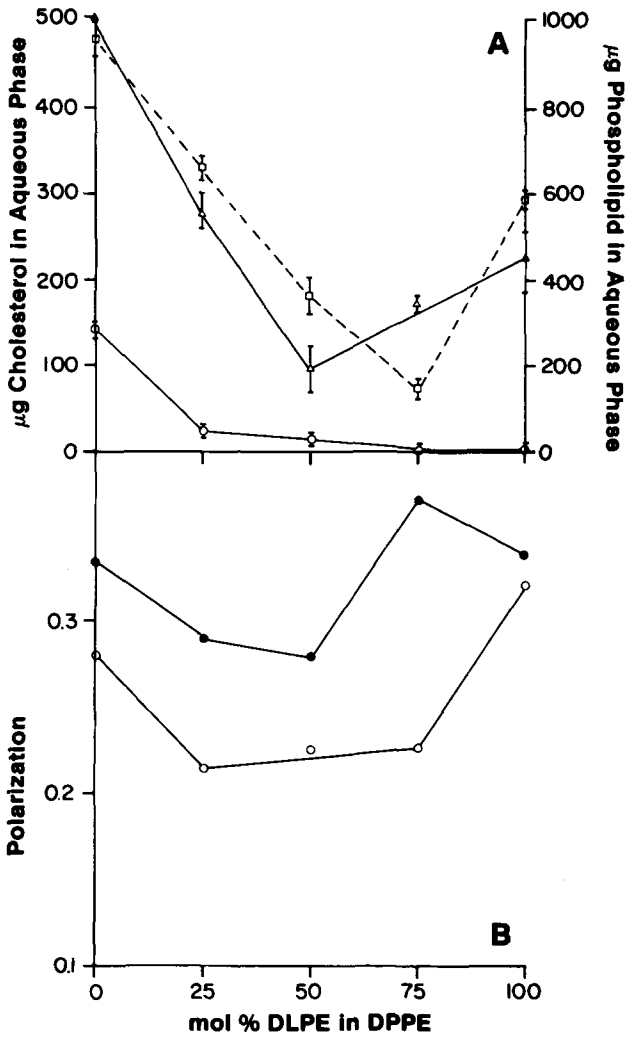

FIG. 2. (A) Cholesterol and phospholipid retention by MLV of DLPE and DPPE with or without cholesterol, in ratio of 1:1 of sterol to phospholipid. Other details are shown in Fig. 1A. (B) Fluorescence polarization of vesicles prepared as in (A). Details are shown in Fig. 1B. O, vesicles with cholesterol; ●, vesicles without cholesterol.

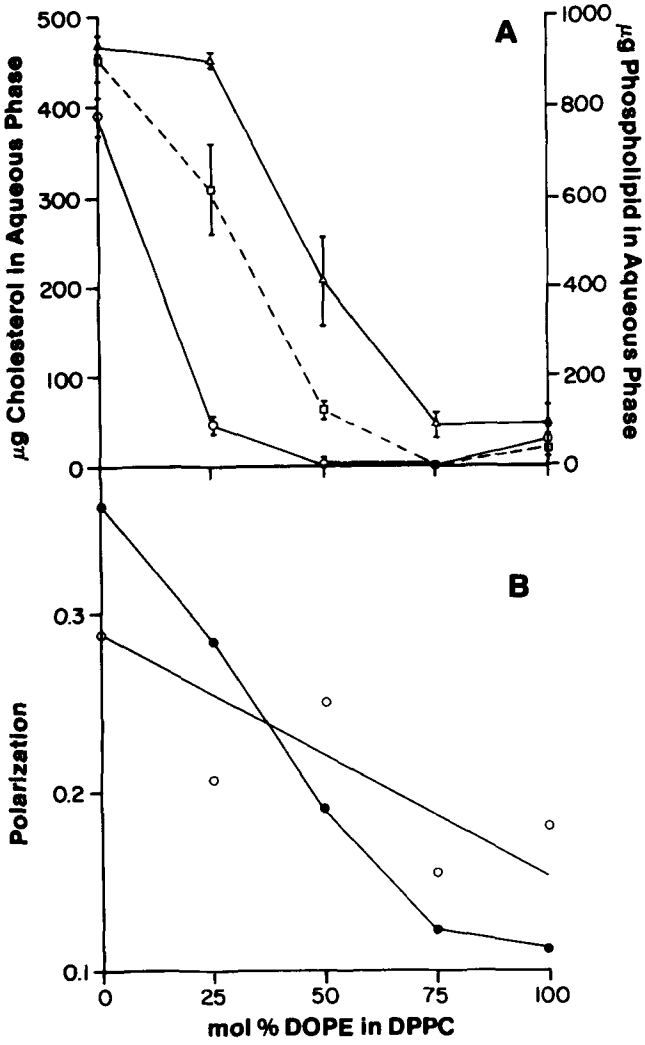

FIG. 3. (A) Cholesterol and phospholipid retention by MLV of DOPE and DPPC with or without cholesterol, in ratio of 1:1 of sterol to phospholipid. Other details are shown in Fig. 1A. (B) Fluorescence polarization of vesicles prepared as in (A). Details are shown in Fig. 1B. O, vesicles with cholesterol; ●, vesicles without cholesterol.

by petroleum ether, but substitution of 25% of DPPC by DOPE caused a substantial increase in phospholipid extraction: 90% could be extracted from vesicles with 75–100% DOPE. When cholesterol was included, extraction of phospholipid from DOPE-containing vesicles was increased and 10% or less of the sterol was retained. Cholesterol modified the fluorescence polarization of all vesicles (Fig. 3B) indicating that acyl side chains of saturated phospholipids became more mobile, whereas unsaturated side chains became less mobile. Cholesterol retention was not proportional to side chain mobility. It appears that DOPE interacts with cholesterol, but the sterol is not protected from the extracting solvent. This observation suggests the presence of an H_{II} phase, because in this configuration, the acyl side chains are exposed to the aqueous phase and would interact with the solvent.

The combination of DOPC and DPPE was difficult to study. The 50 mol% mixture formed waxy particles which stuck to the walls of the tube and dispersed poorly, no matter how long they were agitated. Cholesterol retention by these vesicles did not vary with phospholipid composition (Fig. 4A). Cholesterol moderated the fluorescence polarization of the vesicles, but the p value for all vesicles containing DOPC was the same (Fig. 4B). Cholesterol retention for vesicles containing a large proportion of DPPE was unrelated to side chain mobility.

Effect of pH on cholesterol retention by PC and PE MLV. Cholesterol retention by vesicles of single species of phospholipid was studied as a function of pH. Figure 5A shows cholesterol retention by DPPE vesicles. There was a marked transition between pH 6.0 and 6.8, where cholesterol retention was doubled from 45% to 90%. An increase in the pH to 10.0 or 12.5 caused a slight decrease of sterol retention. Cholesterol retention at pH 1.1 and 2.0 was less than the amount retained at pH 3.5 to 6.0. Cholesterol retentions by DOPE and DLPE were similar, Figure 5B and 5C, and showed a marked increase above pH 8.0: cholesterol retention was 90% for both types of vesicles. Below pH 8.0, the vesicles retained almost no sterol. Studies with DPPC, Figure 5D, yielded a different pattern. Retention of cholesterol by MLV of DPPC above pH 8.0 was over 90%. Between pH 4.0 to 5.5, cholesterol retention varied from 75 to 80%, but at pH 2.0, 95% of the sterol was retained. The effect of pH on DOPC vesicles was similar but more striking. Cholesterol retention rose from 45% at pH 5.0 to 90% at pH 10, Figure 5E. At pH 2.0, the amounts of sterol retained was 85%. DLPC was unlike its long chain analogs at low pH. At pH 1.0 to 2.0, it retained less than 20% of cholesterol. Between pH 2.0 and 7.0, the amount of sterol retained rose from 0 to 30%, but above pH 7.0, cholesterol retention increased to 90%, Figure 5F. Phospholipid remaining in the aqueous phase above pH 8.0 was nearly 100% for all lipids. Phospholipid retention in the mid-pH range varied with each lipid. Values can be read from Figures 1 to 4 and from Figures 1 and 5 (A) in (1). At pH 2 and below, phospholipid retention was between 80 and 100% for DPPC, DOPC, DPPE and DLPE. DOPE retained no phospholipid and DLPC retained about 50%.

We considered the possibility that differences in size of vesicles at various pHs might influence cholesterol extraction. The average size of DOPC and DPPE vesicles with cholesterol at pH 2.0, 6.0 and 10.0 was determined by Doppler effect light scattering analysis. The results are shown in Table 1. The sizes of vesicles differed considerably with pH, ranging from 620 nm to over 6000 nm.

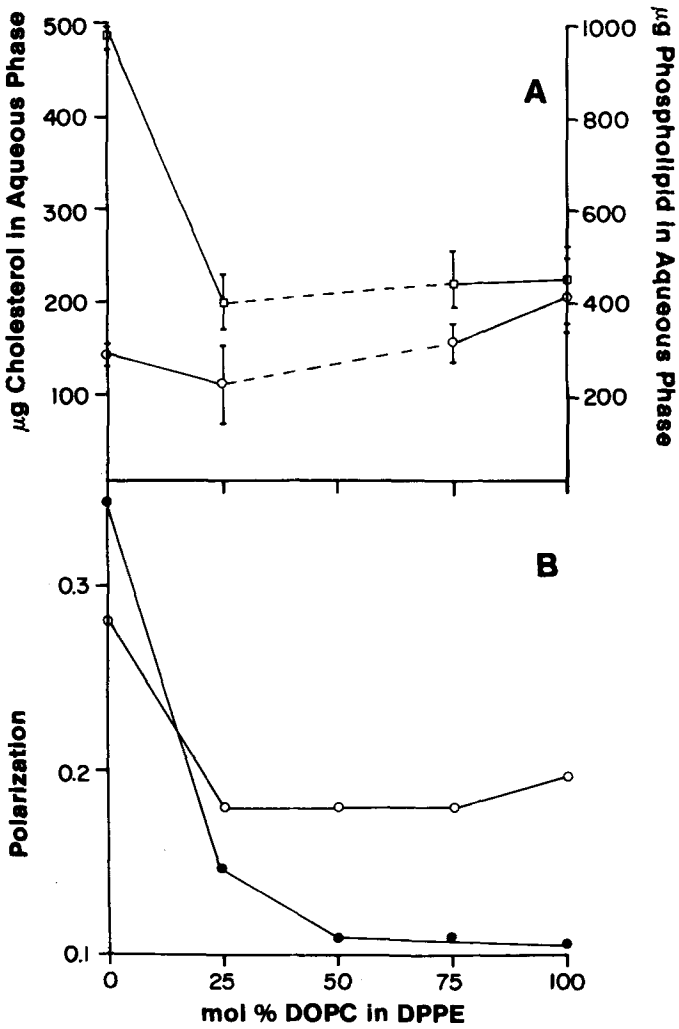

FIG. 4. (A) Cholesterol and phospholipid retention by MLV of DOPC and DPPE with or without cholesterol, in ratio of 1:1 of sterol to phospholipid. Other details are shown in Fig. 1A. Data for 50 mol% DOPC in DPPE were not obtained. (B) Fluorescence polarization of vesicles prepared as in (A). Details are shown in Fig. 1B. ○, vesicles with cholesterol; ●, vesicles without cholesterol.

TABLE 1

Diameters of Vesicles

	pH		
Vesicle composition	2.0	5.8	10.0
DPPE + cholesterol	2598 nm	6432 nm	1230 nm
DOPC + cholesterol	620	1414	2800

Diameters of DOPC and DPPE vesicles with cholesterol in a 1:1 molar ratio measured at pH 2.0, 5.8 and 10.0. Vesicles were prepared as described under Materials and Methods with unlabeled sterol. Vesicle size was determined by Doppler Effect Light Scattering Analysis (9).

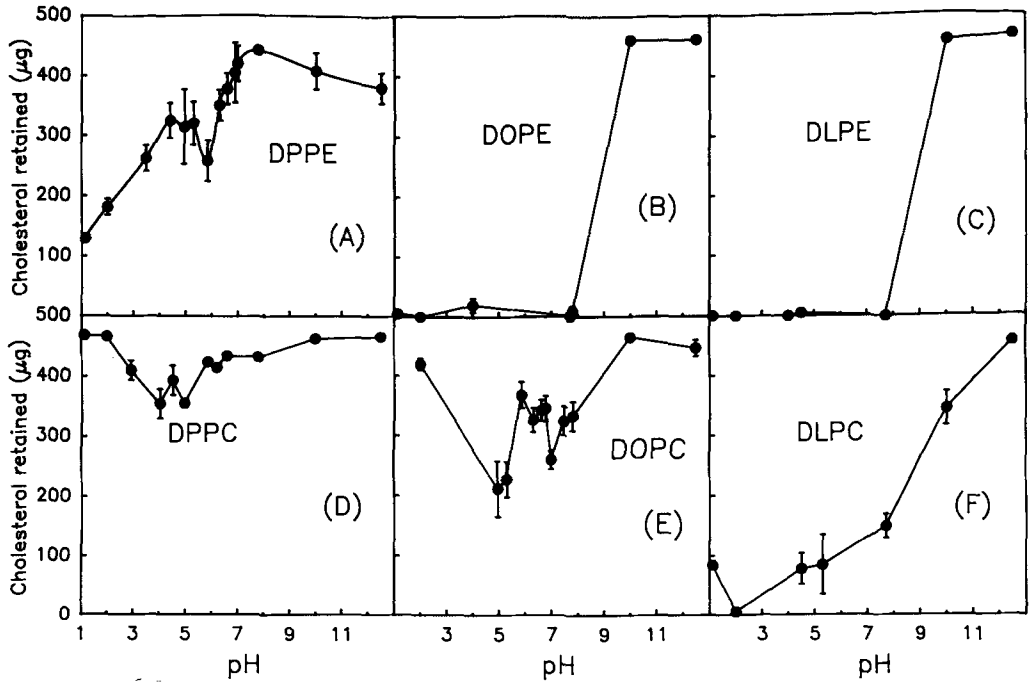

FIG. 5. Cholesterol retention by phospholipid vesicles as function of pH. Vesicles were prepared with cholesterol in molar ratio of 1:1 of sterol to phospholipid in solutions of pH 1.1 to pH 12.5, as described under Materials and Methods. Other details are shown in Fig. 1A. Each point is the mean of 3 to 6 samples, with S.D. shown by bars or included within symbols. (A) DPPE; (B) DOPE; (C) DLPE; (D) DPPC; (E) DOPC; (F) DLPC.

DOPC vesicles prepared with equimolar cholesterol retained 90% of the sterol at both pH 2 and pH 10, but the average size of the vesicles at pH 10 is more than 4 times greater than at pH 2. For DPPE vesicles, cholesterol retention at pH 2 and 6 was about the same, but the vesicles at pH 6 are 2.5 times larger than at pH 2. It does not appear that vesicle size affects cholesterol retention.

Sonicated vesicles. Vesicles of DPPE, DPPC and DOPC with equimolar amounts of cholesterol and trace amounts of 4-[^{14}C]cholesterol were prepared by sonication as previously described. The resultant vesicles were extracted three times with petroleum ether. The amounts of cholesterol retained in the aqueous phase by each type of vesicle are shown in Table 2 and compared with the amounts of sterol retained by unsonicated vesicles at acid and basic pH and in unbuffered sodium chloride solution. Sterol retention by sonicated vesicles of DPPC and DPPE was in a 1:1 molar ratio, but DOPC vesicles retained sterol in a 0.75:1 cholesterol to phospholipid ratio.

TABLE 2

Cholesterol Retention by Vesicles

	0.9% NaCl		KH_2PO_4-K_2HPO_4 buffer	
	Unsonicated	Sonicated	pH 5.8	pH 7.8
DOPC	211 ± 47	368 ± 28	358 ± 25	333 ± 25
DPPC	390 ± 28	465 ± 3	415 ± 6	432 ± 4
DPPE	142 ± 6	455 ± 4	218 ± 32	442 ± 7

Vesicles were prepared from DPPE, DPPC or DOPC with cholesterol in a 1:1 molar ratio and trace amounts of [4-^{14}C]cholesterol, as described in Materials and Methods. Values for cholesterol retention by similar vesicles in unbuffered NaCl and in phosphate buffer at pH 5.8 and 7.8 are given for comparison. Values in μg.

DISCUSSION

Between pH 3.5 and 6.0, MLV of PE retain less cholesterol after extraction with a nonpolar solvent than MLV of PC. These differences are eliminated at pH values between 6.5 to 8.0, depending on the acyl side chain composition of the phospholipid. The difference also disappears for vesicles prepared by sonication. Mixed phospholipid vesicles retain cholesterol in proportion to the amount of PC incorporated into the bilayers. PC with saturated 16-C side chains were more susceptible to ordering than the unsaturated compounds. The influence of saturated 16-C side chains extends to PE lipids also. DPPE exhibited the greatest capacity of all PE lipids examined to retain cholesterol in the aqueous phase (Fig. 1). The ability of saturated acyl side chains to enhance cholesterol retention does not apply to short chain phospholipids. Neither DLPE (Fig. 2) nor DLPC (1) retained significant amounts of cholesterol in the aqueous phase. Fluorescence polarization studies indicate that mobility of DLPC side chains was modified by cholesterol whereas the mobility of DLPE side chains was not (Fig. 2 and [1]); however, the configuration assumed by the DLPC-cholesterol complex did not protect the sterol from extraction. Low pH had different effects on PC and PE lipids. Long chain PC, saturated or unsaturated, retained cholesterol in a nearly 1:1 molar ratio at pH 2, whereas DLPC, DLPE, DPPE and DOPE retained little or no sterol.

Two factors must be considered in explaining the behavior of cholesterol in aqueous phospholipid-containing

media. One, cholesterol can intercalate itself into the hydrocarbon domain of structures assumed by phospholipids (17) and two, once intercalated, cholesterol maintains its relationship to the phospholipid because of thermodynamic constraints placed upon amphipathic molecules in an aqueous phase. The hydrophobic interactions and van der Waals forces that characterize the association between the sterol and paraffin side chains of phospholipids are applicable only to the extent that phospholipids maintain a relationship to one another. This relationship is dependent on the polarity of the bulk phase (5,18), the hydrogen ion concentration (15), intermolecular hydrogen bonding (6–8) and the presence of inorganic cations (19,20).

The effect of pH on the phase structure of phospholipids has been examined previously. Saturated PE, below their transition temperatures, pack more closely in bilayer structures at low pH than at high pH (15,21). At pH less than 3, the phosphate groups are fully protonated (16). This results in a loss of hydration around the head groups and a reduction of their space requirements (13,21). At room temperature, which is below the T_c for saturated PE (22), the acyl side chains are in the gel state and can be pushed closer together. The space occupied by the molecule becomes cylindrical and the bilayer conformation would be maintained. At high pH, the phosphate groups are ionized and hydrated, occupying a larger space than at low pH. The head groups are spread apart, allowing more mobility among acyl side chains. Introduction of cholesterol into the bilayers reduces the mobility of the side chains (15) and the bilayer structure remains stable. Unsaturated PE, such as DOPE, form an H_{II} phase at low pH (11–13,21,23) because the space requirements for the acyl side chains exceed those of the head groups and the molecules occupy conical spaces. Haines (6) has proposed that acid-anion pairs may form among head groups in the mid-pH range. Chaotropic agents, which disrupt hydrogen bonding, stabilize lamellar phases of PE, and this finding led to the conclusion that hydration of the bilayer surface determines phase state (5). At high pH, the space requirements of head groups and side chains are more nearly equal and the molecules occupy a cylindrical space, assuming a bilayer conformation (14).

Above pH 3, PC occurs as zwitterion but it is not clear why pH of 8 and higher caused an increased cholesterol retention, indicative of a spreading of head groups. PC form bilayers throughout the pH range (15). Cevc (21) reported that the T_c of DPPC decreases by 8°C between pH 0 and pH 13 and suggested a decrease in the ordering of acyl side chains at the higher pH. Hsia and Boggs (15), using ESR studies, found similar evidence of disorder at high pH but ascribed it to the effect of pH on the spin label. Introduction of cholesterol into PC bilayers moderates the mobility of acyl side chains (17), but does not alter the phase structure, except when unsaturated PC and PE lipids are mixed (24).

At the temperature of these experiments and at low to neutral pH, all of the PC and PE phospholipids, except DOPE, were below their transition temperatures (22) and were expected to be in the bilayer state (15,21). Mixed vesicles containing unsaturated PC and PE are unstable and may form H_{II} phases (24). The bilayer conformation should protect phospholipid from extraction by a nonpolar solvent and the data show this to be the case. The exception was DLPE, for which half the phospholipid was extractable. Incorporation of cholesterol into bilayers, particularly if two species of phospholipid were present, increased extractability of phospholipids. Fluorescence polarization and extraction data for the pH range of 3.5 to 5.8 show that DLPE does not retain or interact with cholesterol. The p value was the same for DLPE with or without sterol (Fig. 2B). DLPC interacts with the sterol but does not prevent its extraction (1). DPPE interacts with the sterol, but only retains it in a 0.33:1 cholesterol to phospholipid molar ratio (Fig. 1). DOPC and DOPE both interact with the sterol, but the association is unstable, as evidenced by the fact that sterol and phospholipid are both extracted by the solvent in the same molar proportions. DPPC interacts with cholesterol and sequesters it from the extracting solvent.

Above pH 8.0, all of the phospholipids retained cholesterol in a 1:1 molar ratio, but below pH 3.0, only DPPC and DOPC protected the sterol from extraction. The latter is in agreement with the findings of van Blitterswijk *et al.* (25), who showed that the affinity of phospholipids for cholesterol increases with the fraction of sphingomyelin or PC in the vesicles and that PC with saturated 16-C side chains were more susceptible to ordering than unsaturated side chains.

Charge repulsion and hydration do not suffice to explain all of the effects of high pH. Monovalent cations from the bulk phase may be shared among negatively charged groups as pH is increased. McLaughlin (26) has calculated that if 20% of the lipid in a membrane is anionic, then the molar concentration of cation at the membrane surface will be 1.0 M. If monovalent cations were to replace hydrogen ions, which were shared among PE or anionic head groups at low pH, they would spread the head groups further apart. Li and Haines (20) observed that unilamellar vesicles made from dioleoylphosphatidic acid by titration to pH 9.0 or 10.0 in sucrose-TRIS buffer were approximately half the size of vesicles made in buffered potassium chloride solution. Vesicles diluted in sucrose-buffer solutions swell more than vesicles diluted in KCl-buffer solutions (19), indicating that monovalent cations prevent vesicle expansion. In our experiments, vesicles were made up in media which contained either Na^+ or K^+ ions. The sharing of monovalent cations among the anionic forms of PE affects the space requirements of head groups and the ability of the entire molecule to interact with cholesterol.

The role of acyl chain length in determining cholesterol intercalation into a bilayer at high pH is illustrated by DLPC and DLPE. DLPC retains 20 mol% or less of cholesterol on extraction by a nonpolar solvent at pH less than 8.0, whereas DLPE retains none (Fig. 5B). It has been suggested that the 12 carbon acyl chain of dilauroyl lipids is too short to fit the sterol (27); however, we found that both lipids retain 90 mol% of cholesterol above pH 8.0. The space between head groups afforded by charge repulsion and hydration or by monovalent cation sharing does not afford more space to fit the sterol into the hydrocarbon domain of the bilayer. The negative charge within head groups or increased hydration may draw the hydroxyl group of cholesterol closer to the surface of the bilayer. The sterol ring structure would then be pulled into the hydrocarbon domain and intercalate with paraffin side chains. This consideration can also explain why

DLPC, unlike its long chain analogs, does not retain cholesterol at low pH. Loss of the negative charge on the head group decreases the attraction for the 3-hydroxy group of the sterol.

The behavior of DOPC in the mid-pH range raises an interesting question. Between pH 4.5 and pH 8.0, DOPC retains less than 50 mol% of cholesterol, losing both sterol and phospholipid to the extracting solvent (Fig. 5F and [1]). The acyl chain is long enough to accommodate cholesterol, and the *cis* double bond at C-9 does not interfere with cholesterol interaction (28). Both phosphate and choline groups are charged so that the head group is fully hydrated. However, removal of cholesterol from these vesicles increased with each successive petroleum ether extraction (unpublished observations), indicating that the vesicles were initially in the lamellar state. Solvent may enter the hydrocarbon domain and disrupt the ordered arrangement of cholesterol and acyl side chains. The disorder created by presence of the solvent would increase the space requirement of the hydrocarbons beyond the umbrella provided by the polar head groups.

Why would solvent enter the hydrocarbon domain? Although it is generally assumed that a net charge is necessary for head group repulsion, this may not always be the case. PC head groups are oriented with phosphate groups at an angle of 50° to the plane of the bilayer and choline groups nearly parallel to the plane (29). In this configuration, positively charged groups will lie adjacent to each other and will occupy maximal space due to repulsion, and negatively charged groups will behave correspondingly. Solvent may penetrate between head groups and enter the hydrocarbon domain.

Sonication of vesicles at pH 3.5–5.0 has the same effect as raising pH (Table 2). Sonication for long periods produces small vesicles with highly curved surfaces (30). The space occupied by choline head groups on the outer surfaces of such vesicles has been calculated to be 0.74 nm^2, which is greater than the area occupied by the same head group on the inner surface, 0.61 nm^2 (31). The increase in effective diameter of head groups in the outer layer of the vesicle allows for greater spreading of all acyl side chains and would permit cholesterol to fit into both leaflets of the bilayer.

The present studies, some of which were performed at extremes of pH, are applicable to physiological states. Subcellular organelles such as inner mitochondrial membranes, chloroplasts and bacterial cell membranes are the site of proton pumps and have been shown to contain high proportions of anionic and PE lipids (32–34). Neither of these membranes incorporate significant amounts of sterol. Natural and artificial membranes rich in PE give up cholesterol to membranes with high proportions of PC and/or sphingomyelin (35–37). Low pH has been reported to facilitate the fusion of lipid vesicles (38) and Ca ion displacement from phosphatidyl serine monolayers *in vitro* (39).

In the present work, the effect of pH on the cholesterol retaining characteristics of PE and PC vesicles can be explained partly by the influence of repulsion of like charges and differences in degree of hydration of head groups. These two factors influence the space requirements of head groups but other factors need to be taken into account. Data suggest that acyl chain length, interaction of negatively charged groups with cationic species, juxtaposition of charged groups and charge state of the head group anion are pertinent to the affinity of phospholipids for cholesterol.

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Changes in Lipid Composition of Liver Microsomes and Fatty Acyl-CoA Desaturase Activities Induced by Medium Chain Triglyceride Feeding

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Changes in fatty acid composition, microsomal Δ^9- and Δ^6-desaturase activities and liver contents of cholesterol and phospholipids were studied in rats fed medium chain triglyceride-supplemented diets. Weanling rats were divided into four groups and fed for three weeks a basal diet with different 10%-fat supplements: corn oil, medium chain triglyceride–corn oil, olive oil and medium chain triglyceride–olive oil. The highest relative content of saturated fatty acids corresponded to corn oil-fed animals. Both monounsaturated fatty acid content and Δ^9-desaturase activity were higher in the animals fed olive oil diets than in corn oil-fed rats. The long chain polyunsaturated fatty acids of the n-3 series were increased in the olive oil and medium chain triglyceride–olive oil-fed groups probably due to the lower linoleic/α-linolenic ratios found in these two diets. The cholesterol/phospholipid molar ratio was unaffected by diet and the unsaturation index was only slightly changed in the four groups. Thus, some mechanism may be operative under these conditions to maintain the homeostasis of the membrane.
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Changes in lipid composition have been observed in mitochondrial and other membranes when feeding animals diets supplemented with higher levels of saturated or unsaturated fatty acids (1-6). Changes in membrane lipid composition are convenient means of changing membrane physical properties, and these physical changes modulate many membrane-associated enzymes (7).

Medium chain triglycerides (MCT) are a type of fat not common in human nutrition. MCT are absorbed via the portal system, do not require chylomicron formation and are not carnitine-dependent for oxidation. MCT are absorbed and metabolized as rapidly as glucose and have better than twice the caloric density of protein and carbohydrate. They are easily oxidized and utilized as fuel and energy with little tendency to deposit as body fat (8). MCT appear to be less lipogenic in adipose tissue than long chain triglycerides (LCT) in rats, and the mechanism for the diminished adiposity of MCT-fed rats is related to the extensive oxidation of MCT and the enhancement of thermogenesis leading to lessened energy efficiency (9).

As MCT are important components of diets used in many clinical conditions, in the present work, we have investigated whether the addition of MCT to olive or corn oil can modify the fatty acid composition of liver microsomes in rats. At the same time, we have determined the activities of Δ^9- and Δ^6-desaturases, as tightly bound membrane enzymes involved in fatty acid unsaturation, to determine possible modifications in their activity induced by dietary fat.

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Abbreviations: C, corn oil; LCT, long chain triglyceride; MCT, medium chain triglyceride; O, olive oil; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL, phospholipid; PUFA, polyunsaturated fatty acid; GLC, gas liquid chromatography; P/S, polyunsaturated/saturated fatty acid ratio; TLC, thin layer chromatography; UNID, unsaturation index.

MATERIAL AND METHODS

Animals and diets. Male weanling Wistar rats weighing 50-55 g were housed in wire-bottom cages with a 12-hr light/dark cycle. Rats were randomly assigned to 4 groups each containing 10 animals. Each group was fed the same basal diet but with different 10%-fat supplements: 10% corn oil (C), 5% MCT and 5% corn oil (MCT-C), 10% olive oil (O), and 5% MCT and 5% olive oil (MCT-O). In addition to fat, the diets contained 65.6% starch, 10% vitamin-free casein, 8% cellulose, 4.5% mineral mix, 1.5% vitamin mix, 0.55% D,L-methionine and 0.33% choline chloride. The fatty acid composition of the four diets is shown in Table 1.

The diets were stored at 4°C and the rats were fed fresh food daily. Food and water were provided ad libitum. The amount of food consumed by animals was comparable for all groups, and all animals had similar weights at the end of the experiments. After consuming the experimental diets for 3 weeks, the rats were anesthetized with 0.5 ml/100 g rat weight of 20% urethane, and the livers were excised.

Reagents and substrates. [1-^{14}C] Palmitic acid (16:0) (sp. act. 54 mCi/mmol, 97.1% radiochemical purity) and [1-^{14}C] linoleic acid (18:2n6) (sp. act. 58.7 mCi/mmol, 98.8% radiochemical purity) were purchased from Amersham International (Amersham, U.K.). Unlabeled fatty acids, ATP, NADH, and Coenzyme A were purchased from Sigma Chemicals Co. (St. Louis, MO). All other reagents and chemicals were of analytical grade.

Preparation of liver microsomes. Immediately upon removal, the livers were rinsed in cold saline solution and microsomes were prepared as described by Philipp and Shapiro (10).

TABLE 1

Fatty Acid Composition of Diets

Fatty acid	Olive oil	MCT-Olive oil	Corn oil	MCT-Corn oil
8:0	—	14.4	—	14.4
10:0	0.2	33.6	—	34.3
12:0	0.5	1.9	0.1	0.9
14:0	0.5	0.4	0.4	0.5
16:0	12.5	6.3	12.6	6.3
18:0	3.0	1.6	1.8	1.1
20:0	0.3	0.2	0.4	0.2
16:1n7	1.1	0.5	0.2	0.2
18:1n9	71.9	36.1	24.1	12.1
20:1	0.2	0.1	0.2	0.1
18:2n6	9.1	4.6	59.2	29.3
18:3n3	0.8	0.4	1.0	0.5

Results are expressed as the percentages of total fatty acid methyl esters.

Analytical methods. Lipids of liver microsomes were extracted with chloroform/methanol (2:1, v/v) (11). The phospholipids were separated by thin layer chromatography (TLC) on Silica Gel G 60 using two different solvent systems, hexane/ethyl ether/acetic acid (80:20:1, v/v/v) (12) to isolate total phospholipids and ethyl acetate/chloroform/n-propanol/methanol/0.25% KCl (25:25:25:10:9, v/v/v/v/v) (13) to separate individual phospholipid fractions. Total phospholipids (PL), phosphatidylethanolamine (PE), phosphatidylcholine (PC) and phosphatidylinositol (PI) were immediately scraped and analyzed for fatty acid composition.

Methylation of fatty acids from each phospholipid class was done according to Morrison and Smith (14). Fatty acid methyl esters were stored at −30°C under nitrogen until analyzed.

Fatty acid analysis was performed by gas liquid chromatography (GLC) using a Hewlett-Packard 5880 A gas chromatograph equipped with a 4 m length × 2 mm i.d. glass column packed with 10% SP 2330 on 100–120 mesh Chromosorb WAW. Peaks were identified by comparison with known standards (Supelco, Barcelona), and the results were reported as area percentages.

Cholesterol in the liver microsomal extracts was determined by the enzymatic method of Röschlau et al. (15).

Phospholipid phosphorus was measured by spectrometry according to the Fiske and Subarrow method as modified by Bartlett (16).

Microsomal proteins were determined by the Lowry method (17) on the aqueous liver microsomal suspensions.

Enzymic assay. Δ^9- and Δ^6-Desaturase activities were assayed according to methods previously described (18–22). The assay was initiated by adding 0.5–1.0 mg of microsomal protein to the incubation mixture which contained 250 μM NADH, 100 μM reduced glutathione, 130 μM ATP, 1 mM $MgCl_2$, 20 μM coenzyme A and 75 μM ^{14}C-palmitic (sp. act. 0.49–2.45 μCi/μmol) or 75 μM ^{14}C-linoleic acid (sp. act. 0.49–2.45 μCi/μmol) in a final volume of 1 ml. Fatty acids were used as sodium salt-albumin-complex (1 μg free fatty acid/11.5 μg bovine serum albumin). Incubations were carried out at 37°C for 20 min in a metabolic shaker. Enzyme assays were terminated by addition of 1 ml of 10% (w/v) KOH followed by saponification at 100°C for 30 min and addition of 1 ml of 4N H_2SO_4. Lipids were extracted twice with 5 ml chloroform/methanol (2:1, v/v). Both organic phases were combined and evaporated under nitrogen. Before methylation 2 μmol of both 16:0 and palmitoleic (16:1n7) acids were added to the substrate and products of fatty acid mixtures in case of Δ^9-desaturase, and 2 μmol of both 18:2n6 and γ-linolenic (18:3n6) acids were added in case of Δ^6-desaturase to serve as unlabeled carriers for TLC separation of substrate and products. Methylation of fatty acids was carried out with 14% boron trifluoride in methanol (14). Fatty acid methyl esters were separated on Silica Gel G-60 TLC plates containing 5% (w/w) $AgNO_3$, using chloroform for Δ^9-desaturase and ethyl ether/hexane/formic acid (25:75:2, v/v/v) for Δ^6-desaturase as solvents. Plates were sprayed with a 0.1% Rhodamine G solution in acetone and visualized under ultraviolet light. Bands of product, unreacted substrate and origin were collected and eluted with ethyl ether. The ether was evaporated under nitrogen and the radioactivity in the residues was counted in a Beckman LS 7500 liquid scintillation spectrometer (Beckman Instruments, Palo Alto, CA) using as fluor toluene/ethyl acetate/PPO/POPOP (934/665/0.3, v/v/w/w). The percentage of conversion of substrate to product was calculated.

Statistical assay. Comparisons between mean values for fatty acids and related indices were made by a two-way analysis of variance and an a posteriori Scheffe test (23).

RESULTS

The relative fatty acid composition of diets is shown in Table 1. The saturated fatty acid percentages were higher in the two MCT diets. Differences in the percentages of major unsaturated fatty acids among diets were apparent for oleic acid (18:1n9) at a higher level in the olive oil diets and linoleic acid (18:2n6) at a high level in the corn oil diets. It should be noted that the linoleic/α-linolenic acid ratio was different among the diets: 59.1 in the corn oil diets and 11.3 in the olive oil diets.

The relative fatty acid composition of liver microsomal total PL, PC, PE, and PI of rats maintained on the diets for 3 weeks is shown in Tables 2–5. Palmitic (16:0) and stearic (18:0) were the major saturated fatty acids in lipid microsomes. In PL, PC and PE, these fatty acids represented 25%, whereas the others such as lauric (12:0), myristic (14:0) and lignoceric (24:0) acids only represented 1% of the total fatty acids. In PI, myristic acid represented 2–4% of total fatty acids. This fraction also showed an elevated proportion of stearic acid. The level of total saturated fatty acids in liver microsomes was slightly increased in rats fed the C and MCT-C diets. The addition of MCT to olive or corn oil did not significantly modify the saturated fatty acid level.

The proportion of monounsaturated fatty acid was different in each type of phospholipid and was influenced by the type of dietary lipid treatment. Microsomes from animals fed O and MCT-O diets had a higher relative content of oleic and palmitoleic acids in PL, PC and PE. For PI, the relative content of these two fatty acids was similar in microsomes from animals of all groups.

The relative content of linoleic acid was higher in the different phospholipid fractions of microsomes from animals fed C and MCT-C diets. Eicosatrienoic acid (20:3n6) was present in greater proportions in the MCT-O and O groups than in the corn oil groups. PL, PC and PI had higher contents of arachidonic acid (20:4n6) in the MCT-C and C groups. In PE, the highest level of arachidonic acid was in the MCT-O and O groups. Docosatetraenoic acid (22:4n6) and docosapentaenoic acid (22:5n6) were higher in the corn oil groups than in the olive oil groups. The highest values of the n6 > 18 index were obtained for the MCT-C and C groups.

The polyunsaturated fatty acids from the n3 series include α-linolenic (18:3n3), eicosapentaenoic (20:5n3), docosapentaenoic (22:5n3) and docosahexaenoic (22:6n3) acids. The highest values were generally for 22:5n3 and 22:6n3. In general these four fatty acids were at higher levels in the olive oil groups than in the corn oil groups. The n3 > 18 index was highest in the olive oil groups.

The unsaturation index (UNID) was decreased in PL of O-fed rats, but was mostly unaffected by diet in PC, PE and PI.

Table 6 shows the results for the Δ^9- and Δ^6-desaturase activities. Δ^6-Desaturase activity was higher in MCT-C

TABLE 2

Fatty Acid Composition of Liver Microsomal Phospholipids in Rats Fed High-Fat Diets for Three Weeks

Fatty acid	Olive oil	MCT-Olive oil	Corn oil	MCT-Corn oil
12:0	0.0 ± 0.0	0.3 ± 0.1[a]	0.0 ± 0.0	0.0 ± 0.0
14:0	0.3 ± 0.0	0.7 ± 0.1[a]	0.3 ± 0.0	0.4 ± 0.1
16:0	24.9 ± 0.3	23.8 ± 0.6	25.5 ± 0.5	26.2 ± 0.7[c]
18:0	24.2 ± 0.9	22.1 ± 0.5[a]	26.2 ± 0.6	26.5 ± 0.6[d]
24:0	0.0 ± 0.0	0.5 ± 0.0[b]	0.4 ± 0.0[b]	0.5 ± 0.1
16:1n7	2.4 ± 0.2	2.3 ± 0.2	1.1 ± 0.1[b]	1.5 ± 0.1[d,e]
18:1n9	17.3 ± 0.4	14.9 ± 0.5[b]	5.8 ± 0.3[b]	7.0 ± 0.4[d,e]
20:3n9	1.8 ± 0.1	1.8 ± 0.2	0.0 ± 0.0[b]	0.0 ± 0.0[d]
18:2n6	7.0 ± 0.4	7.8 ± 0.2	12.7 ± 0.5[b]	11.8 ± 0.4[d]
18:3n6	0.3 ± 0.0	0.4 ± 0.0	0.5 ± 0.0	0.4 ± 0.1
20:2n6	0.1 ± 0.0	0.2 ± 0.0[a]	0.7 ± 0.0[b]	0.4 ± 10.0[c,f]
20:3n6	1.8 ± 0.1	2.5 ± 0.1[b]	0.8 ± 0.0[b]	1.8 ± 0.1[d,f]
20:4n6	15.6 ± 0.5	15.9 ± 0.7	19.0 ± 0.8[b]	17.2 ± 0.8
22:4n6	0.3 ± 0.0	0.7 ± 0.0[b]	1.7 ± 0.2[b]	1.2 ± 0.1[d,e]
22:5n6	1.0 ± 0.1	2.1 ± 0.1[b]	3.2 ± 0.4[b]	3.2 ± 0.5
18:3n3	0.6 ± 0.1	0.5 ± 0.1	0.2 ± 0.0[b]	0.3 ± 0.1
20:5n3	1.0 ± 0.1	0.4 ± 0.0[b]	0.0 ± 0.0[b]	0.0 ± 0.0[d]
22:5n3	0.3 ± 0.0	0.5 ± 0.0[a]	0.5 ± 0.0[a]	0.4 ± 0.0
22:6n3	0.9 ± 0.1	2.6 ± 0.2[b]	1.4 ± 0.1[b]	1.2 ± 0.1[d]
n6 > 18	19.2 ± 0.5	21.8 ± 0.8[b]	25.9 ± 1.1[b]	24.1 ± 0.5[e]
n3 > 18	2.3 ± 0.1	3.5 ± 0.2[b]	1.9 ± 0.1[a]	1.7 ± 0.1[d]
Rn > 18[g]	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
SAT[h]	49.4 ± 0.9	47.3 ± 0.5[a]	52.3 ± 1.1[a]	53.6 ± 0.8[d]
UNID	128.4 ± 2.3	145.9 ± 3.2[b]	148.0 ± 4.9[b]	139.3 ± 2.3

Results are mean percentages ± S.E.M. of total fatty acid methyl esters.
[a]$p < 0.05$, [b]$p < 0.01$, significance with respect to olive oil-fed rats.
[c]$p < 0.05$, [d]$p < 0.01$, significance with respect to MCT-olive oil-fed rats.
[e]$p < 0.05$, [f]$p < 0.01$, significance with respect to corn oil-fed rats.
[g]Rn > 18 ratio, n3 > 18/n6 > 18.
[h]SAT, total saturated fatty acids.

TABLE 3

Fatty Acid Composition of Liver Microsomal Phosphatidylcholine in Rats Fed High-Fat Diets for Three Weeks

Fatty acid	Olive oil	MCT-Olive oil	Corn oil	MCT-Corn oil
12:0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
14:0	0.4 ± 0.0	0.5 ± 0.0	0.4 ± 0.0	0.4 ± 0.1
16:0	24.6 ± 0.7	24.4 ± 0.4	24.4 ± 0.8	26.2 ± 0.8
18:0	20.1 ± 0.7	20.3 ± 0.6	22.7 ± 0.4[b]	23.1 ± 0.3[d]
24:0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0[a]	0.2 ± 0.0[d]
16:1n7	2.3 ± 0.2	2.7 ± 0.2	1.0 ± 0.0[b]	1.5 ± 0.1[d,f]
18:1n9	16.5 ± 0.5	15.4 ± 0.5	5.4 ± 0.2[b]	6.3 ± 0.4[d]
20:3n9	2.5 ± 0.2	2.3 ± 0.2	0.0 ± 0.0[b]	0.1 ± 0.0[d]
18:2n6	7.9 ± 0.5	9.6 ± 0.4[a]	14.1 ± 0.6[b]	13.9 ± 0.5[d]
18:3n6	0.5 ± 0.0	0.5 ± 0.0	0.6 ± 0.0	0.5 ± 0.1
20:2n6	0.4 ± 0.0	0.4 ± 0.0	0.7 ± 0.1[b]	0.4 ± 0.0[f]
20:3n6	2.5 ± 0.1	2.9 ± 0.1[a]	1.0 ± 0.1[b]	2.1 ± 0.1[d,f]
20:4n6	16.6 ± 1.0	14.8 ± 0.8	22.3 ± 0.6[b]	20.0 ± 0.6[d,e]
22:4n6	0.5 ± 0.0	0.5 ± 0.0	1.3 ± 0.1[b]	0.8 ± 0.2[e]
22:5n6	1.8 ± 0.1	1.9 ± 0.1	3.3 ± 0.4[b]	2.4 ± 0.5
18:3n3	0.5 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.3 ± 0.0
20:5n3	0.4 ± 0.0	0.6 ± 0.1[a]	0.4 ± 0.0	0.3 ± 0.1[d]
22:5n3	0.5 ± 0.0	0.4 ± 0.1	0.5 ± 0.0	0.3 ± 0.1[e]
22:6n3	2.0 ± 0.1	2.3 ± 0.1[a]	1.1 ± 0.1[b]	0.9 ± 0.1[d]
n6 > 18	22.2 ± 0.9	21.0 ± 0.8	29.2 ± 0.8[b]	26.2 ± 1.0[d,e]
n3 > 18	2.9 ± 0.1	3.4 ± 0.2[a]	2.0 ± 0.1[b]	1.6 ± 0.2[d]
Rn > 18[g]	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
SAT[h]	45.1 ± 0.6	45.2 ± 0.5	47.8 ± 0.9[a]	50.0 ± 0.7[d]
UNID	147.3 ± 2.5	146.4 ± 2.4	164.3 ± 3.5[a]	149.6 ± 4.7[e]

Results are mean percentages ± S.E.M. of total fatty acid methyl esters.
[a]$p < 0.05$, [b]$p < 0.01$, significance with respect to olive oil-fed rats.
[c]$p < 0.05$, [d]$p < 0.01$, significance with respect to MCT-olive oil-fed rats.
[e]$p < 0.05$, [f]$p < 0.01$, significance with respect to corn oil-fed rats.
[g]Rn > 18 ratio, n3 > 18/n6 > 18.
[h]SAT, total saturated fatty acids.

TABLE 4

Fatty Acid Composition of Liver Microsomal Phosphatidylethanolamine in Rats Fed High-Fat Diets for Three Weeks

Fatty acid	Olive oil	MCT-Olive oil	Corn oil	MCT-Corn oil
12:0	0.0 ± 0.0	0.2 ± 0.1	0.1 ± 0.0[a]	0.1 ± 0.0
14:0	0.2 ± 0.1	0.5 ± 0.1	0.9 ± 0.2[a]	1.1 ± 0.2[c]
16:0	19.5 ± 0.6	20.3 ± 0.6	20.6 ± 0.7	20.2 ± 0.7
18:0	24.6 ± 0.5	23.6 ± 0.6	25.8 ± 0.9	25.7 ± 1.0
24:0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.4 ± 0.1[d]
16:1n7	0.5 ± 0.0	0.8 ± 0.0[b]	0.3 ± 0.0[a]	0.4 ± 0.0[d]
18:1n9	7.2 ± 0.3	6.8 ± 0.3	4.3 ± 0.5[b]	4.8 ± 0.4[d]
20:3n9	1.6 ± 0.1	1.3 ± 0.1	0.0 ± 0.0[b]	0.0 ± 0.0[d]
18:2n6	3.4 ± 0.2	3.9 ± 0.1[a]	6.4 ± 0.2[b]	6.1 ± 0.3[d]
18:3n6	0.2 ± 0.0	0.3 ± 0.0	0.4 ± 0.1	0.4 ± 0.1
20:2n6	0.1 ± 0.0	0.0 ± 0.0	0.7 ± 0.1[b]	0.4 ± 0.1[d,e]
20:3n6	1.4 ± 0.1	1.6 ± 0.1	0.9 ± 0.1[b]	1.8 ± 0.2[f]
20:4n6	27.7 ± 0.4	27.6 ± 0.7	22.7 ± 1.0[b]	24.7 ± 1.0[c]
22:4n6	1.0 ± 0.1	0.9 ± 0.1	3.8 ± 0.3[b]	2.6 ± 0.3[d,e]
22:5n6	4.0 ± 0.2	3.6 ± 0.2	7.8 ± 0.7[b]	6.3 ± 0.5[d]
18:3n3	0.4 ± 0.0	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
20:5n3	0.7 ± 0.1	0.7 ± 0.0	0.2 ± 0.1[b]	0.5 ± 0.1[e]
22:5n3	1.7 ± 0.1	1.1 ± 0.1[b]	1.5 ± 0.2	1.1 ± 0.1
22:6n3	5.7 ± 0.3	6.3 ± 0.2	3.0 ± 0.1[b]	2.8 ± 0.2[d]
n6 > 18	34.5 ± 0.4	34.0 ± 0.9	36.3 ± 1.2	36.2 ± 0.8
n3 > 18	8.1 ± 0.3	8.0 ± 0.2	4.7 ± 0.3[b]	4.4 ± 0.3[d]
Rn > 18[g]	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
SAT[h]	44.4 ± 0.6	44.6 ± 1.0	47.5 ± 1.5	47.6 ± 0.9[c]
UNID	206.6 ± 2.8	205.0 ± 4.4	195.8 ± 6.6	191.9 ± 4.3[c]

Results are mean percentages ± S.E.M. of total fatty acid methyl esters.
[a]$p < 0.05$, [b]$p < 0.01$, significance with respect to olive oil-fed rats.
[c]$p < 0.05$, [d]$p < 0.01$, significance with respect to MCT-olive oil-fed rats.
[e]$p < 0.05$, [f]$p < 0.01$, significance with respect to corn oil-fed rats.
[g]Rn > 18 ratio, n3 > 18/n6 > 18.
[h]SAT, total saturated fatty acids.

TABLE 5

Fatty Acid Composition of Liver Microsomal Phosphatidylinositol in Rats Fed High-Fat Diets for Three Weeks

Fatty acid	Olive oil	MCT-Olive oil	Corn oil	MCT-Corn oil
12:0	0.0 ± 0.0	1.5 ± 0.7	0.2 ± 0.1	0.0 ± 0.0[c]
14:0	2.4 ± 1.0	4.3 ± 1.2	2.3 ± 0.9	1.8 ± 0.6
16:0	13.0 ± 2.1	13.7 ± 0.9	13.0 ± 0.9	18.3 ± 3.6
18:0	47.3 ± 8.1	38.3 ± 4.0	41.5 ± 3.2	32.7 ± 6.6
24:0	1.2 ± 0.3	1.1 ± 0.3	1.1 ± 0.5	0.3 ± 0.2[c]
16:1n7	0.5 ± 0.2	0.7 ± 0.1	0.7 ± 0.2	0.9 ± 0.3
18:1n9	5.9 ± 1.8	6.4 ± 0.9	5.8 ± 0.8	5.2 ± 0.9
20:3n9	4.1 ± 1.0	3.6 ± 0.4	0.0 ± 0.0[b]	0.0 ± 0.0[d]
18:2n6	1.4 ± 0.4	2.0 ± 0.3	3.1 ± 0.4[a]	5.4 ± 1.1[c]
20:2n6	0.2 ± 0.1	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0
20:3n6	2.6 ± 0.4	3.0 ± 0.4	1.5 ± 0.3[a]	1.0 ± 0.3[d]
20:4n6	14.4 ± 2.7	17.5 ± 1.8	23.8 ± 2.5[a]	23.3 ± 2.4
22:4n6	0.0 ± 0.0	0.0 ± 0.0	1.4 ± 0.2[b]	1.0 ± 0.3[c]
22:5n6	2.5 ± 0.4	2.4 ± 0.4	3.2 ± 0.2	5.0 ± 1.3
18:3n3	0.8 ± 0.4	2.0 ± 0.7	0.0 ± 0.0	0.0 ± 0.0[c]
20:5n3	0.3 ± 0.1	0.0 ± 0.0[a]	0.0 ± 0.0[a]	0.0 ± 0.0
22:5n3	2.3 ± 0.6	1.7 ± 0.2	1.7 ± 0.2	3.9 ± 1.4
22:6n3	1.1 ± 0.3	1.7 ± 0.2	0.5 ± 0.3	1.2 ± 0.5
n6 > 18	19.7 ± 3.4	22.9 ± 1.8	29.9 ± 2.7[a]	30.3 ± 2.3[c]
n3 > 18	3.7 ± 0.6	3.4 ± 0.3	2.2 ± 0.3	5.1 ± 1.7
Rn > 18[e]	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.1
SAT[h]	64.0 ± 6.2	58.9 ± 2.5	58.2 ± 2.9	53.0 ± 3.4
UNID	121.8 ± 20.9	137.8 ± 8.9	145.6 ± 11.8	168.8 ± 14.7

Results are mean percentages ± S.E.M. of total fatty acid methyl esters.
[a]$p < 0.05$, [b]$p < 0.01$, significance with respect to MCT-olive oil-fed rats.
[c]$p < 0.05$, [d]$p < 0.01$, significance with respect to olive oil-fed rats.
[g]Rn > 18 ratio, n3 > 18/n6 > 18.
[h]SAT, total saturated fatty acids.

TABLE 6

Δ^6- and Δ^9-Acyl-CoA Desaturase Activities of Liver Microsomes of Rats Fed High-Fat Diets for Three Weeks

	Olive oil	MCT-Olive oil	Corn oil	MCT-Corn oil
Δ^6	0.27 ± 0.03	0.29 ± 0.04	0.59 ± 0.03[a]	0.32 ± 0.02[c]
Δ^9	0.53 ± 0.03	0.38 ± 0.02[a]	0.15 ± 0.01[a]	0.12 ± 0.01[b,d]

Δ^6- and Δ^9-desaturase activities are expressed as nmol of product produced per min per mg protein.

[a] $p < 0.01$ significance with respect to olive oil-fed rats.

[b] $p < 0.05$, [c] $p < 0.01$ significance with respect to corn oil-fed rats.

[d] $p < 0.01$ significance with respect to MCT-olive oil-fed rats.

TABLE 7

Microsomal Cholesterol and Phospholipid Content and Cholesterol/Phospholipid Molar Ratio (C/P) in Rats Fed High-Fat Diets for Three Weeks

	Olive oil	MCT-Olive oil	Corn oil	MCT-Corn oil
Cholesterol μg/mg protein	29.63 ± 1.74	30.14 ± 0.46	28.23 ± 0.70	24.91 ± 0.87[a,b]
Lipid phosphorus μg/mg protein	14.32 ± 0.63	15.03 ± 0.36	14.30 ± 0.43	13.84 ± 0.62
C/P	0.17 ± 0.01	0.16 ± 0.01	0.16 ± 0.01	0.15 ± 0.01

[a] $p < 0.01$ significance with respect to MCT-olive oil-fed rats.

[b] $p < 0.01$ significance with respect to corn oil-fed rats.

and C groups than in MCT-O and O groups. Δ^9-Desaturase activity changed in a direction opposite to that of the Δ^6-desaturase; it was highest in the MCT-O and O groups.

Table 7 shows the results obtained for microsomal content of cholesterol and phospholipid. Cholesterol was significantly lower in the MCT-C group than in the C and MCT-O groups. There were no differences in the cholesterol/phospholipid molar ratio related to the diet.

DISCUSSION

Many studies have been performed related to the changes occurring in tissue and membrane lipids as a result of feeding a variety of lipid supplements (2,3,21,22). For mammalian membrane lipids, it has been observed that large changes in the level of dietary lipid saturation have little or no effect on the level of saturation present in membrane lipids (2,7,22,25,26). This may reflect on the existence of some homeostatic mechanism that preserves a constant ratio of saturated to unsaturated fatty acids and the fluidity of membranes (6,27).

In the present study, we tried to ascertain if MCT feeding can introduce apparent changes in individual fatty acids or desaturase activities in hepatic microsomes of rats when fed with O or C.

Liver microsomal phospholipids obtained from the C group had a higher relative content of saturated fatty acids than those obtained from the O group, despite the fact that the polyunsaturated/saturated fatty acid ratio (P/S) was higher in the C than in the O diet. This difference cannot be attributed to the saturated fatty acid content of the C and O diets, which is similar for the two major saturated fatty acids, palmitic and stearic. Similar results have been obtained by others (2), who suggested that the increase observed in the level of saturated fatty acids in animals fed a linoleic acid-rich diet compensated for the high deposition of longer metabolites of the n6 series in membrane lipids which occurs with these diets. However, different authors have described that dietary PUFA produce an inhibition of hepatic lipogenesis (28–31).

In liver microsomal phospholipids from the MCT-C and MCT-O groups, the long chain saturated fatty acid level was not significantly modified with respect to their control groups. It is probable that medium chain fatty acids are not present because of their high oxidation rate in liver (8).

Oleic acid and palmitoleic acid contents were higher in the liver microsomes of rats fed O and MCT-O, both diets having a high content of these two acids. Moreover, Δ^9-desaturase activity was lower in the C- and MCT-C-fed groups. This was probably due to the high dietary content of linoleic acid which inhibited this activity (20,22). The increase observed in palmitoleic acid in the two groups fed MCT, when compared with their respective controls could be due to the hepatic lipogenic effect of MCT (32–34).

The relative content of linoleic acid in microsomes was elevated in the two groups fed MCT-C and C because of diet composition. The higher proportion of 20:3n6 fatty

acid in the two groups fed MCT, when compared with their respective control groups C and O, suggests an enhanced Δ^6-desaturase activity as a result of the hyperinsulinemic effect of MCT (35,36). However, our results for in vitro Δ^6-desaturase activity show a decrease or no change in MCT-C and MCT-O, when compared to their respective control C and O. The influence in vivo of MCT could be through a decrease in Δ^5-desaturase activity although levels of arachidonic acid found in microsomes were not significantly modified in MCT-fed animals. The accumulation of 20:3n6 fatty acid is noteworthy because it is precursor of PGE_1, an antiaggregatory factor, and also because 12 and 15 hydroxyeicosaenoic acids formed from 20:3n6 are inhibitors of platelet aggregation (37,38).

The precursor of the n3 series of PUFA (α-linolenic acid), was present in relatively small amounts in the different phospholipid fractions. The higher level of PUFA from the n3 series found in the 2 O-fed animals is interesting to note because of the its important antiaggregatory properties of these fatty acids (39).

The n3/n6 PUFA ratio in tissues depends more on the linoleic/α-linolenic ratio in the diet than on the absolute content of the individual fatty acid. Studies both in vivo and in vitro (40,41) have demonstrated that the competition of 18-carbon unsaturated fatty acids for further desaturation is in the order of n3 > n6 > n9 (42). This would account for the increased synthesis of n3 PUFA series in our groups fed O and MCT-O, diets in which the linoleic/α-linolenic ratio was lowest (11.3) although Δ^6-desaturase determined in vitro was also lower. PUFA n6 > 18 were higher in the C and MCT-C groups in agreement with the Δ^6-desaturase activity shown in vitro. Moreover, these C and MCT-C diets were rich in the precursor, linoleic acid, and had a higher linoleic/α-linolenic ratio (59.1).

Despite the dietary induced changes in PUFA, the value of UNID (which is a general way of expressing membrane unsaturation (7)) was only slightly affected by the nature of the dietary lipid intake.

The microsomal cholesterol content was lower in the MCT-C group. Phospholipid phosphorus and the cholesterol/phospholipid molar ratio showed similar values in the 4 groups. Because the UNID was mostly unaffected by the diet and the cholesterol/phospholipid ratio did not significantly change, we can conclude that these different diets do not produce any profound modifications in factors altering membrane fluidity. Changes observed in the Δ^9- and Δ^6-desaturase activities could be due to the different microsomal fatty acid patterns induced by the diets. This presumed that the response of these desaturase activities to the different diets takes place in order to maintain the homeostasis of membranes.

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Δ6 and Δ5 Desaturase Activities in Liver from Obese Zucker Rats at Different Ages

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Δ6 Desaturation of linoleic acid (18:2 n-6) and Δ5 desaturation of dihomo-γ-linolenic acid (20:3 n-6) were measured in liver microsomes from genetically obese Zucker rats (fa/fa) and from their lean littermates (Fa/—). Both groups were fed a balanced commercial diet. The rats were 6, 9 and 12 weeks old, which corresponded to stages in their active growth period. The content of total fatty acids and n-6 polyunsaturated fatty acids in whole liver and liver microsomes was also determined in order to ascertain how the desaturase activities measured *in vitro* reflected regulation of essential fatty acid metabolism *in vivo*. Contrary to values obtained for Δ6 desaturation, Δ5 desaturation at nonsaturating substrate levels were lower in obese rats than in lean controls. In contrast, at saturating substrate level, the maximal Δ5 desaturase activities were the same in both phenotypes and they increased with age. Study of Δ5 desaturation kinetics (1/V vs 1/S) showed that V_m did not differ between 12-week-old obese and lean rats, whereas K_M in obese rats was much lower than in controls, expressing the very low affinity of the enzyme for the substrate in obese animals. The fatty acid composition of liver lipids reflected the results of desaturase activities *in vitro*. In particular, the ratios 20:4 n-6/20:3 n-6 were lower in obese rats than in lean rats, which can be explained by the lower conversion of 20:3 n-6 into 20:4 n-6 by Δ5 desaturation. However, the total amount of 20:4 n-6 in the whole liver did not differ between phenotypes, whatever their age. This work presents evidence for a relationship between the changes in fatty acid compositional data in hepatic total lipids, total lipids of liver microsomes and modifications of fatty acid desaturase activities in the genetically obese Zucker rat.
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Obesity in the genetically obese Zucker rats is associated with hyperinsulinemia and hyperlipidemia (1–4). Rates of lipogenesis and Δ9 desaturase activity in the liver of obese rats are generally elevated when compared with their lean littermates. Oleic acid (18:1 n-9) and particularly palmitoleic acid (16:1 n-7) contents are thus increased both in liver and in adipose tissue. This increase, however, depends on the dietary lipids (8,9). Modifications in the composition of n-6 polyunsaturated fatty acids, especially linoleic acid (18:2 n-6) and arachidonic acid (20:4 n-6), are also observed in liver and blood lipids of obese compared with lean rats (8,11), with a decreased ratio of 20:4 n-6/18:2 n-6. This altered essential fatty acid composition may be related to differences in desaturation activity in livers of obese rats when compared with their lean littermates.

In rats, Δ6 desaturase, which converts 18:2 n-6 into 18:3 n-6 (γ-linolenic acid), and Δ5 desaturase, responsible for the transformation of 20:3 n-6 (dihomo-γ-linolenic acid) into 20:4 n-6, are present in the liver cell endoplasmic reticulum (12,13). Desaturation reactions are slower than the intermediary step of elongation (14–16) and are influenced by hormonal and nutritional factors (17–22). Insulin seems more specifically involved in the regulation of Δ6- and Δ5 desaturase activities since injection of the hormone into diabetic rats increases these activities (23–25). The enzyme activities can also depend on the amount of ingested 18:2 n-6, as we have previously observed (22,26).

In the present work, we studied the biosynthetic rate of 20:4 n-6 from 18:2 n-6 *in vitro* and its consequence on liver fatty acid composition *in vivo* in both obese and lean Zucker rats at different ages. The aim of this work was to obtain a better insight into the control of this biosynthetic pathway by factors such as hyperphagia and hyperinsulinemia. To ascertain how the enzyme activities measured *in vitro* were regulated *in vivo*, the fatty acid composition of total liver and liver microsome lipids was determined. Six-, nine- and twelve week-old rats were chosen because this corresponds to their active growth period during which morphological and biochemical parameters rapidly change (2) as do the enzymatic activities of hepatic lipogenesis (27).

MATERIALS AND METHODS

Chemicals. [1-^{14}C] linoleic acid and [2-^{14}C] dihomo-γ-linolenic acid (55 mCi mmol^{-1}, 97% radiochemically pure) were purchased from the Radiochemical Center (Amersham, U.K.). Coenzymes and unlabelled fatty acids were provided by Sigma Co. Inc. (St. Louis, MO). All other chemicals used, issued from Sigma and Merck (Darmstadt, Germany), were of analytical grade.

Animals and diet. Three experiments were carried out with Zucker rats from the Centre de Sélection et d'Elevage d'Animaux de Laboratoire (Centre National de la Recherche Scientifique, Orléans la Source, France). In each experiment, 4 obese rats (fa/fa) and the same number of their lean littermates (Fa/—) were used. In the first two experiments, the 4-week-old obese and lean rats weighed 112 ± 8 g and 91 ± 7 g, respectively, at their arrival. They were allowed free access to tap-water and were fed ad libitum on the standard diet (AO4 from UAR, Villemoisson sur Orge, France) for 2 weeks or for 5 weeks and were thus sacrificed at 6 and 9 weeks of age, respectively. In the third experiment, the obese and lean rats were 11 weeks old at their arrival and weighed 317 ± 18 g and 226 ± 14 g, respectively. They were given the commercial diet for one week before being sacrificed (12 weeks old). The weight composition of the commercial diet received before and after arrival at the laboratory was as follows: proteins 17%, carbohydrates 58.7%, lipids 3%, cellulose 4.3%, mineral salts 4%, vitamins 1% and water 12%. Linoleic acid which represented 52.7% of the total fatty acid of the diet was in sufficient amount to avoid fatty acid deficiency likely to modify the conversion rate of 18:2 n-6 into 20:4 n-6.

Isolation of microsomes. At the end of the experiment, obese and lean rats were killed by decapitation between

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8 and 9 a.m. Livers were removed, weighed and washed. About 3 g of each liver were cut into thin slices and homogenized in 6 volumes of 0.25 M sucrose and 0.05 M phosphate (pH 7.4) solution. The homogenate was centrifuged at 13,000 × g for 20 min to precipitate cell fragments, nuclei and mitochondria. After the floating lipids were removed with filter paper, the supernatant was centrifuged at 100,000 × g for 60 min. The pellet, principally composed of microsomes, was resuspended in 0.4 ml supernatant and 0.8 ml homogenizing solution. The microsomal protein content was estimated, according to Layne (28), to be about 30 mg ml^{-1}. All the operations wre conducted at 4°C. Four separate assays were performed for each type of rat.

Enzyme assays in vitro. In each assay, 5 mg microsomal protein were incubated at 37°C and with continuous shaking with 60 nmol [1-^{14}C] 18:2 n-6 or 20 nmol [2-^{14}C] 20:3 n-6. Each radioactive substrate was diluted with the corresponding unlabelled fatty acid to obtain an ethanol solution with a specific activity of 5 mCi $mmol^{-1}$ (20 nmol 10 μl^{-1} of solution). The incubation medium contained 72 mM phosphate buffer, pH 7.4, 4.8 mM $MgCl_2$, 0.5 mM coenzyme A, 3.6 mM ATP and 1.2 mM NADPH, in 2.1 ml of total volume, as reported elsewhere (26). The duration of incubation was 10 min for Δ6 desaturation and 5 min for Δ5 desaturation, corresponding to linearity between the transformed amount of substrate and the amount of microsomal protein. The substrate levels were nonsaturating in order to gain a better appreciation of the influence of microsomal lipids on the desaturation rate (29,30). For Δ5 desaturation, a saturating substrate level (40 nmol) was also used in order to determine the maximum Δ5 desaturation capacity of liver microsomes.

The reactions were stopped with KOH in ethanol and the fatty acid methyl esters were prepared as previously reported (26). The conversion of the substrates was determined by measuring the radioactivity of the newly formed fatty acids after fractionation by gas-liquid chromatography and collection according to Bézard et al. (31). A Packard model 470 gas chromatograph (Packard Instr., Downers Grove, IL) was equipped with a 180-cm × 4-mm i.d. coiled glass column filled with 24% DEGS (Applied Science Lab., State College, PA) on 80–100 mesh Chromosorb (Johns-Manville, Denver, CO). The fraction collector was from Packard and the radioactivity was measured by liquid scintillation spectrometry in a 300 C Tricarb Packard Spectrometer.

The percentage of conversion of substrate was deduced from the ratio of the radioactivity recovered in the newly formed fatty acids to the total radioactivity recovered in the analysis. Desaturation activity was calculated from the conversion percentage and expressed as nmol of transformed substrate under the conditions used. With the saturating level of 20:3 n-6, the maximal capacity of Δ5 desaturation was expressed as nmol of desaturated substrate per min and per whole liver.

Fatty acid analysis. Lipids from total liver and from liver microsomes were extracted according to Delsal (32). The amount of total lipids per g of liver was determined gravimetrically from an aliquot. Another aliquot was transmethylated, and the fatty acid methyl esters were analyzed by capillary gas-liquid chromatography on a model 419 Becker-Packard apparatus equipped with a 30-m × 0.3-mm i.d. glass capillary column coated with Carbowax 20 M. Before methylation, a known amount of heptadecanoic acid was added to the lipid extract as an internal standard to calculate the amount of total and individual fatty acids. The separation of lipid classes was achieved by liquid column chromatography following the procedure of Hirsch et al. (33).

Statistics. Statistical evaluation of the means was performed by analysis of variance and by classification of the means using the Duncan's multiple range test (34).

RESULTS

Data reported in Table 1 show that the body weight of obese rats was higher than that of lean controls whatever the age studied. It increased in both phenotypes with age. Liver weight was also greater in obese rats compared with lean at the 3 ages and it also increased with age in both phenotypes. When expressed as a proportion of body weight, liver weight was still generally higher in obese rats and it decreased with age in both phenotypes. The higher liver weight in obese rats was partly due to higher lipid content and this content increased with age, contrary to the liver lipid content of lean rats.

As shown in Table 2, at nonsaturating level of substrate, the Δ6 desaturation activity was found to be different in obese and lean rats only at 9 weeks. Δ5 Desaturation, on the contrary, was lower in obese rats whatever the age but it was more evident at 12 weeks. However, when measured at saturating level of 20:3 n-6 (Table 3), the maximal Δ5 desaturation activity in obese rats was not significantly different from that in lean controls except at 6 weeks. Because of enlargement of the liver, the capacity of the whole organ to convert 20:3 n-6 into 20:4 n-6 was nonetheless higher in obese rats.

When comparing the data reported in Table 2 and Table 3 for Δ5 desaturation, it appears that the amount of 20:4 n-6 formed from 20:3 n-6 only clearly increased in 12-week-old rats when the amount of substrate increased from 20 to 40 nmol. At this age, the determination of V_m and K_M for Δ5 desaturation (Fig. 1) shows that V_m was

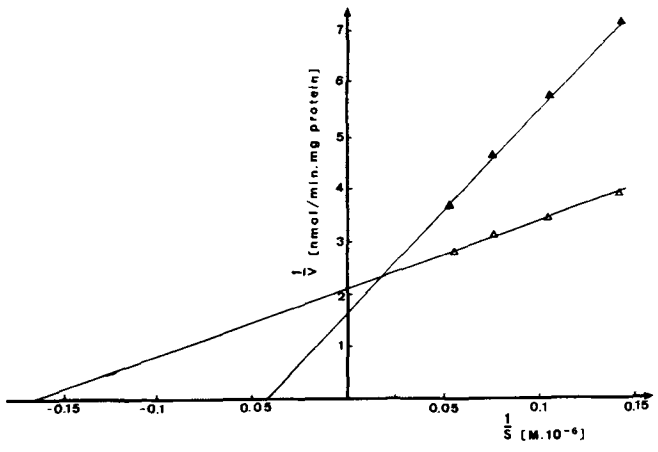

FIG. 1. Lineweaver-Burk plot of dihomo-γ-linolenic acid (20:3 n-6) desaturation to arachidonic acid (20:4 n-6) in liver microsomes of 12-week-old obese (▲—▲) and lean (△—△) rats. The details of animal groups and analytical procedure are given in Materials and Methods and in the legend to Table 1. Approximate kinetic parameters: K_m = 2.5 10^{-5}M; V_{max} = 0.58 nmol/min/mg protein (obese rats); K_m = 0.6 10^{-5}M; V_{max} = 0.48 nmol/min/mg protein (lean rats).

TABLE 1

Body Weight, Liver Weight and Liver Lipid Content of Obese and Lean Zucker Rats at Different Ages

	Phenotype	Age (weeks) 6	9	12
Body weight (g)	Obese	202 ± 6^{c}	251 ± 5^{b}	373 ± 32^{a}
	Lean	136 ± 7^{z}	219 ± 3^{y}	290 ± 9^{x}
	Significance	$p < 0.001$	$p < 0.001$	$p < 0.01$
Liver weight (g)	Obese	9.7 ± 0.5^{c}	11.9 ± 0.3^{b}	16.1 ± 2.4^{a}
	Lean	6.1 ± 0.3^{y}	9.0 ± 1.0^{x}	11.0 ± 1.4^{x}
	Significance	$p < 0.001$	$p < 0.01$	$p < 0.05$
Liver weight (g/100 g of body weight)	Obese	4.82 ± 0.08^{a}	$4.74 \pm 0.20^{a,b}$	4.30 ± 0.30^{b}
	Lean	4.48 ± 0.28^{x}	$4.11 \pm 0.15^{x,y}$	3.80 ± 0.17^{y}
	Significance	NS	$p < 0.01$	$p < 0.05$
Lipid content (mg/g of liver)	Obese	52.5 ± 2.1^{c}	60.3 ± 3.9^{b}	68.5 ± 1.6^{a}
	Lean	48.9 ± 0.6^{y}	45.8 ± 1.5^{x}	$50.0 \pm 3.5^{x,y}$
	Significance	$p < 0.02$	$p < 0.001$	$p < 0.001$

Results are expressed as means ± S.D. for 4 rats of each phenotype. Statistical evaluation of the means was performed by analysis of variance and by classification of the means using the Duncan's multiple range test. For each age means were compared between phenotypes and the differences estimated at different threshholds (NS = nonsignificant; $p < 0.05$.) Means were also compared between ages for each phenotype. Values not followed by the same superscript were significantly different ($p < 0.05$): a, b, c in order of decreasing magnitude for obese rats; x, y, z for lean rats.

TABLE 2

Δ6- and Δ5-Desaturase Activities of Hepatic Microsomes of Obese and Lean Zucker Rats at Different Ages (at nonsaturating substrate levels)

	Phenotype	Age (weeks) 6	9	12
Δ6 desaturation[a] (60 nmol)	Obese	4.70 ± 0.65^{a}	4.90 ± 0.25^{a}	5.40 ± 0.40^{a}
	Lean	4.50 ± 0.10^{z}	7.40 ± 0.70^{x}	6.00 ± 0.30^{y}
	Significance	NS	$p < 0.01$	NS
Δ5 desaturation[b] (20 nmol)	Obese	$3.93 \pm 0.42^{a,b}$	3.68 ± 0.15^{b}	4.35 ± 0.50^{a}
	Lean	5.12 ± 0.45^{y}	5.05 ± 0.52^{y}	7.55 ± 0.32^{x}
	Significance	$p < 0.01$	$p < 0.01$	$p < 0.001$

Means values ± S.D. given for three rats (Δ6 desaturation) or four rats (Δ5 desaturation) of each phenotype and of each age.

[a]nmol 18:2 n-6 fatty acid converted to 18:3 n-6/10 min/5 mg of microsomal protein.

[b]nmol 20:3 n-6 fatty acid converted to 20:4 n-6/5 min/5 mg of microsomal protein.

For statistical evaluation, see footnote in Table 1.

only slightly higher in obese rats (0.58 nmol of substrate transformed per min per mg of microsomal protein compared with 0.48 nmol in lean rats). On the contrary, K_M was distinctly higher in obese rats (2.5 10^{-5}M) than in lean controls (0.6 10^{-5}M). The determination of K_M for Δ6 desaturation was not made in this work for 12-week-old rats. However, determinations of Δ6 desaturase activity, at saturating substrate level (120 nmol), gave the following figures: 8.1 ± 0.3 and 8.3 ± 0.4 nmol 18:2 n-6 converted to 18:3 n-6/10 min/5 mg of microsomal protein, for obese and lean rats, respectively. Since the activities were not significantly different at nonsaturating (Table 2) and saturating substrate levels, K_M could not be distinctly different in obese and lean rats.

The quantities per g of liver of total and n-6 fatty acids are reported in Table 4. The concentration of total fatty acids was higher in liver from obese rats than from their lean littermates and increased with age in both phenotypes. Since the obese rat livers were larger than the controls, the differences, expressed as mg per whole liver, are still more important between the two types of rats. The amount of 18:2 n-6 and 20:4 n-6 per g of liver was generally lower in obese rats, but the amount per whole liver was not significantly different between the

TABLE 3

Δ5 Desaturase Activities and Capacities of Hepatic Microsomes of Obese and Lean Zucker Rats at Different Ages (at saturating substrate level)

		Age (weeks)		
	Phenotype	6	9	12
Activity[a]	Obese	4.13 ± 0.32^{b}	5.75 ± 0.25^{a}	6.75 ± 0.83^{a}
	Lean	5.25 ± 0.58^{y}	5.93 ± 0.80^{y}	8.50 ± 0.20^{x}
	Significance	$p < 0.02$	NS	NS
Capacity[b]	Obese	24.9 ± 4.2^{c}	41.6 ± 4.4^{b}	57.2 ± 7.0^{a}
	Lean	17.8 ± 3.3^{z}	27.1 ± 3.3^{y}	37.1 ± 1.0^{x}
	Significance	$p < 0.05$	$p < 0.001$	$p < 0.01$

[a]nmol 20:4 n-6 formed/5 min/5 mg of microsomal protein.

[b]nmol 20:4 n-6 per min and per whole liver (= 20:4 n-6 formed × mg^{-1} × min^{-1} × amount of microsomal proteins isolated per liver).

For statistical evaluation, see footnote in Table 1.

TABLE 4

Concentrations of Total and n-6 Fatty Acids in Total Liver Lipids from Obese and Lean Rats at Different Ages (mg/g liver)

		Age (weeks)		
Fatty acids	Phenotype	6	9	12
Total fatty acids	Obese	20.5 ± 1.2^{c}	29.5 ± 1.8^{b}	45.0 ± 1.7^{a}
	Lean	17.3 ± 1.1^{y}	18.7 ± 0.4^{y}	25.8 ± 1.3^{x}
	Significance	$p < 0.01$	$p < 0.001$	$p < 0.001$
18:2 n-6	Obese	1.8 ± 0.1^{c}	2.6 ± 0.2^{b}	4.3 ± 0.2^{a}
	Lean	2.3 ± 0.2^{z}	2.9 ± 0.1^{y}	4.1 ± 0.3^{x}
	Significance	$p < 0.01$	$p < 0.05$	NS
20:3 n-6	Obese	0.32 ± 0.01^{c}	0.46 ± 0.04^{b}	0.56 ± 0.04^{a}
	Lean	0.11 ± 0.01^{y}	0.16 ± 0.02^{x}	0.16 ± 0.01^{x}
	Significance	$p < 0.001$	$p < 0.001$	$p < 0.001$
20:4 n-6	Obese	2.5 ± 0.2^{c}	3.4 ± 0.3^{b}	4.5 ± 0.4^{a}
	Lean	4.0 ± 0.5^{y}	4.1 ± 0.2^{y}	6.2 ± 0.9^{x}
	Significance	$p < 0.01$	$p < 0.01$	$p < 0.02$
n-6 fatty acids	Obese	5.9 ± 0.3^{c}	6.7 ± 0.4^{b}	9.7 ± 0.4^{a}
	Lean	6.7 ± 0.7^{y}	7.6 ± 0.4^{y}	10.8 ± 1.3^{x}
	Significance	NS	$p < 0.02$	NS
20:4 n-6 / 18:2 n-6	Obese	1.30 ± 0.08^{a}	1.31 ± 0.07^{a}	1.05 ± 0.05^{b}
	Lean	1.74 ± 0.10^{x}	1.41 ± 0.07^{y}	1.51 ± 0.10^{y}
	Significance	$p < 0.001$	NS	$p < 0.001$
20:4 n-6 / 20:3 n-6	Obese	7.8 ± 0.5^{a}	7.4 ± 0.8^{a}	8.0 ± 1.2^{a}
	Lean	36.4 ± 4.8^{x}	25.6 ± 5.1^{y}	38.8 ± 6.0^{x}
	Significance	$p < 0.001$	$p < 0.001$	$p < 0.001$

Results are expressed as means ± S.D.

For statistical evaluation, see footnote in Table 1.

two phenotypes because of the larger liver of obese rats. However, the amount of 20:3 n-6 per g of liver was clearly higher in obese rats compared with lean. The difference was even greater in the whole organ. The lower ratio of 20:4 n-6/18:2 n-6 in obese rats reflects a lower rate of the overall conversion of 18:2 n-6 into 20:4 n-6 (Δ6 desaturation, elongation and Δ5 desaturation). This seems primarily due to a decrease in the rate of Δ5 desaturation of 20:3 n-6 since the ratio of 20:4 n-6/20:3 n-6 was found to be even lower. This ratio was not modified with age in obse rats.

Table 5 shows the quantities per g of liver of total and n-6 fatty acids in liver phospholipids from obese and lean rats. Comparison with data reported in Table 4 allows

TABLE 5

Concentrations of Total and n-6 Fatty Acids in Liver Phospholipids from Obese and Lean Rats at Different Ages (mg/g liver)

Fatty acids	Phenotype	Age (weeks) 6	9	12
Total fatty acids	Obese	16.6 ± 0.9^{b}	20.7 ± 0.6^{a}	19.8 ± 0.9^{a}
	Lean	14.4 ± 0.9^{y}	14.8 ± 0.8^{y}	19.1 ± 1.4^{x}
	Significance	$p < 0.02$	$p < 0.001$	NS
18:2 n-6	Obese	1.5 ± 0.5^{b}	1.6 ± 0.1^{b}	2.4 ± 0.1^{a}
	Lean	1.8 ± 0.2^{z}	2.2 ± 0.1^{y}	2.8 ± 0.2^{x}
	Significance	NS	$p < 0.001$	$p < 0.02$
20:3 n-6	Obese	0.31 ± 0.04^{b}	0.40 ± 0.06^{a}	0.48 ± 0.04^{a}
	Lean	0.10 ± 0.02^{x}	0.13 ± 0.02^{x}	0.15 ± 0.02^{x}
	Significance	$p < 0.001$	$p < 0.001$	$p < 0.001$
20:4 n-6	Obese	2.4 ± 0.2^{c}	2.8 ± 0.1^{b}	3.8 ± 0.2^{a}
	Lean	3.9 ± 0.3^{y}	3.7 ± 0.3^{y}	4.9 ± 0.5^{x}
	Significance	$p < 0.001$	$p < 0.01$	$p < 0.01$
n-6 fatty acids	Obese	4.9 ± 0.3^{b}	5.0 ± 0.3^{b}	6.9 ± 0.2^{a}
	Lean	6.0 ± 0.4^{y}	6.2 ± 0.4^{y}	8.1 ± 0.7^{x}
	Significance	$p < 0.01$	$p < 0.01$	$p < 0.02$
20:4 n-6 / 18:2 n-6	Obese	1.67 ± 0.10^{a}	1.75 ± 0.09^{a}	1.58 ± 0.14^{a}
	Lean	2.17 ± 0.06^{x}	1.68 ± 0.11^{y}	1.75 ± 0.08^{y}
	Significance	$p < 0.001$	NS	NS
20:4 n-6 / 20:3 n-6	Obese	7.7 ± 0.5^{a}	7.0 ± 0.8^{a}	7.9 ± 1.1^{a}
	Lean	39.0 ± 5.7^{x}	28.5 ± 5.1^{y}	$32.7 \pm 6.3^{x,y}$
	Significance	$p < 0.001$	$p < 0.001$	$p < 0.001$

Results are expressed as means ± S.D.

For statistical evaluation, see footnote in Table 1.

appreciation of the proportion of phospholipid-bound fatty acids in total lipids. This proportion was high and of the same magnitude in obese and lean rats (more than 80% at 6 weeks of age). It decreased with age in both phenotypes but to a greater extent in obese rats, reflecting enrichment of the liver with triacylglycerols. Contrary to 18:2 n-6, the quasi totality of 20:3 n-6 and 20:4 n-6 was present in phospholipids at 6 weeks of age in obese and lean rats. The contribution of phospholipids to the total n-6 fatty acids then decreased with age, except for 20:3 n-6 in lean rats. Thus, the higher proportion of 20:3 n-6 in the total lipids of the obese rat liver became less and less a reflection of its proportion in phospholipids which was contrary to that found in lean rat liver. The proportion of 20:4 n-6 in phospholipids also decreased with age, but this was similar in both phenotypes. As above, the higher ratio of 20:4 n-6/20:3 n-6 in obese compared with lean rats reflects a lower rate of Δ5 desaturation in these animals.

Concentrations of total fatty acids and n-6 fatty acids of liver microsomes (mg per g of liver) are reported in Table 6. Differences which were observed between obese and lean rats in total liver lipids (Table 4) were not generally observed in microsomal lipids. Only the ratios 20:4 n-6/20:3 n-6 were found to be significantly lower in obese rats. However, the amount of 20:3 n-6 and 20:4 n-6 in microsomes per g of liver in obese rats was higher than in lean rats at 12 weeks of age. In these 12-week-old rats, the neutral lipid fatty acids represented 32% of total microsomal fatty acids in obese rats and 17% in lean rats. The saturated and monounsaturated fatty acids were predominant in this fraction amounting to 0.47 and 0.12 mg/g of liver in obese and lean rats, respectively.

DISCUSSION

This work shows that results obtained on desaturation rates *in vitro* (Tables 2 and 3) correlate with the fatty acid composition of total liver and liver microsomes (Tables 4 and 6) in obese rats. *In vivo,* the lower ratios of 20:4 n-6/18:2 n-6 and especially of 20:4 n-6/20:3 n-6 observed in obese rats suggest a lower rate of overall desaturation of 18:2 n-6 into 20:4 n-6, mainly due to a lower rate of Δ5 desaturation. The amount of 20:4 n-6 increased in liver with age, also suggesting that desaturation rates increase. However, persistence of a low 20:4 n-6/20:3 n-6 ratio in obese rats suggests that the Δ5 desaturation step remained the most affected in these animals.

These results were corroborated by results obtained by direct enzyme assays *in vitro* on the Δ6 desaturation of 18:2 n-6 and the Δ5 desaturation of 20:3 n-6 by liver microsomes. At nonsaturating substrate levels, which better reflect physiological conditions (29), the Δ6 desaturation rate was not different between obese and lean rats. Such a result agrees with recent observations by Wahle (35). In contrast, the Δ5 desaturation rate of

TABLE 6

Concentrations of Total and n-6 Fatty Acids in Total Microsomal Liver Lipids from Obese and Lean Rats at Different Ages (mg/g liver)

Fatty acids	Phenotype	Age (weeks) 6	9	12
Total fatty acids	Obese	1.27 ± 0.10^{b}	1.88 ± 0.31^{a}	2.43 ± 0.28^{a}
	Lean	1.34 ± 0.14^{y}	1.71 ± 0.03^{x}	$1.17 \pm 0.41^{x,y}$
	Significance	NS	NS	$p < 0.01$
18:2 n-6	Obese	0.094 ± 0.004^{b}	0.193 ± 0.053^{a}	0.202 ± 0.046^{a}
	Lean	0.132 ± 0.020^{y}	0.222 ± 0.002^{x}	$0.186 \pm 0.060^{x,y}$
	Significance	NS	NS	NS
20:3 n-6	Obese	0.015 ± 0.003^{b}	0.034 ± 0.001^{a}	0.035 ± 0.012^{a}
	Lean	0.011 ± 0.004^{y}	0.021 ± 0.005^{x}	0.005 ± 0.002^{z}
	Significance	NS	NS	$p < 0.01$
20:4 n-6	Obese	0.128 ± 0.006^{c}	0.251 ± 0.005^{b}	0.547 ± 0.055^{a}
	Lean	0.214 ± 0.025^{x}	0.288 ± 0.023^{x}	0.329 ± 0.095^{x}
	Significance	$p < 0.01$	$p < 0.05$	$p < 0.01$
n-6 fatty acids	Obese	0.263 ± 0.001^{c}	0.520 ± 0.060^{b}	0.810 ± 0.095^{a}
	Lean	0.400 ± 0.035^{y}	0.570 ± 0.030^{x}	$0.460 \pm 0.155^{x,y}$
	Significance	$p < 0.001$	NS	$p < 0.01$
20:4 n-6 / 18:2 n-6	Obese	1.36 ± 0.03^{b}	1.30 ± 0.36^{b}	2.71 ± 0.72^{a}
	Lean	1.62 ± 0.24^{x}	1.30 ± 0.10^{y}	1.77 ± 0.28^{x}
	Significance	NS	NS	NS
20:4 n-6 / 20:3 n-6	Obese	8.5 ± 1.5^{b}	7.3 ± 0.15^{b}	15.6 ± 1.2^{a}
	Lean	19.5 ± 0.7^{z}	13.7 ± 1.2^{y}	65.8 ± 3.2^{x}
	Significance	$p < 0.001$	$p < 0.001$	$p < 0.001$

Results are means of 4 determinations ± S.D.

For statistical evaluation, see footnote in Table 1.

20:3 n-6 at nonsaturating substrate level was found to be lower in obese rats, whatever the age tested. The same tendency existed with saturating level of 20:3 n-6 but the difference between obese and lean rats was not statistically significant. Values of K_M and V_m for Δ5 desaturation found in 12-week-old obese rats (Fig. 1) suggested that the affinity of the enzyme for its substrate was lower in obese than in lean rats. Obviously, the measured Δ5 desaturation assay incorporates several reactions, i.e., activation of the substrate to acyl-CoA, desaturation itself and incorporation of both the substrate and of reaction product into microsomal lipids. The measured K_M and V_m values are approximate but they nonetheless indicate that the overall Δ5 desaturation rate was lower in obese rats. The desaturation activity also depends on the lipid environment (30). The activity can be modified by the fatty acid composition of the membrane phospholipids of microsomes. It can also be partially inhibited by certain free fatty acids present in neutral lipids acting as substrates for the enzyme. Pugh and Kates (36) provided evidence that arachidonic acid can be synthesized either from the CoA derivative or from the phospholipid-bound form of eicosatrienoic acid. The decrease in Δ5 desaturase activity in obese rats can thus reflect a lower affinity for the phospholipid substrate or a reduced capability to form the CoA derivative. Our data do not resolve these options.

Our results also show that hepatic desaturase activity and the amount of 20:4 n-6 in liver lipids increased with age in obese rats. Since hyperphagia takes place after weaning in these rats, an increase in 18:2 n-6 intake could in part explain the desaturase stimulation during the 6 to 12 weeks growth period. However, the same variations were also observed in lean rats which do not exhibit hyperphagia. The explanation could be that the amount of 18:2 n-6 ingested also increases with increased dietary consumption in both types of rats during growth. Moreover, growth activity is accompanied by modifications of hormonal status which can in turn influence desaturase activities. In particular, the hormonal status in obese rats, which are characterized by hyperinsulinemia and hypothyroidism (1-4), could afford an explanation for the differences observed in desaturase activities between obese and lean phenotypes. Thyroid hormones are known to inhibit microsomal Δ6 desaturation in rats (37,38) but nothing is known about Δ5 desaturation. In diabetes, Δ9, Δ6 and Δ5 desaturations are reduced but are restored by insulin injection (23-25). Our results could, in this view, be explained by a lower sensitivity of Δ5 desaturation to the stimulatory effect of insulin in obese rats. However, Weekes et al. (9) demonstrated that changes in the plasma insulin level illicited by changes in the diet in obese and lean rats were not correlated with changes in Δ9 desaturation. Is it the same with Δ6 and Δ5 desaturations? Moreover, despite a low level of plasma insulin in lean rats, the Δ6 desaturation rate in these animals at nonsaturating levels of substrate was identical to that observed in obese

rats. Lastly, the maximal Δ5 desaturation rate measured at saturating substrate level was the same in both phenotypes. It can be concluded from these observations that insulin level does not constitute a major factor in the regulation of Δ6 and Δ5 desaturation activities in obese Zucker rats.

In these animals the total amount of liver arachidonic acid was generally close to that found in lean rats, suggesting that liver Δ6 and Δ5 desaturation rates were high enough in obese rats to satisfy the requirement of the liver for 20:4 n-6. This does not prove, however, that the requirement for 20:4 n-6 for secretion of VLDL phospholipids by the liver is also satisfied.

Results reported in this work which complement others previously reported (11,39,40) enforce the idea that liver Δ6 and Δ5 desaturases are two distinct enzymes, since genetic obesity does not similarly affect their activity.

A lower ratio 20:4 n-6/20:3 n-6 was also found in platelet phospholipids from 12-week-old obese rats compared with lean rats (11). This can be correlated to what was observed in this work on total liver and microsome lipids since plasma phospholipids principally originate from liver and since they exchange with platelet phospholipids. In obese rats, this ratio in microsomal lipids was the same at 6 and 9 weeks whereas it is increased at 12 weeks but much less than in lean rats (Table 6). However, the amount of 20:4 n-6 in total liver microsomes increased in obese rats at a higher level than in lean rats. The same was true for platelets in which the amount of 20:4 n-6 was higher than in their lean littermates, despite a lower 20:4/20:3 ratio (11). In platelets, 20:4 n-6 is the precursor of thromboxane A_2 which induces aggregation. This would then agree with the influence of aging, associated with obesity, in increasing platelet aggregability and in decreasing vessel prostacyclin production in genetically obese rats (41).

To our knowledge, this work constitutes the first insight in the relations between Δ6 and Δ5 desaturation activities in liver microsomes and n-6 fatty acid content of liver lipids in obese Zucker rats. In contrast, in the genetically obese mouse (ob/ob), it was demonstrated that liver phospholipids contained a higher amount of 20:4 n-6 than in lean controls (42). The Δ6 and Δ5 desaturation activities were also higher (43). These observations seem to prove that essential fatty acid metabolism and its regulation by hormones are different in the obese mouse (ob/ob) and in the obese rat (fa/fa).

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Molecular Species of Glycerophospholipids and Sphingomyelins of Human Erythrocytes: Improved Method of Analysis

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This study reports the application of modern methods of molecular species analysis in determination of the structure of both major and minor glycerophospholipids and sphingomyelins of human erythrocytes. Individual phospholipid classes were resolved from total lipid extracts by thin-layer chromatography. Diradylglycerols were released by phospholipase C and converted into trimethylsilyl ethers, which were resolved into the alkenylacyl, alkylacyl and diacylglycerol subclasses by normal phase high performance liquid chromatography. Molecular species of diradylglycerols and ceramides were quantitated according to carbon and double bond number by gas liquid chromatography using a fused silica capillary column wall-coated with bonded RTx-2330. The molecular species of ceramides were determined by GC/MS. The diradyl glycerophosphocholines contained 93.0% diacyl, 4.6% alkylacyl and 2.5% alkenylacyl, while the diradyl glycerophosphoethanolamines were made up of 48.8% diacyl, 47.8% alkenylacyl and 3.4% alkylacyl subclasses. Analysis of the molecular species showed that the long chain polyunsaturated acids were mainly combined with C_{16} in all diradyl GPC subclasses and in diacyl GPE, while in the alkylacyl and alkenylacyl GPE and in diacyl glycerophosphoinositol and diacyl glycerophosphoserine they were combined mainly with C_{18} saturated fatty chains. In addition to the C_{16} and C_{18} alkyl and alkenyl, the ether fractions also contained significant proportions of C_{20}, C_{22} and C_{24} chains. The molecular species of the ceramide moieties of the SPH were made up largely of mono- and diunsaturated species. Over 200 molecular species were identified and quantitated in a representative sample of human red blood cells.
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During the past several years specific physiological functions have been recognized for minor molecular species of glycerophospholipids (GPL) of plasma and subcellular membranes of cells. Thus, specific diacyl GPL classes have been shown to provide characteristic diacylglycerol species as second messengers for the activation of protein kinase C (1), while other diacyl GPL species donate their arachidonic acid residues for prostanoid formation (2). In the retinal rods minor amounts of very long chain polyunsaturated species of glycerophosphocholines (GPC) have been discovered to provide highly organized membrane structures for light perception (3). The ether-linked GPL have long been known to be present in increased amounts in neoplastic tissues (4), where they may serve both as promoters and inhibitors of invasiveness (5). The identification of *sn*-glycero-1-palmityl-2-acetyl-3-phosphocholine as the platelet activating (6) and antihypertensive (7) factor has served to focus further attention on the physiological function of specific molecular species of GPL. In addition, the recent identification of specific molecular species of glycerophosphoinositol (GPI) as structural components of the lipid anchor of specific cell membrane proteins (8) has stimulated interest in the identification of the minor molecular species of GPL of all cell membranes and lipoproteins.

In the present study we have combined several chromatographic and mass spectrometric techniques to identify and quantitate over 200 molecular species of GPL and over 50 molecular species of sphingomyelins (SPH) in a representative sample of human red blood cells. A preliminary report has appeared (9).

MATERIALS AND METHODS

Erythrocytes. The erythrocytes for all analyses of the GPL classes and molecular species were obtained from the same healthy adult male (26 years) in the fasting state. The subject also provided the red cell SPH which, however, were examined only for the fatty acid composition and carbon number distribution of the ceramide moieties. The molecular species of erythrocyte SPH had been obtained previously (J. J. Myher and A. Kuksis, unpublished results) on another healthy adult male, who had a closely similar fatty acid and ceramide composition for its erythrocyte SPH. Comparable carbon number distributions of the ceramide moieties of erythrocyte SPH were obtained on three other healthy adult males (26–50 years). Plasma and cells were separated by centrifugation, and cells were washed to remove the buffy coat (10).

Lipid isolation and derivatization. Total lipid extracts were obtained with chloroform-methanol (2:1, v/v) (11). Individual GPL classes and SPH were isolated from the total lipid extracts by thin-layer chromatography (TLC) using chloroform-methanol-acetic acid-water 75:45:12:6 as solvent. The phosphatidylserine/phosphatidylinositol (PS/PI) fraction was resolved using chloroform-methanol-3 M ammonia 65:35:6.5 (12). Diradylglycerols and ceramides, released from the GPL and SPH, respectively, by phospholipiase C *(B. cereus)* were converted into the trimethylsilyl (TMS) and tBDMS ethers (13). Aliquots of the TMS ethers of the diradylglycerols were converted to the fatty acid methyl esters and dimethylacetals by a 2-hr reaction at 80°C with fresh methanol-H_2SO_4 (94:6, v/v). Alkylglycerol diacetates were prepared with acetic anhydride/pyridine (1:1, v/v) after transmethylation of the alkylacylglycerol TMS ether with 1N sodium methoxide in methanol-toluene (3:2, v/v) for 15 min at 20°C. Following methanolysis, the mixture was neutralized with 1% acetic acid in hexane and extracted by adding 200 μl

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Abbreviations: CV, coefficients of variation; FAME, fatty acid methyl esters; FID, flame ionization detection; GC, gas chromatography; GC/MS, gas chromatography/mass spectroscopy; GLC, gas-liquid chromatography; GPC, glycerophosphocholines; GPE, glycerophosphoethanolamines; GPI, glycerophosphoinositol; GPL, glycerophospholipids; GPS, glycerophosphoserine; HPLC, high performance liquid chromatography; PI, phosphatidylinositol; PS, phosphatidylserine; SPH, sphingomyelins; TLC, thin-layer chromatography; TMS, trimethylsilyl; BDMS, tertiary-butyldimethylsilyl.

chloroform, 0.5 ml water and 50 μl 3 M aqueous ammonia. The organic phase was washed with 250 μl water and then dried by passing it through a small column of anhydrous sodium sulfate. After drying under nitrogen, the sample was acetylated for 0.5 hr at 80°C with 75 μl acetic anhydride-pyridine (1:1, v/v). The reagents were removed by evaporation under nitrogen and the products purified by high performance liquid chromatography (HPLC).

Chromatographic analyses. The TMS ethers of the diradylglycerols were separated within 10 to 15 min into alkylacyl, alkenylacyl and diacylglycerol fractions by HPLC on a Supelcosil LC-Si (5 μ) column (250 cm × 4.6 mm i.d.) using 0.3% isopropanol in hexane (1 ml/min) (14). The analyses were done at 30°C and the lipid solutes were monitored at 214 nm. After evaporation of the solvent under nitrogen the samples were redissolved in a small volume of hexane and quantitated by gas liquid chromatography (GLC). Fatty acid methyl esters (FAME) (5.2–7.2 min) and dimethylacetals (7.7–10.7 min) were resolved by normal phase HPLC, using 0.25% isopropanol in hexane, while FAMEs (3.5–4.3 min) and alkylglycerol acetates (7.3–9.9 min) were separated with 0.8% isopropanol in hexane. SPH was converted to FAMEs by acidic transmethylation as described for the diacylglycerols. The molecular species of the di-tBDMS ethers of ceramides were analyzed by gas chromatography/mass spectroscopy (GC/MS) as previously described (15). The species were first quantitated by carbon number by gas chromatography (GC) using flame ionization detection (FID), and the individual species within each carbon number were determined by GC/MS. The composition by molecular weight was determined from the $[M - 57]^+$ ions. The composition of the long chain bases was derived from the $[CH_3(CH_2)_n(CHCH)_mCHO\text{-}tBDMS]^+$ and the $[CH_3(CH_2)_n(CHCH)_mCHO(tBDMS)CHCHOSi(CH_3)_2]^+$ ions, whereas the composition of the fatty acids was determined from the $[CH_3(CH_2)_nCONHCHCH_2O\text{-}tBDMS]^+$ ion. By solving a set of simultaneous equations it was possible to quantitate as many as 9 molecular species within a single GC peak. Separation of the di-TMS ethers of ceramides according to carbon number and degree of unsaturation in the long chain base or fatty acyl chain could also be obtained by GLC using the RTx 2330 (Restek Corp., Port Matilda, PA) capillary column. Diradylglycerol TMS ethers and alkylglycerol diacetates were resolved on the basis of carbon number by means of a fused silica capillary column (8 m × 0.32 mm i.d.) coated with permanently bonded SE-54 liquid phase. Samples were injected on-column and temperature was programmed in 4 ramps from 40 to 350A°C as previously described (16). The molecular species were separated according to carbon number and degree of unsaturation on a polar capillary column as previously reported (14,17), except that a fused silica column (15 m × 0.32 mm i.d.) coated with cross-bonded RTx 2330 (Restek Corp., Port Matilda, PA) was substituted for the glass column. Diradylglycerol TMS ethers were separated isothermally at 250°C using a split injection (split ration 7:1) and H_2 carrier gas at 3 psi head pressure. Fatty acid methyl esters and alkylglycerol diacetates were resolved on the same column using two temperature programs. Injections were made at 100°C and after 0.5 min the oven temperature was programmed at 20°C/min to either 130°C or 180°C and then to 240°C at 5°C/min. Determinations of major components (greater than 10%) had coefficients of variation (CV) of 2% or less, whereas minor components (less than 1%) had CV of approximately 10%. The overall analytical scheme is outlined in Figure 1.

RESULTS

Total lipid composition. The phospholipid class composition was similar to that determined previously by TLC separation and phosphorus analysis (18) or GLC analysis of the component fatty acids (19,20). As shown below the diradyl glycerophosphoethanolamines (GPE) contained 51% and the diradyl GPC about 7% of total as ether lipids. Figure 2 shows the HPLC separation of alkenylacyl, alkylacyl and diacylglycerol subclasses of the diradyl GPE. The erythrocyte diradyl GPE was made up of 48.8% diacyl, 47.8% alkenylacyl and 3.4% alkylacylglycerols, which were similar to values reported previously (21,22), except that alkylacyl GPC was not detected in the earlier work. The normal phase HPLC yielded

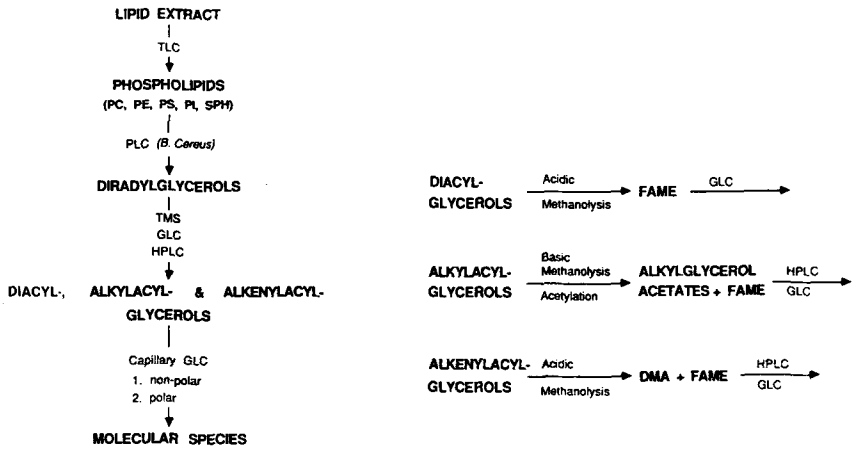

FIG. 1. Overall scheme of separation and identification of molecular species of diradylglycerophospholipids. DMA, dimethylacetals; FAME, fatty acid methyl esters; other abbreviations as given in text.

essentially a complete separation and purification of the diradylglycerol subclasses when run as the TMS ethers, which were slightly better resolved than the t-BDMS ethers. The HPLC procedure is clearly superior to the TLC method used previously (13), especially with respect to the resolution of the alkylacyl and diacylglycerol fractions. There was also a partial resolution of the molecular species within each diradylglycerol class. The broadening of the diacylglycerol peak observed at higher sample loads did not affect the resolution of the ether fractions. Derivatization of the diradylglycerols immediately after phospholipase C digestion is needed to prevent isomerization during the subsequent chromatographic analyses. The separation of the TMS ethers by normal phase HPLC is unique. Attempts to resolve these derivatives by TLC led to significant breakdown of the TMS ethers. The overall fatty acid compositions of the various phospholipid classes of the erythrocytes were similar to those reported in the literature (18-21,23). The detailed fatty acid composition of the various diradylglycerol fractions derived from them is discussed below under molecular species.

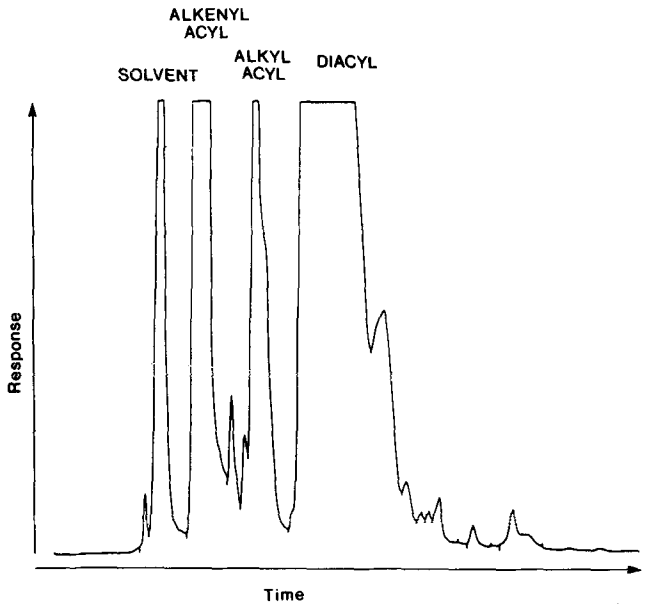

FIG. 2. Normal-phase HPLC separation of alkenylacyl, alkylacylacyl and diacylglycerol moieties of human red cell diradyl GPE. Peak identification as given in figure. HPLC conditions: column, Supelcosil LC-Si (5 μ), 250 cm × 4.6 mm i.d.; solvent, hexane/isopropanol (99.7:0.3, v/v), 1 ml/min; temperature, 30°C, isothermal. Sample: diradylglycerol TMS ethers.

Analysis of molecular species. The detailed analyses of molecular species using the improved methodology are presented from one sample of red cells only. The new fused silica capillary columns are easier to install, provide higher column efficiency and stability at elevated temperatures (250-260°C) and permit higher sample loads than the glass capillaries used previously (13,17). Less complete analyses on other samples were obtained using the older procedures (13), but these results are not shown. Figure 3 illustrates the carbon number resolution of the diacyl, alkylacyl and alkenylacylglycerol subclasses of the diradyl GPC. It is seen that the subclasses differ significantly in the relative proportions of the chain lengths, with the alkylacylglycerol species possessing a much higher proportion of the longer chain lengths. Of particular interest here is the presence of appreciable amounts of C_{20}, C_{22} and C_{24} alkyl chains (total carbon numbers 40-44). There is even a small amount of C_{26} species. A comparable increase in the chain length was not seen for the alkylacyl GPE. There were also chain

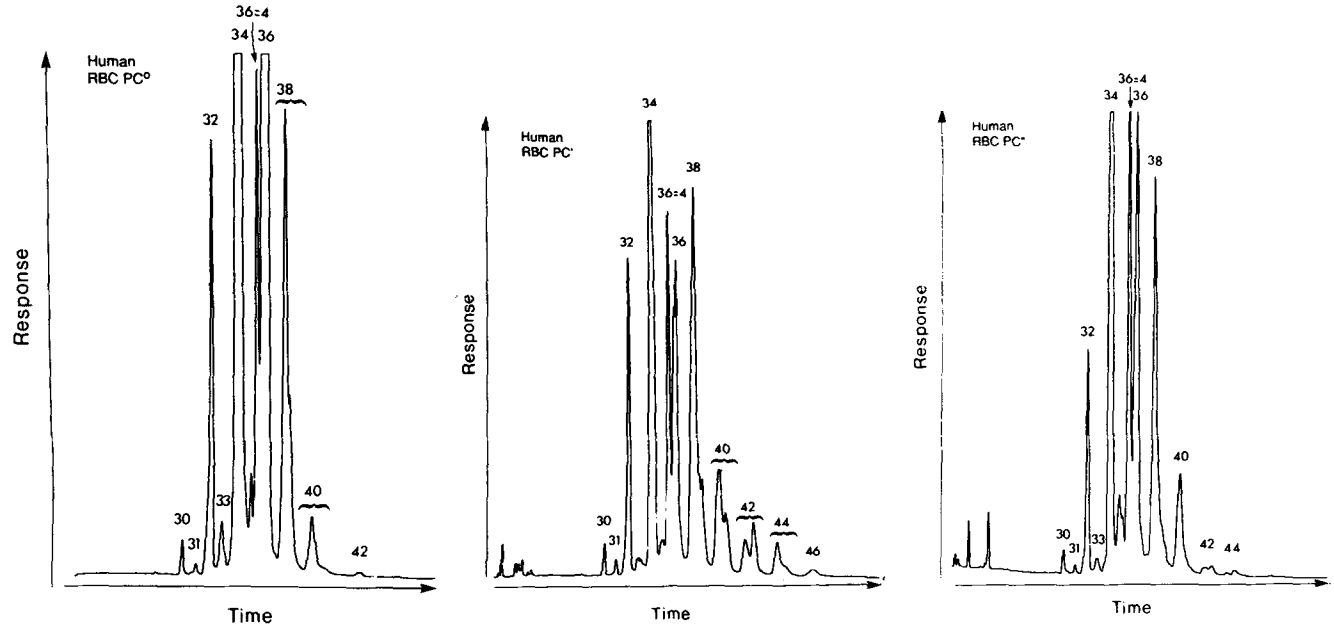

FIG. 3. Carbon number resolution of diacyl, alkylacyl and alkenylacylglycerol moieties of human red cell diradyl GPC. Peak identification as given in figure. GLC conditions: column, 8 m × 0.32 mm fused silica capillary coated with cross-linked 5% phenylmethyl silicone (HP-5), 0.17 μ film thickness; carrier gas, H_2, 6 psi; instrument and other operating conditions as given in text. Sample: diradylglycerol TMS ethers.

length differences among the various diradyl GPE, as well as among the diacyl GPC, GPE, glycerophosphoserine (GPS) and GPI, as shown in Table 1. These data, when combined with the data derived from polar capillary GLC (see below), yielded the quantitative composition of the molecular species of the diradylglycerols.

Figures 4 and 5 show the polar capillary GLC profiles of the inositol and serine phosphatides of the red blood cells, which contain only the diacylglycerol subclass. The quantitative composition of the molecular species of red cell diacyl GPI and GPS is given in Table 2 along with that of the diacyl GPC and GPE. The molecular species seen are consistent with the fatty acid composition shown in Table 3. There are marked differences between the major and minor molecular species among the different GPL classes. Thus, while the diacyl GPC and GPE contain closely similar amounts of 16:0-18:1ω9 (18.0 vs 15.1%), 18:0-18:1ω9 (5.8 vs 4.3%) and 16:0-20:3ω6 (5.2 vs 3.5%), they dffer greatly in the content of 16:0-18:2ω6 (26.9 vs 6.2%), 18:0-18:2ω6 (11.2 vs 2.1%), 16:0-20:4ω6 (5.1 vs 13.5%), 18:0-20:4ω6 (3.9 vs 12.6%) and 18:1ω9-20:4ω6 (0.6 vs 8.4%). In contrast, the diacyl GPI and GPS contained closely similar amounts of 18:0-20:4ω6 (18:0-20:3ω6) (45.3 vs 47.4%), 18:0-18:1ω9 (3.6 vs 5.9%) and 18:1-20:3 + 18:1t-20:4 (4.5 vs 4.9%), but differed in the content of 18:0-22:4ω6 (2.3 vs 11.3) and 18:0-22:5 plus 18:0-22:6ω3 (2.8 vs 21.0).

Figures 6 and 7 compare the elution patterns obtained on polar capillary GLC for the TMS ethers of the alkylacylglycerols derived from the erythrocyte diradyl GPC and GPE. The corresponding quantitative values are given in Table 4. There are marked differences in both qualitative and quantitative composition, which indicate that the alkylacylglycerols represent different pools of molecular species. While the alkylacyl GPC and GPE contain nearly identical proportions of 16:0′-20:4 and

TABLE 1

Carbon Number Distribution of the Diacyl, Alkylacyl and Alkenylacylglycerol Subclasses of Diradyl GPL of Human Red Cells

Carbon number	Diradyl GPC			Diradyl GPE		
	PC°	PC′	PC″	PE°	PE′	PE″
	Mole %					
30	0.4	0.8	0.6			
31	0.2	0.5	0.3			
32	5.2	9.0	6.2	1.0	0.3	0.1
33	0.9	1.1	0.8	0.3	—	0.2
34	51.5	26.1	31.9	23.7	4.1	4.7
35	—	1.6	3.7	—	0.8	0.6
36:4	5.1	9.5	14.6	13.5	9.5	9.7
36	26.9	15.4	17.7	16.5	6.2	9.3
38	8.3	21.5	17.8	35.1	43.2	47.7
40	1.4	8.2	5.3	8.9	33.9	26.1
42	—	3.9	0.8	1.2	2.3	1.5
44	—	1.9	0.3	—	—	0.2
46	—	0.6	—	—	—	—
% PL class	93.5	4.3	2.2	48.8	3.4	47.8

PC° and PE° diacyl GPC and diacyl GPE; PC′ and PE′, alkylacyl GPC and alkylacyl GPE; PC″ and PE″, alkenylacyl GPC and alkenylacyl GPE.

TABLE 2

Molecular Species of Diacyl GPL of Erythrocytes

GLC peak	Molecular species	Diacyl GPL classes				GLC peak	Molecular species	Diacyl GPL classes			
		PC	PE	PI	PS			PC	PE	PI	PS
		Mole %									
1	14:0-16:0	0.4				30	17:0-20:4	0.2	0.4	0.6	
2	15:0-16:0	0.1				33	18:0-20:4ω6	3.9	12.6	45.3	47.4
4	16:0-16:0	4.6	0.6	0.4	0.1		18:0-20:3ω6				
5	16:0-16:1	0.9	0.4	0.6	0.1	35	16:0-22:4ω6	0.5	5.2		
8	16:0-17:0	0.2				35A	18:1-20:3 + 18:1t-20:4			4.5	4.9
10	16:0-18:0	2.1	0.3	1.0	2.1	36	18:1ω9-20:4	0.6	8.4	2.7	
11	16:0-18:1ω9	18.0	15.1	3.8	1.3	37	18:1ω7-20:4	0.2	0.9		
12	16:0-18:1ω7	2.9	1.0	0.9		38	18:0-20:5ω3	0.3	0.6		
13	16:0-18:2	26.9	6.2	5.5	0.2	39	16:0-22:5ω3	2.0	4.7	0.8	0.3
14	17:0-18:1	0.4	0.3				16:0-22:6ω3				
18	17:0-18:2	0.3	0.3			40	18:2-20:4	0.3	1.8	0.3	
19	18:0-18:0	0.6	0.1	1.1		41	18:0-22:4ω6	0.4	1.9	2.3	11.3
20	18:0-18:1ω9	5.8	4.3	3.6	5.9	42	18:0-22:5ω6		0.7		
21	18:0-18:1ω7	0.3	0.2	0.8		43	18:1ω9-22:4ω6		1.3		
22	18:1ω9-18:1ω9	1.8	4.6	1.4	0.4	44	18:0-22:5 + 18:0-22:6	1.0	2.0	2.8	21.0
23	18:1ω9-18:1ω7		0.5			45			0.2		
24	18:0-18:2	11.2	2.1	6.6	2.9	46	18:2-22:4		0.2		
25	16:0-20:4	5.1	13.5	7.7	0.7	47	18:1ω9-22:5 + 18:0-22:6		1.6		
	16:0-20:3	5.2	3.5	2.7		Other		2.2	3.8	4.3	1.4
	18:1ω9-18:2										
26	18:1ω7-18:2	0.6				% PL class		93.5	48.8	100	99
29	18:2-18:2	1.0	0.9								
	16:0-20:5			0.3							

PC, PE, PI and PS, choline, ethanolamine, inositol and serine phosphatides.

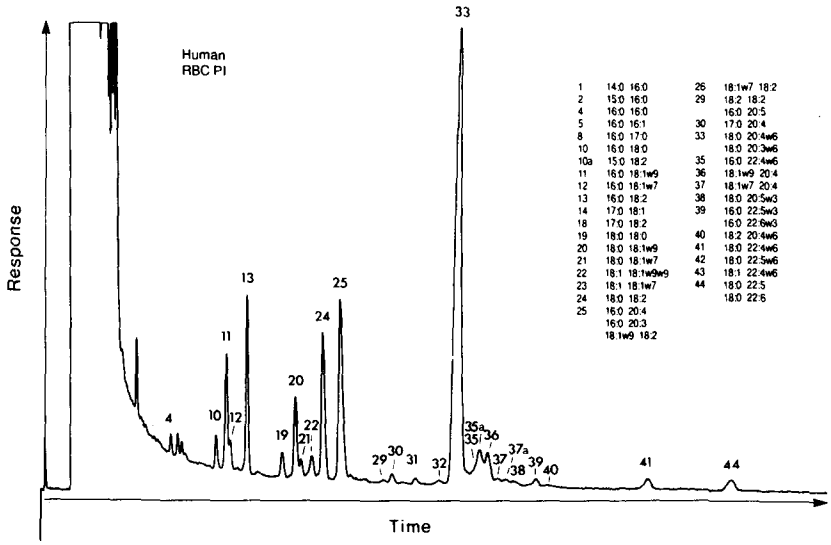

FIG. 4. Polar capillary GLC profiles of the diacylglycerol moieties of human red cell diacyl GPI. Peak identification as given in figure. GLC conditions: column, 15 m × 0.32 mm fused silica capillary coated with cross-bonded RTx 2330; carrier gas, H_2, 3 psi; temperature, 250°C, isothermal; instrument and other operating conditions as given in text. Sample: 1 μl of 0.1% diacylglycerol TMS ethers in hexane. Split ratio 7:1.

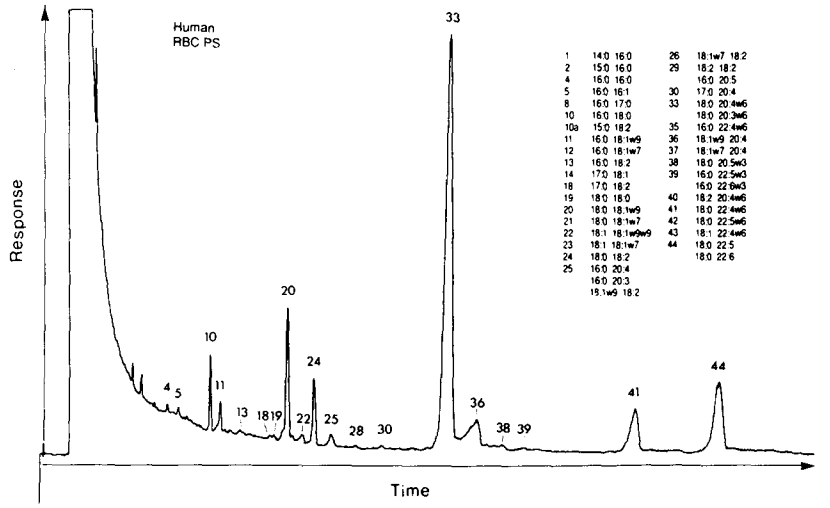

FIG. 5. Polar capillary GLC profiles of the diacylglycerol moieties of human red cell diacyl GPS. Peak identification as given in figure. GLC conditions as given in Fig. 4. Sample: 1 μl of 0.1% diacylglycerol TMS ethers in hexane.

TABLE 3

Fatty Acid Composition of Diacyl GPL of Human Red Cells

Fatty acids	Diacyl GPL classes				Fatty acids	Diacyl GPL classes			
	PC	PE	PI	PS		PC	PE	PI	PS
					Mole %				
14:0	0.4	0.2			18:3	0.1	—		
15:0	0.3	0.2			20:1	0.1	0.6		
16:0	35.6	22.5	13.3	3.3	20:2ω6	0.4	0.4	—	—
16:1ω9	0.4	0.3	0.8	—	20:3ω6	1.7	1.2	2.5	1.6
16:1ω7	0.4	0.3	0.5	—	20:4ω6	4.6	17.7	26.3	22.9
17:0	0.6	0.4	0.7	0.2	20:5ω3	0.2	0.3	0.2	—
18:0	11.7	11.1	33.3	44.3	22:4ω6	0.2	3.3	1.8	6.2
18:1t	0.7	1.1	2.4	1.7	22:5ω6	0.2	0.8	—	1.7
18:1ω9	16.4	23.6	7.2	5.7	22:5ω3	0.3	1.6	1.2	2.9
18:1ω7	1.9	1.4	1.6	0.6	22:6ω3	1.2	3.6	1.3	7.0
18:2ω6	22.6	9.2	7.0	2.0					

PC, PE, PI and PS as defined in Table 2.

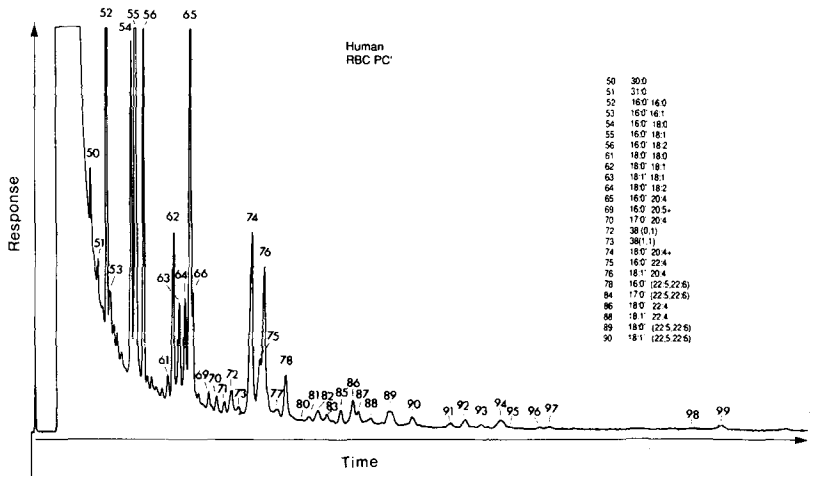

FIG. 6. Polar capillary GLC profiles of the alkylacylglycerol moieties of human red cell alkylacyl GPC. Peak identification as given in figure. GLC conditions as given in Fig. 4. Sample: 1 μl of 0.1% diradylglycerol TMS ethers in hexane.

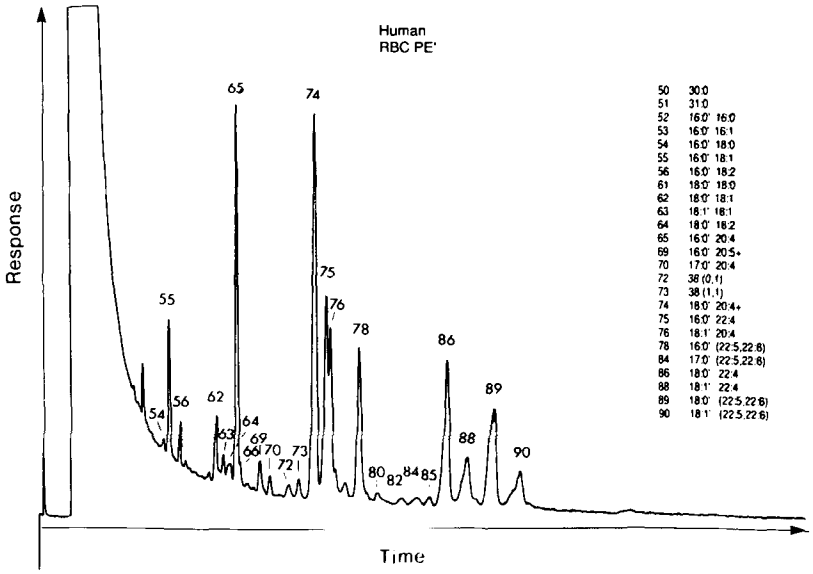

FIG. 7. Polar capillary GLC profiles of the alkylacylglycerol moieties of human red cell alkylacyl GPE. Peak identification as given in figure. GLC conditions as in FIG. 4. Sample: 1 μl of 0.1% diradylglycerol TMS ethers in hexane.

18:1′-20:4 species, the alkylacyl GPC contains much more of the saturated and monoenoic and less of the 18:0′-20:4, 16:0′-22:4, 18:0′-22:4 and other polyunsaturated species than alkylacyl GPE. This identification of the molecular species is supported by independent analysis of the alkylglycerol and fatty acid composition of the alkylacyl GPC and GPE (see below).

Figures 8 and 9 illustrate the separations obtained for the diacetates of the alkylglycerol moieties of the red blood cell alkylacyl GPC on nonpolar and polar capillary columns, respectively. From Figure 8, it is seen that the major carbon numbers range from 16 to 24 and that all, except 16, show partial splitting due to a slightly earlier elution of the corresponding mono- and diunsaturated species. From Figure 9, it can be seen that the saturated, monoenoic and dienoic alkylglycerol chains are resolved. It can now be appreciated that the 18:1′ peak contains a significant quantity of the *trans*-isomer, which is eluted earlier, and the ω7 isomer, which is eluted later, than the 18:1′ ω9 isomer. The corresponding quantitative compositions of the alkylglycerols and the FAMEs are given in Table 5.

Figures 10 and 11 give the polar capillary GLC elution patterns recorded for the alkenylacylglycerol moieties of the diradyl GPC and GPE. The corresponding quantitative values are given in Table 6. There are significant differences between two GPL classes in the composition of the alkenylacyl species, indicating the existence of either separate pools of precursors or independent transformation mechanisms. For each phospholipid type there

TABLE 4

Molecular Species of Alkylacyl GPL of Erythrocytes

GLC peak	Molecular species	Alkylacyl GPL PC′	Alkylacyl GPL PE′	GLC peak	Molecular species	Alkylacyl GPL PC′	Alkylacyl GPL PE′
		Mole %l				Mole %	
50	30:0	0.8		74	18:0-20:4+	7.6	16.3
51	31:0	0.5		75	16:0-22:4	2.6	8.5
52	16:0-16:0	8.8		76	18:1-20:4	6.3	7.2
53	16:0-16:1	0.8		77		1.0	1.2
54	16:0-18:0	6.3	0.3	78	16:0-22:5 + 16:0-22:6	2.2	7.1
55	16:0-18:1	14.9	3.2	79-83		1.9	2.2
56	16:0-18:2	5.8	0.9	84	17:0-22:5 + 17:0-22:6+	0.2	0.9
57-60			0.4	85		0.8	0.8
61	18:0-18:0	0.6	0.2	86	18:0-22:4	1.5	10.0
62	18:0-18:1	4.1	2.1	87		0.7	
63	18:1-18:1	2.9	0.9	88	18:1ω9-22:4 + 18:1ω7-22:4	0.8	5.3
64	18:0-18:2	3.1	1.0	89	18:0-22:5 + 18:0-22:6	1.4	9.6
65	16:0-20:4	10.1	9.5	90	18:1-22:5 + 18:1-22:6	0.6	5.3
66		2.8	1.9	91-98	42	4.4	2.3
69	16:0-20:5+	0.6	1.0	99-102	44	1.4	
70	17:0-20:4	0.4	0.5	Other		2.7	0.1
72	38 (0,1)	1.1	0.6				
73	38 (1,1)	0.3	0.7	% PL class		4.3	3.4

PC′ and PE′, as defined in Table 1.

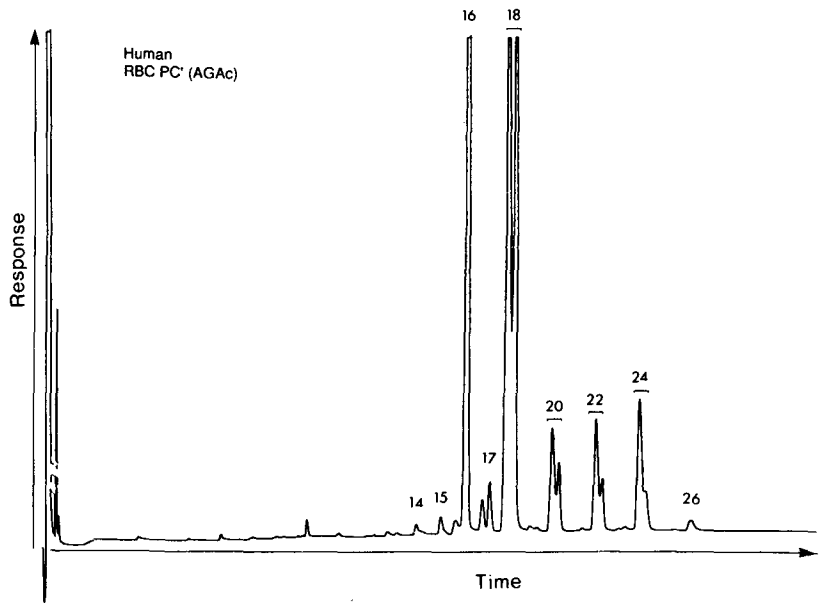

FIG. 8. Nonpolar capillary GLC separation of the alkylglycerol moieties of alkylacyl GPC. Peak identification as given in figure. GLC conditions as in Fig. 3. Sample: alkylglycerol diacetates.

appears to be a much greater similarity between the alkylacyl and alkenylacyl subclasses. The alkenylacyl GPC species are much richer in the saturated, monoenoic, dienoic and trienoic and poorer in tetraenoic and other polyunsaturated components than the alkenylacyl GPE. The above identities of the molecular species of the alkenylacylglycerol moieties of the erythrocyte diradyl GPC and GPE are supported by independent determination of the composition of the alkenyl and fatty acid chains by polar capillary GLC of the dimethylacetals and the FAMEs, respectively, as shown in Table 7. The present data indicate much less oligoenoic fatty acids (16:0, 18:0, 18:1, 18:2) in the alkenylacyl GPE than previously reported (24).

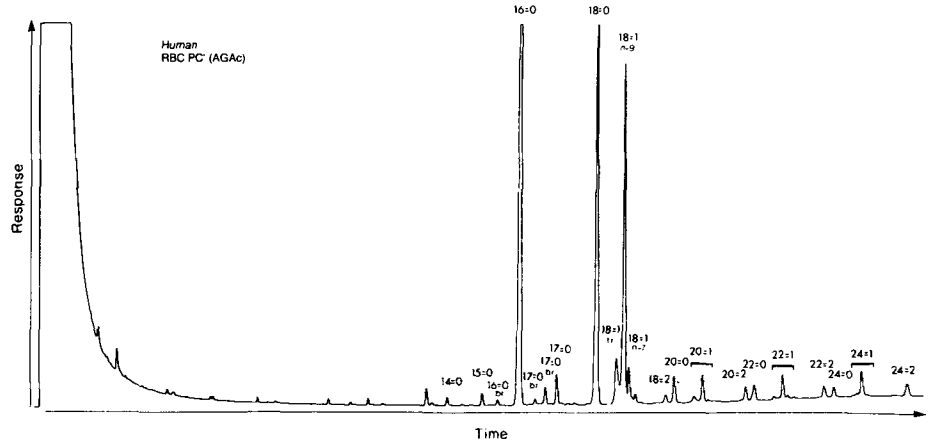

FIG. 9. Polar capillary GLC resolution of alkylglycerol moieties of alkylacyl GPC. Peak identification as given in figure. Column and carrier gas as in Fig. 4. Temperature programmed from 100°C (0.5 min) to 180°C at 20°C/min, then to 240°C at 5°C/min. Instrument and other GLC conditions as given in text. 24:2 eluted in 17.5 min. Sample: alkylglycerol diacetates.

TABLE 5

Composition of Alkylglycerols and Fatty Acid Methyl Esters of Alkylacyl GPL of Human Red Cells

Fatty chain	Alkylglycerols		Fatty acid methyl esters	
	PC'	PE'	PC'	PE'
			Mole %	
14:0	0.4	0.3	0.6	—
15:0	0.6	0.4	0.4	—
16:0	37.0	27.7	25.7	4.0
16:1ω9+ 16:1ω7	0.6	0.8	1.0	0.4
17:0	1.7	1.3	0.1	0.1
18:0	21.6	47.6	2.1	1.6
18:1t	3.8	5.7	0.3	—
18:1ω9	19.1	11.4	17.0	6.9
18:1ω7	2.2	1.9	1.0	0.6
18:2ω6	0.3	—	13.5	3.6
20:0	1.8	1.3	—	—
20:1	2.2	1.1	—	—
20:2ω6	0.9	—	0.5	—
20:3ω6	—	—	2.9	0.9
20:4ω6	—	—	25.7	35.5
20:5ω3	—	—	0.6	1.5
22:0	1.2	0.2	—	—
22:1	1.2	0.3	—	—
22:2	0.9	—	—	—
22:4ω6	—	—	2.9	20.5
22:5ω6	—	—	0.7	1.4
22:5ω3	—	—	1.8	10.9
22:6ω3	—	—	3.3	12.2
24:0	0.9			
24:1	2.5			
24:2	1.0			

PC' and PE', as defined in Table 1.

Table 8 gives the carbon number distribution of the ceramide moieties of red blood cell SPH from three subjects. They all possess a closely similar range and proportions of carbon numbers. The major components are the C_{34} and C_{42} even carbon number species, but odd carbon number components are also present in readily

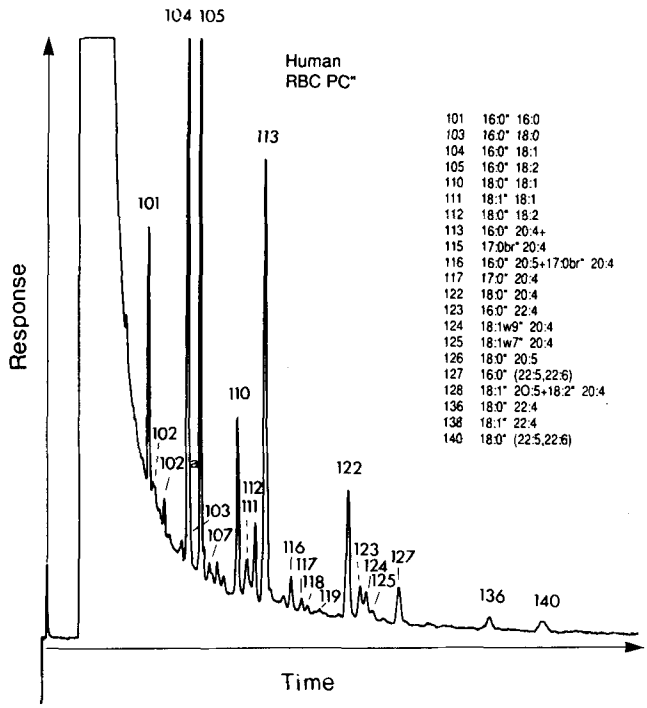

FIG. 10. Polar capillary GLC profiles of the alkenylacylglycerol moieties of human red cell alkenylacyl GPC. Peak identification as given in figure. GLC conditions as in Fig. 4. Sample: 1 μl of 0.1% diradylglycerol TMS ethers in hexane. Split ratio 7:1.

detectable amounts. The molecular species determined by GC/MS are given in Table 9. There has been no comparable previous analysis of the molecular species of red cell SPH.

DISCUSSION

The present study provides the first detailed description of the molecular species of the GPL and SPH from the red blood cells isolated from the same subject. Previously

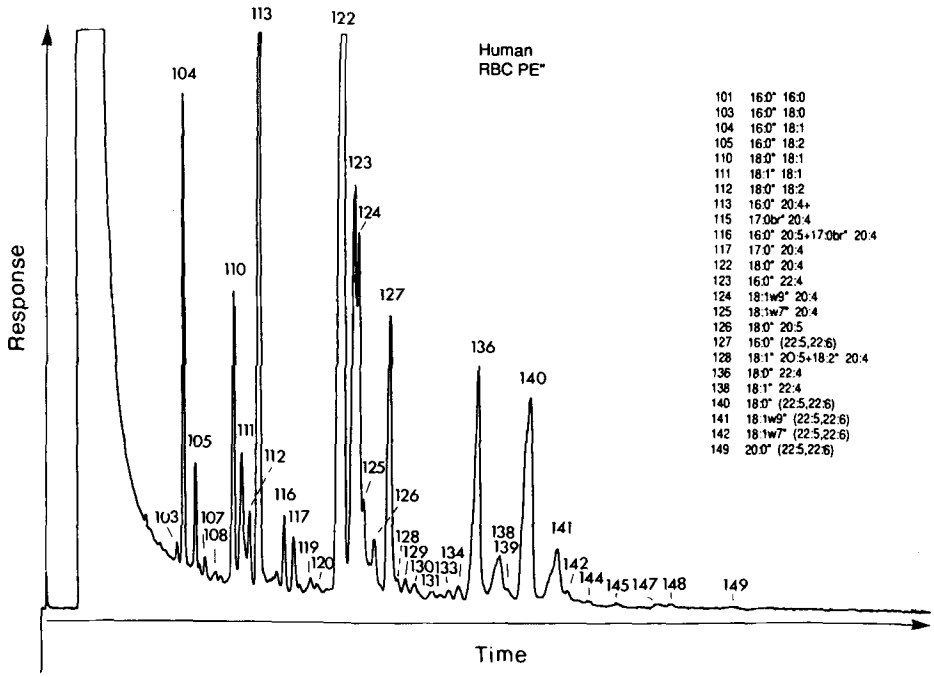

FIG. 11. Polar capillary GLC profiles of the alkenylacylglycerol moieties of human red cell alkenylacyl GPE. Peak identification as given in figure. GLC conditions as in Fig. 4. Sample: 1 μl of 0.1% diradylglycerol TMS ethers in hexane.

TABLE 6

Molecular Species of Alkenylacyl GPL of Erythrocytes

GLC peak	Molecular species	Alkenylacyl GPL PC″	Alkenylacyl GPL PE″	GLC peak	Molecular species	Alkenylacyl GPL PC″	Alkenylacyl GPL PE″
		Moles %				Moles %	
101	16:0-16:0	5.6		124	18:1ω9-20:4	2.0	6.8
102		0.6		125	18:1ω7-20:4	1.0	
103	16:0-18:0	0.5	0.1	126	18:0-20:5	0.5	1.3
104	16:0-18:1	15.8	3.8	127	16:0-22:5 + 16:0-22:6	2.9	6.2
105	16:0-18:2	17.3	1.0	128	18:1-20:5 + 18:2-20:4		0.4
107-109		1.9	0.4	129-135		1.1	2.5
110	18:0-18:1	5.9	3.5	136	18:0-22:4	1.4	8.1
111	18:1-18:1	2.0	2.1	138	18:1-22:4	0.3	2.4
112	18:0-18:2	3.1	0.9	139		0.1	
113	16:0-20:4+	14.6	9.7	140	18:0-22:5 + 18:0-22:6	1.6	9.6
	18:1-18:2 + 16:0-20:3	3.5	0.7	141	18:1ω9-22:5 + 18:1ω9-22:6	0.5	2.9
115	17:0br-20:4	0.6	0.4	142	18:1ω7-22:5 + 18:1ω7-22:6	0.2	0.7
116	16:0-20:5 + 17:0br-20:4	1.5	0.9	143-148		0.6	1.3
117	17:0-20:4	0.6	0.7	149	20:0-22:5 + 20:0-22:6		0.1
118-121		1.4		152-153	44	0.3	
122	18:0-20:4	8.0	23.4	Other		2.1	1.2
123	16:0-22:4	2.5	8.9				
				% PL class		2.2	47.8

PC″ and PE″, as defined in Table 1; br, branched..

only a few of the phospholipid classes have been analyzed for molecular species, and most of the analyses had been based solely on the fatty acid composition. The relative proportions of the various lipid classes found in the present study were similar to those recorded by earlier workers (18,20). Furthermore, the major molecular species of the diacyl GPC compare favorably with selected earlier analyses (19,20,25), but the species of the other classes have not been assessed previously, either qualitatively or quantitatively. There have been no previous analyses of the molecular species of the alkylacyl and alkenylacyl GPC and GPE, although the presence of both alkylacyl and alkenylacyl subclasses in red blood cell GPE had been recognized (22,24).

TABLE 7

Composition of Dimethylacetals and Fatty Acid Methyl Esters of Alkenylacyl GPL of Human Red Cells

Fatty chain	Dimethylacetals		Fatty acid methyl esters		Fatty chain	Dimethylacetals		Fatty acid methyl esters	
	PC″	PE″	PC″	PE″		PC″	PE″	PC″	PE″
	Mole %					Mole %			
16:0	63.0	25.4	22.7	2.9	20:1	0.7	0.9		
16:1ω9 + 16:1ω7	1.8	0.5	0.8		20:3ω6			3.2	1.0
17:0	1.9	3.6			20:4ω6			26.1	44.4
18:0	21.6	47.8	6.0	1.9	20:5ω6			0.1	1.8
18:1t	1.9	3.6		—	22:4ω6			2.7	14.3
18:1ω9	6.0	12.2	29.7	9.7	22:5ω6			2.2	1.9
18:1ω7	2.8	3.4	1.1	0.2	22:5ω3			1.9	8.8
18:2ω6		0.9	—	2.6	22:6ω3			3.4	10.5
20:0	0.4	0.9							

PC″ and PE″, as defined in Table 1.

TABLE 8

Carbon Number Distribution of Ceramide Moieties of Human Red Cell Sphingomyelins

Carbon number	Ceramides		Carbon number	Ceramides	
	Sample A[a]	Sample B[b]		Sample A[a]	Sample B[b]
	Mole %			Mole %	
32	1.7	1.6 ± 0.1	39	0.4	0.4 ± 0.1
33	1.2	1.2 ± 0.1	40	9.4	10.2 ± 1.2
34	29.8	28.7 ± 2.3	41	3.1	3.0 ± 0.4
35	1.0	0.9 ± 0.2	42	46.6	43.9 ± 2.4
36	4.6	5.7 ± 1.0	43	0.8	0.9 ± 0.1
37	0.2	0.2 ± 0.0	44	2.2	2.3 ± 0.1
38	1.5	2.0 ± 0.5			

[a]Single analysis from the subject, who supplied the red cell lipids for the diradylglycerophospholipid analyses.
[b]Mean ± S.D. from 3 subjects.

TABLE 9

Molecular Species of Sphingomyelins of Human Red Cells

Molecular species[a]	Sphingomyelins	Molecular species[a]	Sphingomyelins	Molecular species[a]	Sphingomyelins
	Mole %				
32:0	0.026	36:0	0.084	40:0	0.024
d16:1-16:0	0.920	d16:1-20:0	0.669	d16:1-24:0	0.917
d16:1-16:1	—	d16:1-20:1	0.053	d16:1-24:1	1.108
d17:1-15:0	0.005	d18:1-18:0	2.218	d17:1-23:0	0.005
d18:1-14:0	0.354	d18:1-18:1	0.248	d17:1-23:1	—
d18:2-14:0	0.046	d18:2-18:0	0.586	d18:1-22:0	4.716
33:0	0.001	d18:2-18:1	0.046	d18:1-22:1	0.186
d16:1-17:0	0.041	37:0	0.003	d18:2-22:0	0.776
d17:1-16:0	0.895	37:1	0.147	d18:2-22:1	0.194
d17:1-16:1	0.010	37:2	—	41:0	0.085
d18:1-15:0	0.126	38:0	0.016	d17:1-24:0	0.422
d18:2-15:0	0.011	d16:1-22:0	0.133	d17:1-24:1	0.413
34:0	0.727	d16:1-22:1	—	d17:1-24:2	0.029
d16:1-18:0	1.398	d17:1-21:0	0.756	d18:1-23:0	0.235
d16:1-18:1	0.156	d18:1-20:0	0.220	d18:1-23:1	0.078
d17:1-17:0	0.039	d18:1-20:1	0.021	d18:2-23:0	0.391
d18:1-16:0	25.773	d18:2-20:0	0.078	d18:2-23:1	—
d18:1-16:1	0.214	d18:2-20:1	0.005	42:0	0.042
d18:2-16:0	1.532	39:0	—	d18:1-24:0	19.630
d18:2-16:1	—	d16:1-23:0	0.056	d18:1-24:1	20.670
35:0	0.173	d16:1-23:1	—	d18:1-24:2	2.302
d16:1-19:0	0.060	d17:1-22:0	0.120	d18:2-24:0	5.261
d17:1-18:0	0.303	d17:1-22:1	0.021	d18:2-24:1	—
d17:1-18:1	0.006	d18:1-21:0	0.072	d18:2-24:2	0.391
d18:1-17:0	0.510	d18:2-21:0	0.029	43	0.80
d18:2-17:0	0.046			44	2.0

[a]Molecular species from a single subject but other than the one who supplied the diradylglycerophospholipids of the erythrocytes.

As a result of the sampling and analytical strategy it was possible to compare the molecular species composition of the different diradylglycerol subclasses among the different GPL, without the need to correct for dietary and metabolic differences in different individuals or pooled samples. The extensive differences noted among the different GPL classes emphasize the metabolic heterogeneity of the red blood cell phospholipids, which must reflect some as yet unrecognized physiological requirement. The analyses reveal that the differences among the diradylglycerol moieties of the GPL are present in both major and minor molecular species and between species, which are known to arise from common metabolic pathways. While previous analyses (19,20) of molecular species retained some uncertainty because of a possible error associated with the extensive reconstitution of data from complementary analytical methods, the present study eliminates this doubt because intact molecular species are being analyzed. The positional distribution of the fatty chains in the individual diacyl GPL molecules was not independently determined; thus, the relative proportions of the reverse isomers have not been estimated. The phospholipase A_2 data is available in the literature (18–20) and we have taken advantage of it in compiling the tabulated material. There should be no doubt about the location of the alkyl and alkenyl groups in the *sn*-1 position of the GPL, although 2-alkenyl GPL has recently been reported in rabbit sperm (26).

Unlike other cell types, the red blood cells do not possess enzymes for *de novo* formation of GPL. They must be derived from the original membrane synthesized in the bone marrow (27) or from a limited exchange of intact GPL with plasma lipoproteins, which is believed to be catalyzed by specific phospholipid exchange proteins (28), as well as limited exchange of acyl groups within the red blood cell membrane (29). In a separate paper (30), we have compared the molecular species composition of the red blood cell phospholipids and the plasma lipoproteins derived from the same subject and have discussed any possible precursor-product relationships. Little evidence was found to suggest that the GPL undergo separate and independent metabolism in the red cell membrane. In this connection, it is pertinent to note the recent identification of specific alkylacyl GPI species as lipid anchors of acetylcholine esterase in bovine (31) and human (32) red blood cell membranes. The bulk of the red cell diradyl GPI did not contain any such species, although they could have been present in the form of GPL glycans as now demonstrated for other cell membranes and other lipid anchors (33). In fact, no significant alkylacyl or alkenylacylglycerol species were found in red cell diradyl GPS or GPI, although both types of ether linkages were present in diradyl GPE and GPC. The physiological significance of the ether-linked GPL is not known, but it has been shown that they affect membrane fluidity (34).

The SPH of human red blood cells has been characterized in the past on the basis of fatty acid and nitrogenous base composition and distribution of the carbon numbers. The present analyses provide the first account of the detailed molecular association of the fatty chains as revealed by GC/MS. As shown elsewhere (15), the composition of the ceramide moieties of plasma SPH is clearly different from that of red blood cell SPH, which apparently do not equilibrate with them. The red cell SPH originate in the bone marrow and do not undergo further metabolism in the red blood cell membrane (27).

Finally, it is known that the diradyl GPC and SPH reside mainly in the outer half of the lipid bilayer of the red blood cell, whereas the diradyl GPE, GPS and GPI are found mainly in the inner half (35). Although the flip-flop rate is slow, it has been reported that for GPC and GPE the fatty acid compositions are the same on both sides of the membrane (36). The fatty acids of the SPH pools of the outer and inner layers of the membrane are known to differ in composition (37). However, fatty acid composition alone is not a good indicator of the type of molecular species to be found in any membrane. Fatty acid composition also does not provide any evidence about the distribution of the alkylacyl and the alkenylacylglycerol subclasses, although the dimethylacetals may indicate the presence or absence of plasmalogens. It would therefore be of interest to employ in future work the improved methods of molecular species analysis to determine the sidedness of the distribution of all GPL of the red blood cell membrane.

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Molecular Species of Glycerophospholipids and Sphingomyelins of Human Plasma: Comparison to Red Blood Cells

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In addition to diacyl glycerophosphocholine and sphingomyelin, human plasma also contains small amounts of other glycerophospholipids, which may have special metabolic function. The structure and origin of these minor plasma lipids has not been determined. Knowledge of the detailed composition of the phospholipids of red blood cells (Myher *et al., Lipids 24,* 1989) permits evaluation of one of the possible sources. This study reports the detailed analyses of plasma glycerophospholipids made in parallel to those of the erythrocyte lipids obtained from the same blood using HPLC and GLC methods. The proportions of the major phospholipid classes in the plasma and erythrocytes were similar to published values, including the essential absence of diradyl glycerophosphoserine from plasma. Plasma diradyl glycerophosphocholine contained 93.0% diacyl, 3.4% alkylkacyl and 3.6% alkenylacyl, whereas the diradyl glycerophosphoethanolamine consisted of 71.8% alkenylacyl, 19.9% diacyl and 8.3% alkylacyl subclasses. The diradyl glycerophosphoinositol was 100% diacyl. The content of the minor subclasses of plasma diradyl glycerophosphocholine is similar to that of the red cells, but the ether content of the diradyl glycerophosphoethanolamine is higher in plasma than in cells. The lipid ether subclasses of plasma glycerophospholipids also contained a higher proportion of the C_{20}, C_{22} and C_{24} alkyl and alkenyl chains than those of the cells. Furthermore, the C_{16} and C_{18}-containing species in diradyl glycerophosphoethanolamine subclasses varied with the nature of the polyunsaturated acid, whereas in diradyl glycerophosphocholine subclasses the polyunsaturated acids were combined with the C_{16} and C_{18} acids in equal proportions. The significant differences in the molecular species of glycerophospholipids and sphingomyelin between plasma and red cells would appear to limit any direct transfer or equilibration of their lipid components.
***Lipids 24,* 408-418 (1989).**

The phospholipid class composition of plasma lipoproteins is unusual. It differs from that of the red blood cell membranes and from the plasma membranes of the cells lining the vascular bed. Thus, although diradyl glycerophosphocholine (GPC) and sphingomyelin (SPH) comprise the bulk and the diradyl glycerophosphoethanolamine (GPE) and glycerophosphoinositol (GPI) constitute minor components, the red cell membrane contains the diradyl GPC, GPE, glycerophosphoserine (GPS) and SPH as major and GPI as minor components (1). The glycerophospholipids (GPL) from both sources contain a wide variety of fatty chains, attached by ester or ether linkages, with chain lengths of 16-22 carbons and up to 6 double bonds, whereas SPH is made up of a separate pool of fatty acids and nitrogenous bases joined by amide linkages. This results in several hundreds of chemically distinct species of phospholipids. We have recently determined the detailed molecular species composition of the red blood cells of man (2). The present study extends this work to the plasma GPL and SPH and, along with the accompanying study (2), constitutes the first comprehensive assessment of the composition of GPL species of plasma and erythrocytes from the same blood. A preliminary account on the comparative composition of plasma and red cell GPL has appeared (3). A summary of the SPH data has been published previously (4).

MATERIALS AND METHODS

Blood plasma. The plasma for the diradyl GPL analyses was obtained from the same sample of blood as the erythrocytes analyzed in the accompanying paper (2). This subject also provided plasma SPH, which, however, was analyzed only for fatty acid composition and the carbon number distribution of the ceramide moieties. The molecular species analyses of the sphingomyelins had been performed on 4 other samples of plasma from 3 normolipemic subjects in the fasting state (VLDL, LDL and HDL_3) and one normolipemic subject in the postprandial state (chylomicrons and VLDL), who had shown fatty acid and ceramide compositions of VLDL and LDL similar to those obtained for plasma total SPH from the subject supplying the diradyl GPL. Plasma and cells were separated by centrifugation and cells were washed to remove the buffy coat. Some of the plasma samples were resolved into the major lipoprotein classes by ultracentrifugation as described elsewhere (5).

Lipid analyses. The methods of lipid extraction and chromatographic analyses of molecular species were as previously described in detail (2). Plasma and lipoprotein total lipid profiles were determined by capillary GLC as reported (6).

RESULTS

Total lipid composition. The phospholipid class composition of whole plasma was of the order reported previously on basis of TLC separation and phosphorus analyses (7) or GLC analyses of the component fatty acids (8), with 67.0% diradyl GPC, 17.7% SPH, 2.5% diradyl GPE and 2.1% diradyl GPI. Figure 1 shows the separation of alkenylacyl, alkylacyl and diacyl subclasses of the diradylglycerol moieties of the plasma ethanolamine GPL. A similar separation of the diradylglycerol moieties of plasma choline GPL gave much smaller but readily detectable fractions for the ether-linked species. These separations were performed with the TMS ethers of the diradylglycerols. Based on GLC quantitation the diradyl GPC contained 93.0% diacyl, 3.4% alkylacyl and 3.6% alkenylacyl, whereas the diradyl GPE contained 71.8%

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Abbreviations: GPC, glycerophosphocholine; GPE, glycerophosphoethanolamine; GPI, glycerophosphoinositol; GPL, glycerophospholipids; GPS, glycerophosphoserine; SPH, sphingomyelin; PC° and PE°, diacyl GPC and GPE; PC′ and PE′, alkylacyl GPC and GPE; PC″ and PE″, alkenylacyl GPC and GPE.

alkenylacyl, 19.9% diacyl and 8.3% alkylacylglycerol subclasses. The diradyl GPI was 100% diacyl. Although the ether-linked GPL content was not measured in the individual lipoprotein classes, it has been observed that each lipoprotein contained at least some of this lipid class (9). The overall fatty acid composition of the various plasma phospholipid classes was similar to those reported in the literature (7,8,10). The detailed fatty acid composition of the various diradylglycerol fractions derived from them is discussed below under molecular species.

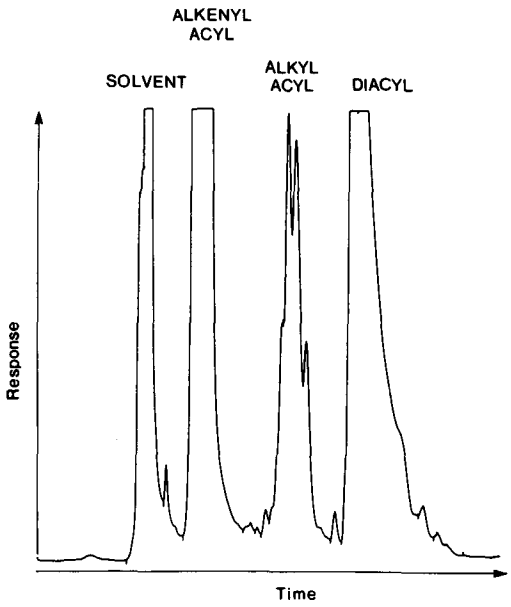

FIG. 1. Normal-phase HPLC separation of alkenylacyl, alkylacyl and diacylglycerol moieties of human plasma diradyl GPE. Peak identification as given in figure. HPLC conditions: column, Supelcosil LC-Si (5 μ), 250 cm × 4.6 mm i.d.; solvent, hexane-isopropanol 99.7:0.3 (v/v), 1 ml/min; temperature, 30°C, isothermal. Sample: diradylglycerol TMS ethers.

Analyses of molecular species. Figure 2 shows the carbon number resolution of the diacyl, alkylacyl and alkenylacyl subclasses of the diradyl GPC. The corresponding quantitative values are given in Table 1. It is seen that the subclasses differ significantly in the relative proportions of the chain lengths, with the alkylacylglycerol species possessing a much greater proportion of the longer chain lengths. Of particular interest here is the presence of appreciable amounts of C_{20}, C_{22} and C_{24} alkyl chains. A comparable increase in chain length was not seen for the alkenylacyl GPC. There were also chain length differences among the diradyl GPE, as well as among the diacyl GPC, GPE and GPI, as shown in Table 1. These data were used for reconstitution of the quantitative composition of the molecular species of the diradylglycerols derived from polar capillary GLC (see following discussion).

Figure 3 shows the polar capillary GLC profile of the diacylglycerol moieties of ethanolamine GPL, whereas Figure 4 shows the polar capillary GLC profile of the diacylglycerol moieties of diacyl GPI. The quantitative composition of the molecular species of the diacyl GPC, GPE and GPI is given in Table 2. The corresponding fatty acid compositions are given in Table 3. It is seen that the diacylglycerol moieties of the three GPL classes differ greatly from each other and from the alkylacyl and alkenylacylglycerol moieties of diradyl GPC and GPE. Thus, combinations of 16:0 with 18:1 and 18:2 form the most abundant species of diacyl GPC, whereas

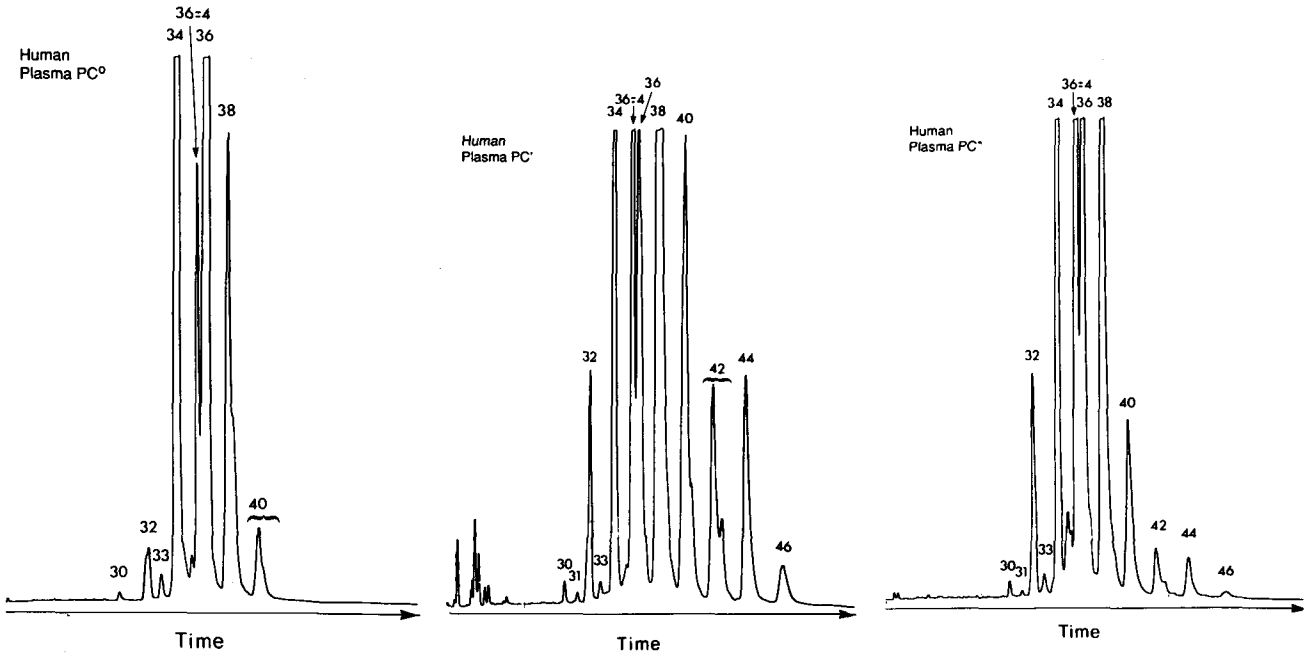

FIG. 2. Carbon number resolution of diacyl (PC°), alkylacyl (PC') and alkenylacylglycerol (PC") moieties of human plasma diradyl GPC. Peak identification as given in figure. GLC conditions: column 8 m × 0.32 mm fused silica capillary coated with cross-linked 5% phenylmethyl silicone (HP-5), 0.17 μ film thickness; carrier gas H_2, 6 psi; instrument and other operating conditions as previously described (2). Sample: 1 μl of 0.1% diradylglycerol TMS ethers in hexane.

TABLE 1

Carbon Number Distribution of Diacyl, Alkylacyl and Alkenylacyl Subclasses in Choline and Ethanolamine Phosphatides of Human Plasma

Carbon number	PC			PE			PI
	Diacyl	Alkylacyl	Alkenylacyl	Diacyl	Alkylacyl	Alkenylacyl	Diacyl
				Mole %			
30	0.2	0.3	0.2	—	—	—	
31		0.2	0.1	—	—	—	
32	1.6	3.8	3.6	0.6	0.3	0.2	0.5
33	0.5	0.4	0.5	0.2	0.3	0.1	—
34	42.4	13.5	24.3	11.1	6.2	4.9	8.2
35	0.7	—	2.5	0.4	1.3	0.8	0.3
36:4	7.7	13.9	21.3	7.5	12.5	11.1	4.0
36	30.4	11.3	17.1	27.4	10.4	14.6	21.5
37	—	—	—	—	—	1.2	1.6
38	14.4	31.6	22.0	43.2	42.1	46.3	59.6
40	2.3	12.1	5.5	9.7	23.3	18.1	3.9
42	—	6.3	1.5	—	2.9	2.0	—
44	—	5.2	1.1	—	0.7	0.7	—
46	—	1.4	0.2	—	—	0.1	—

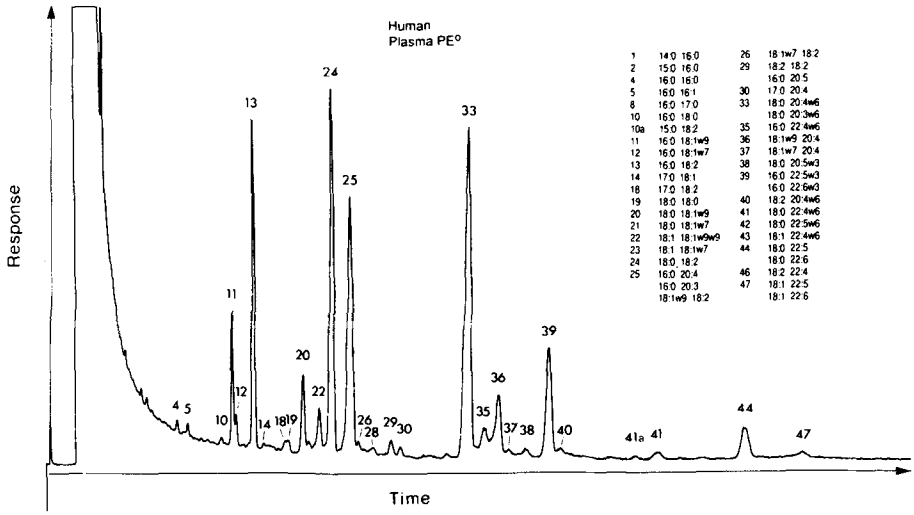

FIG. 3. Polar capillary GLC profile of the diacylglycerol moieties of human plasma diacyl GPE. Peak identification as given in figure. GLC conditions: column, 15 m × 0.32 mm fused silica capillary coated with cross-bonded RTx 2330; carrier gas, H_2, 3 psi; temperature, 250°C, isothermal; instrument and other operating conditions as given (2). Split ratio 7:1. Sample: 1 μl of 0.1% diacylglycerol TMS ethers in hexane.

combinations of 18:0 with 18:2 and/or 20:4 are favored in diacyl GPE and GPI.

Figures 5 and 6 show the elution patterns obtained on polar capillary GLC for the TMS ethers of the alkylacylglycerols derived from plasma choline and ethanolamine GPL, respectively. The corresponding quantitative values are given in Table 4. There are marked differences in both qualitative and quantitative composition, which indicates that the alkylacylglycerols represent different subcellular pools of biosynthetic precursors. Although both choline and ethanolamine GPL contain nearly identical proportions of 16:0′ 20:4 and 18:0′ 20:4 species, the alkylacyl GPC contain much more of the saturated, monoenoic and 18:1′ 20:4 and 16:0′ 22:4, and less of other polyunsaturated species than alkylacyl GPE. Figures 7 and 8 show the separation obtained for the alkylglycerol moieties of the alkylacyl GPC on the nonpolar and polar capillary columns, respectively. From Figure 7, it is seen that the major carbon numbers range from 16 to 24 and that all, except 16, show partial splitting due to a slightly earlier elution of the corresponding mono- and diunsaturated species. From Figure 8, it is seen that the saturated, monounsaturated and diunsaturated alkylglycerol chains are resolved. It can now be appreciated that the 18:1′ peak contains a significant proportion of the *trans* isomer, which is eluted earlier, and the ω7 *cis* isomer, which is eluted later, than the 18:1 ω9 *cis* isomer. The identity of the plasma alkylglycerol diacetates on the polar column was confirmed by GLC analysis of the alkylglycerol fractions resolved by argentation TLC. The

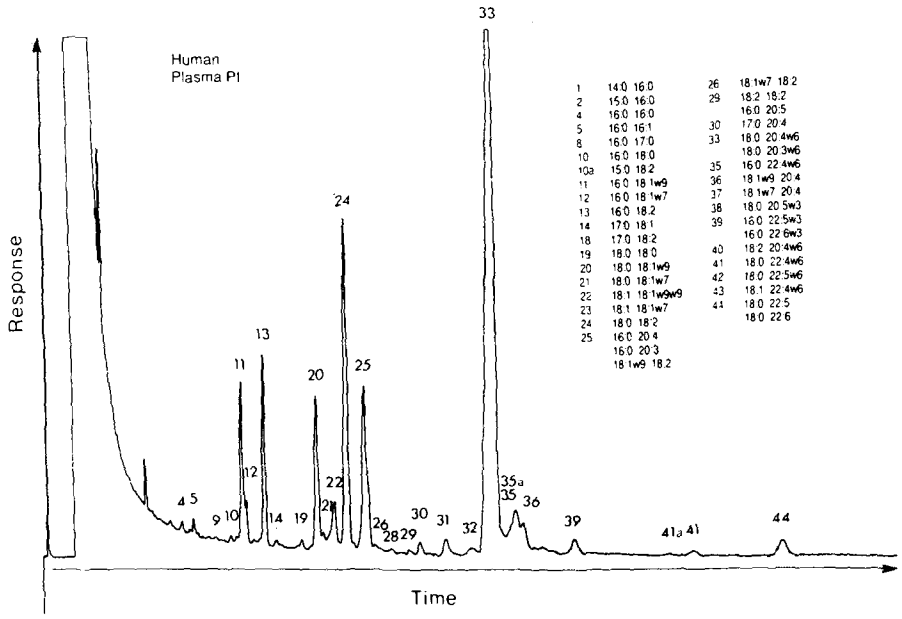

FIG. 4. Polar capillary GLC profile of the diacylglycerol moieties of human plasma diacyl GPI. Peak identification as given in figure. GLC conditions as given in Figure 3. Sample: 1 μl of 0.1% diacylglycerol TMS ethers in hexane.

TABLE 2

Molecular Species of Diacyl Glycerophospholipids of Human Plasma

GLC peak	Molecular species	Phospholipid classes PC	PE	PI	GLC peak	Molecular species	Phospholipid classes PC	PE	PI
		Mole %					Mole %		
1	14:0-16:0	0.2			30	17:0-20:4	0.2	0.3	0.5
4	16:0-16:0	0.8	0.3	0.2	33	18:0-20:4ω6	6.7	22.7	50.0
5	16:0-16:1	0.7	0.3	0.4		18:0-20:3ω6			
8	16:0-17:0	0.2	—	—		16:0-22:4ω6	0.9	2.7	
10	16:0-18:0	0.2	0.2	0.1	35A	18:1-20:3 + 18:1t-20:4			3.9
11	16:0-18:1ω9	9.3	2.8	3.4	36	18:1ω9-20:4	1.3	5.5	1.6
12	16:0-18:1ω7	2.1	0.7	0.8	37	18:1ω7-20:4	0.2	0.7	
13	16:0-18:2	29.4	7.7	4.0	38	18:0-20:5ω3	0.3	1.2	
14	17:0-18:1	0.4	0.1	0.2	39	16:0-22:5ω3	3.5	7.8	1.1
18	17:0-18:2	0.3	0.3			16:0-22:6ω3			
19	18:0-18:0	0.5	0.3	0.2	40	18:2-20:4	0.5	1.1	
20	18:0-18:1ω9	2.1	2.6	5.0	41	18:0-22:4ω6	0.4	1.1	0.8
21	18:0-18:1ω7	0.6	0.3	0.5	42	18:0-22:5ω6	—	—	—
22	18:1ω9-18:1ω9	1.3	1.9	2.5	43	18:1-22:4ω6	0.4	—	—
23	18:1-18:1ω7	—	—	—	44	18:0-22:5	1.5	4.5	2.8
24	18:0-18:2	15.4	13.8	11.6		18:0-22:6			
25	16:0-20:4	7.7	7.5	4.0	46	18:2-22:4		—	—
	16:0-20:3	7.5	5.9	3.5	47	18:1-22:5	1.3		
	18:1ω9-18:2					18:1-22:6			
26	18:1ω7-18:2	1.0	0.5	—	Other		2.9	4.8	3.5
29	18:2-18:2	1.9	0.7	—					
	16:0-20:5			0.2	% PL class		93.0	19.9	100

peaks identified as *trans* isomers migrated well above the long and short chain *cis*-monoenes on the silver nitrate plates. Thus, the GLC and argentation TLC behavior of these compounds is consistent with the known chromatographic properties of the corresponding *trans* acids (11). The GLC retention of alkylglycerol esters was consistent with their known chromatographic properties (12) and the elution of reference standards. The corresponding quantitative composition of the alkylglycerol and the fatty acid methyl esters is given in Table 5.

Figures 9 and 10 give the polar capillary GLC elution patterns recorded for the alkenylacylglycerol moieties of

TABLE 3

Fatty Acid Composition of Diacyl GPC, GPE and GPI From Human Plasma

Fatty acids	Diacylglycerophospholipids			Fatty acids	Diacylglycerophospholipids		
	Choline	Ethanolamine	Inositol		Choline	Ethanolamine	Inositol
	Mole %				Mole %		
14:0	0.3	0.4	0.9	18:3ω3	0.1	—	—
15:0	0.3	0.2	0.3	20:1ω9	0.4	0.2	—
16:0	28.8	14.4	7.7	20:2ω6	0.3	0.3	0.6
16:1ω9	0.4	0.4	0.3	20:3ω6	2.6	1.4	3.3
16:1ω7	0.5		0.5	20:4ω6	7.6	18.6	28.0
17:0	0.2	0.3	0.6	20:5ω3	0.4	0.3	0.1
18:0	12.9	21.1	37.3	22:4ω6	0.2		0.3
18:1t	0.7	1.2	2.3	22:5ω6	0.3		0.2
18:1ω9	11.2	8.7	7.9	22:5ω3	0.7	2.0	0.8
18:1ω7	2.3	1.5	—	22:6ω3	2.5	6.9	1.1
18:2ω6	27.3	20.0	7.8				

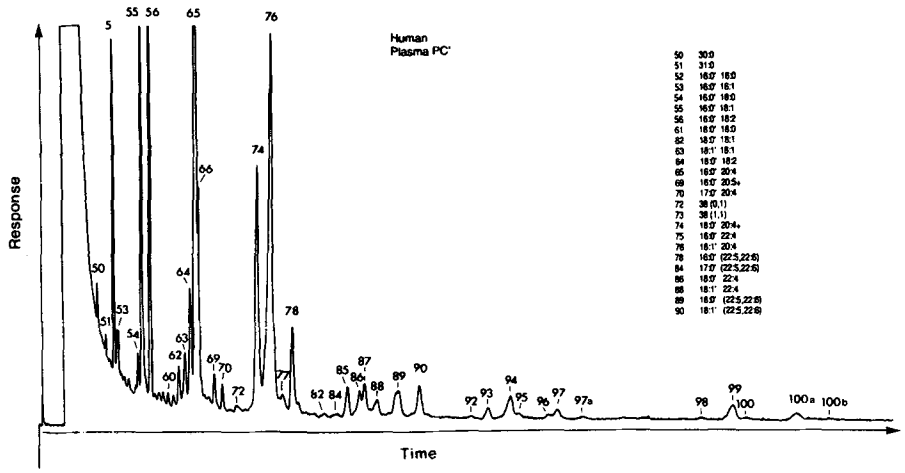

FIG. 5. Polar capillary GLC profile of the alkylacylglycerol moieties of human plasma diradyl GPC. Peak identification as given in figure. GLC conditions as given in Figure 3. Sample: 1 μl of 0.1% diradylglycerol TMS ethers in hexane.

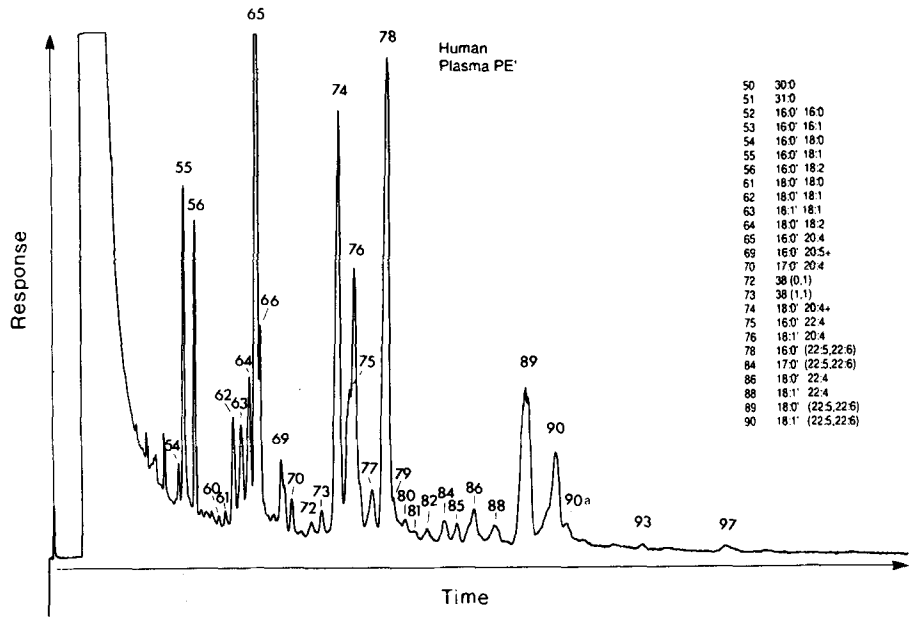

FIG. 6. Polar capillary GLC profile of the alkylacylglycerol moieties of human plasma diradyl GPE. Peak identification as given in figure. GLC conditions as given in Figure 3. Sample: 1 μl of 0.1% diradylglycerol TMS ethers in hexane.

TABLE 4

Molecular Species of Alkylacyl Glycerophospholipids of Human Plasma

GLC peak	Molecular species	Phospholipid classes PC′	PE′	GLC peak	Molecular species	Phospholipid classes PC′	PE′
		Mole %				Mole %	
50	30:0	0.3		74	18:0-20:4+	9.1	11.1
51	31:0	0.2		75	16:0-22:4	17.9	3.2
52	16:0-16:0	3.0		76	18:1-20:4		8.2
53	16:0-16:1	1.0		77		0.9	2.0
54	16:0-18:0	0.6	0.5	78	16:0-22:5 + 16:0-22:6	3.3	16.3
55	16:0-18:1	6.5	3.8	79-83		0.4	3.7
56	16:0-18:2	5.7	2.9	84	17:0-22:5 + 17:0-22:6+	0.2	1.0
57-60		0.7	0.4	85		1.6	0.7
61	18:0-18:0	0.2	0.2	86	18:0-22:4	1.9	1.9
62	18:0-18:1	0.9	1.5	87		1.9	
63	18:1-18:1	1.5	1.9	88	18:1ω9-22:4 + 18:1ω7-22:4	1.2	1.2
64	18:0-18:2	3.1	2.5	89	18:0-22:5 + 18:0-22:6	2.0	8.7
65	16:0-20:4	13.9	11.7	90	18:1-22:5 + 18:1-22:6	2.3	7.0
66		5.9	2.5	91-98	42	6.3	2.9
69	16:0-20:5+	0.9	1.6	99-102	44	4.9	0.7
70	17:0-20:4	0.6	0.5	Other		0.8	0.9
72	38 (0,1)	0.3	0.1				
73	38 (1,1)		0.4	% PL class		3.4	8.3

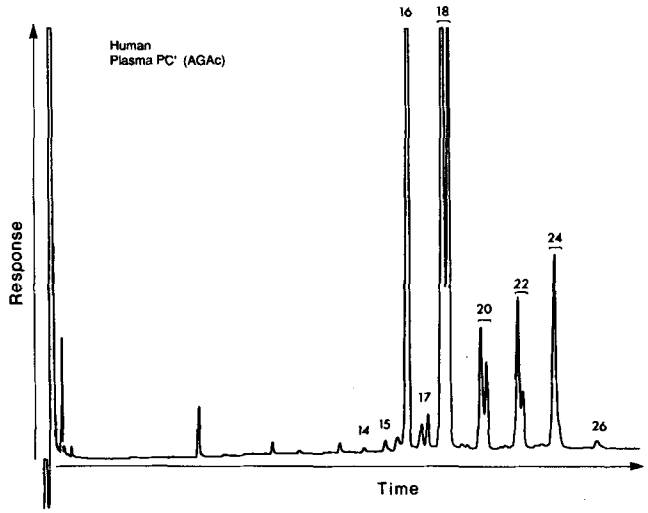

FIG. 7. Nonpolar capillary GLC resolution of alkylglycerol moieties of alkylacyl GPC of human plasma. Peak identification as given in figure. Column and carrier gas as given in Figure 2. Sample: 1 μl of 0.1% alkylglycerol diacetates in hexane.

plasma diradyl GPC and GPE. The corresponding quantitative values are given in Table 6. There are significant differences between the two lipids in the composition of the alkenylacyl species indicating the existence of separate pools of precursors or independent transformation mechanisms. The alkenylacyl GPC is much richer in species in which a 16:0″ alkenylglycerol is combined with saturated, monoeneoic, dienoic and tetraenoic fatty acids, whereas alkenylacyl GPE is much richer in 18:0″ alkenylglycerol combined with dienoic, tetraenoic and hexaenoic fatty acids. The above identities of the molecular species of the alkenylacylglycerol moieties of the diradyl GPC and

TABLE 5

Quantitative Composition of Alkylglycerols and Fatty Acids of Alkylacyl Glycerophosphocholine From Human Plasma

Fatty chains	PC′ Alkylglycerols	PC′ Fatty acids
	Mole %	
14:0	0.16	0.22
15:0	0.43	0.1
16:0	33.14	8.39
16:1ω9	0.89	0.32
16:1ω7		0.39
17:0	1.0	
18:0	16.54	1.8
18:1t	3.59	0.2
18:1ω9	21.37	6.6
18:1ω7	2.10	0.6
18:2	0.41	14.5
20:0	2.48	
20:1	3.0	
20:2ω6	1.8	0.29
20:3ω6		4.14
20:4ω6		42.14
20:5ω3		1.01
22:0	1.53	
22:1	3.0	
22:2	1.5	
22:4ω6		3.2
22:5ω6		1.2
22:5ω3		2.8
22:6ω3		6.4
24:0	0.6	
24:1	3.3	
24:2	2.3	

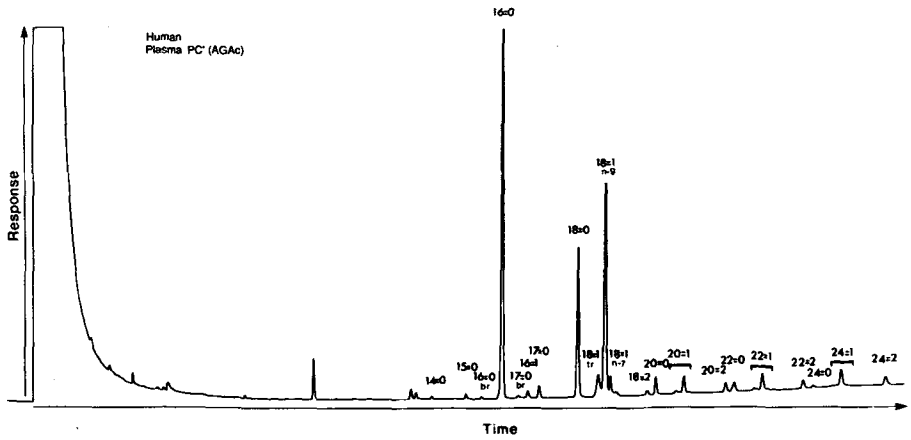

FIG. 8. Polar capillary GLC resolution of alkylglycerol moieties of alkylacyl GPC. Peak identification as given in figure. Column and carrier gas as in Figure 3. Temperature programmed from 100 (0.5 min) to 180°C at 20°C/min, then to 240°C at 5°C/min. Instrument and other GLC conditions as given (2). 24:2 eluted in 17.5 min. Sample: 1 μl of 0.1% alkylglycerol diacetates in hexane.

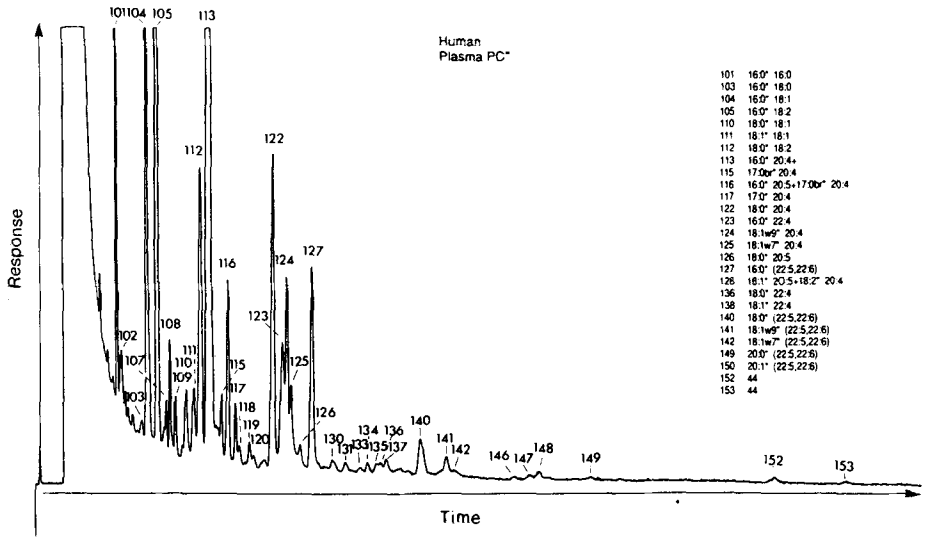

FIG. 9. Polar capillary GLC profile of the alkenylacylglycerol moieties of human plasma diradyl GPC. Peak identification as given in figure. GLC conditions as given in Figure 3. Sample: 1 μl of 0.1% diradylglycerol TMS ethers in hexane.

GPE are supported by the composition of the alkenyl chains, determined independently by polar capillary GLC of the dimethylacetals, and the fatty acid methyl esters derived from the corresponding alkenylacylglycerol fractions, shown in Table 7.

Table 8 gives the molecular species composition of the ceramide moieties of the SPH of VLDL, LDL, HDL_3 and chylomicrons. The SPH from the fasting plasma samples had been obtained from 3 normolipemic subjects other than the one who supplied the plasma diradyl GPL. The SPH from the postprandial plasma was obtained from another normolipemic subject 2 hr after ingesting a fatty meal. Because the plasma total SPH of the present subject had fatty acid and ceramide composition closely similar to that of the VLDL and LDL of the previously analyzed subjects (data not shown), it was assumed that the molecular species would be similar to those already determined, but not previously reported. As a result the data in Table 8 show minor interlipoprotein and intersubject variation, except for HDL_3. They possess closely similar ranges of carbon numbers, but differ significantly in the relative proportions of the short and long chain lengths. The major components are the even carbon number species, especially C_{34} and C_{42}, but odd carbon number components are also present in readily detectable amounts. We have previously pointed out the differences between HDL and LDL in the chain length distribution of the plasma SPH (4,9), but the present report gives the first full documentation of the composition of the major and minor species including VLDL and chylomicrons.

DISCUSSION

The present study provides the first detailed description of the molecular species of the different classes of GPL

TABLE 6

Molecular Species of Alkenylacyl Glycerophospholipids of Human Plasma

GLC peak	Molecular species	Phospholipid classes PC*	PE*	GLC peak	Molecular species	Phospholipid classes PC*	PE*
		Mole %				Mole %	
101	16:0-16:0	3.3		126	18:0-20:5	0.7	2.0
102		0.8		127	16:0-22:5 + 16:0-22:6	4.8	7.9
103	16:0-18:0	0.2	0.1	128	18:1 20:5 + 18:2-20:4	0.2	0.6
104	16:0-18:1	5.7	1.6	129-135		1.5	4.1
105	16:0-18:2	19.4	3.3	136	18:0-22:4	0.4	0.8
107-109		2.1	0.7	138	18:1-22:4	0.3	0.7
110	18:0-18:1	1.1	1.9	139		0.2	0.3
111	18:1-18:1	1.2	1.3	140	18:0-22:5 + 18:0-22:6	1.5	6.6
112	18:0-18:2	4.6	6.7	141	18:1ω9-22:5 + 18:1ω9-22:6	1.0	3.3
113	16:0-20:4+	21.3	11.1	142	18:1ω7-22:5 + 18:1ω7-22:6	0.3	0.8
	18:1-18:2 + 16:0-20:3	6.0	3.2	143-148		0.9	1.2
115	17:0br-20:4	0.8	0.2	149	20:0-22:5 + 20:0-22:6	0.2	0.6
116	16:0-20:5 + 17:0br-20:4	2.1	1.1	150	20:1-22:5 + 20:1-22:6		0.3
117	17:0-20:4	1.0	0.7	152-153	44	1.1	0.7
118-121		1.2	0.6		46		0.1
122	18:0-20:4	6.7	21.8	Other		0.7	2.2
123	16:0-22:4	3.0					
124	18:1ω9-20:4	3.8	13.5				
125	18:1ω7-20:4	1.9		% PL class		3.6	71.8

br, branched

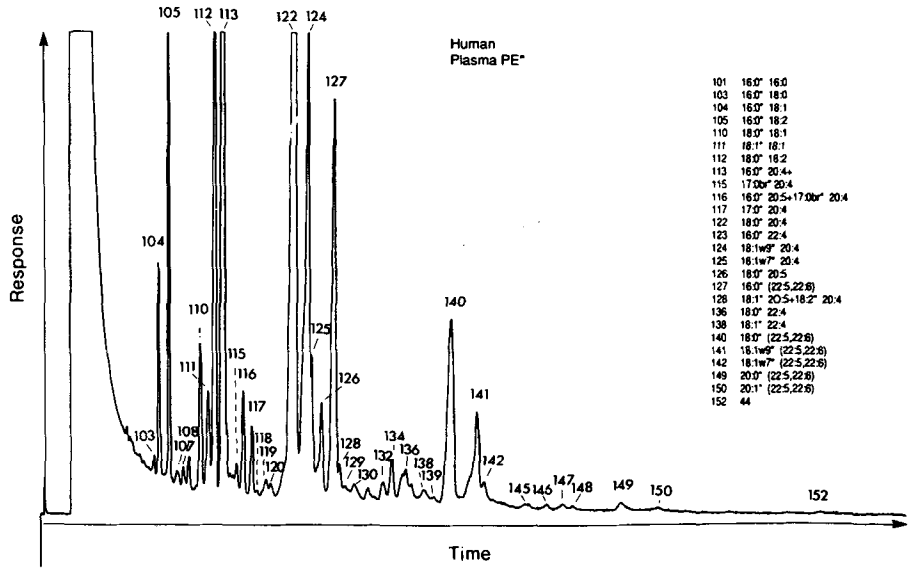

FIG. 10. Polar capillary GLC profile of the alkenylacylglycerol moieties of human plasma diradyl GPE. Peak identification as given figure. GLC conditions as given in Figure 3. Sample: 1 μl of 0.1% diradylglycerol TMS ethers in hexane.

from human plasma isolated from the same subject and of the molecular species of SPH isolated from different lipoprotein classes. Previously only the major diacyl GPC species had been identified (13,14) in pooled or mismatched samples, whereas the analyses of plasma diradyl GPE (15) and GPI (10) had been confined to the estimation of the total lipid amount and fatty acid composition. The presence of ether-linked phospholipids, especially the high proportion of the alkenylacyl GPE, has not been appreciated. The characteristic composition of the molecular species of the diacyl GPI, however, could have been approximately predicted from the fatty acid composition (10). The presence of phospholipids having *trans*-monoenoic alkyl and alkenyl chain in both plasma and erythrocytes presumably arises from dietary *trans*-fatty acid precursors. The metabolic consequences of such substitution, if any, are unknown. We had previously reported (9) the ceramide carbon numbers of the VLDL, LDL and HDL fractions, but molecular species had been given for only 4 of the total of 12 carbon numbers. In another study (4) the ceramide carbon numbers of VLDL, LDL and HDL were determined for subjects on saturated

TABLE 7

Quantitative Composition of Fatty Acids and Dimethylacetals of the Alkenylacyl Glycerophospholipids of Human Plasma

Fatty chains	PC″ Fatty acids	PC″ Dimethylacetals	PE″ Fatty acids	PE″ Dimethylacetals	Fatty chains	PC″ Fatty acids	PC″ Dimethylacetals	PE″ Fatty acids	PE″ Dimethylacetals
	Mole %		Mole %			Mole %		Mole %	
16:0	9.7	66.9	2.3	27.4	20:3ω6	3.7		2.1	
16:1ω9		1.9	0.1	1.4	20:4ω6	33.2		42.7	
16:1ω7					20:5ω6	1.0		2.6	
17:0		2.0	1.3	2.1	22:0				0.6
18:0	3.0	15.3	0.7	40.4	22:1ω9				0.6
18:1t				3.0	22:4ω6	2.7		2.3	
18:1ω9	8.1	10.6	5.9	16.3	22:5ω6	1.0		1.1	
18:1ω7	0.7	3.3	0.1	3.7	22:5ω3	2.1		4.7	
18:2	29.3		17.8		22:6ω3	5.0		16.5	
20:0		tr		2.7	24:0				0.2
20:1ω9				1.2	24:1				0.3
20:2ω6	0.5		0.6						

TABLE 8

Molecular Species of Sphingomyelins of Different Lipoprotein Classes of Human Plasma

Molecular species	Lipoprotein classes Chylos[a]	VLDL[a]	VLDL[b]	LDL[b]	HDL_3[b]	Molecular species	Lipoprotein classes Chylos[a]	VLDL[a]	VLDL[b]	LDL[b]	HDL_3[b]
			Mole %						Mole %		
32:0	0.052	0.043	0.037	0.040	0.070	38:0	0.066	0.072	0.127	0.119	0.071
d16:1-16:0	1.382	1.279	0.976	1.073	0.170	d16:1-22:0	0.910	1.035	1.186	1.714	2.016
d16:1-16:1	0.061	0.054	0.018	0.006	—	d16:1-22:1	0.085	0.074	0.126	0.167	0.083
d17:1-15:0	0.010	0.005	—	0.007	0.029	d17:1-21:0	0.048	0.040	0.100	0.038	0.057
d18:1-14:0	0.750	0.688	0.501	0.607	1.002	d18:1-20:0	1.862	2.054	1.429	2.209	2.280
d18:2-14:0	0.155	0.111	0.057	0.062	0.310	d18:1-20:1	0.120	0.084	0.125	0.154	0.133
33:0	0.089	0.004	0.011	—	0.010	d18:2-20:0	0.879	0.953	0.755	0.792	1.716
d16:1-17:0	0.082	0.070	0.024	0.009	0.030	d18:2-20:1	0.31	0.128	0.051	0.107	0.144
d17:1-16:0	1.461	1.387	0.951	0.491	0.404	39:0	0.017	0.014	0.018	0.010	0.070
d17:1-16:1	0.031	0.027	0.012	0.003	0.015	d16:1-23:0	0.350	0.344	0.512	0.707	0.605
d18:1-15:0	0.086	0.080	—	0.034	0.052	d16:1-23:1	0.067	0.013	0.041	0.049	0.125
d18:2-15:0	0.050	0.030	0.001	—	0.020	d17:1-22:0	0.485	0.384	0.510	0.612	0.622
34:0	0.678	0.676	1.169	1.052	0.383	d17:1-22:1	0.054	0.050	0.078	0.085	0.095
d16:1-18:0	1.909	2.018	1.813	1.324	1.540	d18:1-21:0	0.269	0.229	0.150	0.249	0.239
d16:1-18:1	0.285	0.267	0.190	0.190	0.220	d18:2-21:0	0.190	0.139	0.090	0.089	0.343
d17:1-17:0	0.010	0.053	0.058	0.001	0.028	40:0	0.060	0.042	0.16	0.17	0.04
d18:1-16:0	30.830	29.899	30.453	28.519	17.301	d16:1-24:0	0.169	0.262	0.315	0.723	1.132
d18:1-16:1	0.394	0.392	0.266	0.305	0.297	d16:1-24:1	1.274	1.314	1.499	1.929	2.628
d18:2-16:0	4.104	3.784	3.251	2.217	3.330	d17:1-23:0	0.403	0.425	0.239	0.340	0.438
d18:2-16:1	—	—				d17:1-23:1	0.068	0.098	0.018	0.073	0.047
35:0	0.173	0.025	—	0.019	0.160	d18:1-22:0	5.804	5.062	5.479	8.052	8.835
d16:1-19:0	0.246	0.148	—	—	0.100	d18:1-22:1	0.115	0.161	0.636	0.655	0.417
d17:1-18:0	0.671	0.541	0.729	0.540	0.433	d18:2-22:0	1.934	1.821	2.363	2.241	4.716
d17:1-18:1	0.118	0.046	0.067	0.031	0.008	d18:2-22:1	0.472	0.460	0.388	0.620	0.849
d18:1-17:0	0.940	0.610	0.751	0.828	0.242	41:0	0.014	0.006	—	0.009	—
d18:2-17:0	0.190	0.160	0.064	0.081	0.201	d17:1-24:0	0.365	0.338	0.440	0.487	0.620
36:0	0.225	0.267	0.274	0.200	0.171	d17:1-24:1	0.554	0.549	0.694	0.688	0.996
d16:1-20:0	0.953	1.105	1.168	1.001	0.905	d17:1-24:2	0.051	0.064	0.053	0.076	0.082
d16:1-20:1	0.121	0.098	0.077	0.109	0.026	d18:1-23:0	2.130	2.033	2.633	3.020	2.810
d18:1-18:0	5.766	6.229	4.668	4.165	2.975	d18:1-23:1	0.075	0.219	0.307	0.264	0.384
d18:1-18:1	0.646	0.737	0.690	0.539	0.674	d18:2-23:0	0.777	0.751	0.893	0.814	1.463
d18:2-18:0	2.502	2.487	1.808	1.326	1.941	d18:2-23:1	0.227	0.161	0.080	0.142	0.244
d18:2-18:1	0.338	0.337	0.213	0.159	0.308	42:0			0.042	—	0.015
37:0	0.032	0.041	0.060	0.026	0.029	d18:1-24:0	5.186	4.817	5.046	6.306	5.233
37:1	0.776	0.885	0.740	0.670	0.471	d18:1-24:1	12.885	12.907	12.945	12.076	13.420
37:2	0.202	0.184				d18:1-24:2	0.376	0.551	0.948	0.987	2.043
						d18:2-24:0	2.277	2.130	3.200	1.595	4.588
						d18:2-24:1	3.406	4.076	3.394	3.234	4.799
						d18:2-24:2	0.890	0.648	0.325	0.401	—
						43	nd	nd	1.5	1.5	1.8

[a]Postprandial plasma of a normolipemic subject other than the one supplying the erythrocyte diradylglycerophospholipids.
[b]Fasting plasma from three different normolipemic subjects other than the one supplying the erythrocyte diradylglycerophospholipids.
nd, Not detectable.

and unsaturated fat diets, but molecular species were reported only for LDL. Neither of the previous publications gave the composition of the chylomicron SPH. The present study shows that the chylomicrons and VLDL possess SPH species, which are closely similar to those of LDL and differ from those of HDL by a lower proportion of the longer chain lengths.

The present study also permits the first systematic comparison of the molecular species of GPL and SPH of erythrocytes and plasma isolated from the same blood. The identification and quantitation of the species is based on polar capillary GLC resolution performed in parallel on purified subclasses and total diradylglycerol moieties of the GPL. The method allowed a resolution of most species on the basis of carbon number and degree of unsaturation, as well as of certain positional and geometric double bond isomers. The determined composition of the molecular species was found to be consistent with the independently measured fatty acid methyl ester, dimethylacetal and alkylglycerol composition.

Although the qualitative compositions are similar, marked differences are seen among the quantitative proportions of both major and minor molecular species between the corresponding plasma and red cell GPL, excluding extensive equilibration. The quantitative differences are as great between the choline and ethanolamine GPL within plasma or cells, as between the corresponding GPL of plasma and cells. However, the similarity in the composition of long chain saturated and unsaturated alkyl groups in the alkylacyl GPC from plasma and red cells suggests a common origin. In contrast to the other GPL, the diacyl GPI of plasma and cells possess closely similar qualitative and quantitative composition of molecular species. The serine phosphatide, which was detected in measurable amounts only in the red cells, possessed molecular species that differed greatly from those of all other GPL, and were characterized by a high proportion of the polyunsaturated long chain length species. The use of whole plasma GPL for the comparison is justified on the basis of the known similarity of the major molecular species of choline GPL in the different lipoprotein classes (16). The use of GPL pooled from the inner and outer halves of the red cell membrane is justified on the basis of the known equilibration of the major diacyl GPC and GPE species between the inner and outer halves of the erythrocyte membranes (17). Such an equilibration, however, does not appear to exist for the SPH either between the inner and outer half of the red cell membrane (18) or among the different lipoprotein classes (4,19).

Red cells could be a potential source of longer chain SPH species in HDL, but a comparison of species indicates that it is unlikely. For C_{42} the ratio of d18:1 to d18:2 containing species is slightly lower in HDL (2.3) than in LDL (3.7). If red cells were the source of elevated C_{42} (d18:1/d18:2 = 8.4) an increase of this ratio rather than the observed decrease would be anticipated. The same argument applies to the ratio of d18:1 24:0 to d18:1 24:1. The chain length differences in SPH between HDL and LDL, therefore, must be attributed to the origin of these lipoproteins in liver and intestine, respectively (20).

The major differences in the quantitative and to a lesser extent in the qualitative composition of the GPL species between plasma and red cells can be explained on the basis of differences in the metabolic origin of the species and the absence of significant mass equilibration among the GPL of plasma and red cells. The present results do not exclude, however, selective exchanges of major (21) or nonselective exchange of minor species. The study would appear to eliminate red cell PE as the only source of plasma PE, including the alkylacyl and alkenylacyl subclasses. Dietary fatty acids affect plasma lipids much more rapidly and extensively than the red cell lipids (22), which are subject to much slower turnover (23). Some of the molecular species of GPL and SPH are clearly derived from the clearance of intestinal VLDL and chylomicrons (24), whereas other GPL species are derived from the nascent HDL secreted by liver (25). The GPL transformations resulting from the activity of plasma lipoprotein (26) and hepatic (27) lipases, and lecithin-cholesterol acyltransferase (28) must also be considered. Although the intestinal GPL is relatively rich in the alkylacyl and the alkenylacyl species (29), the liver GPL is essentially free of them (30).

Although the detailed comparisons were performed on blood from one subject only, these results were representative of the GPL analyses from other subjects. The present analyses are of interest in view of the increasing importance of the minor species of GPL in lipoprotein and cell membrane structure and function. A more complete assessment of the origin of the GPL and SPH of plasma must await studies of the kinetics of appropriately labeled molecular species of GPL and SPH.

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Modulating Influence of Dietary Lipid Intake on the Prostaglandin System in Adult Men

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We evaluated the effect of moderate dietary changes on the prostaglandin system by measuring the urinary excretion of 7α-hydroxy-5,11-dioxo-tetranorprostane-1,16-dioic acid (PGE-M). In a crossover design, twenty-four free-living male subjects in good health (24 to 54 years of age) were fed two diets: (i) Regular (R) diet, 41% energy (en%) from fat, P/S 0.59, M/S 0.96; (ii) Experimental (E) diet, 19 en% from fat, P/S 1.31, M/S 1.48. Diet R contained 13.9 g/day of neutral detergent fiber (NDF) and about 600 mg/day of cholesterol per 3200 kcal; Diet E contained 35.5 g/day NDF and about 280 mg/day cholesterol. Each controlled-diet period lasted ten weeks. The menu cycle was 7 days, and all diets were calculated to provide adequate amounts of essential nutrients. The PGE-M excretion rates were determined in 24-hr urine by stable-isotope dilution gas chromatography-mass spectrometry in the selected ion-monitoring mode. Low-fat Diet E, with an intake of 6.6 en% from polyunsaturates, was associated with an average 14.2% reduction in PGE-M daily output, compared to high-fat Diet R with a 9.3 en% from polyunsaturates ($P = 0.046$). These results support the view that dietary lipid changes can significantly alter the *in vivo* production of E-series prostaglandins. We cannot conclude, however, if this apparent diet effect was brought about by the change in linoleate intake alone or was the result of complex biochemical interactions among individual fatty acids, both saturated and unsaturated.
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The molecular basis of nutritionally-induced changes in cardiovascular parameters is a subject of considerable interest. According to the "eicosanoid hypothesis," the putative vascular effects of dietary polyunsaturates result from associated alteration of the endogenous production of prostaglandins. Thus, a quantifiable diet/prostaglandin link is of interest to clinicians, nutritional biochemists and epidemiologists. Progress in this area has been hindered by the limited availability of highly sensitive and specific analytical methods for the quantification of appropriate markers in complex biological matrices.

Biosynthesis of eicosanoids ultimately depends on the availability of their immediate precursors: arachidonate (AA, 20:4ω6), dihomo-gamma-linolenate (DGLA, 20:3ω6), and eicosapentaenoate (EPA, 20:5ω3). Only EPA can be introduced in significant amounts through the diet or through fish oil supplementation. AA and DGLA, the precursors of diene and monoene prostaglandins (PG), respectively, are derived from elongation and desaturation of linoleate (LA, 18:2ω6) which is plentiful in a conventional "Western" diet. That lipid intake should influence PG biosynthesis *in vivo* seems, in general, a reasonable expectation. Direct dietary effects have indeed been demonstrated with arachidonate (1) and eicosapentaenoate (2–5). In contrast, the consequences of dietary LA manipulations on the PG system are much less predictable (6,7). It is probably so because many enzymes are involved in the conversion of linoleate to DGLA and AA. Such enzymes are influenced by several factors, nutritional as well as hormonal (8,9). It has been shown recently in a rat study that *ex vivo* PG biosynthesis can be modulated by dietary alterations involving fatty acids which are non-PG precursors, e.g., saturated fatty acids (10). These observations have enhanced interest in unraveling the complex relationships among dietary fat, eicosanoid production and physiologic effects.

Because present dietary modifications recommended to the general public for the prevention of atherothrombotic disorders and cancer include reduction of total fat and replacement of saturated fats with polyunsaturates, we deemed it relevant to evaluate the comparative effects of two types of diets on the activity of the prostaglandin system in a group of twenty-four healthy male volunteers. As a chemical marker of such activity we selected the whole-body turnover of E-series prostaglandins ($E_1 + E_2$) which was measured by the urinary excretion rate (μg/24 hr) of 7α-hydroxy-5,11-dioxo-tetranorprostane-1,16-dioic acid (PGE-M). Prominent among the physiological properties of PGE_2 is its powerful influence on myocardial and coronary circulation and on peripheral microvessels (11). Thus, any agent that can modify the PGE_2 biosynthetic level is expected to produce an effect on important cardiovascular variables. The two diets considered in this study differ in their percent of energy (en%) from fat, P/S and M/S ratios. Could such dietary manipulation have a quantifiable effect on the prostaglandin system? Based on present knowledge, and given the acute scarcity of similar studies with human subjects, the answer to this question is not obvious. Cholesterol and fiber intakes also differ in the two diets but, for the purpose of this study, they are not regarded as significant variables. This is part of a larger study in which the two diets must approximate, in all their variables, a typical American diet and a diet recommended for prevention of heart attack and cancer.

MATERIALS AND METHODS

Subjects. Twenty-four male volunteers between 24 and 54 years of age (mean 36.1 ± 8.6, SD) were recruited in the area immediately surrounding the Beltsville Agricultural Research Center. The subjects ultimately selected were free from known metabolic abnormalities as determined by a complete physical examination and laboratory tests. None of them had been on a special dietary regimen, e.g. vegetarian, over the previous year, nor had they taken prescription medications, vitamins, aspirin, aspirin-

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Abbreviations: AA, arachidonate; DGLA, dihomo-gamma-linolenate; en%, energy %; EPA, eicosapentaenoate; E, experimental; LA, linoleate; NDF, neutral detergent fiber; PGE-M, 7α-hydroxy-5,11-dioxo-tetranorprostane-1,16-dioic acid; PG, prostaglandin; R, regular.

containing drugs or nonsteroidal anti-inflammatory drugs ten days before the beginning of the study and during the study. Nutrient supplements were also forbidden for the duration of the study. The subjects continued their normal life and normal activities throughout the experiment and were informed of the purpose of the study, procedures to be followed, and samples to be collected. All the procedures were approved by the Human Studies Committees of the U.S. Department of Agriculture, the National Institutes of Health and the Georgetown University Medical School.

Controlled diets. Two diets designated as E (experimental) and R (regular) were formulated from commonly available foods. Diet E contained about 19% of energy (en%) as fat, 35.5 g/day of neutral detergent fiber per 3200 kcal, and a P/S ratio of 1.31. This ratio is more than twice that found in typical Western Diets, and is characteristic of low-risk populations. It has been recommended for prevention of coronary artery disease. Diet R contained about 41 en% as fat, 13.9 g/day of neutral detergent fiber per 3200 kcal, and a P/S ratio of 0.59. This ratio approximates that observed in earlier studies (12) among free-living subjects in the Beltsville area. Tables 1 and 2 show the nutrient contents of two typical diets. The relative distribution of polyunsaturates to saturates and monounsaturates (P:S:M) was approximately 1.31:1:1.48 in the experimental diet and 0.59:1:0.96 in the regular diet. The mean daily cholesterol intake was 280 mg/day on Diet E (3200 kcal) and 600 mg/day on Diet R, with proportional adjustments for different caloric intakes. Total energy input was adjusted to maintain a constant body weight for the duration of the study.

TABLE 1

Composition of a Typical 3200-Kcal Diet: Mean Daily Intake of Nutrients[a]

Nutrient	Diet E	Diet R
Carbohydrate		
g/day	537.6	366.7
en%	67.2	45.8
Lipid		
g/day	67.2	144.7
en%	18.9	40.7
Protein		
g/day	136.8	118.4
en%	17.1	14.8

[a]Calculated from a 7-day menu cycle.

TABLE 2

Fatty Acid Content of a Typical 3200-Kcal Diet[a]

Fatty acid	Diet E	Diet R
Total saturated		
g/day	18.1	51.6
en%	5.1	14.5
Oleic (18:1ω9)		
g/day	26.6	49.7
en%	7.5	14.0
Linoleic (18:2ω6)		
g/day	21.7	30.6
en%	6.1	8.6
α-Linolenic (18:3ω3)		
g/day	2.0	1.9
en%	0.6	0.6

[a]Minor fatty acid constituents: arachidonic (20:4ω6), 0.12 g/day; eicosapentaenoic (20:5ω3), 0.02–0.1 g/day.

We used a seven-day menu cycle to provide variety and maintain acceptability of the diets. All nutrients for which food data are available were provided by the diets in amounts to meet the Recommended Dietary Allowances (13). Nutrient compositions of the diets were calculated by using the Lipid Nutrition Laboratory food database, which provided the latest data on food composition from the USDA, together with data provided by the food industry, the Nutrient Coding Center in Minneapolis, and analysis. The controlled-diet meals were prepared in the Human Studies Facility of the Beltsville Human Nutrition Research Center (BHNRC). On weekdays, all meals were eaten in the Center's dining facility. Meals for Saturdays, Sundays and holidays were prepacked and distributed for home consumption. No food other than what was provided by the study was permitted, and alcohol consumption was not allowed during the controlled-diet periods. Consumption of coffee, tea, and water was unrestricted.

Experimental design. The study employed a crossover design with two phases involving controlled diets: after a pre-diet free-choice period (baseline) lasting two weeks, twelve subjects were placed on the E Diet and the other twelve on the R Diet for ten weeks (phase 1); phase 2 was another ten-week controlled-diet period (switchover from the diet in phase 1). After phase 1 there was a ten-day holiday break during which the volunteers consumed self-selected diets. Subjects in the two groups were balanced on the basis of their body mass index.

Urine collection. Twenty-four-hour urine was collected in silanized glass bottles and kept on ice during the collection period, three times during week 2 of the pre-diet free-choice period and three times during week 10 of phases 1 and 2. After the 24-hr collection was completed, the volumes were measured, portions were taken (*vide infra*), and either analyzed immediately or stored at −22°C for a few days.

Measurement of PGE-M. Two-percent portions of each 24-hr collection, in a given week, were pooled. A 20-ml aliquot of the resulting mixture was analyzed for 7α-hydroxy-5,11-dioxo-tetranorprostane-1,16-dioic acid (PGE-M) to assess the mean daily turnover of prostaglandins $E_1 + E_2$ *in vivo* during the 72-hr period. We used stable isotope dilution mass spectrometry in the selected ion-monitoring mode. Procedures and instrumentation have been described (14). When storage of urine specimens lasted more than one day, PGE-M values were adjusted to compensate for decay according to a first-order rate constant determined in our laboratory (15). PGE-M excretion rates are all expressed as μg/24 hr.

Statistical methods. Twenty-four-hour PGE-M excretion rates and lipid intakes were evaluated by linear regression and by paired t tests with the computer methodology of the Statistical Analysis System (SAS Institute, Inc., Cary, NC). Multiple linear regression with backwards elimination was used to evaluate the potential influence of height, weight, and baseline PGE-M

levels on the differences observed between individual PGE-M levels on Diet E and Diet R.

RESULTS

One subject withdrew for personal reasons at the end of phase 1 and another withdrew later for health reasons leaving 22 subjects for analysis. Before conducting further statistical analyses, we first determined that the order in which the subjects went on the controlled diets (R or E first) did not have an effect on the results. The mean of PGE-M urinary excretion was 13.44 ± 1.63 (SEM) μg/24 hr after ten weeks on controlled Diet R and 11.53 ± 1.24 (SEM) μg/24 hr after ten weeks on Diet E. Paired t testing indicated a significant reduction in the urinary marker after ten weeks on the experimental diet (difference = 1.91 ± 0.90 (SEM) μg/24 hr, $P = 0.046$). Values for individuals are shown graphically in Figure 1 for each of the 22 individuals who completed both study diets. The mean PGE-M excretion rates of subjects who switched from Diet E to Diet R were 11.61 and 13.57 μg/24 hr, respectively; those of subjects who switched from Diet R to Diet E were 13.28 and 11.44 μg/24 hr, respectively. In multiple regression models, weight was shown to have a significant effect on the difference in PGE-M levels between diets: lighter weight individuals (weight range 56.3–75.8 kg) showed greater reduction (16% vs 1%) in PGE-M levels on Diet E compared to Diet R than heavier individuals (weight range 77.4–108.2 kg).

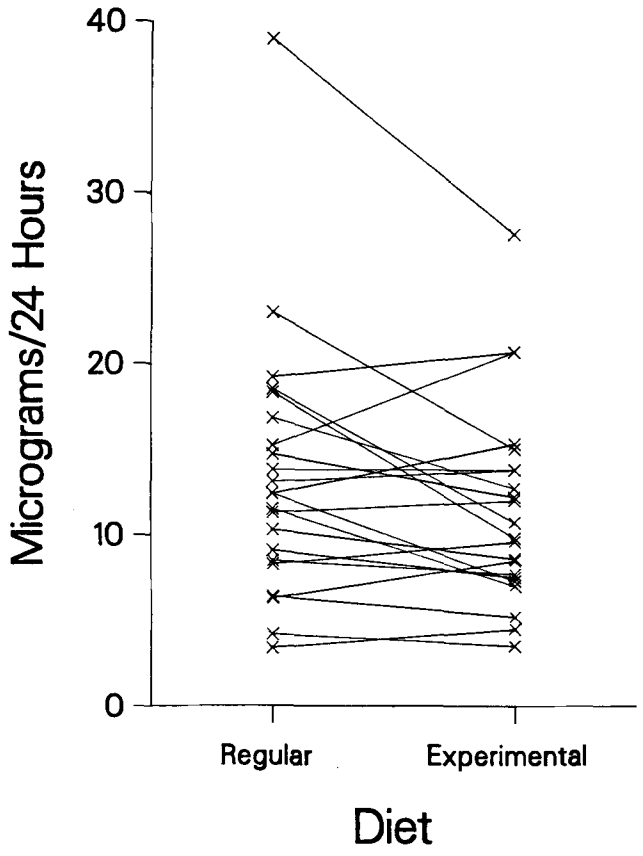

FIG. 1. PGE-M urinary excretion rates of the 22 individuals who completed both study diets.

DISCUSSION

This study was conducted to determine if relatively minor dietary modifications can significantly influence the systemic production of E-series prostaglandins as measured by the urinary excretion rate of 7α-hydroxy-5,11-dioxotetranorprostane-1,16-dioic acid (PGE-M). Because plasma fatty acids do not represent the PG precursor pool (6,7,17–19), we omitted their determination. The most direct and meaningful means of assessing *in vivo* prostaglandin production is by measurement of their urinary catabolites (20). The PGE-M excretion rate provides a measure of the aggregate endogenous synthesis of prostaglandins E_2 and E_1 (21). This methodology neglects the contribution from kidney and seminal vesicles to whole-body turnover of PGE. The rationale for disregarding such contribution has been discussed (22).

Pioneering studies by Zöllner *et al.* (23), Nugteren *et al.* (24) and Adam *et al.* (25,26) demonstrated the possibility of nutritionally altering the endogenous biosynthesis of primary prostaglandins in humans. Those studies, however, involved very substantial variations of the levels of linoleate intake. Similarly, a more recent study by Adam and Wolfram (27) compared the effects of linoleate intake of 0 and 20% energy on the excretion of tetranorprostanedioic acid, an analytical artifact which has been proposed as an index of systemic turnover of primary PG of both the E and F series (28). In contrast, the objective of our study was to compare the effect of a typical American diet (R) with that of a diet which has been recommended for the prevention of coronary artery disease. The main finding here is that—while all the subjects were presumably in a PUFA nondeficient status—variations in lipid intake profile (see Table 3) brought about a measurable and statistically significant change in the biosynthetic level of E prostaglandins. In a recent human diet study from this laboratory (22) we could not detect an effect on the PGE-M excretion rate when two levels of linoleate intake (10 and 30 g/day) were considered in a crossover design in which the energy from fat was maintained constant at 35%. That preliminary study, however, was conducted with only four volunteers. Moreover, the analytical method we used then to measure PGE-M (29) did not afford the same level of accuracy as the method used in this study (14).

We must consider the possibility that the apparent diet effect on the prostaglandin system observed in this study may have not been brought about solely by the different linoleate intakes in the two diets. As shown in Table 3, the intake of monounsaturates in Diet R is twice that in Diet E, while the level of saturates in Diet R is three times that in Diet E. The intake of polyunsaturates (93% of which is linoleate) in the "regular" diet is only 1.4 times

TABLE 3

Energy Percent Contributed by Various Lipid Classes[a]

Lipid class	Diet E	Diet R
Polyunsaturates (P)	6.6	9.3
Saturates (S)	5.0	16.0
Monounsaturates (M)	7.4	15.4

[a]Calculated from data of Table 2.

the level in the "experimental" diet, namely 9.3 en% versus 6.6 en%. It would indeed be extraordinary if the only driving force behind the modulation of the endogenous PGE production were such a modest change in 18:2ω6 intake. Results of whole animal nutritional studies and of biochemical studies performed at the enzyme or subcellular levels have led to the hypothesis, now widely accepted, that competitive inhibition may occur among all dietary unsaturated fatty acids, i.e., those of the ω3, ω6 and ω9 groups, at each step of their metabolic transformation (6,9). Specifically, each of the most important members of dietary unsaturates—linoleic, oleic and α-linolenic—inhibits the elongation and desaturation of the other (30,31). Interactions among saturated and unsaturated fatty acids present in the diet and in the metabolic pool have also been demonstrated (32). The nature of our study does not permit us to conclude whether we are observing the direct and simple effect of linoleate intake or the cumulative effect of complex interactions. Perhaps the more important point to keep in mind is that a diet very similar to the typical American diet is associated with a higher biosynthetic rate of E-series prostaglandins compared to a diet with fewer fatty calories and a higher P/S ratio. The lowering of systolic blood pressure in conjunction with enhanced intakes of polyunsaturates observed in several studies (33,34) may have been brought about at least in part by alteration of the eicosanoid system, possibly involving the E prostaglandins. The effect of a nutritionally-induced alteration of the thromboxane to prostacyclin ratio in those studies cannot be ruled out.

Dietary fiber has been shown to affect cholesterol absorption and metabolism (35). But, to our knowledge, there is no report suggesting a fiber-modulating influence on the prostaglandin system. The effect of dietary fiber in this study was probably insignificant. Similarly, a direct correlation between dietary cholesterol and eicosanoid synthesis *in vivo* has not been demonstrated.

ACKNOWLEDGMENTS

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Eicosapentaenoic and Arachidonic Acid: Comparison of Metabolism and Activity in Murine Epidermal Cells

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The biological activity, including metabolism and modulation of ornithine decarboxylase activity and DNA synthesis, of arachidonic acid (AA) and eicosapentaenoic acid (EPA) were compared in epidermal cells from SENCAR mice. Radiolabelled AA and EPA were found to be similarly incorporated into and released from membrane phospholipids of unstimulated cultures. However, when cells were stimulated with the tumor promoter 12-0-tetradecanoylphorbol-13-acetate (TPA), the release of AA was significantly higher than the release of EPA. The extent of metabolism of AA and EPA to prostaglandins was determined in both freeze-thawed cell preparations and in viable cultured cells. In the freeze-thawed preparations, use of AA as a substrate resulted in significantly more PGF than when EPA was used as the substrate. However, more PGE_3 was formed than PGE_2. PGD levels were the same for either fatty acid precursor. Prostaglandin production was also determined in viable cultured cells since other influences such as phospholipase A_2 activity can modify prostaglandin production. Control cultures prelabelled with either AA or EPA produced similar amounts of the respective PGF, PGE, and PGD. However, TPA-stimulated cultures produced significantly higher amounts of each prostaglandin in cultures prelabelled with AA compared to cells prelabelled with EPA. HETE or HEPE production was the same both for cultured cells prelabelled with AA or EPA and for homogenates from uncultured cells incubated directly with the radiolabelled fatty acids. TPA-induced ornithine decarboxylase (ODC) was significantly higher in AA-treated cultures compared to EPA-treated cultures. AA supports DNA synthesis to a greater extent than EPA, either alone or in the presence of TPA. These findings suggest that AA and EPA do not have equivalent biological activity in mouse epidermal cells.

Lipids 24, 423–429 (1989).

The type of fat in the diet has been shown to modulate tumor development in several organs (1,2). In particular, increasing the amount of dietary linoleic acid (18:2, n-6) is correlated with increased incidence and yield of rat mammary tumors (3,4). In contrast to the tumor enhancement associated with this n-6 fatty acid, animals fed diets high in linolenic acid (18:3, n-3) and other n-3 fatty acids demonstrate reduced tumorigenesis in several animal models (5–8).

Increases in prostaglandin E_2 (PGE_2), a product of arachidonic acid (AA), which is derived from elongated and desaturated linoleic acid, have been associated with increased tumor incidence (9), suggesting that n-6 eicosanoids are potential modulators of tumorigenesis. It has also been reported that the promotion stage of carcinogenesis is particularly sensitive to prostaglandin levels (10,11 and reviewed in 12). The multistage skin carcinogenesis model has been used extensively to study the regulation of tumor development since the initiation and promotion stages can be operationally separated. The promotion stage is achieved by repetitive treatments with tumor promoters, such as the phorbol ester 12-0-tetradecanoylphorbol-13-acetate (TPA), following the application of a subthreshold dose of carcinogen. Hyperproliferation, inflammation and induction of ornithine decarboxylase (ODC) are hallmarks of TPA promotion (13), and are, in part, mediated by the cyclooxygenase and/or lipoxygenase products derived from AA (14, reviewed in 12). This is supported by the evidence that indomethacin and other anti-inflammatory inhibitors of AA metabolism inhibit both TPA promotion and TPA-induced ODC (10,11, 15,16). Verma and Boutwell (16) further demonstrated that exogenous application of PGE_1 or PGE_2 could overcome the indomethacin inhibition of TPA induced ODC. These observations support the hypothesis that AA metabolites are important mediators of the TPA-induced inflammatory and proliferative responses (17).

Eicosapentaenoate (20:5) (EPA), an n-3 fatty acid found in fish oil, competes with AA for incorporation into cell membrane phospholipids *in vivo* (18,19) and *in vitro* (20, 21). Partial substitution of AA by EPA in membrane phospholipids should therefore lead to the replacement of AA-derived cyclooxygenase and lipoxygenase products (2-series eicosanoids) with EPA-derived metabolites (3-series eicosanoids). Competitive inhibition of AA metabolism by EPA has also been indicated in platelets (22). Not only would less AA-derived products be available but the EPA-derived eicosanoids may be less biologically active (23).

Based on the above considerations, we hypothesized that murine epidermal cells would differ in their response to EPA and AA with regard to incorporation and metabolism. In addition, we postulated that the cyclooxygenase metabolites formed from EPA would not be as biologically active as AA-derived prostaglandins in influencing specific responses associated with tumor promoter treatment of epidermal cells. This paper compares several parameters of the biological activity of AA and EPA in murine epidermal cells including: 1) the rate of incorporation into and release of these fatty acids from membrane phospholipids; 2) the amount of PGE, PGD, and PGF produced by cells pre-labelled with each fatty acid; 3) the extent of production of the lipoxygenase products; and 4) the effect of the two fatty acids on TPA-induced ODC activity and DNA synthesis.

MATERIALS AND METHODS

Chemicals. TPA was obtained from Life Systems (Newton, MA) and diluted in acetone. Purchased from

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Abbreviations: AA, arachidonic acid; EPA, eicosapentaenoic acid; TPA, 12-0-tetradecanoylphorbol-13-acetate; ODC, ornithine decarboxylase; HETE, hydroxyeicosatetraenoic acid; HEPE, hydroxyeicosapentaenoic acid; PG, prostaglandin; TLC, thin layer chromatography.

Amersham Corp. (Arlington Heights, IL) were [1-^{14}C]-arachidonic acid, [1-^{14}C]-eicosapentaenoic acid (specific activity of both is 59.6 mCi/mmol), [^{3}H]-AA, [^{3}H]-EPA (specific activities, 94.5 Ci/mmol and 79.0 Ci/mmol respectively), [1-^{14}C]-ornithine hydrochloride (specific activity, 55 mCi/mmol) and [methyl-^{3}H]-thymidine (specific activity, 6 Ci/mmol). Authentic prostaglandin and phospholipid standards were purchased from Sigma Chemical Co. (St. Louis, MO) and the 5-, 8-, 12- and 15-HETE standards from Caymen Chemical (Ann Arbor, MI). Silica Gel 60-254 (0.25 mm; Merck, Darmstadt, West Germany) was used for thin layer chromatograph (TLC). Solvents and other chemicals were of the highest quality commercially available.

Cell isolation. Inbred newborn SENCAR mice (24) were obtained from the Science Park - Veterinary Division breeding colony. Intact epidermal cells were isolated from either 1- or 2-day-old newborn mice by the trypsinization procedure of Yuspa and Harris (25). For culture experiments, the cells (basal-like) were plated at a density of 2×10^6 per 35 mm tissue culture dish for arachidonate or eicosapentaenoate metabolite analysis or at a density of 5×10^6 per 60 mm tissue culture dish for ODC assays. The plating medium was an enriched Waymouth's, containing 10% fetal bovine serum; the growth medium was an enriched MCDB 151 (referred to as SPRD 110), containing 0.1% bovine serum albumin but no serum, as described by Morris *et al.* (26). Following plating, the cultures were incubated at 37°C in 5% CO_2 for 24 hr prior to use in the assays described below.

Freeze-thawed cells. For experiments using non-viable cells, isolated epidermal cells were frozen at −70°C prior to incubation with radiolabelled fatty acids.

Phospholipid extraction and analysis. Incorporation of labelled fatty acids into phospholipids was determined by replacing the plating medium with SPRD-110 medium containing radiolabelled AA or EPA (0.10 μCi/ml media) for 12 hr. At designated times the medium was aspirated from the cells, the cell layer immediately washed once with cold saline, and frozen at −70°C. The thawed cells were scraped into 1.0 ml methanol and the phospholipids extracted by adding 3 ml of chloroform (chloroform:methanol, 3:1, v/v) and 1 ml 2M KCl; the chloroform:methanol extracts were dried under vacuum. Reconstituted extracts were separated by TLC together with authentic standards using a solvent system of chloroform:methanol:acetic acid (65:25:4; v/v/v) (27). Standards were identified with iodine vapors and the corresponding sample phospholipid bands were then scraped and counted by liquid scintillation.

Competition of AA and EPA for incorporation into phospholipids was determined by incubating cultured epidermal cells with SPRD 110 medium containing 0.25 μg/ml AA and EPA and tracer amounts (2.5 μCi or 9 ng/ml) of either ^{3}H-AA or ^{3}H-EPA for designated incubation times. The phospholipids were extracted and quantitated as described above.

Release of the fatty acids from the phospholipids was analyzed by labelling cultures with either ^{3}H-AA or ^{3}H-EPA in SPRD 110 (0.1 μCi/ml medium) after which the labelled media was replaced with fresh SPRD 110 containing either acetone or TPA (1.0 μg/ml). At designated times, the cells were harvested from the plates and phospholipid extraction and quantitation were performed as described above.

Prostaglandin synthesis. To determine whether AA and EPA were metabolized to the same extent by epidermal prostaglandin synthetase, freshly isolated cells (28×10^6) were freeze-thawed several times. The broken cell preparations were incubated with either ^{14}C-AA or ^{14}C-EPA for 1 hr in SPRD 110 and the prostaglandins extracted twice with 3 ml of ethyl acetate. The extraction efficiencies were usually greater than 90%. The pooled extracts were dried under vacuum, redissolved in ethyl acetate, and applied with standards to thin-layer silica gel plates. The plates were developed with the organic phase of ethyl acetate: iso-octane:acetic acid:water (28:13:5:25, v/v/v/v) (28). The metabolites were detected by staining with iodine vapors, and then scraped and counted. Autoradiograms were made to ascertain that the AA and EPA metabolites co-chromatographed.

To determine prostaglandin production from viable cells, cultures were labeled for 12 hr, previously determined to be the time of maximum incorporation into epidermal phospholipids by Fischer *et al.* (29), in SPRD 110 containing ^{3}H-AA or ^{3}H-EPA (0.1 μCi/ml). Following replacement with fresh medium containing either acetone or TPA (1 μg/ml), the medium was collected at the appropriate time points and acidified to approximately pH 3 with 1 N HCl and extracted as described above.

Lipoxygenase activity. Freshly-isolated epidermal cells were homogenized as previously described (30) and centrifuged at $12,500 \times g$ for 10 min, followed by centrifugation of the supernatant for 60 min at $105,000 \times g$. This supernatant was used for lipoxygenase assays. ^{14}C-AA or ^{14}C-EPA (0.5 μCi) was added to the reaction assay containing 1 mg protein/ml Tris buffer (29). After 30 min incubation time at 37°C the reaction was stopped by acidification, and ethyl acetate extraction was performed as above. The HETEs were separated by TLC using a solvent system of diethyl ether:hexane:acetic acid (60:40:1; v/v/v).

Ornithine decarboxylase induction. These assays were performed on viable epidermal cultures treated for 7 hr with solvent (acetone) or TPA. A $12,000 \times g$ centrifugation was performed on homogenized scraped cells, using a Na/K-phosphate buffer containing pyridoxal phosphate. The supernatant was used to determine enzyme activity, as described by Weeks and Slaga (31), and the total protein was measured by the Coomassie blue reaction (Bio-Rad, Richmond, CA).

DNA synthesis. Twenty-four hours after plating epidermal cells at 1.5×10^6/35-mm tissue culture dish, the cultures were treated with 0.1 μg/ml either AA or EPA in the presence or absence of 0.1 μg/ml TPA in SPRD-110 medium. The specific activity of DNA synthesis was measured at the given times after treatment by determining the amount of ^{3}H-thymidine incorporated per μg DNA after a 1 hr pulse label with 2 μCi/ml, using the Schmidt-Thannhauser isolation procedure (32) and Burton assay (33).

Statistical analysis. The data were analyzed using the Student's t-test.

RESULTS

Phospholipid analysis. Since incorporation of fatty acids into cell membrane phospholipids is the first step in fatty acid metabolism, it was important to compare rates of

incorporation of both fatty acids into epidermal cell phospholipids and to determine whether incorporation occurred equally within the same phospholipid classes. Uptake of both ^{3}H-AA or ^{3}H-EPA into total phospholipids increased over time at similar rates and to the same extent, as indicated in Figure 1. Uptake was nearly maximal by 4 hr. As shown in Table 1, incorporation into specific phospholipid classes was similar for both fatty acids.

In order to determine whether EPA could compete equally with AA for incorporation into phospholipids, epidermal cells were incubated with both AA and EPA. Measurement of incorporation of tracer amounts of either ^{3}H-AA or ^{3}H-EPA in the presence of unlabelled fatty acids showed that EPA was incorporated into phospholipids at a slightly higher, although not significant ($p > 0.05$), level at 1, 3, or 6 hr as shown in Figure 2. Overall, AA and EPA appear to be equally competitive for incorporation into epidermal phospholipids.

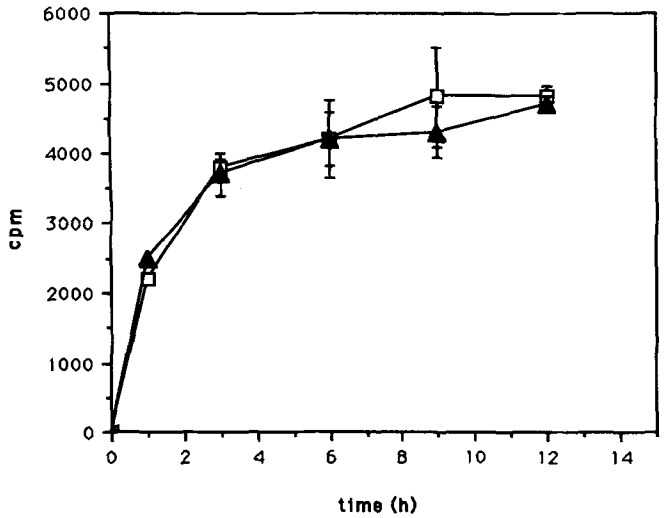

FIG. 1. Rate of incorporation of ^{3}H-AA or ^{3}H-EPA into total phospholipids of cultured mouse epidermal cells. Each point represents cultures (n = 2 to 4) of 2 × 10^6 cells grown for up to 12 hr in serum-free medium containing 0.15 μCi of AA or EPA. Total phospholipids were extracted at the designated times and counted. Results are expressed as mean cpm ± std. dev. of ^{3}H-fatty acid incorporated into 2 × 10^6 cells from two experiments. □, ^{3}H-AA; ▲, ^{3}H-EPA.

TABLE 1

Incorporation of Arachidonate and Eicosapentaenoate into Epidermal Cell Phospholipids

	% total incorporation	
	AA	EPA
PC	19.8 (15.4-24.2)	19.4 (16.4-22.4)
PE	48.3 (46.2-50.3)	50.1 (46.1-54.1)
PI/PS	30.4 (28.1-32.6)	28.5 (22.7-35.9)
Other	1.6 (1.5- 1.7)	2.1 (1.6- 2.5)

Epidermal cells were labelled with either ^{14}C-AA or ^{14}C-EPA (0.1 μCi/ml) for 15 hr. Phospholipids were extracted, separated by TLC and counted. Values represent the means of two experiments (the ranges are shown in parentheses). PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI/PS, phosphatidylinositol and phosphatidylethanolamine (comigration); Other, tentatively identitied as sphingomyelin.

Release of either ^{3}H-AA or ^{3}H-EPA from phospholipids was also found to be similar for both fatty acids (Fig. 3) in unstimulated cultures. However, when the cultures were treated with TPA, ^{3}H-AA prelabelled cells released this fatty acid at a faster rate and to a higher extent than the cells prelabelled with ^{3}H-EPA (Fig. 3).

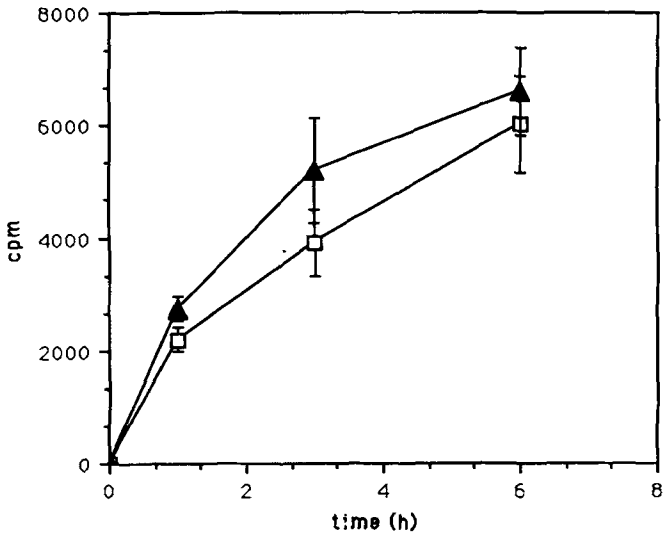

FIG. 2. Competition of ^{3}H-AA and ^{3}H-EPA for incorporation into total phospholipids of mouse epidermal cells as a function of time. Cultures of 2 × 10^6 cells were incubated with tracer amounts of radiolabelled AA or EPA and 0.5 μg/ml of unlabelled EPA and AA respectively. Each point represents the mean cpm ± std. dev. in the extracted phospholipids from four cultures in a representative experiment. □, ^{3}H-AA; ▲, ^{3}H-EPA.

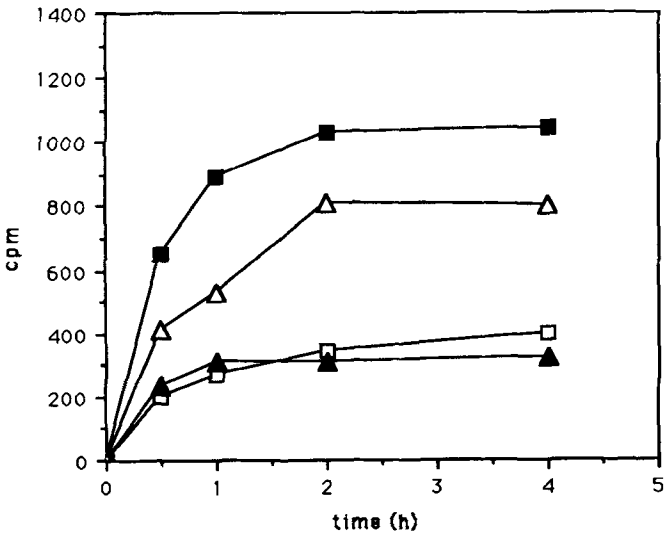

FIG. 3. Rate of release of incorporated ^{14}C-AA and ^{14}C-EPA from phospholipids of solvent control or TPA-stimulated murine epidermal cell cultures. Each point represents the mean of cultures of 2 × 10^6 cells labelled as in Figure 1. Following treatment for the designated times with either solvent (acetone) or TPA (1 μg/ml), the medium was removed and counted. The points are from a representative experiment. The average fold increase in release of radiolabel from acetone-treated cultures at 4 hr was statistically significant from TPA-treated cultures for both fatty acids ($p = 0.002$, n = 10 from 6 independent experiments). □, AA; ▲, EPA; ■, AA-TPA; △, EPA-TPA.

By 4 hr, TPA induced an average increase over control of 3.6 fold for AA and 2.4 fold for EPA prelabelled cultures. This difference in release between AA and EPA was statistically significant ($p < 0.002$).

Prostaglandin synthesis. In order to determine if epidermal prostaglandin-synthesizing enzymes use AA and EPA as substrates equally well, epidermal cells were freeze-thawed (to eliminate competing incorporation processes), and incubated with the appropriate fatty acid, and prostaglandins F, E and D quantitated by liquid scintillation. Autoradiography revealed that PGF was not formed as efficiently from EPA as from AA: the percent PGF production of the total prostaglandin synthesized from EPA was 15.8% compared to 32.9% for AA-derived prostaglandins (Fig. 4). However, EPA appears to be a slightly better substrate for PGE production: synthesis of PGE from EPA was 66.3% while PGE derived from AA was 51.0%. PGD production was found to be equally efficient with either EPA or AA (17.9% and 16.1% respectively).

The products of the cyclooxygenase pathway were also compared between viable cultures prelabelled with ^{3}H-AA or ^{3}H-EPA (Fig. 5). At 4 hr, the major cyclooxygenase metabolite in either solvent- or TPA-treated cultures was PGE followed by PGD and PGF. TPA-treated AA prelabelled cultures produced 8.2 times more PGE than control AA cultures. This is a statistically significant increase ($p < 0.001$; $n = 10$ from five independent experiments). Similarly, cultures prelabelled with EPA and stimulated with TPA produced more prostaglandins than control EPA cultures but the difference was not significant. Both AA-control and AA-TPA cultures produced more PGE than the respective EPA-control cultures or EPA-TPA cultures; the difference between AA and EPA cultures which were stimulated with TPA was significant ($p < 0.001$).

Lipoxygenase activity. HETE and HEPE production were similar in homogenates using either labelled AA or EPA as substrate (Fig. 6): 65% of the HETE products consisted of 12- and 15-HETE for AA, while 67% of the HEPE products were the 12- and 15-HEPE. Both fatty

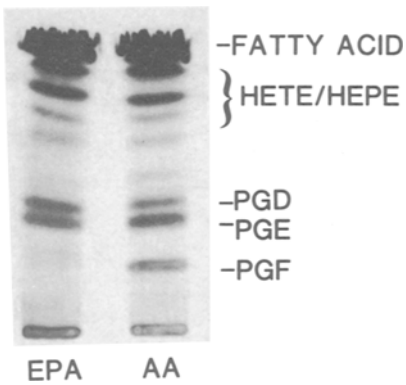

FIG. 4. Autoradiogram of prostaglandin metabolites from either ^{14}C-AA or ^{14}C-EPA incubated with freeze-thawed epidermal cells. The major sample metabolites were identified by co-chromatography with authentic standards and the position of the sample metabolites verified by autoradiography. The experiment was performed twice.

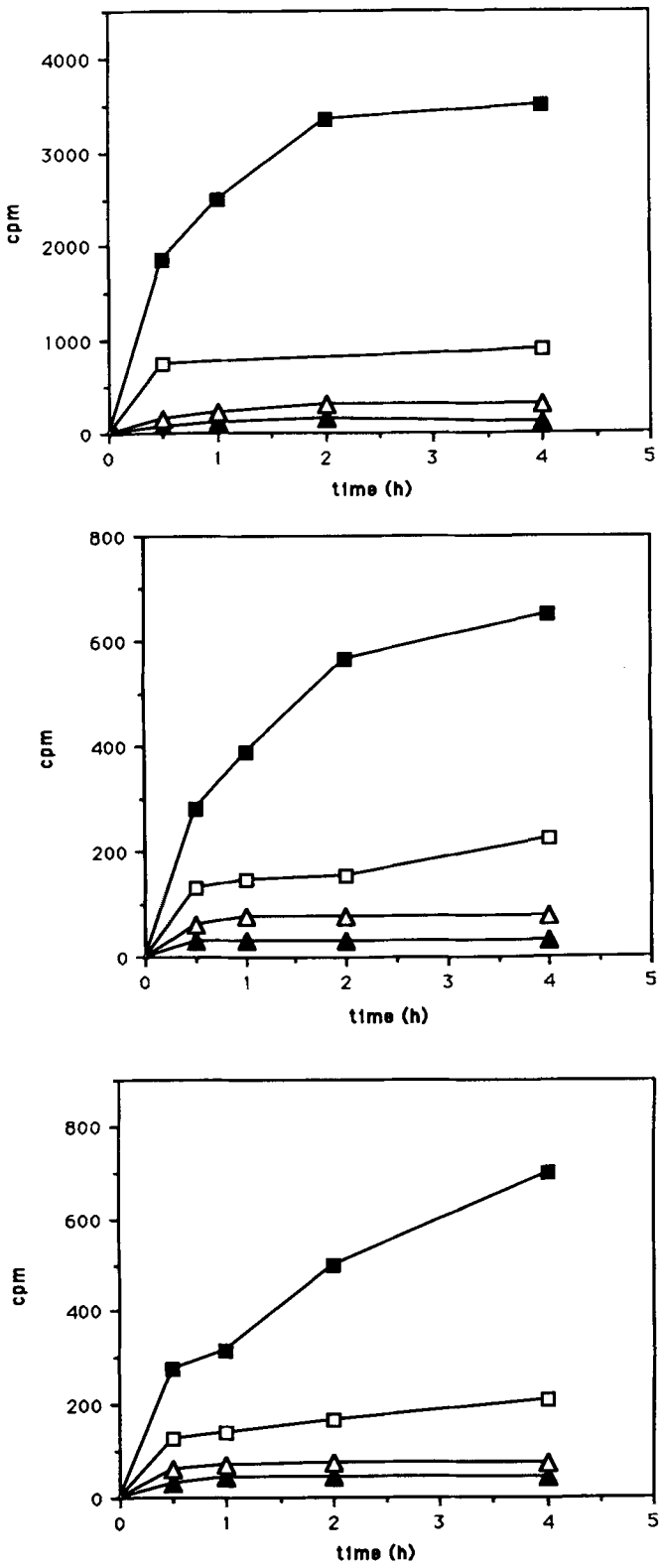

FIG. 5. Prostaglandin synthesis in solvent control and TPA-stimulated cultured epidermal cells. Primary cultures were prelabelled as described with either ^{3}H-AA or ^{3}H-EPA and were treated with either acetone or 1 μg/ml TPA. Cultures were terminated at times specified and the metabolites, PGE (top panel), PGF (middle panel) and PGD (bottom panel) separated by thin-layer chromatography and counted. Five separate experiments were performed. Values represent mean cpm from a typical experiment, performed in duplicate. □, AA; ■, AA-TPA; ▲, EPA; △, EPA-TPA.

acids produced 2% or less of the 5- and 8-hydroxy fatty acids. HETE production in prelabelled cultured cells was also the same as HEPE production (data not shown).

Ornithine decarboxylase activity. The induction of ODC activity by TPA is at least partially dependent on the production of PGE_2 (21). It was therefore of interest to determine whether EPA-mediated PGE_3 production would support TPA-stimulated ODC activity to the same extent as arachidonate-derived PGE_2 (Table 2). ODC activity was not found to differ significantly between cells cultured with either fatty acid in the acetone-control group. A comparison of the (AA + TPA) vs (AA) groups shows a seventeen-fold increase in experiment 1 and a sixteen-fold increase in experiment 2. A similar comparison of the (EPA + TPA) and (EPA) groups shows eight- and twelve-fold increases, respectively. The difference in TPA-induced ODC activity between the two fatty acid groups was statistically significant ($p < 0.01$).

DNA synthesis. Comparisons were made between the specific activity of DNA synthesis in epidermal cells grown in the presence of either AA or EPA, with or without TPA. Employing standard medium formulations, an initial experiment (data not shown) indicated that at 1 μg/ml EPA was toxic since it caused a large number of cells to slough from the culture dishes; AA did not have this effect. For this reason, subsequent DNA synthesis experiments used 0.1 μg/ml for both fatty acids. A three-day incubation time was chosen since it was previously determined that this was optimum for TPA. As shown in Figure 7, AA supported DNA synthesis to nearly twice the level observed with EPA ($p = 0.01$). The DNA content of all dishes was the same and no toxicity was observed. The level of DNA synthesis in the presence of TPA was also significantly higher ($p = 0.01$) for AA than EPA. The effect of TPA on AA was also significantly different ($p = 0.01$); however, TPA did not significantly enhance DNA synthesis in EPA cultures.

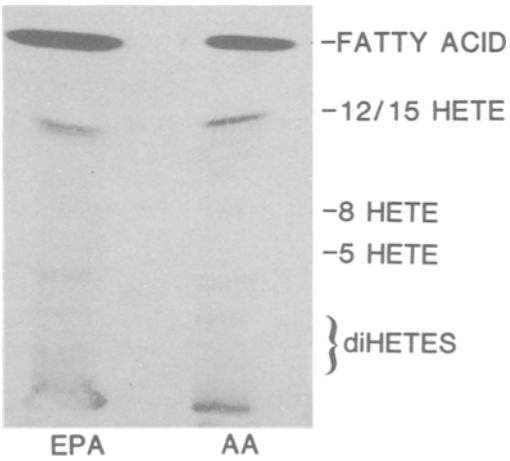

FIG. 6. Autoradiogram of HETE/HEPE synthesis from cytosolic preparations of murine epidermal cells incubated with ^{14}C-AA or ^{14}C-EPA. The major products were identified by co-chromatography, with authentic standards and the position of the sample metabolites verified by autoradiography. The experiment was performed twice.

TABLE 2

Effect of AA versus EPA on TPA-induced ODC Activity in Murine Epidermal Cells *in vitro*

Treatment	nmol CO_2/mg protein/hr[a]	
	Experiment 1	Experiment 2
Control	0.079 ± 0.039	0.070 ± 0.032
TPA (1 μg/ml)	0.424 ± 0.089	0.827 ± 0.195
AA[b]	0.107 ± 0.031	0.054 ± 0.023
AA + TPA	1.309 ± 0.238[c]	1.118 ± 0.243[c]
EPA	0.095 ± 0.042	0.059 ± 0.022
EPA + TPA	0.777 ± 0.215[c]	0.688 ± 0.125[c]

[a]ODC activity.

[b]Fatty acids were used at 1 μg/ml media, complexed to fatty acid free BSA prior to addition to serum free medium. Values represent the mean ± std. dev. of 2 experiments with 4 to 6 dishes each per treatment group.

[c]The difference in ODC activity between AA + TPA and EPA + TPA was statistically significant ($p < 0.01$; student's t-test) for both experiments.

DISCUSSION

Incorporation of AA and EPA into phospholipids appears to be equivalent in non-stimulated murine epidermal cultures with respect to rate and class of phospholipid. Our findings are similar to those found by Morita *et al.* (20) where incorporation of either fatty acid was equal in

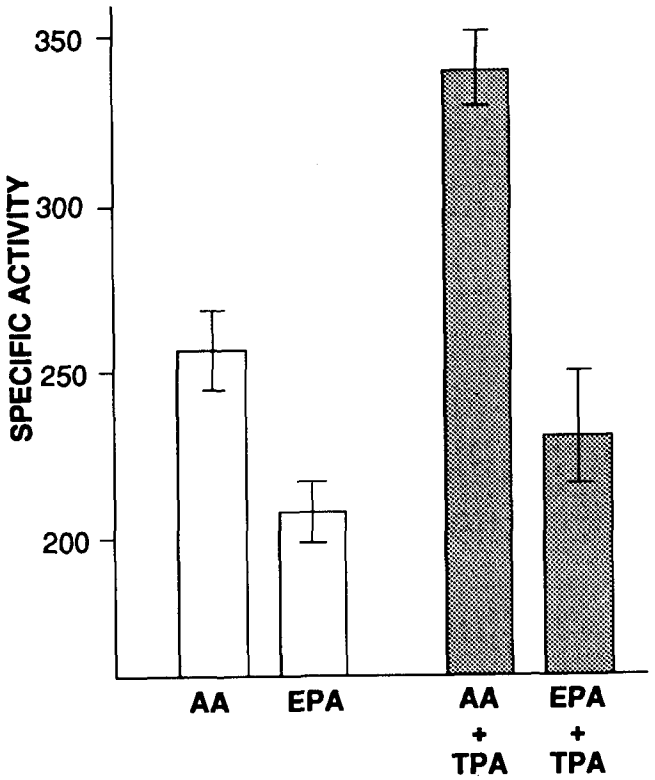

FIG. 7. DNA synthesis in epidermal cells cultured with either AA or EPA. The specific activity of ^{3}H-thymidine incorporation was determined 3 days after continuous treatment of cultures with either 0.1 μg/ml AA or EPA alone or in the presence of 0.1 μg/ml TPA. Values represent the mean ± SEM (n = 3 to 4).

aortic smooth muscle cell cultures. However, Morita *et al.* (20) and Weaver and Holub (21) found that platelets preferentially incorporate AA, suggesting that the extent of incorporation is cell-type specific.

Release of either AA or EPA from phospholipids of non-stimulated epidermal cells was also similar. However, when the cultures were stimulated with TPA, a phorbol ester which activates phospholipase A_2 (34), AA was released from the phospholipids to a greater extent than EPA, suggesting that phospholipase A_2 has a preference for phospholipids containing AA.

To determine if the prostaglandin-synthesizing enzymes can use AA or EPA equally as a substrate, in the absence of the competing reaction of incorporation into phospholipids, freeze-thawed cells were used to study metabolism *in vitro*. We found that PGF was produced to a greater extent when labelled-AA was the substrate. In contrast, PGE production was slightly more efficient with EPA as the substrate. Lands and Byrnes (35) also found less prostaglandin production from EPA when compared to AA. Their data support our findings that in general EPA is not as easily enzymatically oxygenated under the same conditions in which AA is rapidly oxidized.

Viable cell cultures were then used to determine if metabolism was different in living cells in which incorporation and other regulatory processes (i.e. phospholipase A_2 activity) are ongoing. Flower and Blackwell (28) suggest that substrate availability for prostaglandin synthetase is controlled by activated phospholipase A_2. We found that TPA-treated AA-cultures produced more PGE compared to the EPA-TPA cultures. The higher amount of this oxygenated product in the AA-TPA cultures is probably due to more AA liberated and available for cyclooxygenase activity, an effect of TPA treatment. We found similar results with PGD and PGF indicating that even with TPA stimulation, EPA is not as readily converted to cyclooxygenase products as is AA. Similar findings were reported by Magrum and Johnson (36) in studies on zymosan-activated macrophages cultured in the presence of either AA or EPA.

In vivo studies have also demonstrated that diets high in n-3 fatty acids are associated with a reduction in PGE production (37). In the present study, the possible inhibitory effect of EPA on total PGE production was not measured. However, other investigators have found that n-3 fatty acids, including linolenate and eicosatrienoate, can inhibit prostaglandin production (38–40). Mohrhauer and Holman (41) suggest that the inhibition of PGE production by linolenic acid is due to an inhibitory effect of linolenic acid on the conversion of linoleic acid to AA.

When prostaglandin synthesis is inhibited with agents such as indomethacin, the lipoxygenase products can become the predominant oxygenated metabolites (42). Our data indicate that lipoxygenase activity was similar in cytosol preparations to which AA or EPA were added directly, as well as in prelabelled, viable cultured cells. It has been previously reported (43) that 5-HETE synthesis in rat leukocytes was greater in rats fed EPA compared to animals fed AA. Analysis of HETE production is important since high amounts of these lipoxygenase products are associated with the hyperproliferative disease, psoriasis (44). In the mouse skin carcinogenesis model, the lipoxygenase products have been shown to be required in several TPA responses. Specifically, lipoxygenase inhibitors have been reported to reduce TPA tumor promotion in several strains of mice (45–48). These same inhibitors will also depress TPA-induced ODC activity (45–47). In addition, when indomethacin, a cyclooxygenase inhibitor, was applied to TPA-treated skin of SENCAR mice, an enhancement of tumor promotion was observed (15). It was suggested that this was due to inhibition of cyclooxygenase activity which resulted in a shunting of the liberated AA to the lipoxygenase pathway, thereby increasing HETE production.

Verma and Boutwell (16) have shown that indomethacin also inhibited the induction of ODC by TPA in mouse epidermis in a dose-response manner. When exogenous PGE_2 was added back, the inhibitory effect of indomethacin on ODC activity was overcome, suggesting that ODC activity is at least partially mediated by the eicosanoids derived from AA. We found that epidermal cells grown in EPA did not support as large an induction of ODC as cells cultured with AA, further supporting the hypothesis that cyclooxygenase metabolites of AA are involved in ODC induction. This function of AA metabolites on ODC induction is significant to the tumor promotion process since inhibitors of ODC induction have also been shown to inhibit tumor promotion (reviewed in 17).

The induction of ODC is usually followed by elevated polyamine production, which is thought to be a factor in the stimulation of DNA synthesis. On this basis it is reasonable to expect that agents that support higher levels of ODC induction would also support higher levels of DNA synthesis. Our results indicate that this correlation holds for AA and EPA.

Our *in vitro* results indicate that incorporation of either AA or EPA into phospholipids is equal. Since numerous dietary studies have shown that the fatty acid composition of membrane phospholipids of various organ systems can be altered by changing the types of fatty acids in the diet (18,19,37), it should also be possible to alter epidermal membrane composition. Because our results indicate that TPA-induced ODC activity and DNA synthesis are significantly lower in EPA-stimulated cultures, it can be predicted that tumor incidence should be lower in animals fed n-3 enriched diets compared to animals fed diets high in n-6 fatty acids. Studies are currently underway in our laboratory to determine whether such diets do alter the fatty acid composition of epidermal cells, as well as altering the level of cyclooxygenase metabolites and affecting tumor incidence in a multistage carcinogenesis model. A combination of *in vitro* and *in vivo* studies will be useful in elucidating the relationship of specific dietary fatty acids to the modulation of cancer development.

ACKNOWLEDGMENT

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Dipyridamole Inhibits Lipid Peroxidation and Scavenges Oxygen Radicals

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Dipyridamole incubated *in vitro* in systems which peroxidize fatty acids inhibited arachidonic acid, gamma-linolenic acid and linoleic acid peroxidation. In the xanthine-xanthine-oxidase system, which produces superoxide anion, dipyridamole inhibited the reduction of cytochrome-C in a dose-dependent fashion. In systems generating hydroxyl radicals, dipyridamole reduced deoxyribose degradation in a dose-dependent manner. The study suggests that dipyridamole inhibits lipid peroxidation, probably by scavening oxygen free radicals.
***Lipids* 24, 430-433 (1989).**

Dipyridamole is an antiplatelet drug, shown to interrupt platelet consumption in experimental thrombotic models and to prolong platelet survival time in patients with cardiovascular diseases (1-3). The mechanism of action of dipyridamole is yet to be understood. However, elevation of platelet cyclic-AMP and blood adenosine have been proposed to explain its antithrombotic effect (1, 4, 5). Dipyridamole has been shown to possess an antioxidant property as it inhibits *in vitro* lipid peroxidation (6, 7), but the mechanism of its antioxidant action has not been investigated. Biological molecules such as unsaturated fatty acids, sugars, proteins, aminoacids and catecholamines undergo degradative changes mediated by oxygen free radicals, via superoxide (O_2^-) and hydroxyl radicals (OH•) (8—10). Therefore, the present study is aimed at understanding the antioxidant action of the dipyridamole on peroxidation of unsaturated fatty acids, degradation of deoxyribose, and reduction of cytochrome-C, induced by different free radical-generating agents in model *in vitro* systems.

MATERIALS AND METHODS

Methyl esters of arachidonic acid, linoleic acid and gamma-linolenic acid, arachidonic acid sodium salt, cytochrome-C (horse heart, type VI), and butylated hydroxytoluene (BHT) were obtained from Sigma Chemical Co. (St. Louis, MO). Bovine liver superoxide-dismutase and bovine milk xanthine-oxidase were obtained from Boehringer Mannheim GmbH (Mannheim, West Germany) and Calbiochem-Behring (San Diego, CA), respectively. Desferrioxamine and all other reagents of highest quality were available from Ciba-Geigy (Basel, Switzerland) and Merck (Darmstadt, West Germany), respectively. Dipyridamole was a gift from Boehringer Ingelheim (Reggello, Italy). Deionized, double-distilled water was used throughout this investigation.

UV-Visible radiation studies were performed on a Hewlett Packard 8452A Diode Array Spectrophotometer.

Methyl esters of arachidonic acid (40 μM), linoleic acid (50 mM) and gamma-linolenic acid (1 mM) were dispersed in 1 ml 0.5 M phosphate buffer (pH 6.0) by vortexing for 30 seconds. Peroxidation was induced by subjecting these dispersion systems to continuous UV light (Philips TL 40W tube, placed 40 cm above) for 60 min at 37°C in open air.

Sodium arachidonate (100 μM) was dissolved in 10 mM NaH_2PO_4 medium (pH 4.5) containing 10 μM Na_2CO_3. Lipid peroxidation in this system was induced by 38 μM ascorbic acid and 8 μM Fe(II), as ferrous ammonium sulphate, upon incubation for 2 hr at 37°C. While studying the effects of dipyridamole and/or BHT separately, the incubation medium contained 5 μM dipyridamole or 10 mM BHT. Simultaneous controls were established which contained equal amounts of ethanol that served as the solvent of dipyridamole and BHT. At the end of the incubation period, lipid peroxidation in the fatty acid systems was assayed by the thiobarbituric acid (TBA) reaction method according to Cordova et al. (11) as follows: the reaction mixture was mixed with 0.5 ml trichloroacetic acid (TCA) (20% w/v) and 1.0 ml TBA (1% w/v). The solution was mixed thoroughly, boiled for 10 min, cooled to room temperature and the absorbance of the chromophore was measured at 532 nm against an appropriate blank. The extent of peroxidation in the fatty acid system was expressed as moles malondialdehyde (MDA)/mole fatty acid (12).

Effect of dipyridamole on O_2^--mediated reduction of cytochrome-C was studied according to Fridovich (13). The reaction mixture contained 50 mM KH_2PO_4 (pH 5.4), 50 μM xanthine, 30 nM xanthine-oxidase 0.1 mM EDTA, and various concentrations of dipyridamole (30-300 μM) in a final volume of 3.0 ml. Reduction of cytochrome-C was followed by the change in absorbance at 550 nm at 37°C.

Effect of dipyridamole on Fe(II)- and Fe(III) + H_2O_2 + ascorbate-induced deoxyribose degradation was studied according to Halliwell and Gutteridge (14), Gutteridge (15), and Winterbourn (16). The system in which Fe(II) was used as a radical-generating agent contained 1.5 mM 2-deoxy-D-ribose in 1.0 ml of 63 mM NaCl and 44 mM NaH_2PO_4 (pH 4.5). Reaction was initiated by the addition of ferrous ammonium sulphate (244 μM, final concentration).

The system in which Fe(III) + ascorbate + H_2O_2 was used as a radical-generating agent contained 2.8 mM 2-deoxy-D-ribose in 1.0 ml of 10 mM NaH_2PO_4 buffer (pH 4.5). The reaction was initiated by 15 μM Fe(III) as ferric chloride, 100 μM ascorbate, and 1.44 mM H_2O_2. The reaction medium contained various concentrations of dipyridamole. The reaction was carried

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Abbreviations: BHT, butylated hydroxytoluene; EDRT, endothelium-derived relaxing factor; EDTA, ethylene diamine tetraacetic acid; LOOH, lipid hydroperoxide; MDA, malondialdehyde; TBA, thiobarbituric acid; TCA, trichloroacetic acid.

out for 15 min at 37°C. The degradation of deoxyribose was assayed according to Halliwell and Gutteridge (14) as follows: at the end of the incubation period, 1.0 ml of TCA (2.8% w/v) and 1.0 ml of TBA (1% w/v in 50 mM NaOH) were added to the incubation mixture, mixed thoroughly, and boiled for 10 min in a water bath. The pink chromophore formed was measured at 532 nm against a suitable blank. The extent of deoxyribose degradation was expressed as absorbance unit/time.

All the data were subjected to statistical analysis. Significance between two means was calculated employing the Student's t-test.

RESULTS

Lipid peroxidation. UV-induced lipid peroxidation in the fatty acid systems was significantly inhibited by dipyridamole (5 μM) and BHT (10 mM) (Fig. 1) and no further inhibition was observed at higher concentrations of dipyridamole. A similar trend was observed in the Fe(II)/ascorbate system where 5 μM dipyridamole significantly inhibited arachidonate peroxidation (52% inhibition) and did not appear to change upon increasing the concentration of dipyridamole (Fig. 2).

Superoxide anion-mediated reduction of cytochrom-C. The O_2^- flux generated by the xanthine-xanthine-oxidase system was monitored by following the rise in absorbance at 550 nm due to the reduction of cytochrome-C. The effect of added dipyridamole on the absorbance changes was analyzed in terms of a simple competition between dipyridamole (inhibitor) and cytochrome-C (substrate for the O_2^- generated). Data were arranged graphically according to Dixon (17) where the reverse of velocity is plotted against the concentration of inhibitor, keeping the substrate constant (Fig. 3). The plot, where each line represents an experiment made at a fixed substrate concentration, clearly demonstrates a competitive inhibition. Interferences of dipyridamole with the xanthine-xanthine-oxidase system were excluded by measuring urate production at 290 nm in the absence of cytochrome-C. In the system the rate of cytochrome-C reduction was not influenced by catalase (25 μg/ml), but was fully prevented by superoxide-dismutase (5 μg/ml).

Hydroxyl radical-dependent deoxyribose degradation. The influence of dipyridamole on OH•-generating systems is shown in Figure 4. Dipyridamole inhibited the deoxyribose degradation in a dose-dependent manner; when OH• was generated by the direct addition of Fe(II) salts to the reaction mixture containing phosphate buffer, according to the following equations:

$$\begin{array}{lllll} Fe(II) & + O_2 & \rightarrow Fe(III) & + O_2 \\ 2O_2 & + 2H^+ & \rightarrow H_2O_2 & + O_2 \\ Fe(II) & + H_2O_2 & \rightarrow Fe(III) & + OH^- + OH^{\bullet}, \end{array}$$

the inhibition was 21.8%, 49.5% and 64.7% at dipyridamole concentrations of 19.8 μM, 79.2 μM, 158 μM, respectively. Similarly, dipyridamole inhibited deoxyribose degradation in a dose dependent manner in a system where OH• was generated at a higher rate through a modified metal-catalyzed Haber-Weiss cycle according to the following scheme:

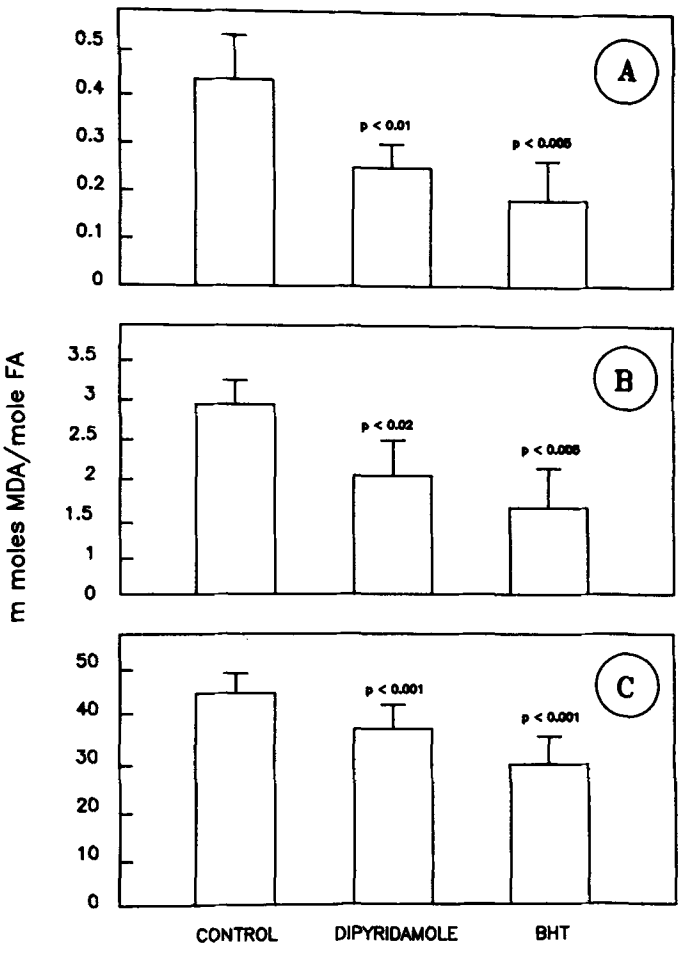

FIG. 1. Effect of dipyridamole and BHT (butylated hydroxytoluene) on UV-induced peroxidation of methyl esters of linoleic acid (A), gamma-linolenic acid (B) and arachidonic acid (C). Control: treated with ethanol (10 μl/ml). Dipyridamole: 5 μM in ethanol. BHT: 10 mM in ethanol. Each value is the average of 5 experiments.

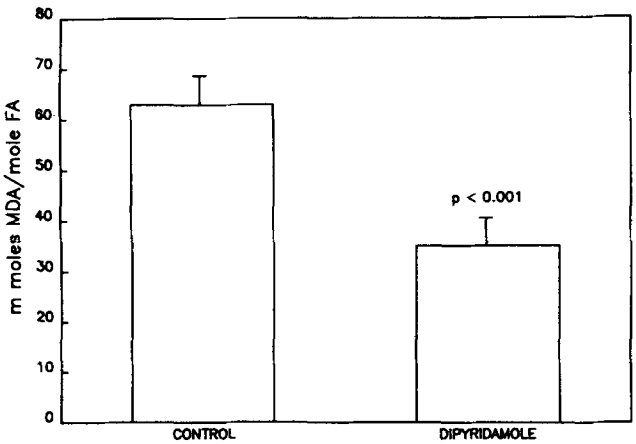

FIG. 2. Effect of dipyridamole on Fe(II) + ascorbate-induced peroxidation of arachidonic acid. Control: treated with ethanol (10 μl/ml). Dipyridamole: 5 μM in ethanol. Each value is the average of 4 experiments.

$$\text{ascorbate} + 2\text{Fe(III)} \rightarrow 2\text{Fe(II)} + \text{dehydroascorbate} + 2\text{H}^+ \quad [1]$$

$$\text{ascorbate} + \text{O}_2 \rightarrow \text{dehydroascorbate} + \text{H}_2\text{O}_2 \quad [2]$$

$$\text{Fe(II)} + \text{H}_2\text{O}_2 \rightarrow \text{Fe(III)} + \text{OH}^- + \text{OH}^\bullet \quad [3]$$

In this pathway ascorbate plays a dual role: it reduces metal ions [1] and generates H_2O_2 [2]. The reduced metal ions react in turn with H_2O_2, giving OH• by a Fenton mechanism [3]. Addition of EDTA to the reaction system removes iron from binding sites in order to produce OH• in "free" solution (15). In this system the inhibition was 15.9%, 31.9% and 43.2% at dipyradamole concentrations of 49.4 μM, respectively.

DISCUSSION

Previous investigations have shown that dipyridamole is an antioxidant that diminishes fatty acid peroxidation in model systems and in tissue culture (6, 7). Our research supports these findings and gives insight into the mechanism leading to inhibition of lipid peroxidation by dipyridamole. Indeed, we have demonstrated that dipyridamole reduces the formation of lipid peroxides in two different in vitro model systems, where fatty acid peroxidation was induced by UV light or by iron/ascorbate.

Lipid peroxidation is a complex chemical process which is started by a highly reactive species (R•), such as O_2^- and OH•. This oxidant (R•) interacts with fatty acids (LH) and, by abstracting hydrogen atoms from their alpha-methylene carbons, gives rise to the formation of a carbon-centered radical; this intermediate compound undergoes a molecular rearrangement to form a conjugated diene (L•) which reacts rapidly with oxygen to give a peroxy radical (LO_2•). The peroxy radical has sufficient energy to abstract a hydrogen atom from another lipid molecule to continue the chain reaction and to convert itself into a lipid hydroperoxide (LOOH):

$$\text{LH} + \text{R}^\bullet \rightarrow \text{L}^\bullet + \text{RH} \quad [4]$$

$$\text{L}^\bullet + \text{O}_2 \rightarrow \text{LOO}^\bullet \quad [5]$$

$$\text{LOO}^\bullet + \text{LH} \rightarrow \text{LOOH} + \text{L}^\bullet \quad [6]$$

In the present investigation we have shown that in three different model systems that produce free oxygen radicals, dipyridamole decreased the detection of both O_2^- and OH• in a dose-dependent fashion.

This effect could be attributed to a scavenging property of dipyridamole, which should inhibit lipid peroxidation by limiting the interaction between fatty acids and oxygen free radicals (reaction [4]).

However, it cannot be excluded that the decrease of lipid peroxides is related to the direct interaction of dipyridamole with intermediate compounds of the lipid peroxidation process such as conjugated diene, peroxy radical or hydroperoxides [5, 6] thus interrupting the autocatalytic chain reaction.

Also, interaction of dipyridamole with metal ions, contained in the model systems, cannot be excluded since lipid hydroperoxides can be decomposed by transition metal ions (M) to give alkoxy (LO•) and peroxy radicals:

$$\text{M}^{(n)+} + \text{LOOH} \rightarrow \text{LO}^\bullet + \text{OH}^- + \text{M}^{(n+1)+} \quad [7]$$

$$\text{M}^{(n+1)+} + \text{LOOH} \rightarrow \text{LOO}^\bullet + \text{H}^+ + \text{M}^{(n)+} \quad [8]$$

The antioxidant activity of dipyridamole could contribute to further explain some of its biological effects. There is increasing evidence that polyunsaturated fatty acids are involved in the control of cell proliferation.

Unsaturated fatty acid intermediates and their derivatives, either of the lipid peroxide pathway or the prostanoid pathway, generate both positive and negative signals for cell proliferation (7). Lipid peroxides

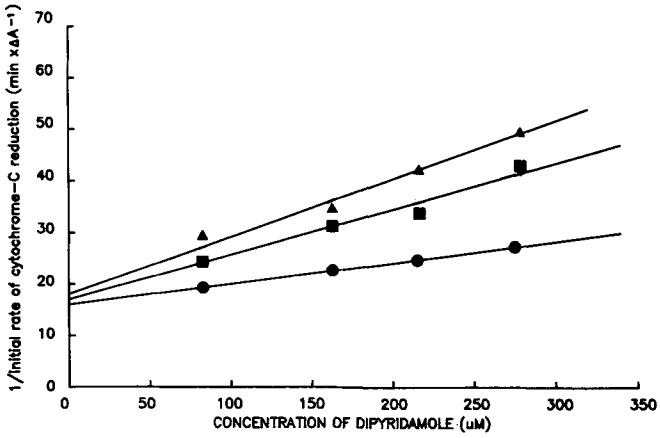

FIG. 3. Effect of dipyridamole on superoxide (O_2^-)-mediated reduction of cytochrome-C. Experiments were carried out at pH 5.40, 37°C, as described in Materials and Methods using horse heart cytochrome-C at a final concentration of ●, 2 μM; ■, 4 μM, and ▲, 6 μM.

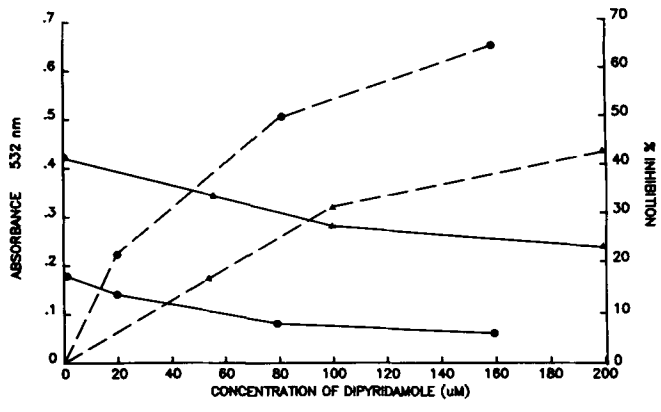

FIG. 4. Effect of dipyridamole on deoxyribose degradation induced by Fe(II) and Fe(III) + ascorbate + H_2O_2. Solid lines: absorbance values. Dashed lines: % inhibition. Circles: Fe(II). Triangles: Fe(III) + ascorbate + H_2O_2. See Materials and Methods for details of incubation conditions and assay procedures.

give inhibitory signals on cell proliferation that can be overcome by antioxidants such as dipyridamole. Thus, dipyridamole was reported to enhance guinea pig SMC proliferation in tissue culture (6), to enhance the proliferative activity of myocardial capillary wall cells (18), and to prolong the survival of experimental skin flaps (19).

The antioxidant properties of dipyridamole may help to explain why this agent enhances (7, 20) prostacyclin synthesis; this was supposed to be due to the protection of prostacyclin synthetase from self destruction by oxygen free radicals (7) or by lipid peroxides (20). This effect could be important in atherosclerotic disease where lipid peroxides accumulate in the vessel plaque and inhibit prostacyclin synthesis (21). The prevention and/or decrease of lipid peroxide accumulation in the endothelium could be the mechanism by which dipyridamole retards the progression of atherosclerotic plaque in patients with peripheral vascular disease (22).

The vasodilating activity of dipyridamole could also be mediated by its antioxidant property. The scavenging of oxygen free radicals by dipyridamole could indeed stabilize endothelium-derived relaxing factor (EDRF), a vasodilating substance which is inactivated by O_2^- (23).

In conclusion, this study provides further evidence of the antioxidant properties of dipyridamole which in turn could contribute to its antithrombotic property.

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Synthesis and Lipase Catalyzed Hydrolysis of Thiolesters of 2-, 3- and 4-Methyl Octanoic Acids

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Racemic 2-, 3- and 4-methyloctanoic acids were synthesized. The 2-mercaptoethanol S-esters of these branched acids, and of octanoic acid, were exposed to lipases of the fungi *Candida rugosa* and *Aspergillus niger*. Reactions were followed spectrophotometrically using Ellman's Reagent. Branching at the 3-position retards lipolysis more severely than at either the 2- or 4-positions. Features of the assay method are discussed with reference to titrimetry.
***Lipids 24,* 434-437 (1989).**

There is considerable interest in enhancing, and understanding the basis for, the several kinds of selectivity (positional, fatty acid and stereo) exhibited by lipases. Recently we synthesized 1,2- and 1,3-dialkylglycerol ethers and developed methods for analyzing their configurations. These compounds and their esters were viewed as complementary to the triglycerides and would avoid some of the ambiguities inherent in the use of the trifunctional substrates (1). As was the case for the major portion of the literature dealing with lipase stereoselection (2), asymmetry resided only in the alcohol portion of the compounds. At this time far less is known of the effects of chirality in the acid residue, despite the inference that one might develop from the literature that lipases primarily bind the acid.

A survey of the effects of methyl branching on the maximum velocity (V_{max}) of ester hydrolysis catalyzed by porcine pancreatic lipase was limited to commercially available, and racemic, fatty acids (3). Little could be learned from these other than that branching close to the acid carbonyl severely retarded reaction. Nevertheless, α-substitution by halogen or ether oxygen does not preclude reaction; and hydrolysis, esterification, and transesterification methods served as the basis for resolving the enantiomers of such acids (2, 4—8). A recent examination of fungal lipases that had been solubilized in organic solvents by derivatization with polyethylene glycol indicated that 4-methylvaleric acid was as reactive as valeric acid itself in esterification of 1-pentanol in benzene, although 2- and 3-methylvaleric acids were not (9). A selection of configurationally pure methyl branched fatty acids could prove useful for learning more of the details of lipase binding, as well as serving to monitor and guide efforts at protein engineering of these enzymes. However, since such compounds are not available they would have to be synthesized.

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Abbreviations: DTNB, 5,5′-dithio-bis-(2-nitrobenzoic) acid; GLC, gas-liquid chromatography; HMPT, hexamethyl phosphoric triamide; IR, infrared; LDA, lithium diisopropylamide; MS, mass spectra; NMR, nuclear magnetic resonance; V_i, initial velocity; V_{max}, maximum velocity.

Before undertaking the preparation of a series of configurationally pure methyl branched fatty acids, we prepared the more readily obtainable racemic 2-,3- and 4-methyloctanoic acids as model compounds. In addition, it was necessary to select 1) a suitable acid derivative to submit to lipolysis, and 2) a lipase assay. The initial velocities (V_i) of several octanoic acid esters were obtained by titrimetry, and those of several thiolesters were measured both titrimetrically and spectrophotometrically (10, 11) using commercial lipase preparations of the fungi *Candida rugosa* and *Aspergillus niger*. The reactivities of the 2-mercaptoethanol S-esters of the racemic acids were then determined spectrophotometrically.

MATERIALS AND METHODS

Infrared spectra (IR) were obtained on a Perkin-Elmer 1310 Spectrophotometer (Norwalk, CT) using 3% solutions in CCl_4. ^1H Nuclear magnetic resonance spectra (NMR) were obtained with a Varian A-60 spectrometer (Florham Park, NJ) using CCl_4 solutions. Gas liquid chromatography (GLC) was accomplished using a Shimadzu GC-Mini 2 instrument (Columbia, MD) using an SBP-1 column (0.25 mm i.d. × 30 m) fitted with flame ionization detection and employing He as carrier gas with a 50:1 split ratio. Mass spectra (MS) were obtained with a Hewlett-Packard 5995 GC-MS system (Avondale, PA) interfaced with an OV-1 column (0.25 mm i.d. × 12 m). Free fatty acid titrations were performed with a Radiometer titrilab II unit (Westlake, OH), and the spectrophotometric assays were conducted with a Perkin Elmer Model 559 UV-Vis spectrophotometer.

All organic solvents were reagent grade; hexamethyl phosphoric triamide (HMPT), 99%, was purchased from Aldrich Chemical Co. (Milwaukee, WI) and was stored over 13A molecular sieves to remove dimethylamine. Ellman's Reagent, 5,5′-dithio-bis-(2-nitrobenzoic) acid (DTNB) was obtained from Aldrich Chemical Co. All other chemicals were reagent grade and were obtained from commercial sources.

2-Methyloctanoic acid (2). The general procedure of Pfeffer and Silbert was followed (13) to alkylate the dianion of octanoic acid 1 with methyl iodide producing 2 (92%): b.p. 131-135°C (20 mm) IR,NMR data consistent with assignment (13).

Methyl E-2-octenoate (5). Hexanal (freshly distilled) (104 ml, 0.87 mol), malonic acid (100 g, 0.96 mol), pyridine (dried over KOH) (100 ml) and diisopropylamine (3 ml) were brought together and allowed to stand for 24 hr. The mixture was then heated to 60°C for about 24 hr. The reaction mixture was acidified with cold 6N H_2SO_4 and extracted with ether. The organic phase was washed with water, dried ($MgSO_4$), and concentrated, stripping with benzene to remove residual water azeotropically. The crude acid was esterified with methanol (500 ml) containing H_2SO_4 (1 ml) by heating under

reflux for 16 hr. The mixture was concentrated and worked up in a standard manner, then distilled to give 5 (72% from hexanal): b.p. 88—93°C (20 mm); IR and NMR were consistent with assignment (14).

3-Methyloctanoic acid (3). Cuprous iodide (23.9 g, 0.125 mol) was stirred in dry ether under an inert atmosphere and cooled in an ice bath as methyllithium (180 ml of 1.4 M in ether) was injected. The mixture was stirred for 0.3 hr at 0-5°C, and then was cooled in dry ice-acetone. Compound 5 (19.5 ml, 0.11 mol) was injected, and the resulting mixture was stirred overnight attaining room temperature. The reaction was worked up by adding a solution of 90 ml of sat. NH_4Cl and 10 ml of conc. NH_4OH (slowly). The resulting mixture was filtered by suction through Celite, and the organic phase was isolated and washed several times with water. After drying ($MgSO_4$) and removal of solvent, the product was distilled to give methyl 3-methyloctanoate (56%): b.p. 84-86°C (20 mm); IR, 1740 cm^{-1}. The ester was saponified with 6N KOH:methanol (1:1) by heating under reflux for 2 hr. Product workup was standard and produced the acid 3 (15) (quantitatively): b.p. 93-96°C (0.5 mm) (16); IR 1700 cm^{-1}; NMR, 0.90 (bt, CH_3), 0.95 (unresolved d, CH_3), 1.28 (CH_2 envelope), 2.2 (2H, m, CH_2 C=0), 11.97 (1H, s, CO_2H).

4-Methyl-E-2-octenoic acid (8). Hexanal (freshly distilled) and *t*-butylamine were converted to N-*t*-butylhexanimine, 6, in 78% yield by the general method of Stork and Dowd (16): b.p. 56—62°C (20 mm); IR, 1670 cm^{-1}. Imine 6 was treated with 1.1 equiv of lithium diisopropylamide (LDA) in dry tetrahydrofuran in a manner completely analogous to the alkylation of octanoic acid. Reaction with excess CH_3I followed by the usual workup procedure gave N-*t*-butyl-2-methylhexanimine, 7, in 84% yield: b.p. 60-70°C (20 mm); IR, 1670 cm^{-1}; NMR, 0.94 (m, $2CH_3$), 1.10 (s, *t*-butyl CH_3), 1.3 (CH_2 envelope), 2.2 (m, CH), 7.35 (d, J=5, N=CH). Imine 7 (30 g, 0.178 mol), malonic acid (27.7 g, 0.178 mol) and pyridine (25 mol) were allowed to react as above for the preparation of 5. Acid 8 was obtained in 81% yield: b.p. 140-144°C (20 mm); IR, 1690, 1650 cm^{-1}; NMR, 0.92 (bt, CH_3), 1.08 (d, J=7, CH_3), 1.3 (CH_2 envelope), 5.72 (d, J=16, C=CHC=0), 6.94 (d of d, J=8, 16, HC=CC=0), 11.95 (s, CO_2H). Anal. calcd for: C,69.19; H,10.33; found: C,68.99; H,10.31.

4-Methyloctanoic acid (4). Acid 8 (5.7 g, 36.5 mmol) was hydrogenated in absolute ethanol (30 ml) over 20% Pd/C at 3 atm in a Paar shaking apparatus. The reaction mixure was filtered, diluted with water, and extracted with ether. Continuing a standard workup procedure, the product was distilled giving the acid 4 (quantitatively): b.p. 94-98°C (0.5 mm) (17), IR and NMR were consistent with assignment.

Thiolesters. Compounds 1a, 1b, 1c (Table 1) were prepared by a general procedure of Renard et al. (11) whereby the requisite thiol was treated with one equiv of octanoyl chloride and 2.4 equiv of pyridine in anhydrous ether (ice bath). The reaction product was obtained by a conventional workup procedure, and the hydric thiolesters (containing O-ester) were chromatographed over silica gel (12). Obtained in this manner were: 1a (33%); IR, 1690 cm^{-1}; NMR 0.90 (bt, CH_3), 1.3 (CH_2 envelope), 2.56 (t, J=7, CH_2C=0), 3.02 (t, J=5, CH_2S), 3.68 (t, J=5, CH_2O); MS, m/e 127 ($C_7H_{15}C$=0)$^+$; b.p. 86-89°C (0.6 mm)-rearranges!

1b (24%); m.p. 59-60°C (hexane); IR, 1690 cm^{-1}; NMR, 0.92 (bt, CH_3), 1.3 (CH_2 envelope), 2.62 (bt, CH_2C=0), 3.04 (d, J=5, CH_2S), 3.6 (m, CHOH, CH_2OH).

1c (98%); b.p. 120-123°C (0.45 mm); IR, 1690 cm^{-1}; NMR, 0.90 (m, CH_3), 1.25 (CH_2 envelope + CH_3), 1.30 (s, CH_3), 2.5 (m, CH_2 C=0), 2.96 (d, J=6, CH_2S), 3.4-4.2 (CHOH, CH_2OH); MS, m/3 259 (M-15)$^+$, 127 ($C7H_{15}C$=0)$^+$. The required thiol for 1c was obtained by converting 3-mercapto-1,2-propanediol to a mixture of the two acetonides and separating these by silica gel column chromatography (19).

Compounds 2a, 3a and 4a, the 2-mercaptoethanol S-esters of the branched octanoic acids, were prepared directly from the acids (1 equiv) and dicyclohexylcarbodiimide (1.1 equiv) in methylene chloride. The product was principally S-esterified and was isolated by precipitating dicyclohexylurea with hexane followed by suction filtration. The solvent was removed from the filtrate, and the crude product was chromatographed as before. Each adduct was obtained in 60-70% yield: IR, 1690 cm^{-1}; MS m/e 141 ($C_8H_{17}C$ = 0)$^+$; NMR consistent with structure. All S-esters containing vic-hydroxyl underwent rearrangement to a mixture of O- and S-esters in the injection port (glass liner) of our GLC.

Lipase assays: Titrimetry. Weighed amounts of commercial lipase were allowed to react in an emulsion created by brief sonication of mixtures containing several concentrations of substrate esters in 5 ml of 10% gum arabic. Free fatty acid release was measured by titration with 0.10 N NaOH using "pH stat" mode at pH 7.3. enzyme solutions were prepared in distilled water; substrate concentrations were varied to maximize the initial reaction rate.

Lipase assays: Spectrophotometry. The general procedure of Renard et al. (11) was followed in which solutions of substrate in HMPT (100 μl of 0.0105 M stock), 100 μl of DTNB (Ellman's reagent) in HMPT (20 mg/ml), HMPT (300 μl) and 2.4 ml of 0.05 M Tris buffered at pH 8.0 were brought together in a cuvette. The absorption at 412 mm was immediately monitored

TABLE 1

Initial Velocities of Lipase-Catalyzed Hydrolysis of Octanoate Esters/Thiolesters Determined Titrimetrically[a]

Compound (No.)	*C. rugosa*[b]	*A. niger*[c]
Trioctanoin (11)	8.7	19.0
Methyl octanoate (12)	0.3	2.5
2,2-Dimethyl-4-hydroxymethyl-1,3-dioxolane octanoate (13)	7.7	12.0
S-2-Mercaptoethanol octanoate (1a)	0.4	5.7
S-3-Mercapto-1,2-propanediol octanoate (1b)	0.5	1.5
2,2-Dimethyl-4-mercaptomethyl-1,3-dioxolane octanoate (1c)	3.2	4.7

[a]Titrimetric velocities were obtained on emulsified mixtures (Experimental Methods). Values are μmol fatty acid min^{-1} mg of powder^{-1} ± 5%).

[b]*C. rugosa* lipase was purchased from Enzyme Development Corp., NY.

[c]*A. niger* was a gift from Amano Co., Troy, VA (Lipase-K).

for background hydrolysis. A solution of the enzyme in buffer (100 μl) was added and the new slope calculated. The Ellman's reagent did not react with the enzyme itself.

RESULTS AND DISCUSSION

Synthesis of methyl branched octanoic acids. Octanoic acid, 1, (Fig. 1) was deprotonated with lithium diisopropylamide (LDA), and the resulting dianion was alkylated with methyl iodide to give 2-methyloctanoic acid, 2. Hexanal was condensed with malonic acid in pyridine and then heated to decarboxylate the adduct producing E-2-octenoic acid. The methyl ester of this acid, 5, was alkylated with lithium dimethylcuprate in ether, and the resulting methyl 3-methyloctanoate was saponified to 3-methyloctanoic acid, 3. The N-*t*-butylimine of hexanal, 6, was deprotonated with LDA and methylated to give 7, and this intermediate was condensed directly with malonic acid to give 4-methyl-E-2-octenoic acid, 8. Hydrogenation of 8 over palladium/carbon in ethanol led to 4-methyloctanoic acid, 4.

Synthesis of thiolesters. Thiolesters 1a, b, c were synthesized from the corresponding thiols using octanoyl chloride and pyridine in ether and purifying the product by column chromatography in the case of 1a, b (11) or by distillation for 1c. The thiolesters of 2-mercaptoethanol and the branched acids, namely 2a, 3a and 4a, were prepared using dicyclohexylcarbodiimide in methylene chloride.

Lipase assays. Earlier we had noted that esters of the 1,2-acetonide of glycerol, 2,2-dimethyl-4-hydroxymethyl-1,3-dioxolane, often were hydrolyzed by lipases with rates comparable to those of the corresponding triglycerides (19). The data of Table 1 indicate again that the octanoate ester of the acetonide of glycerol, 13, does react more rapidly than methyl octanoate and comparably to (trifunctional) trioctanoin. The corresponding thiolester 1c similarly reacted more readily than did the other thiolesters examined (except that 1a was slightly more reactive than 1c), though more slowly than 13. An assay of thiolesters has been devised that makes use of Ellman's Reagent at pH 8 to monitor formation of thiolate ion (10); some examples of its utility have been reported (20)(21). The suggested use of HMPT as a cosolvent (11) can provide a homogenous medium that avoids the preparation of emulsions normally required for studies of lipase catalyzed hydrolysis. The reproducibility of such data and the ability to refer with greater certainty to "substrate concentrations" improves the value of kinetic data obtained. Additionally, this methodology would be more sensitive so that precious substrates might be employed sparingly. Implicit are assumptions that 1) candidate substrates do not hydrolyze under these conditions; 2) S to O migration in compounds such as 1a and 1b does not occur during lipolysis—the assay monitors for thiolate ion generated, hence indirectly for free fatty acid, and 3) the same HMPT concentration will maintain a clear solution for a broad range of substrates.

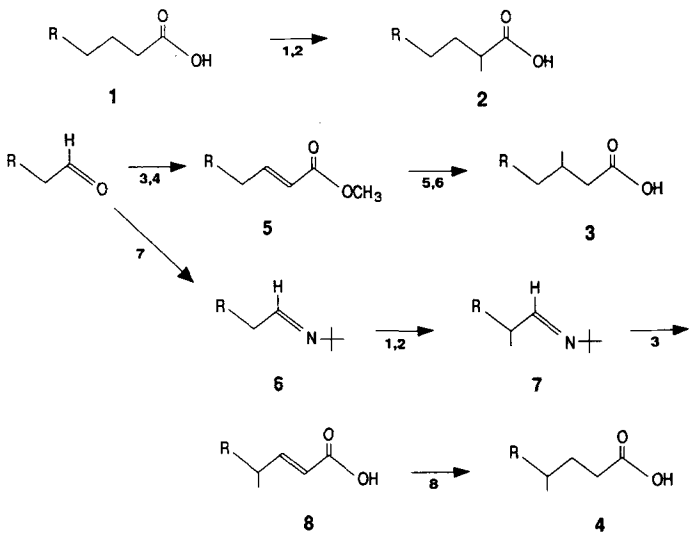

FIG. 1. Synthesis of racemic methyl branched octanoic acids. 1) LDA; 2) CH_3I; 3) malonic acid, pyridine; 4) CH_3OH, H^+; 5) lithium dimethyl cuprate; 6) OH^-; 7) *t*-butylamine; 8) H_2, Pd/C.

The data of Table 2 indicate slower rates of lipolysis in the HMPT containing solutions, with compound 1c faster reacting than 1a and 1b with both lipases. The solution of 1c was cloudy, however, and underwent spontaneous hydrolysis as determined by titrimetry at (the required) pH 8.0. Although solutions of 1a and 1b were homogeneous, a low background hydrolysis was observed as well (about 10%). The values in Table 2 have been corrected for the backgrounds. Although the point was not thoroughly evaluated, synthetic work in our laboratory indicates that S to O migration can occur at high pH, and titrimetry using the HMPT mixtures does not indicate non-enzymatic hydrolysis of compounds 1a and 1b.

The spectrophotometric assay was then performed with the S-mercaptoethanol esters of the branched acids, 2a-4a, (Table 2). The reaction velocities relative to 1a (unbranched analog) showed slower reaction for 2a and 4a, and no reaction at all for 3a. The activity changes are roughly parallel for both commercial lipases.

In summary, we prepared several methyl branched octanoic acids. Several esters and thiolesters of oc-

TABLE 2

Initial Velocities of Lipase-Catalyzed Hydrolysis of Thiolesters by Spectrophotometric Assay (15% HMPT)[a]

Compound	*C. rugosa*[b] V_i	Rel. V_i	*A. niger*[c] V_i	Rel. V_i
1a	0.24 ± 0.01	1.00	0.49 ± 0.06	1.00
1b	0.40 ± 0.01	—	nd[d]	—
1c	0.74 ± 0.01	—	nd[d]	—
2a	0.04 ± 0.01	0.17	0.025 — 0.01	0.05
3a	0	0	0	0
4a	0.13 ± 0.01	0.53	0.09 ± 0.02	0.18

[a]Values given are μmol fatty acid min^{-1} mg of $powder^{-1}$ and are the averaged results of at least three runs corrected for background hydrolysis. Solvents other than HMPT gave these results for 1c (solvent, %, V_i): DMF, 15, 0.55; DMF, 20, 0.60; DMSO, 15, 0.59; DMSO, 20, 0.56; dioxane, 15, 0. All mixtures were heterogeneous.
[b]See Table 1, footnote *b*.
[c]See Table 1, footnote *c*.
[d]Not determined.

tanoic acid itself were hydrolyzed by two commercial lipase preparations, and the 2-mercaptoethanol S-ester was selected as the most conveniently prepared derivative. We then made and assayed the esters of the racemic methyl branched acids. Although faster reacting thiolesters than those originally described (11) are evidently possible, reaction homogeneity may have to be sacrificed. The parallel changes in reactivity of the branched octanoic acid esters observed using two randomly selected (impure) lipases is intriguing, and studies are projected for the corresponding configurationally pure compounds, as well as the remaining methyl branched octanoic acids of the series.

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The Hepatocellular Transport of Sulfobromophthalein-Glutathione by Clofibrate Treated, Perfused Rat Liver

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The hypolipidemic drug clofibrate is known to affect the hepatic transport of various organic anions including bilirubin, fatty acids and sulfobromophthalein. Changes in the rate of metabolism and/or intracellular transport have been claimed responsible for the effect. To evaluate these possibilities, the transport of sulfobromophthalein-glutathione, a model compound that does not require metabolism for biliary excretion, was studied in perfused livers isolated from clofibrate-treated and control rats. Cytosolic fatty acid binding protein and glutathione S-transferase activity were also measured. Clofibrate treatment significantly increased liver weight; as a result glutathione S-transferase activity (toward 1-chloro-2,4-dinitrobenzene) fell if expressed per gram of liver (4560 ± 420 (SE) vs 7010 ± 260 nmoles/min for clofibrate treated and controls respectively, $p<0.002$), but was unchanged when expressed per total liver (60.8 ± 6.5 vs 64.6 ± 3.5 μmoles/min for clofibrate and controls $p>0.5$). Irrespective of how it was expressed fatty acid binding protein was significantly increased by the drug treatment. Steady state sulfobromophthalein-glutathione removal velocity was saturable with increasing concentrations of sulfobromophthalein-glutathione in both control and clofibrate-treated livers. Steady state extraction ratio, as well as Vmax and Km for removal, did not differ between the two groups. In keeping with other observations, these data collectively indicate that the hepatic steady state removal of nonmetabolized compounds is not affected by clofibrate. Because the concomitant decrease in glutathione S-transferase activity only reflects an opposite change in liver weight, it remains to be determined whether clofibrate alters the hepatic transport of sulfobromophthalein and other compounds that are conjugated with glutathione solely by changing their rate of metabolism.
***Lipids 24,* 438–442 (1989).**

Clofibrate and structural analogs such as nafenopin and ciprofibrate are drugs currently used for the treatment of hyperlipidemic disorders. In addition to their lipid-lowering properties, these compounds have been shown to affect various aspects of hepatic structure and function such as bile flow, liver weight, peroxisomal membrane composition and enzymatic activities (1–3). They are also known to affect the hepatic content of cytosolic fatty acid binding protein ($FABP_c$) (4–6) and of various transferases (7–8). Although their mechanisms of action are still poorly understood, it has been recently proposed that the active form of clofibrate and analogs may be their acyl coA thioester (9) and that the drug-induced pleiotropic response may be regulated by a peroxisome proliferator binding protein which binds these agents with high affinity [10].

An interesting phenomenon which follows the administration of many hypolipidemic drugs is a change in the hepatic transport of various compounds including bilirubin, sulfobromophthalein (BSP) and fatty acids (11–15). Several (11, 13, 16, 17), although not all (18), reports suggest that BSP transport is decreased by clofibrate, whereas fatty acids and bilirubin are taken up at a greater rate. It has been proposed that the increased fatty acid uptake in clofibrate-treated livers could be the result of a greater cytoplasmic transport rate which in turn could be mediated by the increased content of $FABP_c$ [14]. Although it is entirely possible that $FABP_c$ plays a role in the hepatic transport of fatty acids (19), it remains to be established whether a change in $FABP_c$ content may affect steady state fatty acid removal rates. Other explanations such as a change in membrane transport, in metabolism and/or in the electrochemical driving forces need to be carefully explored.

On the other hand, the hepatocellular transport of bilirubin and BSP in clofibrate-treated livers seems to parallel their metabolic rates because the drug has been reported to increase the conjugating enzyme activity of bilirubin (UDP-glucuronosyl transferase) and decrease that of BSP (GSH-transferase) (11, 15, 20). Consistent with the hypothesis of metabolism-dependent changes in the hepatic transport of these organic anions are the findings of an increased conjugated bilirubin/unconjugated bilirubin and unconjugated BSP/conjugated BSP ratios in bile following clofibrate treatment (11, 13, 16). Because bilirubin and BSP share the same sinusoidal membrane transport system (21, 22), it is unlikely that clofibrate affects their removal by acting at the membrane level. Nevertheless, this and other explanations (cytoplasmic diffusion and excretion rates) have not been ruled out. To evaluate these possibilities, we studied the effect of clofibrate on the hepatic transport of BSP-glutathione (BSP-GSH), a substance which shares the same transport system with BSP (23), but does not require metabolism for biliary excretion (24, 25). Although preliminary data suggested that BSP-GSH transport may be decreased in clofibrate treated livers (26), a larger number of experiments revealed that this drug does not affect steady state BSP-GSH hepatic removal.

MATERIALS AND METHODS

Materials. Sulfobromophthalein (BSP) bovine serum albumin (BSA, fraction V essentially fatty acid free) reduced glutathione (GSH) and clofibrate (Ethyl 2-[p-chlorophenoxy] isobutyrate) were purchased from Sigma

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Abbreviations: BSA, bovine serum albumin; BSP, sulfobromophthalein; BSP-GSH, glutathione conjugate of sulfobromophthalein; $FABP_c$, cytosolic fatty acid binding protein; GSH, reduced glutathione; GSH-transferase, glutathione S-transferase.

Chemical Co. (St. Louis, MO). Oxypherol FC-43 was obtained from Alpha Therapeutics (Los Angeles, CA). BSP-GSH was prepared by the *in vitro* method of Whelan *et al.* [27]: after collection and purification, thin layer chromatography (28) indicated the resulting material being at least 95% monoconjugate.

Clofibrate Treatment. Sprague-Dawley male rats (55-65 days old, Bantin and Kingman, San Leandro, CA) were fed *ad libitum* powdered standard rat chow with or without 0.5% (w/w) clofibrate for ten days.

Liver Perfusion. Livers were removed under light ether anesthesia, placed in a thermoregulated (37°C) and humidified cabinet and perfused via portal vein with Oxypherol FC-43 as previously described [29]. Perfusate flow rate was adjusted to 2.5-3 ml/min/g liver with a peristaltic pump (LKB, Bromma, Sweden); the pH was controlled and kept constant (7.35-7.45) by modifying pO_2 and pCO_2 of Oxypherol as necessary. Viability was assessed by bile flow, gross appearance, oxygen consumption, perfusion pressure and by remeasuring, at the end of the experiments, the extraction ratio of the initial BSP-GSH solution; experiments in which the extraction was decreased by more than 10% were discarded. After a 30-min equilibration period the circuit was switched from recirculating to single-pass mode and the liver perfused for four min with Krebs Henseleit buffer (containing 1% BSA and previously equilibrated with 95% O_2 and 5% CO_2) to remove Oxypherol from the liver. Thereafter, various BSP-GSH solutions in Krebs Henseleit buffer (concentrations from 5.6 to 100 μM) were infused for 2 min each and effluent samples collected every 30 sec. Each infusion was followed by a 4-min washout with the same buffer containing no BSP-GSH. The concentrations of BSP-GSH in the effluent samples were measured (after alkalinization with 2 M NaOH) by absorption at 580 nm. From the measured concentrations, BSP-GSH extraction ratio was calculated as E = (Ci-Co)/Ci where Ci and Co are the concentrations entering and exiting the liver respectively, and the velocity of removal as V = E•F•Ci, where F is the perfusate flow rate per gram of liver. These calculations assume that no BSP-GSH concentration gradients develop along the sinusoid during the infusion and that all the hepatocytes are exposed to the same BSP-GSH concentration. This potential source of error does not make the adoption of a distributed model strictly necessary in this study because BSP-GSH is a substance with low first pass extraction (~30%; see Results) and, thus, the bias introduced by a lumped model is negligible (30). The relationship between BSP-GSH removal velocities and BSP-GSH concentrations was analyzed by nonlinear least square computer curve fitting as reported (31). Single experiments were individually fitted and the resulting values averaged to give the data presented in the Results section. The quality of the fit was judged on the basis of the sum of squares, correlation coefficient, standard error and uncertainty of the resulting parameters. The statistical significance of differences between the two groups was assessed by the unpaired Student's t test.

Determination of $FABP_c$ concentration and GSH-transferase activity. After perfusion, livers were blotted dry, weighed and homogenized. After centrifugation (20,000 *g* × 20 min and 100,000 *g* × 60 min) the resulting supernatant was stored at −70°C until assayed (24). The $FABP_c$ was measured by radial immunodiffusion using purified $FABP_c$ as standard (32). GSH transferase activity was assessed spectrophometrically toward 1-chloro-2,4-dinitrobenzene (33). The activity, measured with excess of substrate, was linearly related to the protein concentration. Protein concentration was measured according to Lowry (34) using BSA as standard.

RESULTS

Time dependence and kinetic features of BSP-GSH removal. Figure 1 illustrates the time course of BSP-GSH extraction by the single pass perfused rat liver. In control livers, steady state extraction was achieved within 30 sec and maintained throughout the experiment irrespective of the BSP-GSH concentration used. In clofibrate-treated livers, steady state extraction was achieved within 30 sec of infusion only at low BSP-GSH concentrations; at higher concentrations steady state was achieved later in time. This phenomenon may indicate a change in both components of a bidirectional transport step in clofibrate-treated livers (see Discussion). In all cases, extractions at 90 and 120 sec were taken as steady state values, averaged and further analyzed. When velocities of removal were calcu-

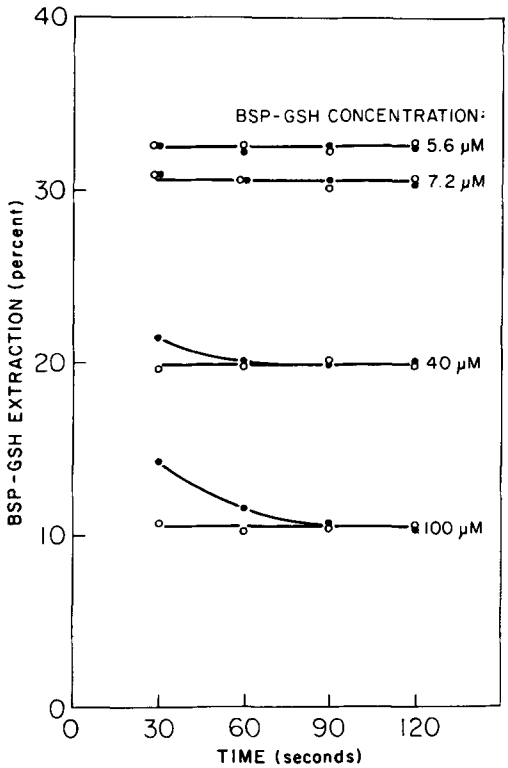

FIG. 1. Representative data illustrating the time course of BSP-GSH extraction by clofibrate-treated livers (closed circles) and controls (open circles). Steady state extraction is rapidly achieved in the control group, whereas clofibrate treated livers require a longer time to reach steady state at high BSP-GSH concentrations.

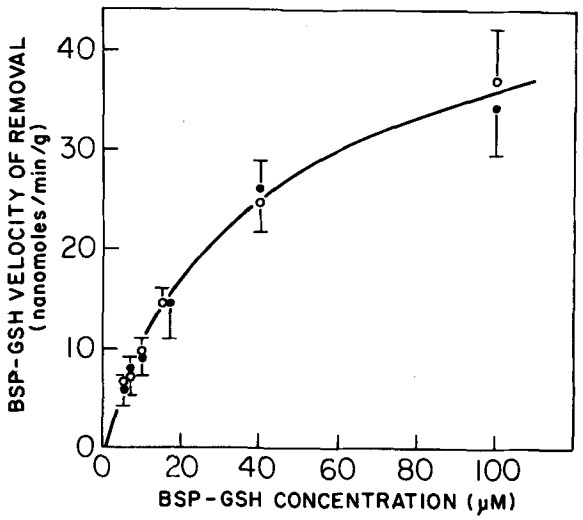

FIG. 2. BSP-GSH removal velocity vs BSP-GSH concentration curve in controls (open circles) and clofibrate-treated livers (closed circles). Single experiments were individually analyzed and averaged as described in Methods. Because the fits are virtually identical for the two groups, for the sake of clarity, only one fit is shown (x ± SE; n = 5).

lated as described in Methods and plotted against BSP-GSH concentrations, the uptake was saturable and conformed to Michaelis-Menten kinetics for both groups (Fig 2). The model best describing the removal process consisted of a single saturable component; other models including additional saturable or nonsaturable components did not improve the quality of the fit and were associated with a large uncertainty of the calculated parameters. The steady state Vmax, Km and intrinsic clearance (Vmax/Km) values did not differ between the two groups (Table 1) since clofibrate treatment significantly increased the liver weight, the perfusate flow was adjusted accordingly so that the two groups were perfused at the same rate (Table 1).

$FABP_c$ levels and GSH-transferase activity. In keeping with prior observations (5), clofibrate treatment increased hepatic $FABP_c$ by 2-3-fold (Table 2). The specific activity of GSH-transferase was determined using 1-chloro-2,4-dinitrobenzene, a substrate conjugated with glutathione by all forms of the enzyme including the BSP-conjugating enzyme [33]. This assay is also a measure of ligandin concentrations [35]. Clofibrate decreased GSH-transferase activity when the latter was expressed per mg of protein or per gram of liver weight (Table 2). Due to the concomitant increase in liver weight, GSH-transferase activity per total liver was not affected by the drug treatment.

DISCUSSION

Like other peroxisomal and microsomal inducers, clofibrate and analogs are known to affect a variety of hepatobiliary functions including bile flow, enzymatic activities and hepatocellular uptake of various compounds. (1-3, 36, 37). Recently, the possibility has been raised that, on the long term, these drugs may act as chemical carcinogens in rats and mice (38, 39). Although the hypolipidemic action of these compounds does not appear to be linked to peroxisomal proliferation (40, 41), it is not known whether other effects represent the end results of a common pathophysiologic mechanism or are diverse in their nature. This uncertainty derives in part from the complexity of many aspects of liver physiology and from the difficulty to dissect and study the events which define them. Thus, hepatic transport function could be affected by these drugs at several levels including membrane transport, intracellular binding and diffusion, metabolism and/or (for cholephilic compounds) biliary excretion. In this respect, previous reports have suggested that changes in BSP and bilirubin transport could be mediated by parallel, concomitant changes in their rate of metabolism (11, 13, 15-17). Other possible explanations, however, were not thoroughly investigated. Furthermore, most previous studies (11, 13, 15-17) were conducted *in vivo*, where additional factors such as blood flow, binding to plasma proteins and extrahepatic elimination may contribute to the overall removal process.

In the present investigation, we studied whether clofibrate treatment affects the transport of BSP-GSH by the single pass perfused rat liver, a system which allows monitoring extraction ratios during steady state and nonsteady state conditions under both first and zero order kinetics. Even though BSP-GSH shares the same membrane transport system with bilirubin and BSP (21-23), and like these organic anions binds (although with less affinity: refs. 24, 42) to cytosolic binding proteins, it is nevertheless rapidly excreted in bile without requiring further metabolism (24, 25). To define their role in any observed difference, we also measured $FABP_c$ levels and GSH-transferase activity (the latter by an enzymatic assay which provided, as well, an indirect estimate of ligandin content).

Steady state BSP-GSH extraction was not affected by clofibrate treatment: for both groups uptake was saturable with increasing BSP-GSH concentrations and

TABLE 1

Steady State Removal of BSP-GSH by Perfused Rat Liver

	Vmax (nmol/min/g)	Km (μM)	Vmax/Km (ml/min/g)	Liver Weight (g/100 g B.W.)	Flow Rate (ml/min/g)
Control	53.7 ± 6.6	41.8 ± 3.7	1.31 ± 0.10	3.56 ±0.13	3.5 ± 0.18
Clofibrate	51.4 ± 3.7	49.8 ± 5.5	1.22 ± 0.13	4.58 ± 0.29	3.2 ± 0.14
P	NS	NS	NS	< 0.02	NS

Vmax, Km and intrinsic clearance (Vmax/Km) were calculated from 90-120-sec average velocities. Data represent x ± SE (n = 5 for each group).

NS Not significant.

TABLE 2

Hepatic $FABP_c$ Concentration and GSH-Transferase Activity

	$FABP_c$			GSH-Transferase		
	μg/mg protein	mg/g liver	mg/liver	nmol/min/mg protein	nmol/min/g liver	μmol/min/liver
Control	41.8 ± 3.2	4.65 ± 0.3	47 ± 2	62.9 ± 3.2	7010 ± 260	64.6 ± 3.5
Clofibrate	113.4 ± 14.3	10.6 ± 0.4	140 ± 3	47.8 ± 5.4	4560 ± 420	60.8 ± 6.5
P	< 0.002	< 0.001	< 0.001	< 0.05	< 0.002	NS

$FABP_c$ was measured by immunodiffusion. GSH-transferase activity was assessed toward 1-chloro-2,4-dinitrobenzene (x ± SE of 5-6 experiments for each group).

the calculated Vmax, Km and intrinsic clearances were the same in drug-treated and control livers. However, although steady state extraction was rapidly achieved in both groups at low BSP-GSH concentrations, under saturating conditions steady state was reached later in clofibrate-treated livers compared to controls. This interesting observation deserves further comment. This phenomenon cannot be accounted for by a change in a single unidirectional BSP-GSH transport rate because, were this the case, the steady state extraction would also be affected. Also, a change in hepatic distribution spaces, due to the greater weight of clofibrate-treated livers, is not a likely explanation because steady state would have been reached later even at low BSP-GSH concentrations. These data rather suggest that clofibrate may decrease to the same extent both components of a bidirectional exchange process located between the extracellular space and the biliary pole of the hepatocyte (e.g., sinusoidal influx-efflux). Such an effect, which becomes pronounced under saturating conditions, would prolong the time required to reach steady state BSP-GSH extraction without affecting the actual steady state extraction value.

As expected, clofibrate significantly induced $FABP_c$, whereas GSH-transferase was reduced only when expressed per unit of liver weight, the total activity being unchanged. The latter finding confirms other reports which indicate that clofibrate and related compounds have only a "dilutional" effect on this enzymatic activity (and on ligandin content) due to the concomitant increase in liver weight (4, 43). Therefore, a decreased transport of BSP (and other compounds conjugated with GSH) in clofibrate-treated animals may not be entirely dependent on a depressed GSH-transferase activity. Indeed, Foliot *et al.* (43) have recently reported that other hypolipidemic agents, related or unrelated to clofibrate, reduce the maximal BSP excretory capacity in rats without affecting total GSH-transferase activity. Nevertheless, a specific (molecule to molecule) interaction between GSH-transferase and the drug could inhibit the enzyme and consequently reduce conjugation rates and the overall transport. This hypothesis is consistent with the finding that ciprofibrate (a clofibrate analog) irreversibly inhibits GSH-transferase both *in vivo* and *in vitro* (44) and with the observation that the hepatic transport of non-metabolized compounds is not affected by hypolipidemic drugs (11, 17, 43). With regard to the effects of clofibrate on bilirubin hepatic transport, our study does not allow speculation. In any event, the association between bilirubin transport and UDP-glucuronosyl transferase activity appears strong (7, 13, 15-17, 45).

Finally, the lack of parallelism between $FABP_c$ content and BSP-GSH transport confirms that $FABP_c$ may not play a major role in hepatic transport processes (18). In fact, $FABP_c$ may not greatly affect the uptake of substances, like fatty acids, which are bound with a much greater affinity than BSP or BSP-GSH. Due to its intracellular location, $FABP_c$ may be expected to affect initial net uptake by decreasing efflux rates as suggested for ligandin (46). However, in female hepatocytes, a 50% greater $FABP_c$ content is associated with a greater oleate efflux rate constant compared with males (D. Sorrentino, unpublished). By contrast, in starvation, a condition of decreased $FABP_c$ content (47) initial net oleate uptake is unchanged suggesting that the primary determinant of hepatic fatty acid flux is transport across the membrane (48).

In conclusion, our study shows that clofibrate treatment does not affect the steady state removal of BSP-GSH by the single pass perfused rat liver. The clofibrate-induced decrease in GSH-transferase does not involve the total enzymatic activity; the latter in turn may not be causally related to the reduced hepatic removal of BSP and other GSH-conjugated compounds.

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Lipids and Fatty Acids of the Horseshoe Crabs *Tachypleus gigas* and *Carcinoscorpius rotundicauda*

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Total lipids from hepatopancreas of the horseshoe crabs, *Tachypleus gigas* and *Carcinoscorpius rotundicauda*, obtained in 7.6 and 3.3% wet weight respective yields, were fractionated by various chromatographic techniques and identified by gas-liquid chromatography and spectroscopic methods. Fatty acid-containing lipids were rich in 16:0 (8.0–25%), 18:1ω9 (6.9–22%) and 18:2ω6 (6.8–18.5%); appreciable amounts of 16:1ω7, 18:3ω3, 20:5ω3 and 22:6ω3 were also present. The level of 26:0 in the hydrocarbon fractions was unusually high (64 and 68%). Carbon chain lengths of major wax esters were 44, 46 and 48 for *T. gigas* and 38, 40 and 42 for *C. rotundicauda*. 1-*O*-Alkyl diglycerides were 7.2 and 9.1% of the total lipids in the two species and contained 14:0(20%), 16:0(60%) and 18:0(20%) alkyl chains along with a relatively higher percentage (32–35%) of saturated fatty acids. High levels of cholesterol (>50% of total sterol) in the free and combined state were encountered in both samples, phospholipid contents being 40 and 35%, respectively, and contained highest levels of unsaturated fatty acids.

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Horseshoe crabs or king crabs, popularly known as "living fossils," belong to the phylum Arthropoda, subphylum Chelicerata, class Meristomata, and subclass Xiphosura. *Carcinoscorpius, Limulus* and *Tachypleus,* the three genera of the subclass, are currently represented only by four species, *C. rotundicauda, L. polyphemus, T. gigas* and *T. tridentatus.* Of them, the first two are important sources for the lectins carcinoscorpin (1) and limulin (2). Because of our interest in the flora and fauna of the Sundarbans mangrove ecosystem from the standpoint of biochemical ecology, we undertook investigation of the various lipid components and fatty acids of the hepatopancreas of two species of horseshoe crabs, namely *C. rotundicauda* and *T. gigas*, which are abundantly available in the coastal region of the Bay of Bengal. Hepatopancreas appears to be the major lipid storage organ in crabs (3–5) and other marine invertebrates, e.g., *Mesodesma mactroides.* However, during reproduction lipids are also distributed to the gonad, and high levels of polyunsaturated fatty acids are found in both organs (6–8). Though preliminary investigation on the lipids of *L. polyphemus* has been reported (9), no data is available on those of *C. rotundicauda* and *T. gigas*.

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Abbreviations: ECL, equivalent chain length; GLS, gas-liquid chromatography; NMID, nonmethylene interrupted dienoic; PL, phospholipids; RRT, relative retention times; TAG, triacylglycerol; TLC, thin-layer chromatography; TMS, tetramethylsilane.

MATERIALS AND METHODS

The two species of horseshoe crabs, *C. rotundicauda* and *T. gigas*, were collected from Prentice Island, between latitudes 21.43° and 21.46°N and longitudes 88.18° and 88.19°E of the Sundarbans mangrove forest, West Bengal, India. Mature animals which do not moult were captured at different times during breeding season (March–June in India) when they came ashore at high tides in pairs, male clinging to the carapace of the female. Five pairs of each species were collected. Sizes of the animals varied between 25—40 cm including caudal spine. Living animals were killed after 24—30 hr following each collection and hepatopancreases were stored in a deep freezer at −25°C. These organs from different collections, regardless of sex, were pooled and extracted for lipids according to Bligh and Dyer (10). Lipids were stored under nitrogen in redistilled hexane at −18°C.

The total lipid was resolved into neutral and phospholipids (PL) by column chromatography on silicic acid (11). The neutral lipid was fractionated by preparative TLC (12) using petroleum ether/diethyl ether/acetic acid (90:10:1, v/v/v). While steryl esters, 1-*O*-alkyl diacyl glycerol, triacylglycerol (TAG) and sterols were separated, the overlapping hydrocarbon and wax ester bands could only be resolved by rechromatography using n-hexane as developing solvent (13). The alkyl diglyceride was further purified by preparative TLC (14) in petroleum ether/benzene (95:5, v/v).

Hydrocarbons were analyzed directly by GLC on a 3% OV-17 packed column (2.1 m × 0.4 cm). Column oven temperature was at 200°C for 4 min and then programmed at 4°/min to 300°C. Identification and quantification were done as described previously (15).

GLC of intact wax esters was carried out on a 3% SE-30 column (1.8 m × 3 mm) (16). Oven temperature was initially at 230°C for 16 min and then programmed (4°/min) to 350°C. Peaks were identified by addition of authentic synthetic compounds (17). Wax esters were hydrolyzed using lipid-free (18) porcine pancreatic lipase on a TLC plate and resulting fatty acids and alcohols were analyzed by GLC as methyl esters and acetates, respectively (17). Steryl esters were similarly lipolysed on a TLC plate (19). Sterols and fatty acids were separated on the same plate, recovered, derivatized (19, 20) and analyzed by GLC (19).

The 1-*O*-alkyl-diglyceride was initially characterized by strong ester bands at 1735 and 1180 cm^{-1} and a *O*-alkyl ether band at 1110 cm^{-1} in its IR spectrum as well as by ^{1}H-NMR spectroscopy. Alkyl glycerol ethers and methyl esters of fatty acids were obtained by refluxing alkyl-diglycerides in methanolic HCl for 2 hr (14). Usual workup followed by preparative TLC (12) separated the components. An aliquot of alkyl glycerol ether was silylated using Trisil-Z (Pierce Chemi-

cal Company, Rockford, IL), and analyzed by GLC on a 3% OV-17 column (14).

For determination of hydrocarbon side chains, a portion of alkyl glycerol ether was converted to alkyl iodide by refluxing with hydriodic acid for 3 hr (21). The iodide was heated with NaCN in DMSO at 90°C for 30 min (22), and the nitrile formed was refluxed with 6% KOH in ethanol for 16 hr (23). The resulting fatty acids were converted to the corresponding methyl esters (20) and analyzed by GLC.

Fatty acids liberated through saponification of triacylglycerols and phospholipids according to Kates (24) were extracted with ether and methylated (20). GLC of the methyl esters, obtained also from various other lipid fractions, was carried out on a 10% DEGS column using a Pye Unicam Model 104 instrument equipped with dual column and dual FID. Peaks were identified and estimated using relative retention times (RRT), equivalent chain lengths (ECL) and cod liver oil fatty acid methyl esters as secondary standard according to Ackman and Burgher (25), confirmed by GLC of the hydrogenated samples. Incidentally, the isomeric monoenoic fatty acids generally present in marine lipids (26,27) and detectable only by open tubular GLC could not be separated and consequently have been represented by the most common isomeric forms known to occur in marine lipids.

Sterols, free or obtained by lipolysis of steryl esters, were analyzed as acetates (28) and trimethylsilyl ether derivatives (29) by GLC on a 3% SE-30 and a 3% OV-17 column, respectively. Aliquots of hydrocarbons, fatty acid methyl esters and wax esters were hydrogenated over Adams' catalyst (30) and were analyzed by GLC.

NMR spectra of 1-*O*-alkyl-diglyceride were recorded on a JEOL FX-100 FT-NMR using $CDCl_3$ as solvent with tetramethylsilane (TMS) as internal standard and IR spectra on a Shimadzu IR-408 spectrophotometer.

RESULTS AND DISCUSSION

The total lipid content of hepatopancreas in *T. gigas* was more than twice that of *C. rotundicauda* with a preponderance of neutral lipids in both the samples (Table 1). The lipid class composition of both species was similar (Table 1), triacylglycerols being the major neutral lipid with hydrocarbons and 1-*O*-alkyl diglycerides also present in appreciable amounts.

Fatty acid methyl esters derived from sterol esters, 1-*O*-alkyl-diglycerides, TAG and PL did not show the presence of conjugation, *trans*-unsaturation or functional groups other than ester carbonyl (Table 2). Alkyl diglycerides contained the highest proportion of saturated fatty acids (32—35%). Palmitic acid (16:0) was the major saturated acid in all the samples, other components being myristic (14:0), stearic (18:0), arachidic (20:0) and behenic (22:0) acids. Oleic acid was present at levels of 20 and 22% in alkyl diglycerides of the two species, almost double the proportion in steryl esters, TAG and PL. Steryl ester of *T. gigas* contained a particularly high proportion of palmitoleic acid (16:1ω7). Alkyldiglyceride contained about 41% and 48% of total fatty acids as polyenoics in *C. rotundicauda* and *T. gigas*, respectively, as against 55–60% in steryl esters and TAG and about 86% in PL. Among polyunsaturated, 18:2ω6, 18:3ω6, 20:5ω3 and 22:6ω3 fatty acids were present in appreciably high proportions in almost all the samples of two animals. Phospholipids of two animals were particularly rich in 18:3ω3 (12–14.5%) and 20:5ω3 (15.5–16.0%) fatty acids. The two dienoic acids with 20- and 22-carbon chains found in the present study might be associated with the nonmethylene interrupted dienoic (NMID) fatty acids, viz., 20:2Δ5, Δ13 and 20:2Δ5, Δ11; 22:2Δ7, Δ15 and 22:2Δ7, Δ13 which were found to occur in the American oyster *Crassostrea virginica* and also in several other marine invertebrates as reported by Paradis and Ackman (31,32). Recently, some unusual *cis*-5-olefinic acids have been found to occur as distinctive lipid components in sea urchins (30), including 18:1, 20:1, 20:2, 20:3 and 20:4 acids.

The overall fatty acid profile is thus similar to those of the invertebrates of this ecosystem studied previously (13, 33, 34) and of the horseshoe crab *L. polyphemus* reported earlier (9). In a marine ecosystem, generally, qualitative similarities are observed in the fatty acid compositions of the organisms which occupy different trophic levels. The first link of the food chain, phytoplankton, is able to synthesize all the fatty acids *de novo* (35). High concentrations of 20:5ω3 and 22:6ω3 acids, generally considered the typical fatty acids of marine life, are contributed by some phytoplanktonic species (diatoms, dinoflagellates, etc.) to the marine ecosystem (36). In a previous study in this laboratory (37), linolenic acid (18:3ω3) was found to occur in substantially high levels (28%) in detritus during a model experiment. Examination of various detritivorous benthic animals, viz. gastropod molluscs and bivalves (partly filter feeder) of this ecosystem (13, 33, 34) revealed the presence of appreciably high levels of 20:5ω3 associated with other polyunsaturates of the -ω3 series. It was thus envisaged that intake of higher levels of the precursor acid, 18:3ω3, through detritus and subsequent chain elongation and desaturation processes *de novo*, would lead to the formation of -ω3 unsaturates in higher levels. Obviously, part of the polyunsaturates in some bivalves are derived from planktons. Intake of these detritivorous animals rich in -ω3 acids may explain accumulation of these polyunsaturates in higher levels in the hepatopancreas of the horseshoe crabs studied.

TABLE 1

Lipid Compositions of Hepatopancreas of *T. gigas* and *C. rotundicauda*

	T. gigas	*C. rotundicauda*
Total lipids[a]	7.6	3.3
Hydrocarbons	11.8	9.6
Wax esters	10.1	9.0
Steryl esters	7.0	6.6
1-*O*-Alkyl diglycerides	7.2	9.1
Triacylglycerols	16.8	19.0
Sterols	7.3	11.7
Total neutral lipids	60.2	65.0
Phospholipids	39.8	35.0

[a]Expressed as percent (w/w) of wet tissue; others as percent (w/w) of total lipids.

TABLE 2

Fatty Acid Compositions[a,b] of various Lipids of *T. gigas* and *C. rotundicauda*

Component acids	Steryl ester		Alkyl diglyceride		Triacylglycerol		Phospholipid	
	1	2	1	2	1	2	1	2
14:0	4.7	1.0	2.0	1.0	2.4	4.0	1.5	2.5
14:1ω7	2.6	0.1	—	—	3.7	3.0	1.7	2.0
16:0	8.0	9.9	17.1	25.0	15.0	9.2	10.0	7.5
16:1ω7	13.4	5.4	1.2	2.2	5.4	9.0	3.0	7.0
18:0	1.8	8.1	4.0	3.0	4.0	7.0	2.0	3.0
18:1ω9	10.1	10.1	20.0	22.0	10.4	6.9	8.5	6.0
18:2ω6	11.4	11.6	15.0	14.0	18.5	6.8	10.0	5.0
18:3ω3	6.6	1.2	9.0	8.0	6.0	2.1	14.5	12.0
18:4ω3	4.4	3.5	—	—	1.1	0.3	1.5	1.0
20:0	1.2	1.0	8.0	5.0	1.9	0.3	0.5	0.5
20:1ω9	2.2	1.0	4.0	3.7	3.0	1.2	3.0	2.0
20:2ω6	—	0.1	—	—	—	0.2	0.5	0.5
20:3ω9	1.4	0.5	1.5	1.0	0.4	1.0	2.0	1.9
20:4ω6	3.3	6.4	2.4	1.9	4.1	10.0	5.0	7.5
20:5ω3	8.0	13.7	8.0	7.0	9.2	14.5	15.5	16.0
22:0	3.1	3.9	1.0	0.7	1.0	0.4	0.5	0.5
22:1ω9	1.1	2.3	1.2	0.9	1.9	3.9	2.5	3.5
22:2	0.3	4.5	2.0	2.5	1.3	1.0	2.5	2.0
22:4ω3	2.0	2.0	—	—	0.9	1.2	2.0	2.5
22:5ω3	3.0	2.5	0.5	0.3	0.5	2.5	4.0	3.6
22:5ω6	3.0	2.3	0.5	0.5	1.1	6.3	1.3	3.0
22:6ω3	6.4	9.0	2.0	1.5	7.5	9.5	8.0	10.5

[a]Expressed as percent (w/w) of total fatty acids.
[b]1, *T. gigas;* 2, *C. rotundicauda.*

TABLE 3

Hydrocarbon Compositions[a] of Hepatopancreas of *T. gigas* and *C. rotundicauda*

Components	*T. gigas*	*C. rotundicauda*
16:0	3.8	3.6
17:0	3.2	3.1
18:0	3.1	2.8
19:0	3.0	2.5
20:0	2.9	2.4
21:0	2.3	2.1
22:0	3.1	2.1
23:0	1.5	1.1
24:0	2.2	1.8
25:0	2.6	2.7
26:0	64.0	68.3
27:0	0.9	1.6
28:0	2.6	1.6
29:0	1.5	2.3
30:0	2.1	0.9
31:0	1.2	1.1

[a]Expressed as percent (w/w) of total hydrocarbons.

Hydrocarbon compositions of the two samples (Table 3) consisted largely of 26:0, amounting to 64% of total hydrocarbon in *T. gigas* and 68.3% in *C. rotundicauda.* Predominance of even carbon chain n-alkanes was also observed in the lipids of the gastropod mollusc *Cerethidea cingulata* (13) and in plant hydrocarbons (18, 38, 39) of this ecosystem unlike terrestrial plants and temperate marine organisms in which odd carbon chain n-alkanes typically predominate. Interestingly enough, preponderance of even carbon chain n-alkanes in 80% of 22 species of Antarctica mid-water zooplankton and fish has been reported recently (40). However, no explanation could be forwarded for the biosynthesis of these unusual n-alkanes. The predominance of even carbon chain n-alkanes might be envisaged via decarboxylation of the excess of odd carbon fatty acids which have been derived from even carbon chain fatty acids by α-oxidation (39). These even carbon chain hydrocarbons are transmitted through food chains to various consumers at different trophic levels.

Though the major fatty acids obtained from the wax esters (Table 4) of the two species were the same, the relative abundance of certain components, e.g., stearic acid (1.2 and 8.7%) varied widely. Alcohols obtained by the hydrolysis of wax esters were from 20:0 to 32:0 in both cases. Odd carbon chains and unsaturated alcohols were also detected, but the number of double bonds in a particular chain could not be determined with the column used. Presence of unsaturated alcohols could, however, be confirmed by reduction and GLC of the products. Major alcohols in the *T. gigas* sample were 21-U, 22:0, 24-U, 24:0 and 28:0. On the other hand, *C. rotundicauda* showed a considerably different abundance with 28:0 over 60% and 29:0 above 14%. Carbon chain lengths of wax esters were determined by direct GLC of the catalytically reduced products to minimize complications regarding identification which might have been caused by the presence of various polyenoic acids. Carbon numbers of the major wax esters of *T. gigas* thus obtained were between 37 and 49, 46 (18%) being the most abundant. On the other hand, major component waxes of *C. rotundicauda* were between 36 and 48 carbon chains, the most abundant being 42 (20%). Even carbon number wax esters predominated over odd carbon chain esters, because of the presence of greater proportions of fatty acids and alcohols with even carbon chain lengths.

Sterols from marine organisms, reviewed recently

TABLE 4

Compositions of Wax Esters and of Fatty Acids and Alcohols Obtained by Hydrolysis of Wax Esters of *T. gigas* and *C. rotundicauda*

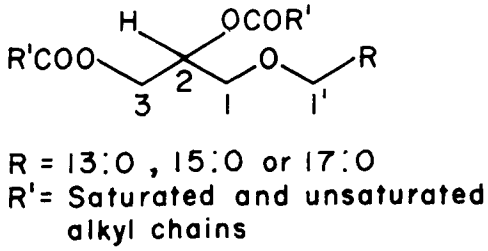
Fatty acids			Alcohols			Wax esters		
Components	1[a]	2	Components[b]	1	2	Components	1	2
14:0	2.5	0.9	20-U	1.6	1.8	34	1.0	1.9
14:1	2.5	0.3	20:O	2.4	0.6	35	3.0	3.5
16:0	6.3	9.5	21-U	7.6	1.9	36	3.0	8.0
16:1ω7	10.6	10.7	21:O	2.3	2.6	37	5.0	6.0
18:0	1.2	8.7	22-U	0.2	1.3	38	4.0	10.5
18:1ω9	18.6	19.5	22:O	6.5	0.4	39	2.0	3.0
18:2ω6	8.1	21.2	23-U	0.9	1.2	40	4.3	18.0
18:3ω3	9.8	2.0	23:O	0.5	0.3	41	5.0	4.1
18:4ω3	0.6	0.6	24-U	24.9	0.4	42	4.5	20.0
20:0	9.1	2.3	24:O	11.8	2.0	43	2.0	0.7
20:2ω6	—	0.4	25-U	0.3	—	44	14.0	5.0
20:3ω9	2.0	0.6	25:O	0.6	3.6	45	7.0	0.5
20:4ω6	0.4	5.6	26-U	0.5	—	46	18.0	8.0
20:5ω3	9.0	6.5	26:O	1.3	0.6	47	8.0	1.0
22:0	1.9	0.6	27-U	0.5	—	48	12.1	8.5
22:2	0.1	0.4	27:O	2.3	3.4	49	5.0	0.5
22:4ω3	2.9	0.5	28-U	1.8	—	50	0.4	0.3
22:5ω3	0.7	1.5	28-O	27.1	60.1	51	0.8	0.3
22:5ω6	3.2	2.1	29:O	1.4	14.3	52	0.9	0.2
22:6ω3	9.6	6.3	30:O	1.5	3.3			
			31:O	0.9	1.5			
			32:O	3.1	0.7			

[a]1, *T. gigas*; 2, *C. rotundicauda*.
[b]U denotes unsaturated components; O denotes saturated carbon chains.

by Djerassi *et al.* (41), may have diversified structures with unusual side chains. Even a sterol isolated from the bivalve of *Macoma* sp. of this ecosystem and partially characterized (33) appears to be a C_{27} compound with unsaturations at the side chain in addition to the one at the 5-position. The sterol profile observed in the present study, whether in the free or ester form, is simple in contrast. As determined by GLC retention parameters supported by MS data, it consists of cholesterol (55%), campesterol (10%), stigmasterol (7%), sitosterol (24%) and 28-isofucosterol (3%). The non-cholesterol distribution resembles that of plant sterols of this ecosystem (19, 38).

^{1}H-NMR (100 MHz) of the alkyl diglyceride (Scheme 1) showed peaks at δ 5.34 ppm (m, -C*H* = C*H*), 5.18 (m, H — 2), 4.24 (AB part of an ABX spectrum with J_{AB} = 12 Hz, J_{AX} = 6 Hz, J_{BX} = 4 Hz; H — 3), 3.54 (d, J = 6 Hz, H — 1), 3.4 (t, J = 6 Hz, H — 1), 2.64 (t, J = 4 Hz, =CH-CH_2-CH=), 2.3 (t, J = 7 Hz, -CH_2COO-), 2.04 (m, -CH_2-CH=CH-), 1.24 (broad s, CH_2) and 0.88 (t, J = 6 Hz, CH_3-CH_2), in good agreement with those reported by Wood and Snyder (42). Fatty acid compositions of 2- and 3-positions have been discussed already. The 1-*O*-alkyl glycerols obtained by the hydrolysis of the diglycerides were silylated (14) and analyzed by GLC. Both the samples contained three major peaks in the ratio of about 1:3:1. The hydrocarbon side chains of alkyl glycerol ethers were subsequently converted via the nitrile derivative to methyl esters of fatty acids with an extra carbon atom. The fatty acids identified were 15:0, 17:0 and 19:0, which confirmed the presence of 14:0, 16:0 and 18:0 hydrocarbon chains in the alkyl group R of this constituents.

Normally the alkyl moieties found in the ether lipids of animal cells are either exclusively saturated or monounsaturated although the acyl moieties of these lipids



Scheme 1. 1-*O*-Alkyl diglycerides from *T. gigas* and *C. rotundicauda*.

contain large proportions of polyunsaturated chains (43). Recently, however, a number of novel 1-*O*-alkyl diglycerides, in which alkyl groups were from 14 to 20 carbon chains, odd and even, and containing saturated, iso, anteiso and monounsaturated moieties, were reported from cod muscle by Ratnayake et al. (44). Nevertheless, in the present study only 1-*O*-saturated alkyl diglycerides were found to occur in high levels in the lipids of both the specimens. Currently naturally occurring ether lipids or alkoxy-lipids, once considered to be "dead ends" of lipid metabolism, have been found to be intimately involved in a variety of physiological reactions (45–48) and are considered to be of value in the therapy of cancer patients (49, 50).

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Increased Plasma Triglyceride Secretion in EFA-Deficient Rats Fed Diets With or Without Saturated Fat[1]

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Metabolic responses to essential fatty acid-deficiency in rats include an increased rate of triglyceride secretion into the plasma, a large reduction in the HDL_1 plasma lipoprotein concentration, and increased concentrations of liver triacylglycerols and cholesteryl esters. Because of differences in the types of EFA-deficient diets used, it is not clear whether these responses were solely due to the absence of EFA from the diet or whether saturated fat, or differences in acyl group chain length in this fat, might be responsible. Therefore, we fed rats diets differing only in amounts and kinds of fat, and measured triacylglycerol secretion rates and liver concentrations of triacylglycerols and cholesteryl esters, for comparison with our earlier measurements of plasma high density lipoprotein subpopulations in rats fed exactly the same diets. The purified diets contained either no fat, 5% by weight hydrogenated coconut oil, 5% hydrogenated cottonseed oil, or each of these three diets supplemented with 1% safflower oil, or 5% corn oil. We also fed some rats a nonpurified stock diet for comparison with literature reports. The present results indicate that the metabolic responses to essential fatty acid deficiency described above are definitely due to essential fatty acid-deficiency and not to the presence or chain length of acyl groups in saturated fat in the diet.
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In rats fed diets adequate in essential fatty acids (EFA), arachidonic acid is approximately 20% of the fatty acids in plasma phospholipids (1) and 50% or more of the fatty acids in cholesteryl esters (CE) of high density lipoproteins (HDL) or in total plasma CE (1, 2). In EFA-depleted rats, the proportion of arachidonate in fatty acids of plasma CE and phospholipids (PL) can fall to 5% or less (1). Such decreases are accompanied by striking changes in HDL subpopulations, i.e., a very large decrease in the HDL_1 subpopulation and a decrease in the peak diameter of the HDL_2 subpopulation (3, 4). These changes occurred in EFA-deficient rats fed a fat-free diet or diets containing 5% fat by weight as hydrogenated coconut oil (HCNO) or hydrogenated cottonseed oil (HCSO), in comparison with controls fed these EFA-deficient diets plus 1% safflower oil or a 5% corn oil diet (3). This evidence (3, 4) indicates that such changes in HDL subpopulations are the result of EFA deficiency and are not merely unusual effects caused by both lack of EFA and fat in a fat-free diet or by lack of EFA in the presence of a saturated fat whose fatty acid composition might produce unique metabolic responses. For example, HCNO is very often used as a fat source in EFA-deficient diets. However, it is unusually rich in lauric (C12:0) and myristic (C14:0) acids, and a larger proportion of these fatty acids may reach the liver by absorption as non-esterified fatty acids via the portal vein, in contrast to longer-chain saturated fatty acids that are absorbed as triglycerides via the lymph.

Increased concentrations of liver triacylglycerols (TG) and CE in EFA-deficient rats (5-7) have been attributed to the possibility of decreased very low density lipoprotein (VLDL) secretion into the plasma, perhaps because of lack of EFA to form phospholipids required for lipoprotein formation and secretion (8, 9). However, we have found (1, 10, 11) that the rate of plasma TG secretion, an index of VLDL secretion, was increased in EFA-deficient rats fed a 5% HCNO diet, in comparison with rats fed a 5% corn oil diet. Liver TG and CE were also increased in these EFA-deficient rats.

Consequently, it was important to determine whether increased plasma TG secretion in vivo could be correlated with increased liver TG and CE, in rats fed other EFA-deficient diets that altered plasma HDL subpopulations, as well as the 5% HCNO diet (3). Such a correlation would establish that the changes in these parameters, which affect or are affected by HDL metabolism, are also the result of EFA deficiency itself. Such information is needed to understand the functions of EFA in lipoprotein metabolism.

Therefore, in the present experiments, we have determined plasma TG secretion in vivo, liver concentrations of TG, phospholipids (PL), and esterified (CE) or non-esterified cholesterol (C), as well as plasma esterified and non-esterified cholesterol, in rats fed a fat-free diet or diets containing 5% fat as HCSO or HCNO, in comparison with controls fed these diets plus 1% safflower oil or a 5% corn oil diet. The corn oil diet was used for comparison with the literature or with some of our own earlier experiments, and for comparison with the diets containing 1% safflower oil. Lipid compositions, but not secretion rates, were also measured in rats fed a non-purified stock diet because stock diet-fed rats are often erroneously reported as "controls" for rats fed EFA-deficient purified diets.

MATERIALS AND METHODS

Rats and diets. Male Sprague-Dawley rats, 45-50 g (about 21 days old) were purchased from Bantin-Kingman (Fremont, CA). The rats were housed individually in wire-mesh-bottom galvanized or stainless steel cages with tap water and diet available at all times. Some rats were fasted overnight (food cups were removed at 5 p.m.) before triglyceride secretion rates

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Abbreviations: C, cholesterol; CE, cholesteryl esters; EFA, essential fatty acid; FRC, fractional catabolic rate, min^{-1}; GLC, gas-liquid chromatography; HCNO, hydrogenated coconut oil; HCSO, hydrogenated cottonseed oil; HDL, high density lipoproteins; LDL, low density lipoproteins; PL, phospholipids; SAFF, safflower oil; TG, triacylglycerols; TLC, thin-layer chromatography; VLDL, very low density lipoproteins.

were measured the next morning from about 9 a.m. to 12 noon. All other rats were allowed access to food until anesthetized. The animal room was kept at 22°C with constant lighting (7 a.m. to 7 p.m.). Rats were assigned to diet groups on the basis of body weight, so that the average starting weights in all groups were equal. Diet compositions are shown in Table 1. The rats were given the diets for 8 weeks and then were used either for measurement of triglyceride secretion rate (using Triton treatment) or for analysis of plasma and liver lipids.

Measurement of triglyceride secretion rate. The right external jugular vein and the left carotid artery were cannulated (10) in rats anesthetized by intraperitoneal injection with Nembutal, 50 mg/kg body weight (Abbott Laboratories, North Chicago, IL).

Blood samples, 0.3 ml, were drawn from the carotid cannula (1) before and 10, 30, 50, 70 and 90 min after injection of Triton WR-1339 into the jugular cannula (1 ml of 10% Triton WR-1339 (Tyloxapol, Sigma, St. Louis, MO) in 0.9% saline). As before (1), lipids were extracted from 0.12 ml plasma and fractionated by thin-layer chromatography (TLC) to isolate TG, and the amount of TG in each sample was measured spectrophotometrically. A measured amount of ^{14}C-glycerol trioleate (Amersham, Arlington Heights, IL) was added to the plasma before extraction; recovery of radioactivity in individual TG samples ranged from 80–95%, and individual sample values were corrected for losses.

TABLE 1

Composition of Purified Diets

Diets[a]	Components, g/100 g of diet	
	Fat	Sucrose[b]
EFA-Deficient		
0% Fat	0	74.5
5% Saturated fat (5% HCNO[c], 5% HCSO[d])	5	69.5
EFA-Adequate		
1% SAFF[e]	1	73.5
5% Saturated fat[c,d] + 1% SAFF	6	68.5
5% Corn[f]	5	69.5

[a]All diets included (g/100 g diet): 20.0 vitamin-free casein; 91.0% crude protein (Teklad, Madison, WI); 1.0 B-vitamin mix in sucrose (12) (vitamins from Sigma, St. Louis, MO); 1.0 vitamin A, D, E mix in sucrose (12) (vitamins from Sigma); 3.5 mineral mix UCB-1Rb (13); Na_2SeO_3 and $KCr(SO_4)_2 \cdot 12H_2O$ added separately in sucrose to give 0.1 mg Se and 2 mg Cr/kg diet. (Mallinckrodt, Paris, KY); 0.18 choline bitartrate (Sigma).

[b]Powdered sucrose, C & H Sugar Co. (San Francisco, CA).

[c]Hydrogenated coconut oil, California Fats and Oils (Richmond, CA). HCNO fatty acids, % by weight: 8:0, 4%; 10:0, 6%; 12:0, 49%; 14:0, 18%; 16:0, 10%; 18:0, 12%.

[d]Hydrogenated cottonseed oil, Wilsey Foods (San Francisco, CA). HCSO fatty acids, % by weight: 16:0, 25%; 18:0, 71%; 18:1n-9, 2%; 18:2n-6, 0.5%; with 1.5% minor unidentified compounds.

[e]Safflower oil, Saffola (Los Angeles, CA). SAFF fatty acids, % by weight: 16:0, 7%; 18:0, 3%; 18:1n-9, 12%; 18:2n-6, 77%; 18:3n-3, 0.5%.

[f]Corn oil, Mazola, Best Foods (Englewood Cliffs, NJ). Corn oil fatty acids, % by weight: 16:0, 13%; 16:1n-7, 0.1%; 18:0, 2.3%; 18:1n-9, 26.1%; 18:2n-6, 58%; 18:3n-3, 1.1%.

The rate of TG secretion for each rat can be calculated from the TG concentrations observed at intervals after administration of Triton WR-1339, which blocks removal of TG from the plasma (14–17). The slope (rate) and correlation coefficient of TG accumulation in each rat were calculated from TG concentrations measured at 6 time points. The fractional catabolic rate, min^{-1} (FCR or k) is rate (mg × min^{-1} × ml^{-1}) divided by TG concentration (mg × ml^{-1}) (11). Comparisons of control and deficient rats were made with Student's unpaired t-test (18).

Analysis of liver and plasma lipids. From rats anesthetized with Nembutal, blood was collected by heart puncture, centrifuged at 4°C, and plasma collected. Lipids from both liver and plasma were extracted into $CHCl_3$/MeOH (2:1, v/v) as described (1).

Total lipid extracts were fractionated by TLC (diethyl ether/petroleum ether/acetic acid, 23:77:1, v/v/v) into TG, total PL and other lipid classes (Merck 0.25 mm silica gel H precoated plates without indicator or binder). Total PL formed a visible spot at the origin and was scraped off without use of dyes. The powder was heated (80°C) in screw-capped tubes, under nitrogen, with methanol, sulfuric acid and a measured amount of heptadecanoic acid (Nuchek Prep, Elysian, MN) as internal standard (1). The methyl esters were analyzed by gas-liquid chromatography (GLC) and the amount of PL was calculated from the amount of fatty acids found by GLC. The calculation assumed that 65% of phospholipid weight is in acyl groups.

TG in liver were analyzed by the same procedure used for plasma TG, except that labelled internal standard was not used during the lipid extraction.

Non-esterified cholesterol and total cholesterol were measured by GLC with stigmasterol (Sigma S-6126, ca. 90% pure, m.p. 164–167°C) as internal standard. An aliquot of total lipid extract was mixed with a measured amount of stigmasterol in solution, and the solvents were evaporated under nitrogen and replaced with acetone. An aliquot was analyzed by GLC. The solvent was evaporated from the remaining sample, which was then saponified with alcoholic KOH for 3–4 hr at room temperature. Water and petroleum ether were then added, and the sample was capped and let stand overnight. The petroleum ether phase, containing cholesterol and stigmasterol, was collected and evaporated to dryness. Acetone was added, and an aliquot analyzed by GLC.

Gas-liquid chromatography. A Varian 2100 Chromatograph (Varian Associates, Instrument Group, Palo Alto, CA), with hydrogen flame detectors and 2-mm i.d. glass columns, was used for analysis of fatty acid methyl esters and cholesterol. Columns about 2 m long, packed with 3% SP2250 on Supelcoport 1-1878 (Supelco Inc., Bellefonte, PA) were used for cholesterol analysis. Columns about 4 m long, packed with 10% SP2330 on 100/120 Chromosorb W AW (Supelco), were used for methyl ester separation. A mixture of known amounts of methyl esters of 16:0, 18:0, 18:1n-9, 18:2n-6, 18:3n-3, 20:4n-6 and 22:6n-3 (Nucheck Prep) was used as a calibration standard. Cholesterol (99+%, Sigma "standard for chromatography," CH-S) was used as standard for cholesterol analysis.

Solvents and chemicals used in lipid extraction

and analysis were reagent grade unless specified otherwise above.

Statistics. The mean values for EFA-deficient rats were compared with those of corresponding control rats by Student's unpaired t-test.

RESULTS

Plasma TG secretion rates. These rates (Table 2) in nonfasted rats were higher in the deficient rats than in their controls. The rates for all the deficient groups were quite similar, as were the rates for all control groups.

We tested plasma TG secretion in rats fasted overnight to evaluate this parameter under conditions similar to those in our experiments (3) on HDL subpopulations, which are customarily obtained from overnight fasted animals. However, fed animals are customarily used in studies of hepatic lipogenesis and plasma TG secretion in order to evaluate the capacity for lipogenesis under dietary conditions promoting lipogenesis. Overnight-fasting decreased the secretion rates in all groups, with somewhat greater decreases in the deficient groups. The rate remained higher in the deficient groups, but statistically significant differences occurred only between the fat-free and 1% safflower oil (SAFF) groups or between the HCNO and corn oil groups.

Plasma TG concentrations. These (Table 2) were significantly lower in the nonfasted deficient groups in comparison with their controls, i.e., the deficient diet plus 1% SAFF. The highest plasma TG concentration was in the corn oil group. Overnight fasting significantly reduced plasma TG concentration in all groups, except the 5% HCSO group, in which plasma TG in the fasted group was slightly higher than in the nonfasted 5% HCSO group or the fasted 5% HCSO + 1% SAFF group. However, for the other pairs of diets, plasma TG concentration remained higher in the EFA-supplemented groups.

Fractional catabolic rates. These rates (Table 2) for fed rats were significantly higher (2.5 to 3 times) in all deficient groups in comparison with their controls. This pattern also occurred after overnight fasting except in the HCSO groups.

Liver lipids (Table 3). With all of the pairs of diets, EFA deficiency significantly increased the concentrations of TG (2-3 times) and CE (4-6 times). EFA deficiency did not affect the concentrations of liver phospholipids or unesterified cholesterol.

The EFA-supplemented groups fed the purified diets had TG values 2-3 times higher and CE values up to 2 times higher than the corresponding values for the chow-fed group. Consequently, the increases in liver TG and CE attributed to EFA-deficiency would be greatly exaggerated if the deficient rats were compared with rats fed a chow diet, rather than a purified diet containing EFA. These results clearly show that a stock diet is not a legitimate control for a purified diet.

Phospholipid fatty acyl group distributions (Table 4). Despite the differences in dietary fat composition, the three EFA-deficient diets produced typical and almost identical distributions of acyl groups, including a high percentage of eicosatrienoate (20:3n-9) and 18:1n-9, and low percentages of 18:2n-6 and 20:4n-6. The EFA-supplemented groups likewise were very simi-

TABLE 2

Plasma Triacylglycerol Concentrations, Rates of Triacylglycerol (TG) Secretion and Fractional Catabolic Rates in Fed or Overnight Fasted Male Rats Given EFA-Deficient Diets or Adequate Diets[a]

Diet	Number of rats	TG secretion rate (mg/ml•min)	TG concentration (mg/ml plasma)	Fractional catabolic rate (min^{-1})
		Fed		
Fat-free	5	0.085 ± 0.024[b]	0.64 ± 0.18[b]	0.155 ± 0.102[b]
1% SAFF	5	0.058 ± 0.008	1.42 ± 0.58	0.049 ± 0.024
5% HCNO	6	0.090 ± 0.020	0.86 ± 0.19[b]	0.114 ± 0.054[b]
5% HCNO + 1% SAFF	4	0.064 ± 0.035	1.59 ± 0.34	0.038 ± 0.014
5% HCSO	5	0.095 ± 0.007[b]	0.55 ± 0.12[b]	0.171 ± 0.030[b]
5% HCSO + 1% SAFF	5	0.063 ± 0.014[c]	0.89 ± 0.24	0.073 ± 0.008
5% Corn	8	0.055 ± 0.018	1.85 ± 0.87	0.034 ± 0.013
		Overnight Fasted		
Fat-free	4	0.050 ± 0.007[b,c]	0.32 ± 0.12[b,c]	0.168 ± 0.044[b]
1% SAFF	5	0.039 ± 0.003[c]	0.59 ± 0.20[c]	0.070 ± 0.017
5% HCNO	6	0.046 ± 0.013[c]	0.46 ± 0.14[b,c]	0.112 ± 0.047[b]
5% HCNO + 1% SAFF	5	0.036 ± 0.06	0.73 ± 0.20[c]	0.050 ± 0.008
5% HCSO	6	0.048 ± 0.008[c]	0.77 ± 0.26	0.067 ± 0.022[c]
5% HCSO + 1% SAFF	5	0.040 ± 0.006[c]	0.56 ± 0.08[c]	0.072 ± 0.012
5% Corn	4	0.033 ± 0.005[c]	0.84 ± 0.28[c]	0.045 ± 0.021

[a]Means ± S.D.
[b]Essential fatty acid-deficient diet significantly different from adjacent control diet, p less than 0.05.
[c]Significant effect of fasting, p less than 0.05.

TABLE 3

Concentrations of Liver Phospholipid (PL), Triacylglycerol (TG) and Unesterified (C) or Esterified (CE) Cholesterol in Rats Given Essential Fatty Acid-Deficient or Adequate Diets*

Diet	Number of rats	mg/g fresh liver			
				Cholesterol	
		PL[a]	TG[b]	C	CE[c]
Fat-free	4	23.4 ± 1.5	36.7 ± 8.3[d]	1.86 ± 0.06	3.69 ± 0.62[e]
5% HCNO	4	23.6 ± 4.1	43.3 ± 15.9[f]	1.82 ± 0.16	2.50 ± 0.78[g]
5% HCSO	4	23.4 ± 4.8	43.1 ± 16.6[h]	1.90 ± 0.35	2.33 ± 1.54[i]
1% SAFF	5	22.0 ± 2.7	17.9 ± 6.0[d]	1.82 ± 0.08	0.62 ± 0.16[e]
1% SAFF + 5% HCNO	4	25.3 ± 6.4	12.8 ± 1.6[f]	1.64 ± 0.23	0.33 ± 0.14[g]
1% SAFF + 5% HCSO	4	23.7 ± 2.9	17.8 ± 6.6[h]	1.65 ± 0.23	0.41 ± 0.06[i]
5% Corn	4	24.0 ± 3.3	14.9 ± 6.4[j]	1.54 ± 0.12	0.49 ± 0.17[k]
Stock	5	27.3 ± 2.6	6.7 ± 1.6[j]	1.68 ± 0.26	0.25 ± 0.08[k]

*Means ± S.D.

[a]Phospholipid calculated from methyl ester weight, assuming that 65% of phospholipid weight is in acyl groups.

[b]Values with the same superscript in a column are significantly different from each other, p less than 0.05.

[c]Calculated as total cholesterol (measured) —unesterified cholesterol (measured).

TABLE 4

Fatty Acyl Group Distributions in Liver Total Phospholipids of Male Rats Fed Essential Fatty Acid-Deficient Diets or Adequate Diets

Diet[a]	Percentage by weight of total methyl esters[b], mean ± S.D.								
	16:0	16:1n-7	18:0	18:1n-9	18:2n-6	20:3n-9	20:4n-6	22:5n-6	22:6n-3
Fat-free	16.5±1.4	4.7±0.6	24.1±1.8	18.7±3.2	1.5±0.5	16.6±2.3	5.7±1.6	1.5±0.5	1.8±0.5
5% HCNO	16.6±3.2	5.4±0.9	25.6±1.5	17.5±2.4	1.7±0.2	18.7±1.7	5.0±0.8	1.4±0.3	1.8±0.5
5% HCSO	18.4±2.5	5.0±0.4	26.8±1.8	18.8±2.1	1.9±0.3	18.0±0.8	4.9±0.2	0.7±0.5	1.2±0.2
1% SAFF	19.9±1.2	tr[c]	25.0±1.8	11.3±0.9	7.3±0.7	0.7±0.4	22.5±1.2	5.6±0.9	0.6±0.1
1% SAFF + 5% HCNO	19.9±1.4	1.5±0.2	28.4±5.2	10.8±1.6	8.7±1.5	0.8±0.4	20.6±3.9	4.0±0.5	1.0±1.0
1% SAFF + 5% HCSO	18.4±2.9	tr	28.7±3.3	10.7±0.8	6.4±1.1	1.4±0.4	23.0±3.1	7.0±2.9	0.5±0.2
5% Corn	18.7±3.4	1.9±0.6	24.4±3.2	10.4±3.8	9.9±1.0	0.5±0.5	23.7±4.6	4.3±2.2	1.6±0.9
Stock	19.9±1.3	0.8±0.5	24.8±4.1	5.6±0.6	15.2±1.0	tr	19.6±1.8	nd[d]	7.4±2.6

[a]4 or 5 animals per group.

[b]Several small components not shown.

[c]Only a trace detected.

[d]nd, Not detected.

lar to each other in the patterns of acyl groups. The expected differences between deficient and control groups were found in the proportions of 16:1n-7, 18:1n-9 and 20:3n-9 (all endogenously formed) and in 18:2n-6, 20:4n-6 and 22:5n-6 (derived from the diet). Stearate (18:0) proportions were similar in all groups, including the stock-diet fed rats, but palmitate was somewhat lower in groups fed the 0 fat and 5% HCNO diets. Stock-diet-fed rats, however, differed from the purified diet-EFA-fed rats in the proportions of 18:1n-9, 18:2n-6, 22:5n-6 and 22:6n-3.

Plasma cholesterol (Table 5). The effects of EFA deficiency on plasma cholesterol varied with diet. Plasma nonesterified cholesterol tended to be lower in the deficient groups, in comparison with their corresponding controls, but the difference was statistically significant only for the HCSO pair. CE was lower in the fat-free and HCSO-fed groups but not in the HCNO-fed group, in comparison with their controls. The lowest values for both nonesterified and esterified cholesterol were in the stock-diet fed group.

DISCUSSION

We had previously found increased rates of plasma TG secretion, together with increased liver TG and CE in EFA-deficient rats fed diets containing 5% HCNO, in comparison with rats fed a 5% corn oil diet (1, 10, 11). In the experiments reported here, our primary aim was to determine whether these increases were unique to EFA deficiency produced with a diet containing 5% HCNO or would occur also with an EFA-deficient diet supplying another type of saturated fat (5% HCSO), as well as with a fat-free diet. The occurrence of similar changes with three different EFA-deficient diets, in comparison with appropriate control groups, would strengthen the conclusion that these changes resulted specifically from EFA deficiency. We had found earlier (3) that all three EFA-deficient diets produced similar changes in HDL subpopulations.

In planning our experiments, we considered it important to have the same amount of saturated fat in the deficient and control diets because of evidence that saturated fat increases the need for EFA (19, 20). Therefore, in designing control diets, we chose to add 1% safflower oil to the EFA-deficient diets containing 5% saturated fat rather than to maintain a constant total fat level by reducing saturated fat to 4%. That no important changes were produced by the 1% difference

TABLE 5

Plasma Cholesterol Concentrations in Rats Given Essential Fatty Acid-Deficient or Adequate Diets*

Diet	Number of rats	Plasma cholesterol, mg/ml	
		Unesterified[a]	Esterified[b]
Fat-free	4	0.16±0.04	0.46±0.08
5% HCNO	4	0.23±0.03	0.58±0.09
5% HCSO	4	0.18±0.04[c]	0.44±0.06[d]
1% SAFF	5	0.24±0.06	0.57±0.12
1% SAFF + 5% HCNO	4	0.27±0.06	0.56±0.13
1% SAFF + 5% HCSO	4	0.28±0.04[c]	0.65±0.18[d]
5% Corn	4	0.27±0.04[e]	0.63±0.18[f]
Stock	5	0.18±0.04[e]	0.36±0.06[f]

*Means ± S.D.

[a]Values with the same superscript are significantly different from each other, p less than 0.05.

[b]Calculated as total cholesterol (measured) —unesterified cholesterol (measured).

in total dietary fat is shown by similar values in the EFA-supplemented groups whose dietary fat levels ranged from 1% to 6%.

Plasma TG secretion, liver TG and CE. From our results it is clear that in comparison with their controls, the three EFA-deficient groups, under fed conditions, showed similar changes in plasma TG secretion and in liver TG and CE, i.e., increased plasma TG secretion, increased FCR and increased liver TG and CE. The differences between deficient and control rats in plasma TG secretion rates and FCR values were reduced but persisted in overnight-fasted rats. Thus these changes are not a unique interaction between EFA deficiency and one particular type of dietary saturated fat but are characteristic of EFA deficiency itself, at least with these relatively low-fat, high carbohydrate diets. Our purified diets contained sucrose, but these changes in lipid metabolism in EFA-deficient rats are not unique to diets containing sucrose, because we have observed them with diets containing glucose (1).

Plasma cholesterol. How EFA deficiency affected total plasma cholesterol and CE concentrations depended on the type of EFA-deficient diet used. Significant differences between deficient and control groups occurred only with the 5% HCSO diets. A decrease in plasma CE might be expected because of the decrease in the HDL_1 subpopulation in EFA-deficient rats (1, 2). However, decreased CE in this lipoprotein fraction could be counterbalanced by increased CE in other lipoprotein fractions. These possibilities are still to be tested. Increased lecithin cholesterol acyltransferase activity has been reported in EFA-deficient rats (21). However, we have found no reports on the distribution of CE and C in plasma lipoprotein fractions of EFA-deficient rats in comparison with controls fed the same diet plus EFA, although changes in fatty acid composition have been reported (21).

Fatty acid composition of liver phospholipids. The proportions of fatty acids in liver phospholipids were similar in deficient rats fed the fat-free diet or the diets containing 5% saturated fat. Likewise, the proportions of fatty acids in liver phospholipids were similar in all control groups. Thus, the presence of dietary saturated fatty acids at a low level (5% by weight) did not alter EFA utilization, at least as indicated by fatty acyl group distribution in liver phospholipid fatty acids. The evidence that saturated fatty acids increase the need for EFA (19, 20) is based mainly on the appearance of more severe skin lesions in rats fed diets containing high levels of HCNO (ca. 20%). However, the levels of EFA in liver phospholipids in these rats did not indicate more severe depletion in comparison with rats fed a 5% HCNO diet (13).

The greater proportion of docosahexaenoate in the liver phospholipid fatty acids of the rats fed the stock diet probably reflects a greater dietary supply of this n-3 fatty acid or its precursors from the nonpurified ingredients, including fish meal, in the stock diet. There is no obvious explanation for the higher proportion of linoleate and the lower proportion of arachidonate in liver phospholipids of the rats fed the stock diet, unless the higher intake of n-3 fatty acids reduced the conversion of linoleate to arachidonate. Note also the lower proportion of oleate in these stock diet-fed rats. These great differences in lipid composition between the rats fed a nonpurified stock diet and those fed the purified control diets (all "adequate" diets) again point out the risks and the worthlessness of using a stock diet as a "control" for a purified diet.

Possible reasons for increased plasma TG secretion and increased liver TG and CE. The increased plasma TG secretion in all of the deficient groups, in comparison with their controls, makes it clear that the increased liver TG in EFA-deficient rats does not occur because their ability to secrete plasma TG is less than that of controls. However, the increased liver TG does show that the ability of deficient rats to make and/or deposit TG exceeded their ability to secrete TG. The reasons for the increased plasma TG secretion are unknown. It may result in part from the increased hepatic lipogenesis in the deficient rats (13, 24) and/or it may be related to the higher metabolic rate of the deficient animals, which have a defective epidermal water barrier and increased epidermal water loss (22, 23). The greater fractional catabolic rates in the deficient groups are consistent with increased energy need. Increased lipoprotein lipase activity has been reported in EFA-deficient rats (21).

Increased liver CE and increased activities of hepatic lipogenic enzymes are early effects of EFA deficiency in rats. These changes have been detected after only 7–9 days of feeding an EFA-deficient diet to weanling rats (6, 24). Hepatic lipogenesis may increase because of the removal of the inhibitory effect of long-chain polyunsaturated fatty acids, which cause both short-term and long-term inhibition (24, 25).

Increased liver CE could result from increased synthesis, from decreased biliary secretion of cholesterol and bile acids, from increased uptake of plasma cholesterol, and/or from reduced cholesterol output in lipoproteins secreted by the liver. There is currently no evidence for increased hepatic synthesis. In contrast, there is evidence for reduced hepatic synthesis, as measured by 1-^{14}C-acetate incorporation, after only one week of EFA deficiency with rats fed a fat-free diet in comparison with controls fed the same diet supplemented with methyl linoleate (6).

How EFA deficiency affects biliary secretion of cholesterol and bile acids is not yet resolved. Decreased biliary secretion has been suggested from the finding that EFA-deficient rats fed a purified, fat-free diet secreted less cholesterol and bile acids and showed reduced hepatic cholesterol synthesis in comparison with rats fed a nonpurified stock diet (8). However, because a stock diet is not a control diet, it is not clear what these observations mean with regard to effects of dietary composition on metabolic processes. Indeed, there is evidence that bile acid secretion and liver cholesterol synthesis are lower in rats fed a purified, EFA-adequate diet than in rats fed a nonpurified stock diet (26, 27). Consequently, the possibility that increased liver cholesterol in EFA-deficient rats results from decreased biliary secretion of cholesterol and bile acids must be reevaluated under conditions of proper dietary control, i.e., with EFA-deficient rats compared with rats fed the same EFA-deficient diet supplemented with EFA.

More rapid removal of ^{14}C-cholesterol from plasma and more rapid accumulation of the labeled cholesterol in liver has been shown in EFA-deficient rats (28). Our recent observation (3) that the apoE containing-HDL_1 subpopulation is greatly reduced in EFA-deficient rats is also consistent with the notion of increased hepatic uptake of cholesterol, presumably via the low density lipoprotein (LDL) (apoB,E) receptor (29). The HDL_1 subpopulation, which is richer than HDL_2 in nonesterified cholesterol, may be a preferred source of cholesterol for bile acid synthesis (30). Increased uptake might be a consequence of increased hepatic LDL receptor activity (31, 32) as a result of reduced hepatic cholesterol synthesis (6) and/or an increased insulin/glucagon ratio in EFA-deficient rats (1).

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The Precursors of Fecapentaenes: Purification and Properties of a Novel Plasmalogen

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Fecapentaene-12 and fecapentaene-14 are genotoxic polyunsaturated ether-lipids produced by the colonic microflora in humans and pigs. Although the fecapentaenes have been extensively characterized, little is known about the nature of the precursors from which they are produced. We purified one form of these precursors from feces of an individual who excreted high levels of fecapentaene-12 and its precursors. Purification was carried out by a series of extractions and precipitation in organic solvents followed by silica and amine high performance liquid chromatography. The purified precursor had identical UV spectral characteristics as the fecapentaenes indicating that it contained the same ether-linked pentaenyl functional group. However, it was not mutagenic. The precursor was amphiphilic in nature, behaving like a synthetic "model" ether-phospholipid on silica and C_{18} thin layer chromatography. When incorporated into phosphatidylcholine micelles it could be hydrolyzed *in vitro* by a combination of lipase and phospholipase C to fecapentaene-12. Our findings indicate that the general structure of this precursor is that of a phospholipid, specifically a plasmalogen—the exact structures of which remain to be determined.
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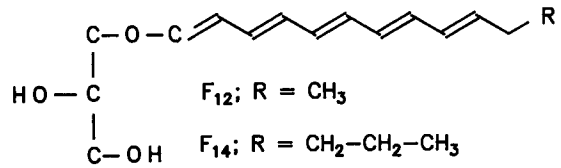
Since their discovery in 1977 (1) and their reported association with increased risk for colon cancer (2), the fecapentaenes have been the subject of intensive studies. Most of the work has been on their formation, structures and biological effects, and there are studies underway to determine the carcinogenic potential of the fecapentaenes in experimental animals. The fecapentaenes are the most prevalent genotoxins found in the colon, accounting for the majority of direct-acting fecal mutagenicity (3, 4). Based on the length of the pentaene chain, two forms of these polyunsaturated 1-*O*-(1-alkenyl) glycerols (Scheme 1) have been isolated from feces—a 12-carbon form, fecapentaene-12, and a 14-carbon form, fecapentaene-14 (5, 6). The biological mechanism by which the pentaene moiety is formed remains unknown. The relative amounts of these mutagens in feces vary considerably among individuals (7), however, the ratio within the feces of any one individual remains constant for years (8). Besides being a potent mutagen in the Ames test (9, 10), fecapentaene-12 is active in a variety of other *in vitro* tests for genotoxicity (11-14). For details of the chemistry and postulated mechanisms of action of these compounds see the excellent reviews of Venitt (15) and Krepinsky (16).

It has been known for years that the levels of fecapentaenes in feces can increase up to 50-fold upon *in vitro* anaerobic incubation of the feces of individuals who excrete fecapentaenes (17, 18). Several species of the common intestinal anaerobes *Bacteroides* are responsible for this increase (19). However, production of fecapentaenes *in vivo* requires not only *Bacteroides* spp., but also requires the presence of precursor compounds with levels in the colon that vary as much as the fecapentaenes (7, 20, 21). *In vitro*, the *Bacteroides* spp. are incapable of producing fecapentaenes unless provided with these precursors, usually in the form of a methanol extract of feces of an individual who excretes fecapentaenes (19, 20). The origin of these precursors is unknown. However, like the fecapentaenes, they are found, in uniform concentrations throughout the colons of certain individuals (21). Recently, the production and excretion of fecapentaenes and their precursors by pigs has been described (21, 22). This is the only other mammal in which these compounds have been detected; and, thus, a potentially useful animal model now exists in which the fecapentaenes and their precursors may be studied.

Recent epidemiological studies have shown that the fecapentaenes and their precursors can be detected in the feces of more than 75% of the individuals in a normal population on a Western diet. The concentrations of fecapentaenes ranged from of < 10 ng/g to > 25,000 ng/g dry feces; the concentration of precursors ranged from < 10 ng/g to 80,000 ng/g dry feces (4, 23). These studies also showed, in contrast to early population studies, that the excretion of fecapentaenes does not appear to be associated with increased risk of colon cancer or intake or dietary fat (23, 24).

We have focused our current work on determining the nature of the precursors, particularly their origin and structures. Purification and structural determination of any form of the precursor has been confounded by the same obstacles encountered during purification and structural determination of the fecapentaenes themselves—their instability to oxygen, light and mild acids, their low concentrations in feces and their molecular heterogeneity. In a preliminary report (20), we described early attempts at purifying precursors and characterizing them as to their chromatographic properties.



SCHEME 1. The general structures of the fecapentaenes.

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Abbreviations: BHT, butylated hydroxytoluene; MTBE, methyl tert-butyl; PBS, phosphate-buffered saline; BHI, brain-heart infusion broth; PRAS, prereduced and anaerobically sterilized; RCM, Radial Compression Module.

In this report, we describe the methods for overcoming the aforementioned obstacles and purifying relatively large quantities of material. We characterize one form of precursor as to its chromatographic and mutagenic properties and demonstrate its ability to be hydrolyzed by a combination of lipolytic enzymes to fecapentaene-12.

MATERIALS AND METHODS

Enzymes, chemicals and reagents. Type XI-E Phosphatidylcholine (egg yolk lethicin), type VII-S lipase (*Candida*), type XI lipase (*Rhizopus*), type XII lipase (*Chromobacterium*), type VI-S lipase (porcine pancreas), phospholipases A_2 (*Naga*, bee venom and bovine pancreas), phospholipase B (*Vibrio*), type XII phospholipase C (*Bacillus cereus*), type XIV phospholipase (*Clostridium perfringens*), type VI phospholipase D (*Streptomyces*), type III phospholipase D (peanut), type I phospholipase D (cabbage) and sphingomyelinases (human placenta and *Staphylococcus aureus*), sodium cholate and crude lyophilized bile (Oxgall) were obtained from Sigma Chemical Co. (St. Louis, MO). Spray reagents for TLC, molybdenum blue, aniline diphenylamine and *N,N*-dimethyl-*p*-phenylenediamine were also obtained from Sigma.

Racemic fecapentaene-12 was synthesized using methods based on the Horner-Wittig reaction as previously described (25, 26). A "model" ether-phospholipid representing "saturated precursor," 1-*O*-dodecyl-2-octadecanoyl-*rac*-glycero-3-phosphocholine), was prepared from 1-*O*-dodecylglycerol (27) by protection of the primary hydroxyl group as the (methoxy-*t*-butylphenyl)silyl derivative, acylation of the secondary hydroxyl group with octadecanoic acid and deprotection of the silyl-protected hydroxyl group under mild conditions (28). Final conversion was achieved by treatment with 2-chloro-2-oxo-1,3,2-dioxaphospholane followed by trimethylamine (29).

The organic solvents used for extractions (reagent grade) and chromatography (HPLC grade) were obtained from American Burdick & Jackson (Muskegon, MI) and were stabilized with 100 μg/ml butylated hydroxytoluene (BHT, Sigma Chemical Co.). HPLC grade chloroform also contained 1% ethanol as a stabilizer.

Brain-heart infusion broth (BHI), phosphate-buffered saline (PBS, pH 7.2) and HEPES buffer (0.01 M, pH 7.5) were prereduced and anaerobically sterilized (PRAS) according to the methods described in the VPI Anaerobe Laboratory Manual (30).

Bacteria and feces. Bacteroides thetaiotaomicron VPI 5482 was obtained from the culture collection of the Department of Anaerobic Microbiology, VPI & SU. Cell-free lysates were prepared by sonication of late-log phase BHI cultures as previously described in detail (7, 20).

Fecal samples from a 36-year-old nonsmoking Caucasian female in good health were used as the source of natural precursors of fecapentaenes. Fecal samples were collected at the subject's home in Zip-lok bags and immediately frozen at −15°C until brought to the laboratory. We placed 5–10 frozen samples in a Zip-lok bag (continually flushing the bag with argon) and allowed them to thaw at room temperature. The samples were thoroughly mixed by kneading, aliquoted into lyophilization flasks and frozen at −80°C. The pooled feces were lyophilized, finely ground (in an anaerobic glove box) and stored in containers sealed under argon at −80°C.

Extraction of precursor from feces. Freeze-dried feces (50 g) were placed in a 1 liter flask and extracted with a series of three reagent grade solvents containing 100 μg/ml BHT. All extractions were performed under argon with vigorous shaking at room temperature for 30 min. All fecal slurries were filtered into a vacuum flask connected to a water aspirator using a Büchner funnel with Whatman #1 filter paper.

First, the feces were extracted with 800 ml hexane, the slurry was filtered and the feces were extracted again with 500 ml hexane; the hexane extracts were discarded. Second, the feces were extracted two times with 500 ml of methyl tert-butyl ether (MTBE); again the extracts were discarded. Third, the feces were extracted three times with 500 ml methanol; the filtrates were pooled and flash evaporated at 55°C to 50 ml. This methanol extract was sealed under argon in stoppered glass tubes and stored at −4°C until it was extracted with hexane.

We performed liquid-liquid extraction on the methanol extract by mixing 5 ml PRAS-distilled water with the 50-ml methanol extract in a 500-ml separatory funnel and then adding 250 ml hexane. The vessel was sealed under argon and shaken vigorously at room temperature for 5–10 min. The phases were separated and the methanol/water phase was extracted four more times, each with 250 ml hexane. The hexane extracts were pooled, flash evaporated at 45°C to 5 ml and stored at 5°C under argon until precipitated with acetone.

The hexane extract was precipitated with acetone by mixing 45 ml of reagent grade acetone containing BHT directly with the 5-ml hexane extract and incubating the mixture without agitation at 4°C for 24—48 hr. The visible precipitate was collected by centrifugation at 2000 *g* for 10 min. We resuspended the precipitate in 2 ml methanol/chloroform (2:1), heated the mixture to 50°C for 10 min and filtered it using a glass prefilter and a 0.5 μm membrane filter (Acrodisc LC25, Gelman Sciences, Ann Arbor, MI). The sample was sealed under argon and stored at −80°C until its components were separated on HPLC.

Throughout the extraction scheme we followed the presence of precursor by incubating the feces or extracts with bacterial cell-free lysates of *B. thetaiotaomicron* 5482 and assaying for the production of fecapentaenes by our standard quantitative HPLC assay as previously described (7, 9, 21). Briefly, the feces (freeze-dried) or extracts (dried under argon) were resuspended in PRAS-BHI supplemented with 10 mg/ml Oxgall. The tubes were inoculated anaerobically with 10% (v/v) cell-free lysate, incubated at 37°C for 24–72 hr, frozen at −80°C, lyophilized and extracted with 20 volumes of acetone. The acetone extracts were chromatographed on silica HPLC using chloroform/isopropanol (92.5:7.5) and areas of the fecapentaene peaks were compared with those of dilutions of synthetic fecapentaene-12.

Purification of precursor by liquid chromatogra-

phy. All HPLC separations were performed on a Waters chromatograph (Millipore Co., Waters Div., Milford, MA) which consisted of: a U6K injector, an M6000 solvent delivery system, a Radical Compression Module (RCM) and a 400 UV detector (340 nm, AUFA = 2.0). All solvents were HPLC grade and were vacuum filtered through a 0.5 μm teflon membrane filter (Schleicher and Schuell, Keene, NH) prior to use. Chromatograms, peak areas and retention times were obtained with an HP 3390 recording integrator. All fraction were collected under argon and the presence of precursor was confirmed by demonstrating the characteristic "pentaene" triplet upon UV spectroscopy (30). Fractions were scanned from 260 to 380 nm (AUFS = 0.5) on a Perkin Elmer Lambda 4B UV/VIS Scanning Spectrophotometer at 120 nm/min.

The first isocratic HPLC system consisted of an RCM silica cartridge (8 mm × 10 cm, 10 μm particle size) and chloroform/methanol (94:6, v/v) containing 100 μg/ml BHT. The dissolved acetone precipitate was injected into the system in 100 μl increments; the flow rate was 3 ml/min. Precursor fractions were pooled and reduced to 2 ml under a stream of argon at 50°C.

The second HPLC system consisted of an RCM μBondapakNH_2 (amine) cartridge (8 mm × 10 cm, 5 μm particle size) and chloroform/methanol/ammonium hydroxide (65:35:1, v/v/v); BHT was omitted. We injected the concentrated pool of silica fractions into the system in 100 μl increments; the flow rate was 2 ml/min. Fractions of purified precursor were stored sealed under argon at −80°C.

Estimations of concentrations of precursor in the purified preparations were calculated by absorbance at 338 nm. Purified precursor was diluted in methylene chloride to yield an absorbance of 0.5 to 1.0. The precursor concentrations were calculated using the fecapentaene-12 extinction coefficient of 74,000 (33) and an estimated precursor molecular weight of 700.

To determine to which form, F12 or F14, the purified precursor was converted we used the RCM μBondapakC_{18} system with a quaternary solvent system of acetonitrile/water/methanol/tetrahydrofuran (36.2:32:25.4:6.4, v/v/v/v) as previously described in detail (6).

The relative purity of our precursor preparations was initially assessed by analyzing purified preparations on the silica and C_{18} HPLC systems (described above) using wavelength monitoring at 254 nm, 280 nm and 313 nm and high sensitivity (AUFS = 0.02).

Thin layer chromatography and UV spectroscopy. TLC was the second major criterion for purity of precursor. It also was used to compare the chromatographic mobilities of purified precursor with those of fecapentaene-12 and the "model" ether-phospholipid. In both cases, we used K6DF "linear K" silica plates (Whatman, Clifton, N.J.) developed in chloroform/methanol/ammonium hydroxide (85:15:1, v/v/v) and KC18F C_{18} plates (Whatman) developed in (1) tetrahydrofuran, (2) acetonitrile or (3) methanol/water (90:10, v/v). All solvents contained 100 μg/ml BHT. Approximately 5–10 μg of compound was spotted on 5 × 20 cm plates under a stream of argon and immediately placed in the developing chamber which had also been flushed with argon. Plates were visualized under long-wave UV light (358 nm) prior to (1) being sprayed with molybdenum blue and heated at 1215°C for 10 min and/or (2) being sprayed with a 25%-sulfuric acid and "charred" at 175°C for 15 min.

To compare the UV spectral characteristics of the purified precursor and synthetic fecapentaene-12, we scanned each from 300 to 380 nm using a Perkin Elmer Lambda 4B UV/VIS scanning spectrophotometer. Stocks of each compound were diluted in methanol, without BHT, to an absorbance 1.0–1.5 at 340 and scanned in 1-ml quartz cuvettes which were flushed with and stoppered under argon.

Enzymatic conversion of purified precursor. Each ten ml of precursor micelles was prepared by adding phosphatidylcholine (100 mg), purified precursor (ca. 500 μg), sodium cholate (100 mg) and BHT (1 mg) to 10 ml of chloroform in a 30–50 ml evaporator flask which was continuously flushed with argon. The solvent was removed by rotary evaporation at 50°C, and the resulting thin film of lipids was suspended in 10 ml PRAS HEPES under argon by gentle vortexing. The suspension was sonicated at 37°C for 10 min in a heated sonic bath (Branson Ultrasonics, Danbury, CT) and was used immediately for enzyme studies or stored at −80°C. As a nonmicelle control, we prepared suspensions of precursor in the same manner as described above for preparation of micelles, however we omitted the phosphatidylcholine.

Combined or individual stocks of lipases (50000 units/ml) and phospholipases (5000 units/ml) were prepared in PRAS HEPES supplemented with 25 mM $CaCl_2$ and stored on ice until used within 1–2 hr of preparation. Reaction mixtures consisted of 0.4 ml of the precursor micelles or nonmicelle precursor controls and 0.1 ml of the enzyme stocks; the mixtures were sealed under argon and incubated at 37°C for up to 18 hr. The reaction mixtures then were frozen in liquid nitrogen and lyophilized at 37°C for 3–5 hr. We extracted the lyophilized reaction mixtures with 2.5 ml acetone supplemented with 100 μg/ml BHT by vigorous shaking under argon for 10 min. The suspensions were filtered (Acrodisc LC13, 0.2 μm) into 10 × 75 mm tubes; and, the extract was blown down to dryness under argon and placed on ice. Each extract was dissolved in 200 μl of chloroform/isopropanol (92.5:7.5, v/v) containing 100 μg/ml BHT and immediately stopped under argon. We analyzed each sample using our standard quantitative HPLC system as described above.

Mutagenicity testing. To test the mutagenic potential of the purified precursor, we used the standard plate incorporation method of the salmonella/microsome mutagenicity assay (31) as previously described for the fecapentaenes and their analogs (7). Five different purified precursor preparations were tested in triplicate (1.0, 10 and 100 μg/plate) on TA98 and TA100 with and without activation with S9 microsome mix. As controls, we used 2-nitrofluorene on TA98, sodium azide on TA100 and dose-response concentrations of synthetic fecapentaene-12 (0.1, 1.0 and 10 μg/plate) on both tester strains.

To confirm that the fecapentaene produced by commercial enzymes from precursor micelles had similar mutagenic activity as synthetic racemic fecapentaene-12, we isolated the fecapentaene produced from precur-

sor in enzyme/micelle reaction mixtures and assayed it for mutagenicity. The fecapentaene was isolated by performing silica HPLC (described above) on the acetone extracts of incubated micelle/enzyme reaction mixtures and collecting the fecapentaene peaks. The fecapentaene fractions were concentrated to 1-5 μg/ml and assayed on TA98 and TA100 without microsomal activation.

RESULTS

Purification of precursor. Neither hexane nor MTBE extracted any precursors from freeze-dried feces as determined by *in vitro* incubation of these preparations with bacterial cell lysates. However, these solvents did remove material which interfered with liquid-liquid hexane extraction and the subsequent precipitation with acetone. If the feces were not extracted with hexane and MTBE prior to methanol, the precursor precipitated poorly in the acetone.

The freeze-dried feces contained ca. 6 μg/g of preformed fecapentaenes. After *in vitro* incubation with bacterial cell lysate and being freeze-dried a second time, the feces contained ca. 18 μ/g fecapentaenes. Thus, we estimated the concentration of precursors in unincubated freeze-dried feces to be 12 μg "fecapentaene-12 equivalents" per gram. Using the estimated molecular weight of 700 for the precursor, we thus estimated 33 μg precursor per gram unincubated freeze-dried feces. From 50 g of these feces, containing ca. 1650 μg of precursors, we purified ca. 570 μg of one form of precursor. Chromatographic profiles from silica and amine HPLC are shown in Figure 1.

When purified precursor was stored anaerobically for extended periods of time (3-5 wks) it would occasionally undergo a "chemical alteration." The fluorescence of the purified precursor fractions changed from lime-green to royal-blue and the characteristic 320-360 nm UV triplet changed to a single broad peak in the 260 nm-290 nm region. Furthermore, the chromatographic properties of this "altered form" of precursor were considerably different than that of fecapentaene-12 or the purified precursor. It migrated with the solvent front on silica and C_{18} TLC systems and eluted in the void volume on normal phase silica and amine HPLC. This "altered form" was stable (retained its royal-blue fluorescence) in air and light and it was not mutagenic. Its formation was not inhibited by BHT or promoted by air and its occurrence was more common when we stored the preparations above −20°C. The problems of sample loss encountered by this chemical change was minimized by storing all purified samples at −80°C and using them for subsequent studies within 1-2 weeks of preparation.

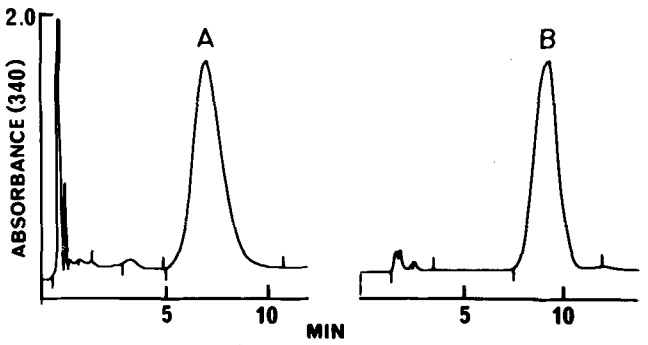


FIG. 1. Purification of a precursor of fecapentaene-12 by HPLC. (A) Initial separation of precursor in the acetone precipitate on normal phase silica HPLC; mobile phase = chloroform/methanol (94:6), flow rate = 3 ml/min, detection at 340 nm (AUFS = 2.0). (B) Final separation of precursor by normal phase HPLC on μBondapakHN$_2$; mobile phase = chloroform/methanol/ammonium hydroxide (60:40:1), flow rate = 2 ml/min, detection at 340 nm (AUFS = 2.0).

Because of the inherent semiquantitative nature of the way in which we estimated levels of precursors, recovery at each stage of purification was only roughly determined. Much of the loss (40-50%) of precursor occurred during the extraction stages where the efficiencies of extraction and precipitation were affected by the high mass loads present in the fecal extracts. However, some loss throughout the final stages of purification resulted from conversion of the precursor to the "altered form." On analysis by TLC of concentrated hexane extracts and silica HPLC fractions we often observed the royal-blue fluorescing spot at the solvent front, which indicated that some of the "altered form" was present.

The isolated precursor was chromatographically pure. No peaks from contaminating material or alteration in the shape of the precursor peak was observed upon reanalyzing purified material by HPLC at several wavelengths using high sensitivity. When we analyzed the purified precursor by both silica and C_{18} TLC we observed a single spot which fluoresced lime-green under UV light. The spot looked like a spot of fecapentaene-12, but it migrated much differently. When the plates were charred with sulfuric acid, only one spot was observed which coincided with the fluorescent spot.

Comparison of precursor with synthetic compounds. The relative mobilities of purified precursor, fecapentaene-12 and the "model" ether-phospholipid on silica and C_{18} TLC are shown in Table 1. Only the purified precursor and the "model" ether-phospholipid reacted with molybdenum-blue spray, a reagent used to detect phospholipids and related compounds. However, the purified precursor did not react with aniline, diphenylamine, benzidine or dimethyphenylene diamine, reagents which have been used to visualize the fecapentaenes (32).

TABLE 1

Relative Mobilities of Fecapentaene-12, Purified Precursor and Synthetic Ether-Phospholipid

TLC system	F-12	Natural precursor[a]	"Model" phospholipid[b]
Silica[c]	0.75	0.32	0.25
Silica[d]	0.65	0.05	0.05
C_{18}[e]	0.88	0.66	0.70
C_{18}[f]	0.84	0.85	0.92
C_{18}[g]	0.73	< 0.05	< 0.05

[a]Natural precursor purified from feces.
[b]synthesized 1-*O*-dodecyl-2-octadecanoyl-rac-glycero-3-phosphocholine.
Solvents: c, chloroform/methanol/ammonium hydroxide (85:15:1); d, chloroform/isopropanol (92:8); e, methanol; d, tetrahydrofuran; g, acetonitrile.

The UV spectra of the purified precursor and fecapentaene-12 in methanol were virtually identical. Absorbance maxima for the precursor were 321, 335 and 353; maxima for fecapentaene-12 were 320, 335 and 352.

The purified precursor was nonmutagenic even when tested at 100 μg/ml, a level 100-fold greater than the level at which the fecapentaenes are mutagenic. However, the fecapentaene-12 purified from enzymatically hydrolyzed precursor micelles was as mutagenic as the synthetic fecapentaene-12, ca. 1000 TA100 revertants per microgram.

Enzymatic hydrolysis of precursor. Maximum hydrolysis of precursor to fecapentaene occurred when the type VII-S lipase (*Candida*) and type XII phospholipase C (*B. cereus*) were incubated with the precursor in micellar form. After 18 hr of incubation at 37°C, 7-10 μg of fecapentaene was produced from the 50 μg of precursor estimated to be in the micelles; no more fecapentaene was produced upon further incubation. The form of fecapentaene produced in all cases was fecapentaene-12; no fecapentaene-14 was detected. Neither of the enzymes alone hydrolyzed the micellar or nonmicellar precursor; nor did the combined enzymes hydrolyze the nonmicellar precursor. Combinations of the other lipases and phospholipases C hydrolyzed micellar precursor with less than 10% of the efficiency as the combination of the *Candida* and *B. cereus* enzymes. The phospholipases A, B and D and sphingomyelinases did not hydrolyze purified precursor to detectable levels of fecapentaenes under any conditions when tested in the presence of the *Candida* lipase.

DISCUSSION

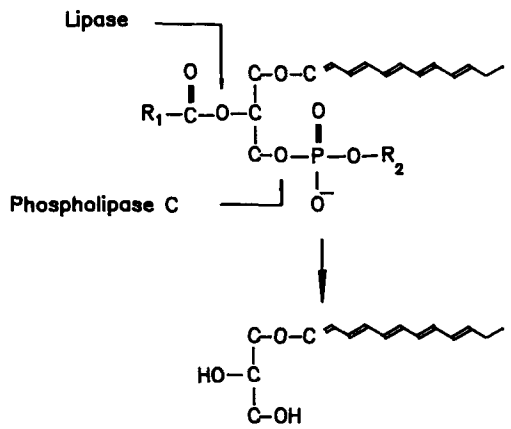
From our experience in purifying the fecapentaenes we knew the importance of using feces in which the fecapentaenes were of primarily one form, either, fecapentaene-12 or fecapentaene-14. When feces were used in which there were high amounts of both forms of fecapentaenes, not only did yields of both forms suffer, but accurate structural analysis also became difficult. Consequently, prior to starting the purification of precursor, we screened feces of several individuals who excreted high amounts of precursors and who were willing to donate fecal samples for years. We chose for this study one individual whose feces contained precursors which were converted by bacterial cell lysates primarily (> 95%) to fecapentaene-12.

The similarities in chromatographic behavior of the purified precursor and the "model" ether-phospholipid on TLC indicated that the precursors of fecapentaenes could be phospholipids. The observation that only combinations of lipase and phospholipase C could hydrolyze the purified precursor (in micelles) to fecapentaene-12 strongly supports, if not proves this possibility. The requirement for precursor to be in micellar form most likely reflects the membrane-like environment in which functioning lipolytic enzymes are most apparent. This is in contrast to the bacterial cell lysates which contain the active enzymes already complexed in membranes and which can hydrolyze precursor alone (7, 20).

The similarities in UV spectra between fecapentaene-12 and the purified precursor indicate that the precursor also contains the pentaenyl moiety, presumably also ether linked at the *sny*-1 position. Based on this observation and the results with the commercial enzymes, we can postulate how the hydrolysis of the precursor to fecapentaene-12 most likely occurs (Scheme 2). The phospholipase C removes the "phosphate" group from the *sn*-3 carbon; the lipase then removes the functional group from the *sn*-2 carbon of the resulting diglyceride. The pentaenyl group remains at the *sn*-1 position, probably because of the inability of the lipase to hydrolyze ether linkages. Thus, we presume the linkage at the *sn*-2 carbon to be an ester and not another ether linkage. Preliminary results (Kingston, unpublished data) obtained by direct chemical analysis (methylation and GC-MS) confirm the presence of an ester group in the precursor. Studies are currently being done to identify this ester group, as well as the phosphoryl functional group at the *sn*-3 position. These studies will be reported elsewhere, together with the full details of the methylation and GC-MS analysis of the purified precursor and synthesis of the "model" ether-phospholipid.

It has been shown that the glycerol backbone of the fecapentaene is not required for mutagenesis (10). Thus, it is noteworthy that even though the precursor contains the pentaene moiety it is not mutagenic. The *sn*-2 and *sn*-3 groups on the glycerol backbone must alter the "remarkable electrophilic properties of the pentaene-ether system" (32) in such a way as to render it nonreactive. We are currently testing the purified precursor for genotoxicity in other *in vitro* systems to determine if the nonreactivity is general or restricted to the salmonella system.

Assuming that the one form of precursor we isolated and characterized is representative of the precursors of fecapentaenes in general, we can assign the precursors to the plasmalogen class of lipids. To our knowledge, this would be the first example of a plasmalogen containing such polyunsaturation and conjugation in the ether-linked sidechain. Biologically active ether lipids, particularly plasmalogens, are found in many tissues in man and are involved in a variety of physiological responses. For an excellent discussion of the structures and functions of natural and "un-



SCHEME 2. Proposed mode of hydrolysis of purified precursor to fecapentaene-12 by lipase and phospholipace C.

natural" plasmalogens (and other ether lipids) the reader is directed to the recent review of Mangold and Weber (34).

It is interesting, but outside the scope of this report, to discuss the three most likely origins of these novel precursors of the fecapentaenes—the diet, the colonic microflora and the host. When and if the origin and natural physiological functions of the precursors are determined, some of the most puzzling questions concerning these compounds may finally be answered: How are these unique ether-phospholipids (particularly the ether-pentaene chain) synthesized? What is their "normal" role in cellular physiology? And, how do they reach the colon where they are converted to potent mutagens?

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Black Currant Seed Oil Feeding and Fatty Acids in Liver Lipid Classes of Guinea Pigs

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Guinea pigs were fed one of three diets containing 10% black currant seed oil (a source of gamma-linolenic (18:3 n-6) and stearidonic (18:4 n-3) acids), walnut oil or lard for 40 days. The fatty acid composition of liver triglycerides, free fatty acids, cholesteryl esters, phosphatidylinositol, phosphatidylserine, cardiolipin, phosphatidylcholine and phosphatidylethanolamine were determined.

Dietary n-3 fatty acids found esterified in liver lipids had been desaturated and elongated to longer chain analogues, notably docosapentaenoic acid (22:5 n-3) and docosahexaenoic acid (22:6 n-3). When the diet contained low amounts of n-6 fatty acids, proportionately more of the n-3 fatty acids were transformed. Significantly more eicosapentaenoic acid (EPA) (20:5 n-3) was incorporated into triglycerides, cholesteryl esters, phosphatidylcholine and phosphatidylethanolamine of the black currant seed oil group compared with the walnut oil group.

Feeding black currant seed oil resulted in significant increases of dihomogamma-linolenic acid (20:3 n-6) in all liver lipid classes examined, whereas the levels of arachidonic acid (20:4 n-6) remained relatively stable. The ratio dihomo-gamma-linolenic acid/arachidonic acid was significantly (2.5-fold in PI to 17-fold in cholesteryl esters) higher in all lipid classes from the black currant seed oil fed group.
***Lipids 24,* 460-466 (1989).**

The enrichment of tissue lipid classes with the 20-carbon chain length fatty acids that are precursors of the various eicosanoids is basic to the idea that dietary fats can affect eicosanoid synthesis and those biological functions they mediate. The eicosanoids derived from these different fatty acids can have differing and even opposing effects. For example, eicosanoids of the 1 series derived from dihomo-gamma-linolenic acid (DHLA; 20:3 n-6) have antiaggregatory effects on platelets (1), whereas eicosanoids of the 2 series derived from arachidonic acid (AA; 20:4 n-6) are proaggregatory (2, 3). Thus, tissue fatty acids favorably modulated by dietary fat intake might lead to synthesis of a complement of eicosanoids having a positive influence on health. Dietary supplementation with DHLA has been shown to result in an increased PGE^1/PGE^2 ratio production by human platelets (4) and a decreased aggregatory response (4, 5).

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Abbreviations: AA, arachidonic acid; BCO, black currant seed oil; CE, cholesteryl esters; CL, cardiolipin; DHA, docosahexaenoic acid; D HLA, dihomo-gamma-linolenic acid; EPA, eicosapentaenoic acid; FFA, free fatty acid; LA, linoleic acid; LN, alpha-linolenic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SA, stearidonic acid; TG, triglyceride; WO, walnut oil; HPTLC, high performance thin layer chromatography.

Black currant seed oil (BCO) is an excellent source (15-19%) of gamma-linolenic acid (GLA; 18:3 n-6), the Δ6-desaturase product of linoleic acid (LA; 18:2 n-6). Because the activity of this enzyme can be compromised under certain conditions by hydrocortisone, adrenalin, glucagon (6), aging (7, 8), cirrhosis (9, 10), diabetes (11) and malnutrition (10, 12), it is hypothesized that certain of the undesirable effects of these conditions could be due to a relative deficit of GLA, DHLA, AA, or their cyclooxygenase metabolites. BCO also contains 12-14% alpha-linolenic acid (LN; 18:3 n-3), a nutritionally essential fatty acid, and 2-4% stearidonic acid (SA; 18:4 n-3), its Δ6-desaturase product. The elongation product of SA, by virtue of its competition for the Δ5 desaturase, may be important not only by inhibiting the transformation of GLA to AA but also because it can be elongated and desaturated to eicosapentaenoic acid (EPA, 20:5 n-3), the precursor of eicosanoids of the 3 series. BCO is thus an interesting oil in several respects.

In the rat, feeding sources of GLA results in increased incorporation of GLA in triglycerides (TG) (13) or DHLA in total lipids (14) but no or slight incorporation into the phospholipids. Phospholipids of the mouse (15-16) are similarly intractable to the influence of dietary GLA. Lipids and phospholipids of humans are more amenable to dietary modulation of n-6 fatty acids. With a single 1 g oral dose, DHLA is incorporated into blood triglycerides within a few hours and into phosphatidylcholine (PC) within 24 hr (17). Sustained oral dosing results in incorporation of DHLA into the membrane of red blood cells and into all lipid classes (17). DHLA levels in serum phospholipids and cholesteryl esters are also sensitive to LA intake (18).

Stone et al. (17) compared the Δ5-desaturase activity from different species and found that in the rat and mouse it is especially active, whereas liver homogenates from man, rabbit (17) or guinea pig (17, 19) show only slight ability to desaturate DHLA to AA. Therefore, in the present experiment, we considered that the guinea pig would be preferred over the rat as an animal model which better reflects the situation in man. The liver being the major site of fatty acid conversions was chosen as the tissue in which to study the effect of diet on fatty acid composition.

The formation of eicosanoids requires the mobilization of substrate as fatty acid, preferentially in a nonesterified form (20, 21). If this is true, there must be *a priori* hydrolysis of esterified lipid classes before prostanoid synthesis can take place (20, 22). Phospholipids are major contributors of fatty acids for prostaglandin synthesis (23) and the consensus is that substrate supply is a limiting factor (24-26). The fatty acid content of triglycerides reflects to a large extent the dietary fat but may also contribute to those pools of free fatty acids susceptible to oxygenation.

The aim of this study was to investigate whether guinea pig liver lipids could be enriched in DHLA by

feeding BCO and if some of these lipid classes were more sensitive than others.

MATERIALS AND METHODS

Animals and diets: Male Dunkin-Hartley guinea pigs weighing ca. 300 g (KFM, Fullinsdorf, Switzerland) were randomly allocated into 3 groups of 7 animals and maintained in individual Macrolon (type III) cages. Each group was fed a nutritionally adequate, semisynthetic diet containing BCO (F.I.S., Chatel St. Denis, Switzerland), walnut oil (WO) (Du Baron, France), or lard (Table 1) *ad libitum* for 40 days. BCO was stabilized with ascorbyl palmitate (200 ppm). The diets were refrigerated and fed fresh each day to the animals. Any leftover diet was discarded. The fatty acid compositions of the diets are shown in Table 2. Walnut oil was chosen as a control for BCO because it is lacking in GLA and SA, but compensates with more LA such that the total quantity of n-6 and n-3 fatty acids, as well as their ratios, are similar in the two oils. All animals had free access to water which contained 250 mg/liter of ascorbic acid. They were weighed weekly.

After an overnight fast, animals were anaesthetized with pentobarbital. Livers were quickly excised and freeze clamped with tongs which had been cooled in liquid nitrogen. Frozen tissue was ground to a fine powder with dry ice and conserved at −80°C until analysis.

Analytical methods. Sample extraction: A 1-g sample of frozen liver tissue was homogenized in 20 vol of chloroform/methanol (2:1, v/v) and total lipids were extracted according to the method of Christiansen (27).

Separation of lipid fractions: Neutral lipids were separated from polar lipids on SepPak cartridges, (Waters Associates, Framingham, MA) (28). Nonacidic and acidic polar lipid fractions were separated on ion-exchange DEAE-Sephadex A-25 (Pharmacia, Sweden) disposable minicolumns (29, 30). The acidic polar lipid fraction was then desalted using a minicolumn of Sephadex G-25 (Pharmacia, Sweden) (31).

Separation of lipid classes: Separation of the lipid classes was performed in duplicate. Neutral lipids were separated by linear high performance thin layer chromatography (HPTLC) on washed silicic acid plates (Merck, ref. 60F254). The plates were developed with petroleum benzine/diethyl ether/acetic acid (85:15:0.5, v/v/v), air-dried, sprayed with 0.005% primuline (w/v) in acetone; the lipid bands were visualized under ultraviolet light. Cholesteryl esters (CE), triglycerides and free fatty acids (FFA), identified by comparison with standards, were scraped off and collected into glass tubes sealed with teflon lined caps.

Polar lipid extracts were separated by linear chromatography (HPTLC) on silicic acid plates (Merck, ref. 60F254) impregnated with 2% boric acid in absolute methanol (w/v) and developed with chloroform/methanol/triethylamine/water (30:25:34:8, v/v/v/v) (29). The acidic phospholipids (phosphatidylserine [PS], phosphatidylinositol [PI], cardiolipin [CL] and the nonacid phospholipids: phosphatidylethanolamine [PE], and phosphatidylcholine [PC]) were visualized as for the neutral lipids, identified via standards, and collected as described above.

Transesterification: All transesterifications were carried out in the presence of silica gel plate materials. For triglycerides, the method was adapted from Shehata et al. (32). Triglycerides were extracted from the silica powder with 1 ml diethyl ether/hexane (2:1, v/v) and 1 ml of 2 M methanolic sodium methoxide was added. The mixture was ultrasonicated for 15 min at room temperature and subsequently washed with 1 ml distilled water.

Cholesteryl esters were extracted with 1.5 ml of diethyl ether/hexane (2:1, v/v) and transesterified with 0.5 ml of the above reaction mixture for 20 min at 50°C. Fatty acid methyl esters were taken up in hexane. We found no selectivity for the various fatty acids in the transesterification process under the given conditions as has been proposed by Epps and Kaluzny (33).

TABLE 1

Composition of the Basic Diet Fed to the Experimental Guinea Pigs

Ingredient	g/100 g
Casein	30.0
Starch	20.0
Sucrose	10.0
Glucose	3.8
Cellulose	15.0
Mineral mix[a]	6.0
Vitamins[b]	2.2
Potassium acetate	2.5
Magnesium oxide	0.5
Fat[c]	10.0

[a]As per M.R.S. Fox and G.M. Briggs (1960) *J. Nutr. 72*, 243-250.
[b]Vitamin fortification mixture, I.C.N. Biochemicals, Cleveland, OH.
[c]Black currant seed oil, walnut oil or lard.

TABLE 2

Fatty Acid Composition of the Guinea Pig Diets (Molar %)

Fatty acid[a]	BCO[b]	WO[b]	Lard
14:0	—	—	2.7
16:0	7.4	8.6	32.4
16:1	—	—	3.4
17:0	—	—	0.5
17:1	—	—	0.3
18:0	0.8	1.9	14.2
18:1	10.4	20.8	36.5
18:2 n-6	48.1	57.7	8.4
18:3 n-6	17.1	—	—
18:3 n-3	12.7	11.0	0.7
18:4 n-3	2.6	—	—
20:1 n-9	0.6	—	0.7
20:2 n-6	0.3	—	—
n-6 sum	65.5	57.7	8.4
n-3 sum	15.3	11.0	0.7
n-6/n-3 ratio	4.3	5.2	12.0
Unsat index[c]	207.6	169.2	59.8

[a]Fatty acids are designated by the number of carbon atoms followed by the number of double bonds. n indicates the position of the first double bond relative to the methyl end of the molecule.
[b]BCO; black currant seed oil. WO; walnut oil.
[c]Unsaturation index: sum of (a × b); a is the relative molar % of each unsaturated fatty acid, b is the number of double bonds for that particular fatty acid.

Free fatty acids were extracted with 1 ml of diethyl ether/hexane (2:1, v/v) and esterified with 1 ml of 2% sulfuric acid in methanol for 20 min at 80°C (34). The fatty acid methyl esters were then neutralized with potassium carbonate.

Polar lipids were transesterified in the same manner as were TG, except that they were extracted with methanol.

Fatty acid methyl esters from all lipid classes were extracted into hexane (Baker resianalyzed) for gas chromatographic analysis.

Gas chromatography: All gas chromatographic analyses were performed on a CARLO-ERBA model 5160 Mega series gas chromatograph (Milan, Italy) equipped with an automatic cold on-column injector, a tailor-made capillary column, coated with immobilized polyethylene glycol (35) and a flame ionization detector. The gas chromatographic conditions were: a fused silica column of 27 m × 0.32 mm I.D. coated with Carbowax 20M; a fused silica precolumn of 1 m × 0.53 mm I.D. The hydrogen inlet pressure was 60 kPa. The oven temperature program was: 80°C, 2 min isothermal, 15°C/min to 140°C, 1 min isothermal, 4°C/min to 220°C (36). The detector was set at 320°C. Chromatograms were recorded using a Spectra Physic 4270 integrator (San Jose, CA). Identification of peaks was made by comparison of retention times with those of a standard (NuChek Prep, Elysian, MN) run under the same conditions.

Statistics: Effect of diet was submitted to analysis of variance and comparison between diets was made using Newman-Keuls studentized range test.

Comparisons between liver fatty acids for a given diet were performed using paired t-tests. Values of $p<0.05$ were considered statistically significant.

RESULTS

Among the three groups of animals, neither the final body weights (BCO = 479 ± 35 g; WO = 454 ± 55 g; and lard = 445 ± 50 g), nor the total liver weights (BCO = 21.6 ± 1.5 g; WO = 18.6 ± 4.1 g; and lard = 17.5 ± 4.3 g) were different.

n-6 Fatty acids. Of the n-6 fatty acids (Figs. 1-3), LA incorporated in the greatest amounts in all lipid classes except PE where AA was also incorporated to a large extent.

The content of LA in the liver TG from guinea pigs

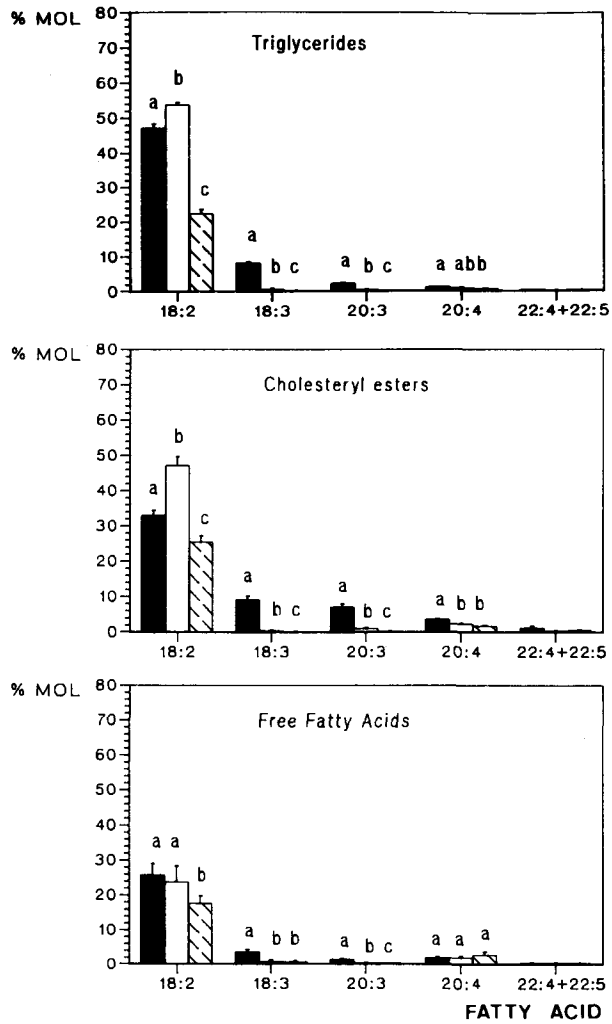

FIG. 1. n-6 fatty acids (mol %) in triglycerides (TG), cholesteryl esters (CE) or free fatty acids (FFA) in livers of guinea pigs fed black currant seed oil (dark bars), walnut oil (open bars) or lard (hatched bars). Values (means for 7-8 animals ± SEM) with different letters are significantly different.

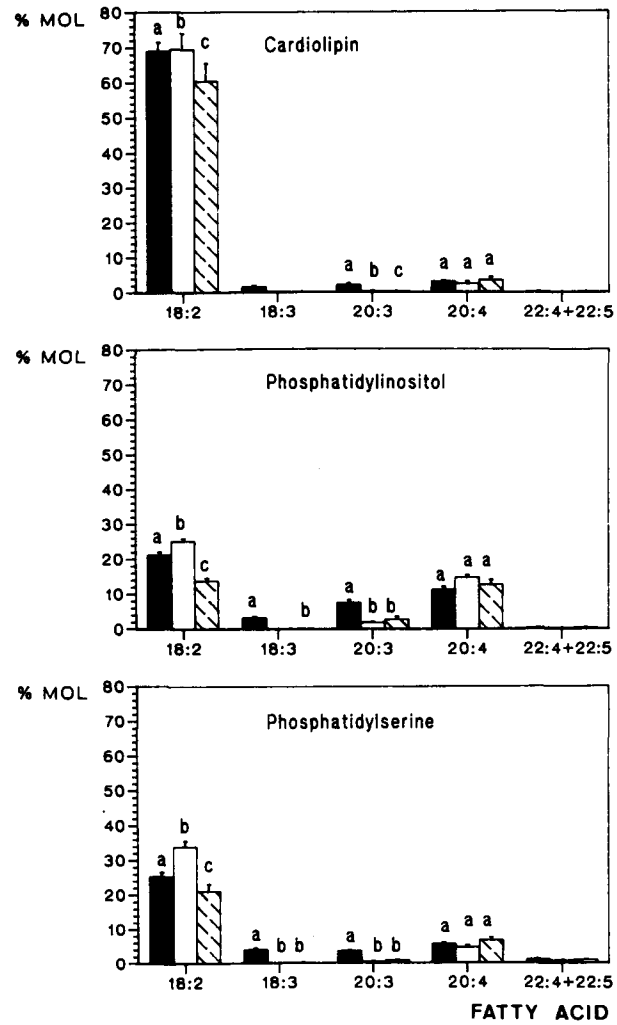

FIG. 2. n-6 fatty acids (mol %) in cardiolipin (CL), phosphatidylinositol (PI) or phosphatidylserine (PS) in livers of guinea pigs fed black currant seed oil (dark bars), walnut oil (open bars) or lard (hatched bars). Values (means for 7-8 animals ± SEM) with different letters are significantly different.

eating the BCO and WO diets was proportionally similar to the content (Table 2) of the diets. In the TG of the lard-fed group, LA proportion exceeded by about 2.5-fold that of the diet. In most of the other lipid classes except for CL and FFA, the levels of LA were roughly related to the dietary proportion: greater in liver of animals eating the WO diet compared with the BCO fed group, and least in the lard-fed group. As with the TG, lipids from the lard group contained more LA than would be expected from the proportion in the diet. In most lipid classes, LA was found at levels of 13–33%, the exception being in CL levels which were much higher (60–70%), even in livers from those animals which ate the lard diet.

The content of both GLA and DHLA was also affected by dietary fat, being greater in liver lipids from BCO fed animals compared with the WO- and lard-fed animals. This was true for all lipid classes. In the BCO group, more GLA (available from the diet) than DHLA was found in TG, FFA and PC. In the other phospholipids, the content of these two fatty acids was similar except in PI and in CL, where DHLA incorporation exceeded that of GLA.

AA, although varying in quantity from lipid class to lipid class, did not show the large differences between dietary groups noted for other fatty acids. This acid was only slightly elevated in PC, PE and CE by BCO feeding.

The shifts upward of DHLA and no change or slight upward shift of AA resulted in ratios of DHLA/AA (Fig. 4) being significantly increased in the group of guinea pigs fed BCO compared with the other groups.

The longer chain n-6 fatty acids, 22:4 and 22:5, were found in very small amounts and, while differing between lipid classes, did not significantly vary with respect to dietary treatment.

n-3 Fatty acids. The n-3 fatty acid data are shown in Figures 5–7. LN was found to a much lesser extent in the liver lipids of the guinea pig than had been LA.

Where the diet contained substantial quantities of LN (BCO and WO), its content was greater than when the diet contained little LN (lard). In the livers of animals fed the lard diet, there was less conservation of LN as there had been for LA. The levels of LN in the neutral lipids (TG, FFA and CE) were the greatest, indicating that these lipid classes have the most direct relation to dietary intake. In the phospholipids, however, the level of this acid was low (0.1–5.4%); of these, CL incorporated the most, as it did of LA.

In the phospholipids of animals fed the lard diet, and in PE and PS from the animals fed the other diets, the content of the longer chain n-3 fatty acids exceeded that of LN, with docosahexaenoic acid (DHA; 22:6 n-3) being the fatty acid found to the greatest extent. EPA content was significantly greater in TG, CE, PC and PE of the BCO fed group compared with the WO fed animal. Significantly, more EPA was also found in the TG and CE of the BCO group than of the lard group.

Unsaturation index. The liver unsaturation index, or the sum of the molar % of each fatty acid multiplied by its number of double bonds (Fig. 8), did not reflect the gross differences in saturation found in the dietary fatty acids (Table 2). This similarity was not found in neutral lipids, and a lesser unsaturation index was also noted in CL and PC in the animals eating the highly saturated lard diet.

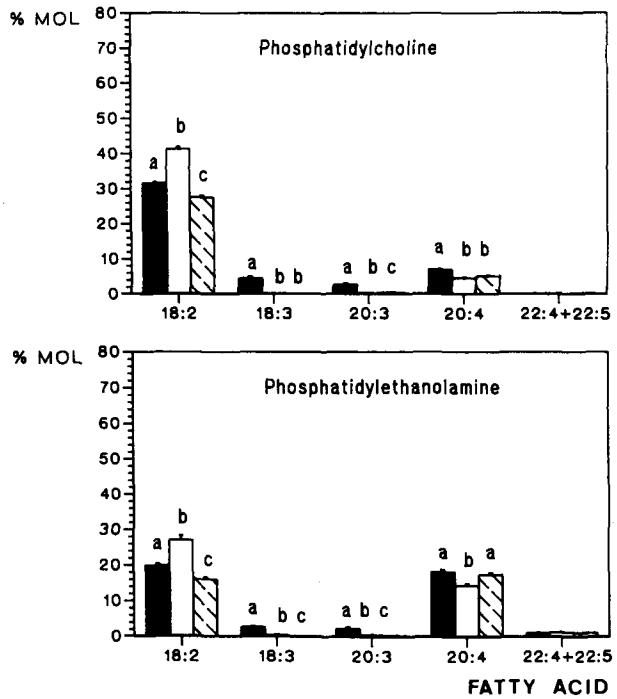

FIG. 3. n-6 fatty acids (mol %) in phosphatidylcholine (PC) or phosphatidylethanolamine (PE) in livers of guinea pigs fed black currant seed oil (dark bars), walnut oil (open bars) or lard (hatched bars). Values (means for 7–8 animals ± SEM) with different letters are significantly different.

DISCUSSION

The observation that LA incorporation into liver lipids was in proportion to the dietary content is consistent with results obtained in human plasma (18). In the lard-fed group, incorporation of LA into liver was dis-

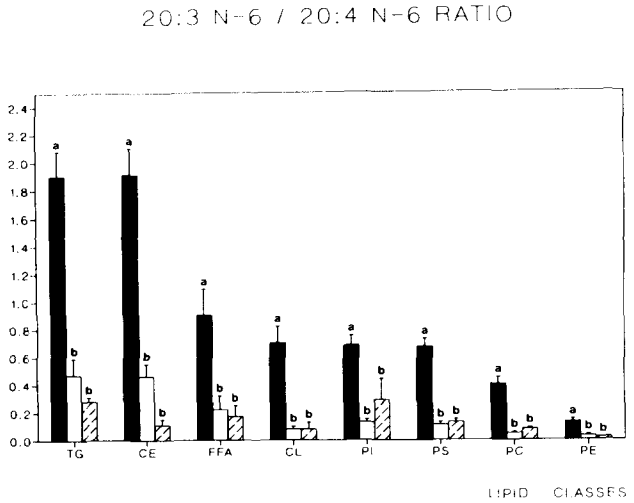

FIG. 4. The ratio of molar % dihomo-gamma-linolenic acid over molar % arachidonic acid in liver lipid classes of guinea pig fed diets containing 10% black currant seed oil (dark bars), walnut oil (open bars) or lard (hatched bars). Values (means for 7–8 animals ± SEM) with different letters are significantly different.

proportionately high compared with the diet content and may indicate a conservation of this nutritionally essential fatty acid.

The LA content of CL was higher than dietary content due to the special affinity of CL in mammalian tissue for 18-carbon fatty acids (37). Although the fatty acid profile of CL can be influenced by a number of factors such as aging (38), alcohol ingestion (39, 40), hyperthyroidism (41), fat-free diets (42, 43), it appears that, for the guinea pig, the low content of LA in the lard diet was not a treatment severe enough to depress significantly the incorporation of this fatty acid in liver CL. The LA content of cardiolipin may be protected because of its role in the integrity of the mitochondrial inner membrane (44–46) and in the electron transport system (47).

The stability of the levels of AA in liver lipids of the guinea pig observed in this experiment after variable LA intakes has also been noted in human plasma phospholipids (18, 48) and in guinea pig liver triglycerides and total phospholipids (19). The importance of maintaining the levels of this acid relates perhaps to its importance in membrane structure and as a precursor of the eicosanoids of the 2 series.

The ratio of DHLA/AA in the phospholipids of most species, including humans, is normally 1/3 to 1/50 (49) and this may be one reason why the synthesis of the dienoic eicosanoids dominates over that of the monoenoic. In this experiment, the DHLA/AA ratio in the guinea pig fed BCO was notably greater in every liver lipid class, as is also true in liver TG and phospholipids of guinea pigs fed evening primrose oil (19, 57).

This supports the idea (19) that, in the guinea pig, Δ5 desaturase becomes a limiting enzyme in the further transformation of GLA when it is supplied in the diet. If the synthesis of eicosanoids is partly regulated by substrate availability as has been suggested (22, 26), then the potential for increased synthesis or increased relative synthesis of the monoene eicosanoids compared with the diene eicosanoids is greater in the BCO fed group. the observation that feeding BCO, which contained SA, increased EPA content in liver

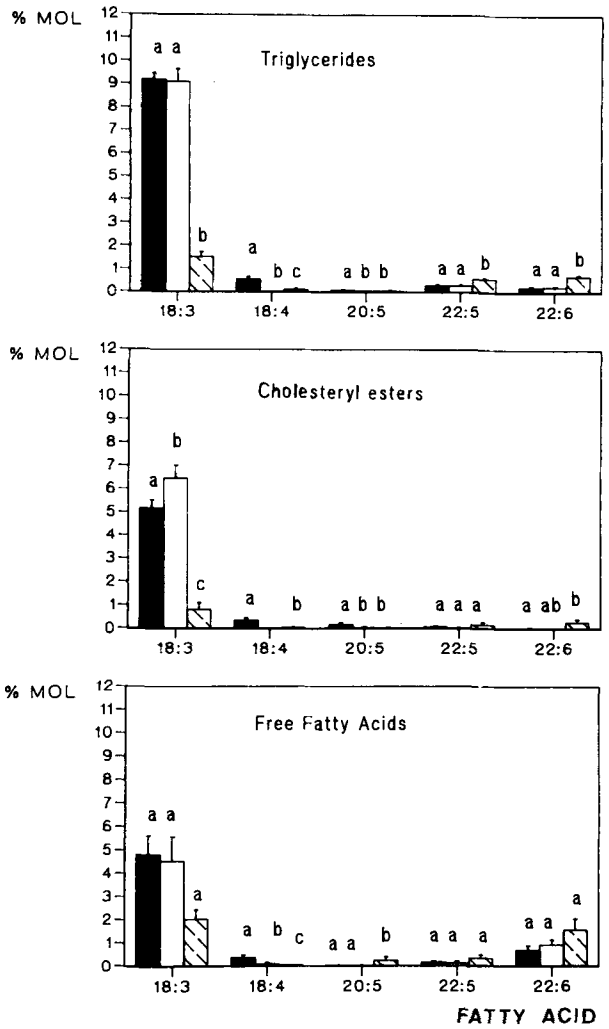

FIG. 5. n-3 fatty acids (mol %) in triglycerides (TG), cholesteryl esters (CE) or free fatty acids (FFA) in livers of guinea pigs fed black currant seed oil (dark bars), walnut oil (open bars) or lard (hatched bars). Values (means for 7–8 animals ± SEM) with different letters are significantly different.

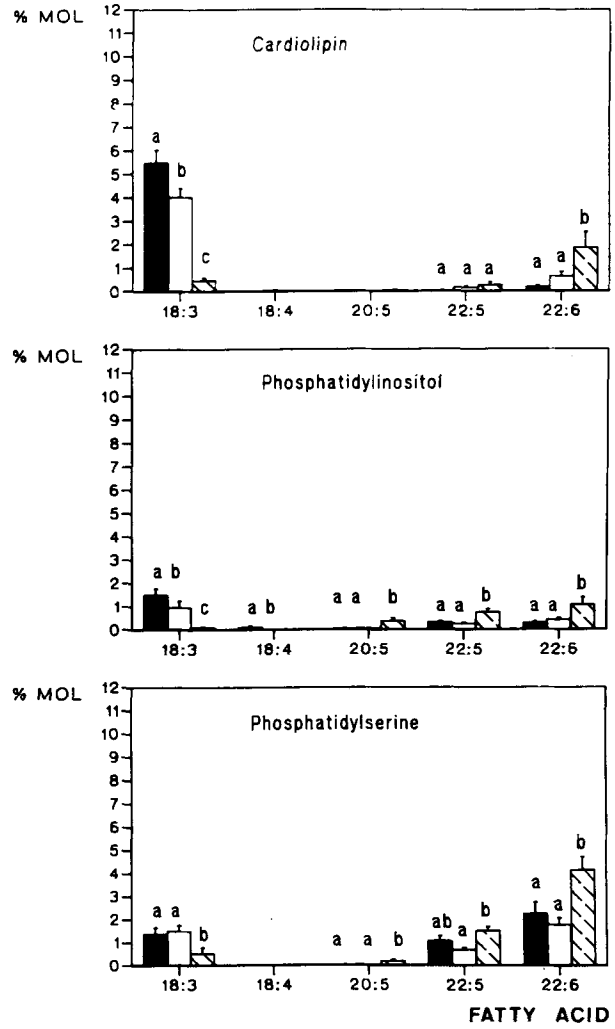

FIG. 6. n-3 fatty acids (mol %) in cardiolipin (CL), phosphatidylinositol (PI) or phosphatidylserine (PS) in livers of guinea pigs fed black currant seed oil (dark bars), walnut oil (open bars) or lard (hatched bars). Values (means for 7–8 animals ± SEM) with different letters are significantly different.

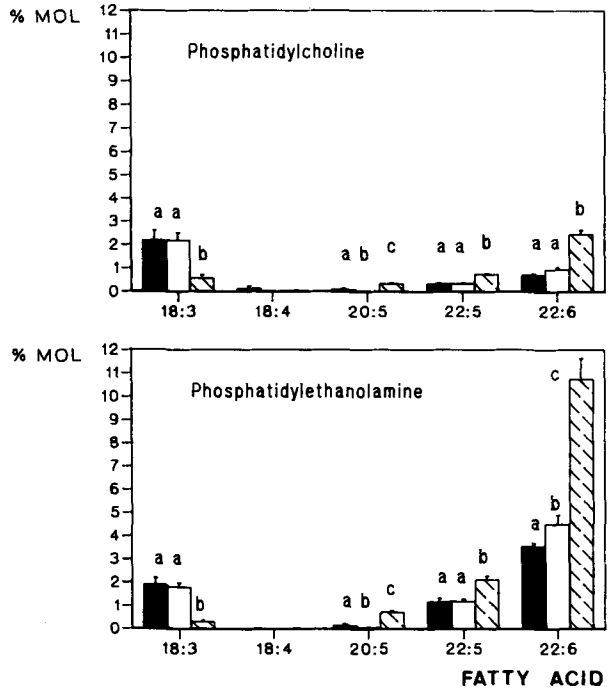

FIG. 7. n-3 fatty acids (mol %) in phosphatidylcholine (PC) or phosphatidylethanolamine (PE) in livers of guinea pigs fed black currant seed oil (dark bars), walnut oil (open bars) or lard (hatched bars). Values (means for 7-8 animals ± SEM) with different letters are significantly different.

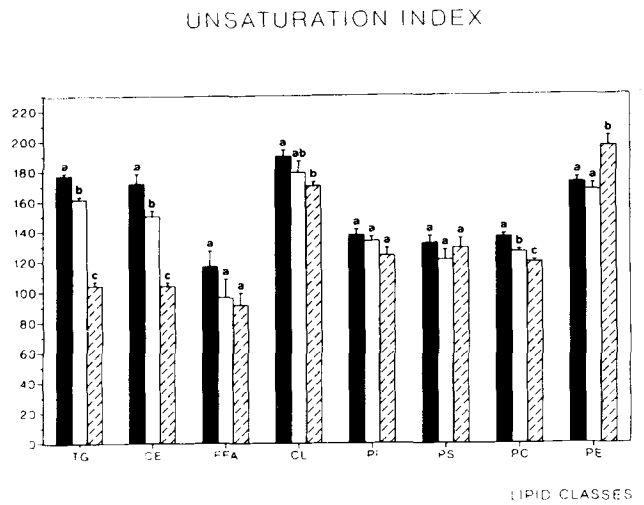

FIG. 8. The unsaturation index of the liver lipid classes from guinea pigs fed diets containing 10% black currant seed oil (dark bars), walnut oil (open bars) or lard (hatched bars). The unsaturation index is the sum of (a × b); a is the relative molar % of each unsaturated fatty acid, b is the number of double bonds for that particular fatty acid. Values (means for 7-8 animals ± SEM) with different letters are significantly different.

indicates that BCO feeding could possibly affect the synthesis of the triene eicosanoids in the guinea pig.

Ingested n-3 fatty acids were to a large extent transformed to higher chain analogues. This was noticeable in the BCO- and WO-fed groups of guinea pigs but was particularly evident in the lard-fed animals. A similar observation has been made in phospholipids of rat liver (50, 51) and muscle (52) and in human serum phospholipids (18).

Fatty acids of the different families compete for the same desaturases with an order of priority n-3 > n-6 > n-9 (53-55). When n-6 fatty acids are provided at low levels in the diet, lack of competition may permit desaturation of the n-3 fatty acids to occur at rapid rates. Gibson et al. (50) have remarked on the compensatory incorporation of DHA where dietary n-6 fatty acids are low.

This highly unsaturated fatty acid contributes to the maintenance of the unsaturation index, one determinant of membrane fluidity (56). When rats were fed diets containing fats of widely varying levels of unsaturation (50), liver mitochondrial and microsomal membrane phospholipid fatty acids maintained the same unsaturation index. In the present study, it was observed that, in the guinea pig, there was a tendency to maintain a constant unsaturation index, but differences were still found in many of the lipid classes, particularly the neutral lipids, generally in the direction to be expected based on dietary unsaturation.

In summary, the results of the present experiment demonstrated that feeding the guinea pig black currant seed oil resulted in important levels of incorporation of DHLA in liver lipid classes and higher ratios of DHLA/AA in all the lipid classes studied. The potential for increased or proportionately increased synthesis of the eicosanoids of the 1 series and possibly the 3 series compared with the 2 series is greater with black currant seed oil as a dietary lipid source than with the other two fats.

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Chemical Synthesis and Spectroscopic Characteristics of C_{18} 1,2-Disubstituted Cyclopentyl Fatty Acid Methyl Esters

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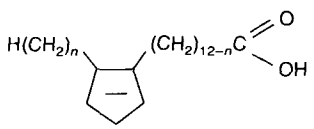
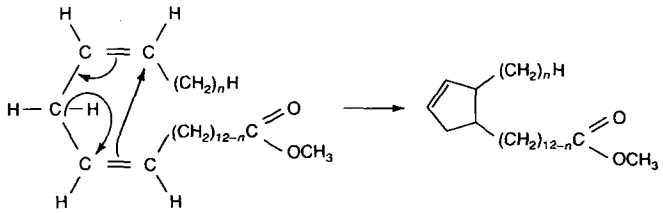
The chemical synthesis of methyl 9-(2′-n-butylcyclopentyl)nonanoate and methyl 10-(2′-n-propylcyclopentyl)-decanoate was performed using a Wittig reaction between 2-alkylcyclopentanones and the corresponding ω-carbomethoxyalkyl triphenylphosphonium bromides. The intermediate alkylcyclopentylidene alkanoate esters were isolated from the reaction mixture, characterized by spectrometric methods, and then catalytically hydrogenated to the desired cyclopentyl esters. Gas chromatography-mass spectrometry (GC-MS) showed that the final products contained a mixture of *trans*- and *cis*-ring isomers with small amounts of other cyclic by-products. The GC-retention features, the mass fragmentation pattern, and the spectroscopic characteristics of both the final and the unsaturated intermediate products are discussed.
***Lipids 24,* 467-476 (1989).**

Cyclopentyl fatty acids, formed during the heating of polyunsaturated fats and oils under various conditions, appear to be mainly C_{18} saturated and monounsaturated 1,2-disubstituted cyclopentyl acids (1,2) of the general formula:



The presence of a double bond in the ring may be due to a thermally promoted intramolecular rearrangement of a methylene interrupted diene system to allow cyclization as proposed by Gast *et al.* (1):



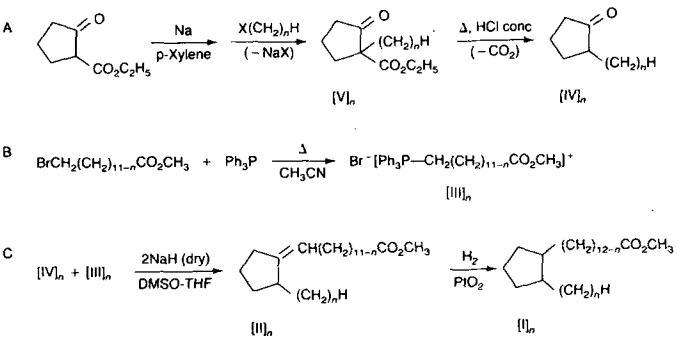
Any proposed mechanism should explain the various geometrical and positional 5-membered ring isomer structures found in oils of different composition. The structural isomers identified in monomeric fractions of heated fats are, respectively, those with an n-propyl n = 3 or an n-butyl n = 4 group as the alkyl side chain substituent (2-5). Recently, Sebedio *et al.* (2) proposed a monounsaturated isomer with an n-hexyl side chain (n = 6).

Other structural studies within either highly unsaturated oils (6,7) or fatty acids (8,9) have not identified C_{18} fatty acids isomers containing 5-membered rings. This suggests that their formation might be favored under certain heat treatment conditions, be dependent on the composition of the oil under study, or may be due to misassignment of structures during the analysis. However, no mention of chemical synthesis of these types of compounds has been found in the literature.

In this paper we report on the synthesis and chemical characterization of the two most important saturated disubstituted cyclopentyl fatty acids found in heated fats and oils. Intermediate 2-alkylcyclopentanones were reacted with corresponding ω-carbomethoxylkyl triphenylphosphonium bromide in a Wittig reaction. Catalytic hydrogenation of the purified unsaturated cyclic ester product afforded the desired cyclopentyl fatty acid methyl esters.

RESULTS AND DISCUSSION

Methyl 9-(2′n-butylcyclopentyl)nonanoate and methyl 10-(2′-n-propylcyclopentyl)decanoate were prepared following the reactions illustrated in Scheme 1. The alkylated keto ester $[V]_n$ was obtained by direct condensation of the sodium salt of ethyl 2-oxo-cyclopentanecarboxylate with the corresponding n-alkylhalide (Reaction A, Scheme 1). Iodo- and bromo-alkanes were used for this purpose. However, the iodoalkanes gave consistently higher product yields.



SCHEME 1. Basic synthetic reactions.

Attempts to produce the alkylated keto ester $[V]_n$ in one step (using sodium methoxide in dry ethanol) produced cyclopentanone ring cleavage (Mayer, 1963), which afforded adipic acid ethyl ester and its α-substituted alkyl derivative as demonstrated by GC-MS analysis:

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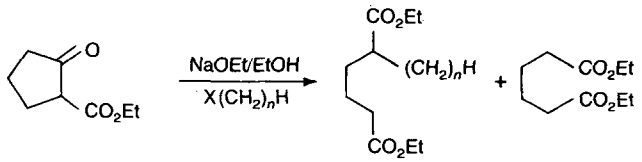
Abbreviations: $AgNO_3$, silver nitrate; $CDCl_3$, deuterated chloroform; CI, chemical ionization; DMSO, dimethyl sulfoxide; ECL, equivalent chain length; FAME, fatty acid methyl ester; GC, gas chromatography; *GLC*, gas liquid chromatography; HP, Hewlett-Packard; IR, infrared; Me_4Si, tetramethyl silane; MS, mass spectrometry; NaCl, sodium chlorine; NMR, nuclear magnetic resonance; RRT, relative retention time; THF, tetrahydrofuran; TLC, thin layer chromatography.



None of the cleavage products were formed using powdered sodium to produce the salt (Reaction A, Scheme 1). This held true even after decarboxylation of the keto ester with concentrated acid was added to produce the intermediates 2-alkyl-cyclopentanones $[IV]_n$ in high yield. However, after 24 hr of decarboxylation close to 4% of the keto esters $[V]_n$ remained intact. Fractional distillation under a vacuum afforded a relatively pure fraction of $[IV]_n$ (ca. 90%). This fraction was used for reaction C (Scheme 1).

The phosphonium bromide intermediates $[III]_n$ were readily prepared from the corresponding ω-bromoalkanoate esters, as described by Awl and Frankel (10) (Reaction B, Scheme 1). The methyl ester of 9-bromononanoic acid was prepared according to Davis *et al.* (11), from the monoester of sebacic acid by replacement of the non-esterified carboxylic group with a bromine atom.

Preparation of the unsaturated butyl substituted cyclopentylidene methyl ester $[II]_4$ was initially attempted by reacting 2-n-butyl-cyclopentanone $[IV]_4$ and the phosphoylid prepared from $[III]_4$ with sodium methoxide, under conditions similar to those described by Awl and Frankel (10). However, as evidenced by GC-MS analysis on samples withdrawn during the course of the reaction, no carbonyl olefination reaction took place. The lack of reactivity of the 2-alkylcyclopentanone, as compared to the alkylated cyclohexenals used by Awl and Frankel (10), was probably due to stabilization of the ketone as an enolate in the presence of sodium methoxide and/or to the steric hindrance of the ring carbonyl carbon by the bulk of the alkyl group preventing the attack of the ylid carbanion. Furthermore, ketones are generally less reactive than aldehydes in carbonyl olefination reactions (12).

The Wittig reaction succeeded under more drastic conditions as was suggested by Corey *et al.* (13). Unsaturated propyl substituted cyclopentylidene methyl ester $[II]_3$ was obtained when a mixture of 2-propylcyclopentanone $[IV]_3$ and the corresponding triphenylphosphonium salt $[III]_3$, which was dissolved in 1:1 DMSO-tetrahydrofuran, was added to dry sodium hydride (Reaction A, Scheme 1). The overall yield of $[II]_3$, however, was around 10% even though a 2:1 molar ratio of phosphonium salt to ketone was used to both favor product yields (14) and compensate for losses of reactants by side reactions. GC-MS analysis of the product showed two peaks corresponding to the double bond geometrical cyclic isomers and unreacted triphenylphosphine ($P(Ph)_3$). Several lower molecular weights components (two peaks M^+ at m/e 250 and two peaks M^+ at m/e 266 by CI GC-MS) and two compounds (apparently isomers eluting after PPh_3 with M^+ at m/e 388) also present.

The origin of these products was attributed to side reactions involving the self-condensation product of 2-n-propylcyclopentanone $[II]_3$ with the methyl sulfinyl carbanion similar to those reactions described by Comer and Temple (15).

Further purification of the reaction product was obtained after a second elution through neutral alumina. The fractions richer in cyclics $[II]_3$ were combined, analyzed by GC-MS, and their ^{1}H-NMR and IR spectra were obtained. The synthetic product $[I]_3$ was prepared by catalytic hydrogenation of the unsaturated intermediate.

The second synthetic product $[I]_4$ was prepared by treating the unreacted product of the initial Wittig reaction attempt with NaH after removal of the solvent. In this case only one molar amount of NaH was used per mole of reactants. Purification of the unsaturated cyclic intermediate was carried out in two steps, first by elution through alumina, and then by silicic acid/$AgNO_3$. In contrast with the previous case, the reaction did not afford a significant amount of high molecular weight side products, and the overall yield and purity of final product were substantially better.

Capillary GC retention characteristics. The chromatographic retention profiles of saturated and monounsaturated cyclopentyl methyl ester final products are illustrated in Figure 1 (A and B). The capillary GC retention data on bonded polyethylene oxide phase (Supelcowax-10) for the main components are specified, in terms of relative retention time (RRT) and equivalent chain length (ECL), in Table 1. The isomer configuration has been assigned based on previous stereochemical studies (16,17) and the steric course of the Wittig reaction.

The retention data of the ring isomers of $[I]_4$ and $[I]_3$, expressed as ECL, agree very closely with those pre-

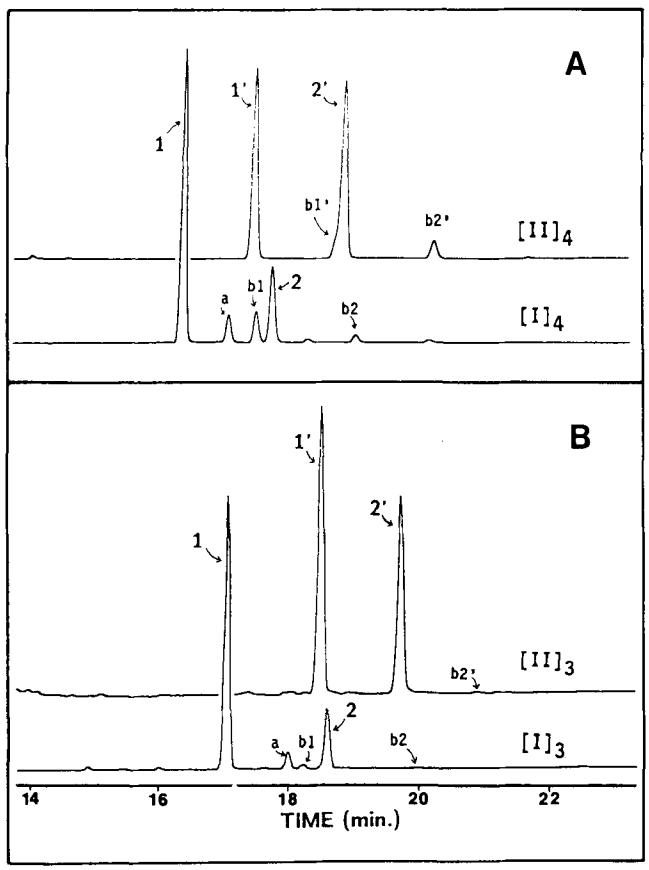


FIG. 1. Chromatograms of disubstituted cyclopentylidene ($[II]_n$) and cyclopentyl ($[I]_n$) fatty acid methyl esters. (A) Butyl substituted cyclic esters, n = 4. (B) Propyl substituted cyclic esters, n = 3.

TABLE 1

Capillary GLC Retention Data and Percent Yields of Cyclopentylidene $[II]_n$, and Cyclopentyl $[I]_n$, Fatty Acid Methyl Esters[a]

Cyclic fame[b]	Peak[c] #	Peak[c] Config.	Retention data[d] RRT	Retention data[d] ECL	% Yield Actual	% Yield Relative
$[II]_4$	1′	(Z)	1.175	18.477	43.5	46.4
	2′	(E)	1.328	18.838	50.2[e]	53.6[e]
$[I]_4$	1	*trans-*	1.049	18.142	65.0	77.7
	2	*cis-*	1.203	18.545	18.7	22.3
$[II]_3$	1′	(Z)	1.276	18.719	38.0	50.6
	2′	(E)	1.412	19.536	37.1	49.4
$[I]_3$	1	*trans-*	1.111	18.311	58.0	80.8
	2	*cis-*	1.284	18.738	13.8	19.2

[a] GC conditions: Column—30 m × 0.25 mm ID, fused silica coated with Supelcowax-10, film thickness 0.25 μm; isothermal at 190°C.

[b] Structure codes according to Scheme 1.

[c] Peak identification according to Figure 1.

[d] GC retention data (corrected for non-retained peak): RRT = relative retention time (separation factor) to methyl stearate; ECL = equivalent chain length according to Miwa *et al.* (32).

[e] Includes coeluting peak b1′ (see Fig. 1).

viously reported (5) when fresh and heated partially hydrogenated soybean oil were analyzed for cyclic monomers. RRT of the same isomers in Table 1, however, are consistently higher than the reported values (5) because the data, in this case, was obtained under isothermal conditions (190°C) and corrected for nonretained peak (methane), which give more reliable values for comparison purposes. The *cis*-isomers of $[I]_3$ and $[I]_4$ have not been previously reported, probably because their relatively higher retention times could result in overlap with some of the disubstituted cyclohexyl fatty acids that are present in the sample when other stationary phases or less efficient columns and chromatographic conditions are used for the analysis.

In the capillary column used, the *cis*-disubstituted butylcylopentyl nonanoate, *cis*-$[I]_4$ (peak 2 in Fig. 1A), is eluted just between the *trans-* and *cis*-disubstituted butylcyclohexyl octanoate and slightly after the *cis*-pentylcyclohexyl heptanoate. This can be deduced from the corresponding ECL data at 190°C, which was obtained from purified mixtures of cyclohexyl isomers. Similarly, *cis*-propylcyclopentyl decanoate, *cis*-$[I]_3$ (peak 2 in Fig. 1B), is eluted between *trans-* and *cis*-propylcyclohexyl nonanoate with total baseline resolution. On the other hand, the *trans-* disubstituted cyclopentyl isomers (peak 1 in Fig. 1A and B) may present the problem of coelution with some disubstituted cyclohexyl isomers. This may be true particularly when higher concentrations of the later compounds are expected in the sample (e.g., highly unsaturated oils or fatty acids).

As anticipated, the unsaturated cyclopentylidene geometric isomers for both $[II]_4$ and $[II]_3$ eluted at higher retention times than their correspondent saturated *trans*-disubstituted cyclopentyl esters (peak 1 of $[I]_4$ and peak 1 of $[I]_3$). However, the saturated *cis*-isomers (peak 2 in Fig. 1A and B) were retained longer than the Z unsaturated isomer (peak 2‡). In addition, the *cis*-isomers were less favored during catalytic hydrogenation of both $[II]_4$ and $[II]_3$, containing approximately equal amounts of **Z** and **E** isomers as shown in Table 1.

The preponderance of the *trans*-ring isomers (peak 1 in Fig. 1A and B) after catalytic hydrogenation may be explained when the influence of steric interactions during heterogeneous metal catalysis is considered. The highly substituted cyclopentylidene double bond is expected to show decreased reactivity during hydrogenation as compared to the more exposed normal side chain or ring double bonds. Therefore, steric factors play an important role in determining the configuration of the resultant reduced product. For both cyclopentylidene isomers **Z** and **E**, suprafacial *syn*-addition of hydrogen (18) is favored when the alkyl side chain substituent is as far from the catalyst surface as possible. This is also required to facilitate chemisorption and formation of activated catalyst-substrate complex. Under these circumstances, both monounsaturated isomers (**Z** and **E**) will afford mainly saturated *trans*-ring isomers upon completion of the surface reaction. Similar conclusions were reported by Awl *et al.* (16) when considering relative reactivities of diunsaturated cyclic esters under different hydrogenation conditions.

Several unexpected minor peaks (coded with lower case letters in Fig. 1A and B) due to side reaction products were observed when examining the chromatograms of the final product $[I]_n$ and $[II]_n$.

GC-MS characterization. Both butyl and propyl cyclopentanones $[IV]_n$ gave a relatively simple mass spectrum with clearly defined molecular ions (relative abundances > 15%) at m/e 140 and 126, respectively. As expected, the base peak for both ketones was the McLafferty rearrangement of the molecular ion, a transferring of a γ-hydrogen from the alkyl side chain to the carbonyl oxygen followed by β-cleavage, affording the relatively stable fragment at m/e 84. The typical ion series 41, 55, 69, and 83 of cycloalkyl compounds (C_zH_{2z-1}) was also observed for both ketones, which confirmed the cyclic structure of the synthetic 2-alkylcyclopentanones.

The unsaturated cyclopentylidene ester isomers **Z** and **E** of the butyl substituted ester $[II]_4$ (peak 1′ and 2′ in Fig. 1A) presented exactly the same mass fragmentation pattern with only minor differences in relative abundances of few ion fragments. This was equally true for the unsaturated isomers of $[II]_3$. However, when comparing the mass spectra of $[II]_4$ and $[II]_3$ (Fig. 2A and B), either for **Z** or **E** isomers, the observed differences in fragment ions were clearly correlated with their structures. The rationalization of the fragmentation process of the butyl and propyl structural isomers is illustrated in Figure 3. When examining the high mass range of the spectra, the most characteristic ions appeared to be those due to α-cleavage at the ring branching point of the alkyl substitutent side chain producing the ion fragment D, and those resulting from radical site rearrangement via labile hydrogen of this fragment to produce D-32 (loss of CH_3OH) followed by loss of water D-32-18. Of similar abundance to D an D-32 are their protonated counterparts with even masses (D+1 and D-31), which may have been formed via γ-H/β-cleavage rearrangement involving the transfer of a hydrogen from the alkyl side chain to the double bond with elimination of an olefin (19). In addition, cleavage of the n-alkyl side chain at the β position to the ring produces a homologous series of ions with

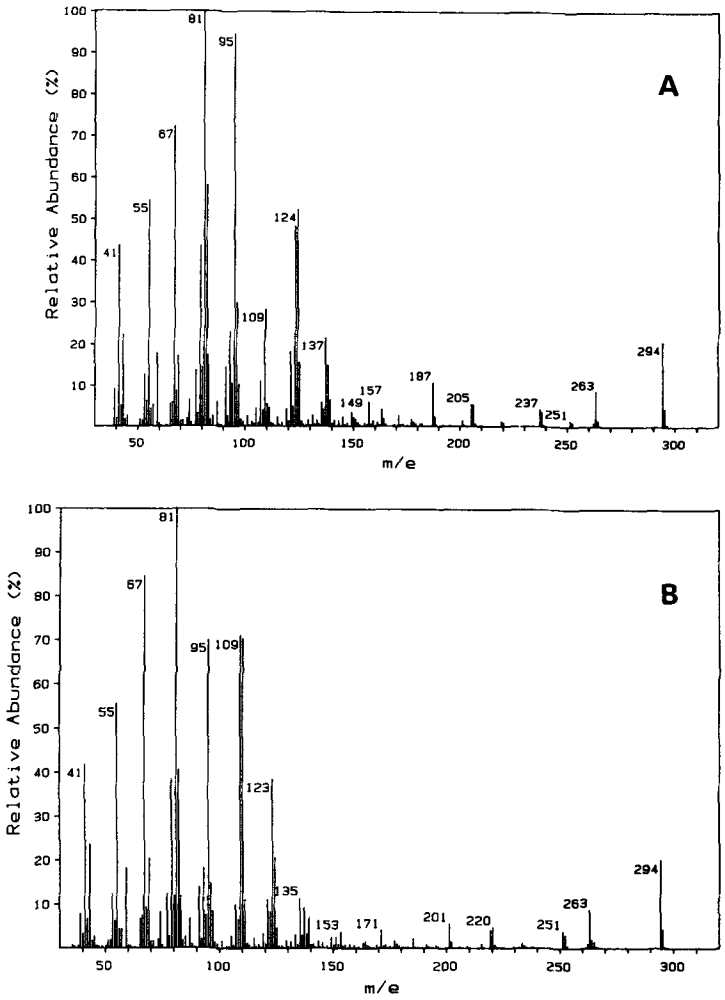


FIG. 2. EI mass spectra of Z-cyclopentylidene fatty acid methyl esters. (A) Methyl 9-(2′-n-butyl-(Z)1′-cyclopentylidene)nonanoate. Peak $[II]_4$-1′ in Fig. 1A. (B) Methyl 10-(2′-n-propyl-(Z)1′-cyclopentylidene)-decanoate. Peak $[II]_3$-1′ in Fig. 1B.

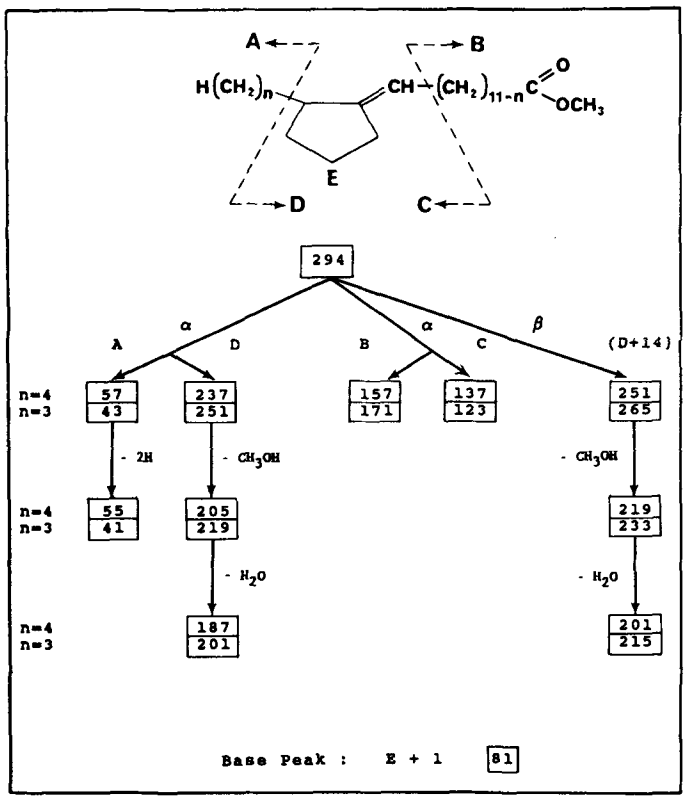


FIG. 3. Mass fragmentation diagram of methyl ω-(2′-n-alkyl-1′-cyclopentylidene) alkanoates $[II]_4$ (n = 4) and $[II]_3$ (n = 3).

relatively low abundance, starting with D+14 and followed by successive loss of the ester moiety affording (D+14)-32 and (D+14)-32-18. The existence of β-cleavage in closely related compounds was originally proposed by Christie *et al.* (20) for monosubstituted cyclopentenyl esters and more recently reported by Sebedio *et al.* (2) for several disubstituted cyclopentenyl and cyclopentyl ester isomers identified in heated sunflowerseed oil.

The base peak at m/e 81 was observed for both cyclopentylidene isomers and resulted from the protonation of the remaining unsaturated cyclic moiety E after cleavage of the saturated alkyl and ester side chain substituents. Ions at m/e 67, 55, and 41 confirmed the cyclopentane ring structure in both disubstituted esters $[II]_4$ and $[II]_3$. In addition, the low mass range of the spectra of both isomers showed a cluster of ions separated by one mass unit, for each member of the cycloalkene fragment series C_zH_{2z-3}, from z = 5 to 11 (Fig. 2A and B). It was interesting to note that the most characteristic members of this series were those ion fragments located at m/e 123 for the butyl cyclopentylidene isomers (Fig. 2A), and at m/e 109 for the propyl cyclopentylidene isomers (Fig. 2B). Each one of these occurred with their equally abundant protonated counterparts. However, none of these ions were accounted for in Figure 3. This observation again confirms the complex nature of the mass spectra of alkyl cyclopentane compounds previously noted by several authors (19–21).

McCloskey and Law (22) observed a similar set of ions with significantly lower intensity while studying the position of the side chains in branched esters that were derived from cyclopropane esters. The authors suggested that simple α-cleavage of the ester side chain with rearrangement of one or two hydrogens would account for these ions. However, the present situation appears to be more complex. Initially, the ejection of ethylene fragments from ion C, due to ring cleavage, can produce only ions at m/e 109 for the butyl substituted ester, and ions at m/e 95 for the propyl isomer. This may suggest another pathway via double bond migration either in ion C or in the molecular ion (M^+), followed by cleavage at the position α to the ring. The product might be an open chain radical ion in which various hydrogen migrations may occur. If this is the case, both the relatively intense ions at m/e 123 (in $[II]_4$ mass spectra, Fig. 2A), and m/e 109 (in $[II]_3$ mass spectra, Fig. 2B), and their protonated counterpart ions can be adequately explained.

No significant difference was observed between the mass spectra of the *trans*- and *cis*-ring isomers of the main synthetic products $[I]_4$ and $[I]_3$. However, major differences in fragmentation were evidenced when comparing the mass spectra of structural isomers $[I]_4$ and $[I]_3$ (Fig. 4A and B). The alternative α-cleavage of the ring substitutents, which is the fragmentation pattern for

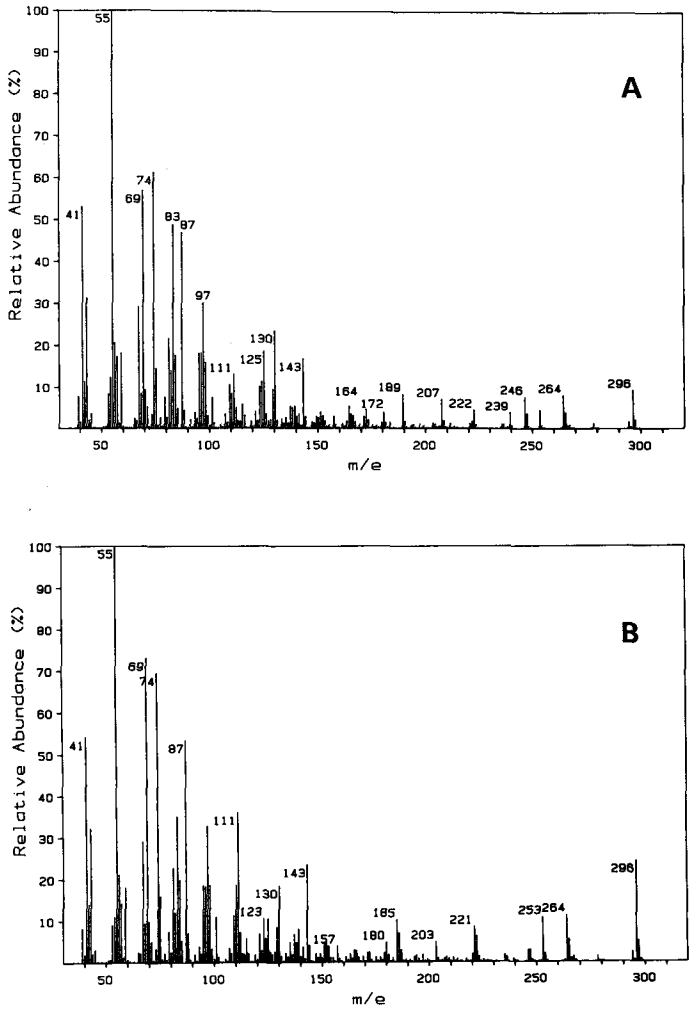


FIG. 4. EI mass spectra of *trans*-disubstituted cyclopentyl fatty acid methyl esters. (A) Methyl *trans*-9-(2′-n-butylcyclopentyl)-nonanoate. Peak $[I]_4$-1 in Fig. 1A. (B) Methyl *trans*-10-(2′-propyl-cyclopentyl)decanoate. Peak $[I]_3$-1 in Fig. 1B.

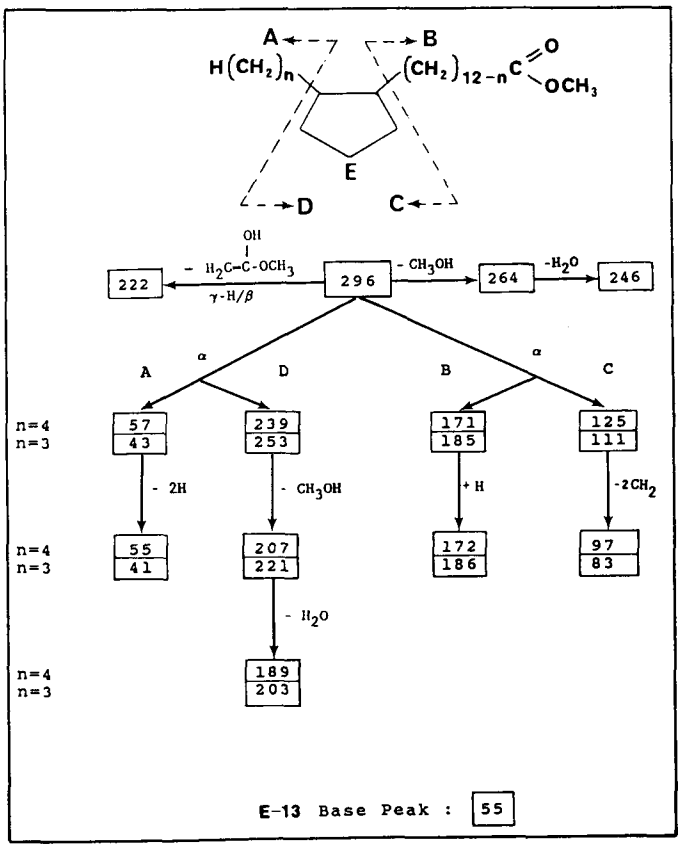


FIG. 5. Mass fragmentation diagram of methyl ω-(2′-n-alkyl-1′-cyclopentyl) alkanoates $[I]_4$ (n = 4) and $[I]_3$ (n = 3).

disubstituted cyclohexyl ester isomers (8,23), appeared to explain the major set of characteristic ions for both cyclopentyl isomers as illustrated in Figure 5. The successive loss of small neutral fragments (methanol and water), from ion D produces fragments D-32 and D-32-18, which, in connection with ion B, yields enough information to determine the location of the cyclopentyl rings within the ester molecule.

All the isomers $[I]_n$ clearly showed a defined molecular ion (M^+) at m/e 296 with relative abundances exceeding 9% (Table 2). This highly significant abundance for M^+ as well as for other ions in the high mass range confirmed previous studies (5,24) in which similar high abundances were observed. These studies had been carried out with the same GC-MS system and ionization conditions (70 eV, soure at 200°C).

The presence of the low mass ion homologous series C_zH_{2z-1} at m/e 41, 55 (base peak), 69, 83, 97, and 111, typical of cycloalkyl compounds, confirmed the cyclopentane structure. However, the occurrence of a prominent McLafferty rearrangement ion at m/e 74 and the ions at m/e 59, 87, 101, 115, 129, 143, and 157 from the series

TABLE 2

Partial Mass Spectra of 1,2-Disubstituted Cyclopentyl Fatty Acids Methyl Esters[a]

	Relative abundance (%)				
	$[I]_4$		$[I]_3$		
m/e	*trans*	*cis*	*trans*	*cis*	Fragment assignment
296	9.3	9.6	24.1	20.1	M^+
295	0.4	0.4	0.5	0.2	M^+-1
294	1.6	2.3	2.5	2.4	M^+-2
267	0.8	0.7	1.3	0.9	D_3+14
264	8.1	11.5	11.1	10.9	M^+-32
253	4.4	3.9	10.6	9.1	D_3 & D_4+14
246	7.7	9.6	2.8	2.8	M^+-32-18
239	3.8	5.3	0.8	0.6	D_4
235	1.0	1.4	1.8	1.7	(D_3+14)-32
222	4.5	6.5	6.2	7.3	M^+-74
221	1.8	2.4	8.4	8.4	D_3-32
217	0.3	0.4	0.7	0.8	(D_3+14)-32-18
207	7.0	9.4	0.9	1.3	D_4-32
203	1.3	1.4	4.8	5.4	D_3-32-18
189	8.3	10.2	0.6	0.6	D_4-32-18
186	0.2	0.5	7.0	6.4	B_3+1
185	0.4	0.7	10.1	8.6	B_3
180	3.8	5.8	4.7	5.0	M^+-116
172	4.6	5.4	2.3	2.2	B_4+1
171	2.8	3.7	2.3	2.1	B_4
164	5.3	5.9	0.8	0.9	M^+-132

[a]Structure codes according to Scheme 1.

$C_zH_{2z-1}O_2^+$ indicated characteristic aliphatic methyl ester fragments.

The most important observation in the high mass range of the spectra of both saturated cyclopentyl esters was the appearance of several even mass ions (Table 2) which were not seen in similar synthetic cyclohexyl ester isomer. Ion peaks at m/e 264 (M^+-32), 246 (M^+-32-18), 222 (M^+-74), and 180 (M^+-116) were present in significant abundances in all the spectra. This group of ions is the most characteristic in the mass fragmentation of C_{18} monounsaturated straight chain FMAE (e.g., methyl oleate), and their occurrence can be explained through the loss of the ester moiety from M^+ via energetically favorable hydrogen rearrangements. The presence of important peaks at M^+-2 and, in lesser extent, at M^+-1 in the spectra of disubstituted cyclopentyl esters (Table 2) indicated labile hydrogen atoms, probably those attached to the tertiary carbons of the ring, which might lead to successive concerted H-rearrangement reactions. In a similar fashion to monounsaturated linear FAME, these reactions will afford relatively more stable even mass fragment ions, and therefore can be proposed as the mechanism to explain this peculiar group of ions.

In the case of saturated disubstituted ester isomers $[I]_4$ and $[I]_3$, cleavage in the β position to the ring of the alkyl substituent (2,20) affording an ion that can undergo loss of the ester moiety like ion D, appeared to be less important than for their unsaturated counterparts, when considering the low abundance of the β-cleavage product ions in the spectra, especially those for $[I]_3$ ring isomers. In this respect the β-cleavage ion of $[I]_4$ at m/e 253 (M^+-43) can be regarded as the most significant one, and the same fragmentation can be explained by loss of a propylene fragment involving cleavage of two bonds during ring decomposition of M^+.

The formation of side reaction products was observed during the synthesis of both $[I]_4$ and $[I]_3$ final products. The compounds that are characterized with letters in Figure 1 accounted for the most important impurities in the final products. The retention parameters and the most probable structures of these by-products were included in Table 3. The compounds responsible for peaks a in Figure 1 were tentatively identified as the monounsaturated disubstituted cyclopentenyl methyl esters with the double bonds between carbons 1 and 2 of the cyclopentane ring (Table 3).

The pair of peaks **b1′** and **b2′** observed for $[II]_4$, and at a very low levels for $[II]_3$ (Figure 1), are apparently due to monounsaturated cyclic isomers with molecular ion at m/e 308 and with very similar fragmentation pattern to the major cyclopentylidene isomers. These isomers formed the components (M^+ at m/e 310) labeled as **b1′** and **b2′** upon catalytic hydrogenation.

Spectroscopic characteristics. The most important IR absorption bands for each particular product were specified below in the experimental section under synthetic methods. IR spectra of intermediate 2-alkylcyclopentanones were very similar and presented all the expected absorption bands for these compounds. Their corresponding ^{1}H-NMR spectra showed practically one signal downfield per each group of equivalent protons within the cyclopentane ring and the characteristic chemical shifts for the protons in the linear n-alkyl side chain: δ 0.90 (triplet, 3) for $-C\underline{H}_3$ and δ 1.28-1.45 (multiplet) for $-C\underline{H}_2-$.

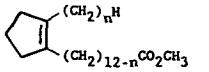
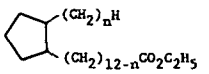
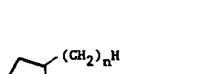
When intermediate methyl 9-(2′-n-butylcyclopentylidene)nonanoate $[II]_4$ was isolated, the IR spectra of the purified product did not show the expected absorption bands for olefinic C-H stretching or bending vibrations. However, the ^{1}H-NMR spectra for the same product showed a multiplet signal at δ 5.11 ppm corresponding to the chemical shift of one olefinic proton in the molecule (Fig. 6A). The presence of unsaturation in the disubstituted cyclopentylidene molecule was finally confirmed when closer examination of the IR spectra of $[II]_4$ showed a small shoulder around 1670 cm^{-1}. This was assigned to weak C=C stretching vibration of a trisubstituted double bond and a sharp but weak absorption band at 850 cm^{-1}, which was due to olefinic C-H out-of-plane deformation. The absence of typical olefinic C-H IR absorption bands at higher frequencies may be explained by the hindrance effect of the ring when double bonds are directly attached to it (25).

Catalytic hydrogenation of $[II]_4$ to obtain the final product, $[I]_4$, resulted in the elimination of the olefinic multiplet in the corresponding ^{1}H-NMR spectra of this product (Fig. 6B). Similarly, the IR spectra of $[I]_3$ did not present the absorption bands attributed to C=C stretching vibrations and C-H out-of-plane deformation.

The following common characteristic chemical shifts wre distinguished in the ^{1}H-NMR spectrum of disubstituted cyclopentylidene methyl esters $[II]_n$ (Fig. 6A for n = 4): δ 0.92 (triplet, 3H) for terminal $-C\underline{H}_3$, δ 1.3 (broad singlet) for $-C\underline{H}_2-$; δ 1.62-1.90 (multiplet, ca. 6H) for cyclopentyl-$C\underline{H}_2-$; δ 1.99 (multiplet, 2H) for

TABLE 3

Capillary GLC Retention Data and Probable Chemical Structures of Side Reaction Products[a]

Main product [ ]$_n$	Peak ID		Retention data[b] RRT	ECL	M$^+$ (m/e)	Possible structure
$[I]_4$	→	a	1.128	18.357	294	
$[I]_3$	→	a	1.216	18.577	294	
$[I]_4$		b1	1.174	18.475	310	
		b2	1.345	18.876	310	
$[I]_3$		b1[c]	1.248	18.655	310	
		b2[c]	>1.30	>19.00	310	
$[II]_4$		b1′	1.321	18.821	308	
		b2′	1.483	19.164	308	
$[II]_3$		b1′[c]	1.410	19.530	308	
		b2′[c]	>1.50	>19.70	308	

[a] GC conditions same as Table 1. Structure coes and peak identification according to Scheme 1 and Figure 1, respectively.

[b] Retention data (corrected for non-retained peak): RRT = relative retention time to methyl stearate; ECL = equivalent chain length.

[c] Traces only.

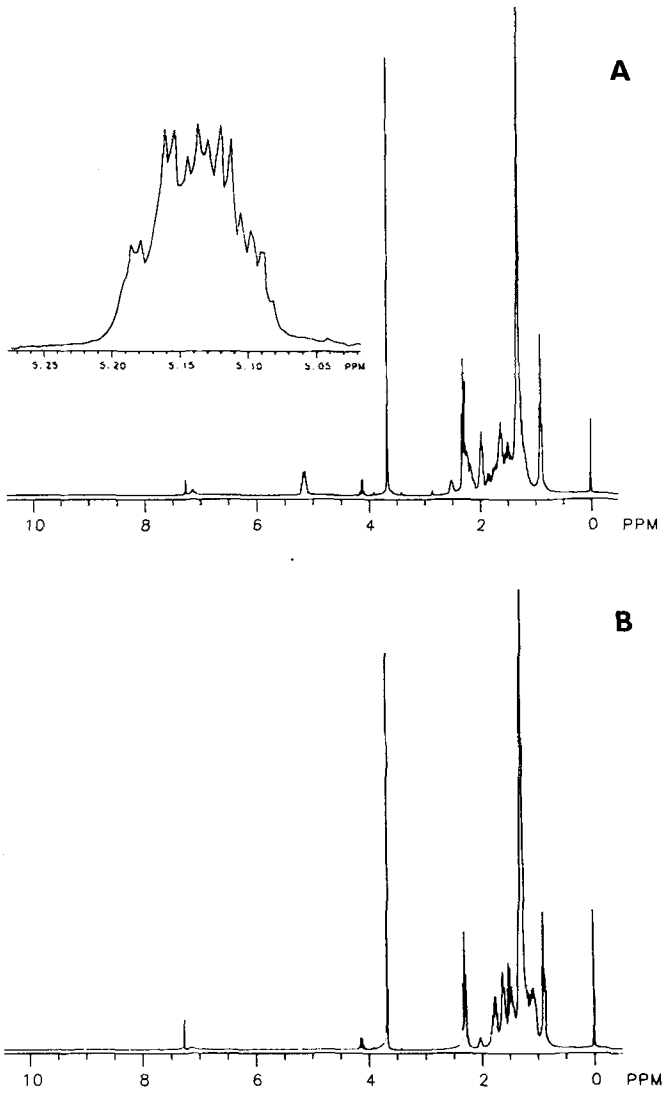


FIG. 6. ^{1}H-NMR specta at 300 MHz. (A) Methyl 9-(2′-n-butyl-1′-cyclopentylidene)nonanoate, [II]$_4$. (B) Methyl 9-(2′-n-butylcyclopentyl)nonanoate, [I]$_4$.

-C<u>H</u>$_2$-C=C, δ 2.30 (triplet, 2H) for -C<u>H</u>$_2$-COO-, δ 2.56 (broad singlet, 1H); δ 3.66 (singlet, 3H) -OC<u>H</u>$_3$, and δ 5.11 (multiplet, 1H) for C=C-<u>H</u>. The assignments for characteristic chemical shifts in the proton NMR spectra of the saturated final products [I]$_n$ (Fig. 6B for n = 4) were as follows: δ 0.89 (triplet, 3H) terminal -C<u>H</u>$_3$; δ 1.28 (broad singlet) for -C<u>H</u>$_2$-; three multiplet signals at δ 1.51, 1.62 and 1.77 ppm for nonequivalent protons in the cyclopentane ring; δ 2.30 (triplet, 2H) for -C<u>H</u>$_2$-COO-; and δ 3.66 (singlet, 3H) for -OC<u>H</u>$_3$.

Other less important, low intensity signals were observed in the IR and ^{1}H-NMR spectra of cyclopentylidene and cyclopentyl substituted esters, which originated from solvent impurities and some of the side reaction products previously described (Table 3).

EXPERIMENTAL

General data. Infrared spectra were recorded on a Beckman IR 4210 spectrophotometer; liquid samples were analyzed as thin films between polished sodium chloride plates. Proton nuclear magnetic resonance (^{1}H-NMR) spectra and one pulse experiments at 300 MHz were recorded on a QE-300 General Electric high resolution NMR spectrometer. Deuterated chloroform ($CDCl_3$) was used as solvent containing 3% tetramethyl silane (Me_4Si) as internal reference. The boiling points are uncorrected.

Thin layer chromatography. Preactivated commercial precoated plates of silica gel (LK-6, 0.25 mm layer thickness, Whatman) were used. The developing solvent was either 4:1 n-hexane-diethyl ether for the cyclic C_{18} esters, or 9:1 methylene chloride/n-hexane for the intermediates. Once dry, the developed plates were visualized by charring with a solution containing 55% H_2SO_4 and 0.6% $K_2Cr_2O_7$.

Gas liquid chromatography. A Hewlett Packard 5790A capillary gas chromatograph (Hewlett Packard, Avondale, PA) equipped with an inlet splitter system fitted with a Jennings glass liner, flame ionization detector, and electronic integrator (HP 3390A) was used. A 30 m × 0.25 mm i.d. fused silica WCOT capillary column coated with Supelcowax-10® (polyethylene oxide bonded phase, Supelco Inc., Bellefonte, PA), 0.25 μm film thickness, was used isothermally or with temperature program conditions. For low molecular weight intermediates, the program ran from one minute at 80°C to 13°C at a rate of 3°C/min. The injection port and detector temperatures for this program were set at 150 and 170°C, respectively. For the final products a two ramp program ran from 175°C (1 min) to 200°C at 1.5°C/min (kept at 5 min at 200°C and then raised to 250°C at 5°C/min). The injector port was at 250°C and the detector at 270°C. The carrier gas was hydrogen at a 1:100 split ratio.

Gas chromatography-mass spectrometry (GC-MS). A Hewlett Packard 5985B GC-MS system was used with the CI-EI source set at 70 eV and 200°C. The same capillary column specified above was also used with this system under the same conditions. Peaks not resolved on this column were analyzed with a 25 m × 0.31 mm i.d. fused silica capillary column coated with HP-5® (5% phenyl methylsilicone bonded phase), 0.17 μm film thickness (Hewlett Packard, Avondale, PA), and operated from 180 to 250°C at a program rate of 2°C/min. The columns were interfaced directly into the mass spectrometer and helium was used as the carrier gas. Methane was used as the reactant gas for chemical ionization (CI) with the source temperature at 100°C.

Special reagents. The powdered sodium reagent was prepared as described by Hauser and Hudson (26). The silver nitrate impregnated with silicic acid (Silicic acid/$AgNO_3$) was prepared as described by DeVries (27).

Ethyl 1-n-alkyl-2-oxocyclopentane carboxylates. The n-propyl and the n-butyl homolog was prepared by a combination of the methods reported by Case and Reid (28) and Chatterjee (29,30). A molar amount (500 ml/mol Na) of ethyl 2-oxocyclopentane carboxylate was added dropwise from a pressure equalizing funnel to an equivalent molar amount of powdered sodium in p-xylene-cooled in ice. The reaction mixture was continuously agitated and allowed to remain overnight at room temperature. The product (sodium salt of the keto ester) was refluxed for 12 hours under agitation after addition of 8-10% excess of a molar amount of alkylhalide (1-iodopropane and

1-bromobutane). After cooling, the mixture was filtered and the residue washed several times with small amounts of p-xylene. The combined washings and the filtrate were concentrated under reduced pressure to remove the p-xylene and any unreacted alkyl halide. GC-MS analysis of the product showed over 85% yield of keto ester condensation product. The crude product yields for n-propyl and n-butyl keto ester homologs were 80% and 71%, respectively. IR (thin film) spectra of alkylated keto esters wre very similar. Ethyl 1-butyl-2-oxocyclopentane carboxylate showed two overlapping strong bands at 1755 cm^{-1} (C=O, ester) and 1722 cm^{-1} (C=O, ketone); two weak absorptions at 1675 cm^{-1} and 1630 cm^{-1} arising from the chelate structure of the enol form; and strong absorption bands at 1255 cm^{-1}, 1222 cm^{-1} and 1145-1165 cm^{-1} (ethyl ester, C-O-C).

2-n-Alkylcyclopentanones. Crude alkylated keto esters were decarboxylated by boiling a mixture of one part of product with six parts of concentrated hydrochloric acid (37%) under reflux for 24 hours. Diluted sulfuric acid (density 1.42 g/ml) was equally useful for decarboxylation, but the final yields of the decarboxylated product were generally lower. The treated product was extracted three times with ethyl ether after saturation with ammonium sulfate. The combined ethereal extracts were thoroughly washed with 5% aqueous sodium carbonate solution, then with by distilled water, and finally dried over anhydrous sodium sulfate. Crude 2-n-propylcyclopentanone was obtained as a yellowish product after evaporation of the ether under vacuum, with a 60% overall yield. A relatively pure colorless ketone fraction (93% pure by GC-MS, M^+ at m/e 126) was obtained after fractional distillation under vacuum (b.p. 54.0°C at 9 mm). The IR (thin film) spectrum showed a sharp strong band at 1740 cm^{-1} (ketone C=O); a sharp but weak peak at 3455 cm^{-1} (overtone of C=O stretch absorption); medium band at 1460 cm^{-1}, sharp bands at 1410 cm^{-1}, and 1380 cm^{-1} (cyclopentane CH_2); and medium absorption at 1155 cm^{-1} [C-C(=O)-C]. Similarly, crude 2-n-butylcyclopentanone was obtained as a slightly yellowish liquid which was shown to be 89% pure (55% crude product yield) by GC-MS analysis (M^+ at m/e 140). The crude product was used as such without further purification. The IR (thin film) spectrum was very similar to 2-n-propylcyclopentanone: 3460 cm^{-1} (weak C=O overtone); 1740 cm^{-1} (ketone C=O); [1462, 1410, and 1380 cm^{-1} $(CH_2)_n$-cyclopentane)]; 1155 cm^{-1} [C-(C=O)-C].

Methyl-9-bromononanoate. Bromodecarboxylation of methyl hydrogen sebacate was carried out by a modification of the Hunsdiecker reaction as described by Davis *et al.* (11). A solution of 0.05 mol of bromine in 20 ml of carbon tetrachloride was added dropwise to a warm stirred mixture containing 0.05 mol of methyl hydrogen sebacate, 10% molar excess of red murcuric oxide, and 70 ml of carbon tetrachloride. The reaction mixture was refluxed for 3 hr. After cooling, the mixture was filtered through a fritted glass Büchner filter funnel, and the residue in the funnel washed with small portions of carbon tetrachloride. The filtrate and the washings were transferred to a separate funnel and washed with 50 ml of 5% sodium hydroxide solution, two times with 30 ml of distilled water, and then dried over anhydrous sodium sulfate. The solvent was removed under vacuum, affording 8.8 grams of colorless crude product. This product was distilled under vacuum using a small Vigreux fractionation column. The fraction boiling at 130-132°C at 4 mm (71% yield) proved, by GC analysis, to be 91% pure methyl 9-bromononanoate. IR (thin film) showed a sharp strong band at 1740 cm^{-1} (ester C=O); three medium bands at 1255, 1198 and 1172 cm^{-1} (C-O-C methyl ester); 725 cm^{-1} [$-(CH_2)_n-$] and 642 cm^{-1} (C-Br).

Methyl 10-bromodecanoate. This bromo ester was prepared by direct methylation of its corresponding free acid as follows: 50 ml of esterification reagent containing 2% sulfuric acid in 3:1 methanol-benzene were added per each gram of bromo acid, and the mixture was then refluxed for 5 hr. The end of the reaction was verified by TLC. The cooled reaction mixture was extracted three times with n-hexane, and the combined organic extracts were washed with distilled water until free of acid. After drying over anhydrous magnesium sulfate, the solvent was removed under vacuum and the final ester analyzed by GC. Crude methyl 10-bromodecanoate yield was 91% and contained 85% pure ester. This product was used for preparation of phosphonium salt without further purification. IR (thin film) of the product showed the same absorption bands as its 9-bromoalkanoate ester homolog.

ω-Carbomethoxyalkyltriphenylphosphonium bromides. The phosphonium salts of the two bromo esters were prepared with the procedure described by Awl and Frankel (10). A molar amount of bromo ester mixed with 15% excess of triphenyl phosphine in acetonitrile was refluxed under nitrogen for 36 hr. The solvent was removed in a rotary evaporator under reduced pressure. The product was extracted at least five times with diethyl ether by maceration with a stirring bar, followed by decantation of the solvent. Most of the unreacted triphenyl phosphine and unreacted bromo ester were removed in this way. The two phosphonium salts were isolated as viscous transparent glasses. The yields of crude products in both cases were over 95%.

Methyl 9-(2'-n-butylcyclopentyl)nonanoate. This product was prepared by a Wittig reaction between the corresponding phosphonium salt and 2-n-butylcyclopentanone followed by catalytic hydrogenation of the purified reaction product. The Wittig reaction conditions used were based on a combination of the procedures described by Awl and Frankel (10) and Corey *et al.* (13). The 8-carbomethoxyoctyl triphenylphosphonium bromide (9.9 g) was dissolved in 25 ml of 1:1 dimethyl sulfoxide (DMSO)-tetrahydrofuran (THF). This solution was added dropwise under nitrogen to magnetically stirred dry sodium hydride (0.73 g as 60% dispersion in mineral oil, previously washed with n-pentane to remove the oil), and then cooled in an ice bath. The reaction became dark brown and the mixture was stirred under N_2 for 20 hr after removal from the ice bath. The final product was treated as described by Greenwal *et al.* (31) with modifications. The reaction mixture was poured into 150 ml of distilled water and then extracted three times, each time with 40 ml of n-pentane. The combined organic extracts were washed with 100 ml of 1:1 water-DMSO, 100 ml of NaCl saturated solution, and twice with 100 ml of distilled water. The washed extract was dried over anhydrous sodium sulfate and the solvent removed in a rotary vacuum evaporator. GC-MS analysis of the crude dry extract showed two peaks (65%) with the same mass spectrum, molecular ion at m/e 294 and all of the expected

fragments for C_{18} monounsaturated cyclic methyl ester, and some unreacted triphenyl phosphine (36%) m/e 262. The molecular weights were confirmed by chemical ionization (CI) GC-MS. The reaction product was passed through a neutral alumina (activity 1) column (20 g) and eluted with n-hexane. The combined eluate fraction containing the unsaturated cyclic ester were still contaminated with triphenyl phosphine. The solvent was evaporated under nitrogen and the residue was further purified through a small column packed with 4 g of silicic acid/$AgNO_3$ by eluting successively with 15 ml of n-hexane and 15 ml of 10% ethyl ether in hexane. The fractions richer in the cyclic compounds (as determined by GLC) were combined and the solvent evaporated under a stream of nitrogen. The yield of the final colorless liquid product was 70 mg which corresponded to an overall reaction yield of 8%. GC-MS analysis and ^{1}H-NMR spectra were obtained for this product. IR (thin film) showed strong band at 1742 cm^{-1} (C=O ester); but surprisingly no absorption bands for olefinic C-H stretching or bending vibrations were observed; 1245, 1200 and 1170 cm^{-1} absorption band owing to stretching vibrations of carbon-oxygen ester bonds (C-O-R); 850 cm^{-1} C-H out-of-plane deformation (cyclic-C=CH-); and 725 cm^{-1} [-$(CH_2)_7$-]).

The saturated disubstituted cyclopentyl ester (*cis*- and *trans*-isomers) was obtained by catalytic hydrogenation of the monounsaturated purified product with platinum dioxide (10-15 mg/0.1 g material). The solvent used was methanol and the reaction was carried out at 20 psi hydrogen pressure at room temperature for two hours. The catalyst was separated by filtration and the solvent was removed at reduced pressure. The final product was analyzed by GC-MS (CI and EI) and the IR and ^{1}H-NMR spectra were obtained. GC-MS showed a total of five peaks with the two main peaks (84%+) corresponding to the ring isomers (*c/t*) of the desired cyclic ester. Both peaks showed very similar mass spectra with M^+ at m/e 296.3. The IR (thin film) spectrum showed 1742 cm^{-1} (ester C=O), [1240-1250, 1195, and 1170 cm^{-1} (methyl ester, C-O-C)]; 725 cm^{-1} [-$(CH_2)_8$-]).

Methyl 10-(2'-n-propylcyclopentyl) decanoate. Using a similar approach, methyl 10-(2'-n-propylcyclopentylidene) decanoate was prepared by the Wittig reaction and catalytically hydrogenated to yield the saturated product. The phosphorane was generated from the corresponding thiphenylphosphonium bromide in presence of the ketone as suggested by Corey *et al.* (13), with several modifications. Sodium hydride (30 mmol as a 60% dispersion in mineral oil) in a 50 ml three necked flask was washed several times with n-pentane to remove the oil. The system was equipped with a condenser, a magnetic stirrer, and a pressure equalizing funnel containing a mixture of 15 mmol of 9-carbomethoxynonyltriphenylphosphonium bromide and 7.5 mmol of 2-n-propylcyclopentanone dissolved in 20 ml of 2:1 dimethyl sulfoxide (DMSO)-tetrahydrofuran (THF) which had been dried on molecular sieves. The system was alternatively evacuated and filled with nitrogen. The mixture was added dropwise to the stirred dry sodium hydride under nitrogen and cooled with an ice bath. After two hours the color of the mixture became brown-orange. The reaction mixture was stirred under nitrogen for 16 hours at room temperature, and then treated as previously described for the butyl substituted unsaturated cyclic ester. Before purification, the overall yield of the crude product was 1.43 g (65%), which contained mainly the desired unsaturated cyclic compounds (two isomers showing M^+ at m/e 294), unreacted triphenyl phosphine (M^+ at m/e 262), and two highly retained compounds with the same mass spectra (M^+ at m/e 388) as shown by GC-MS. Purification of the product was attempted in one step through a column packed with a bottom layer of silicic acid/$AgNO_3$ (10 g) and a second (top) layer of neutral alumina (2 g) which had been previously conditioned with n-hexane. The product was successively eluted with 50 ml each of n-hexane and 10% ethyl ether in n-hexane. At this point the product, though free of triphenylphosphine, still contained significant amounts of the undesired compounds with M^+ at m/e 388. Separation of a 75%$^+$ purity fraction of two unsaturated cyclic isomers was achieved after a second elution through neutral alumina (4 g). IR (thin film) spectra of this product showed a strong band at 1742 cm^{-1} (ester C=O); one sharp medium band at 1710 cm^{-1}, apparently due to the presence of an unsaturated free acid C=O stretch vibration; a medium band at 1640 cm^{-1} (olefinic C-H stretch); 1254, 1195 and 1175 cm^{-1} (C-O-C methyl ester); 855 cm^{-1} owing to C-H out-of-plane bond deformation in a trisubstituted double bond (cyclic-C=CH-CH_2); and 725 cm^{-1} [-$(CH_2)_8$-].

Hydrogenation of the partially purified product was carried out as previously described. GC-MS of the hydrogenated product showed two main peaks with M^+ at m/e 296.3 and very close fragmentation patterns accounting for 72% of the total GC peak areas. IR (thin film) showed complete disappearance of characteristic strong bands of the olefinic C-H vibrations and the expected absorption bands for a cyclic methyl ester were observed: 1740 cm^{-1} (ester C=O); [1245-1250, 1195 and 1168 cm^{-1} (methyl ester, C-O-C)]; 725 cm^{-1} [-$(CH_2)_9$-].

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The Effect of a Fish Oil Diet on the Fatty Acid Composition of Individual Phospholipids and Eicosanoid Production by Rat Platelets

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When rats were fed a diet containing chow or fish oil for six weeks, the platelet phospholipid content and percent distribution were similar. In the fish oil fed animals there was a 54, 40, 41, and 24% reduction, respectively, in the levels of 20:4(n-6) in the choline-, ethanolamine-, inositol- and serine-containing glycerophospholipids. Dietary fish oil increased the total (n-3) polyunsaturated fatty acid content in all lipids. This effect was most pronounced in the ethanolamine glycerophospholipids which now contained 26, 11, and 4 nmols of 20:5(n-3), 22:5(n-3), and 22:6(n-3) in 10^9 cells. Ionophore A23187 stimulation of platelets from the chow fed rats resulted in the synthesis of 7, 64, and 3.5 nmols of 12-hydroxy-5,8,10-heptadecatrienoic acid, 12-hydroxy-5,8,10,14-eicosatetraenoic acid and 12-hydroxy-5,8,10,14,17-eicosapentaenoic acid, respectively, from 1×10^9 cells. The values from animals fed fish oil were 4, 18, and 27 nmol/10^9 platelets. It was not possible to detect any lipoxygenase products from 22:5(n-3) or 22:6(n-3), even though both acids are readily metabolized by lipoxygenase when added directly to platelets. These findings suggest that 22-carbon (n-3) fatty acids are not liberated when phospholipases are activated by calcium mobilization.
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Platelets metabolize unesterified arachidonic acid via cyclo- and lipoxygenase pathways into a family of eicosanoids with important physiological functions (1,2). Eicosapentaenoic acid can also be metabolized by these cells to thromboxane A_3 and hydroxy fatty acids (3,4). Epidemiological data suggests that a high intake of fish oil is correlated with a low incidence of thrombotic disorders (5,6). This finding is frequently correlated with the level of 20:5(n-3) in platelet phospholipids (7-10). According to this hypothesis both arachidonic acid and 20:5(n-3) are liberated from platelet phospholipids. Both acids compete with cyclooxygenase to depress the synthesis of thromboxane A_2. Thromboxane A_3 is a poor agonist for inducing platelets to aggregate (11,12).

Fish oils contain not only 20:5(n-3), but also significant amounts of other (n-3) fatty acids such as 22:5(n-3) and 22:6(n-3). The levels of these two acids in platelet lipids also increase when (n-3) acids are added to the diet (5,6). Our previous studies have shown that exogenous 22:5(n-3) and 22:6(n-3) are both metabolized by platelets into an isomeric pair of hydroxy fatty acids via an indomethacin insensitive pathway (13,14). Both 22:5(n-3) (13) and 22:6(n-3) (15) inhibit cyclooxygenase when arachidonic acid is used as the substrate. Exogenous 22:6(n-3) reduces platelet aggregation and arachidonic acid metabolism *in vitro* (16). When platelets are incubated with radioactive 22:6(n-3) it is incorporated into phospholipids (17). However, it was not possible to detect the release of radioactive 22:6(n-3) when the prelabeled cells were exposed to agonists which activate phospholipases (18). These findings suggest that the phospholipids containing 22:6(n-3) are not substrates for phospholipase. However, it is possible that the radioactive 22:6(n-3) was incorporated into a different pool of phospholipids than that which was used as a substrate for phospholipases.

The purpose of the present work was to study the effect of fish oil supplementation on the lipid metabolism of rat platelets in order to provide a more comprehensive assessment of the value of dietary fat change. We analyzed the fatty acid composition of individual platelet phospholipids and now present evidence showing that 22-carbon (n-3) acids are not released from ionophore A23187 stimulated platelets.

MATERIALS AND METHODS

Materials. Disodium EDTA, Tris HCl, Tris base, and ionophore A23187 were products of the Sigma Chemical Company (St. Louis, MO). Formic Acid was purchased from the J.T. Baker Chemical Co. (Phillipsburg, NJ). Aquasil was used as a siliconizing agent and was obtained from the Pierce Chemical Company (Rockford, IL).

12(S)-Hydroxy[5,6,8,9,11,12,14,15(n)-^{3}H]eicosatetraenoic acid (Sp. Act. 119 Ci/mmol) was obtained from Amersham International. Phospholipid standards were purchased from Avanti Biochemicals, Inc. (Birmingham, AL). Fatty acid methyl esters were obtained from Nu-Chek Prep, Inc. (Elysian, MN). 12(S)-Hydroxyeicosatetraenoic acid was obtained from Biomol Research Lab, Inc. Dimethylacetal standards were prepared in our laboratory, as previously reported (19).

Thin-layer chromatography was performed on Whatman LK5 precoated plates (Whatman Inc., Clifton, NJ). Formula-963 (NEN Research Products, Boston, MA) was used as a scintillation cocktail.

All solvents for extraction and chromatography were HPLC grade.

Animals and dietary conditions. Male weanling Sprague-Dawley rats were fed *ad libitum* either the regular chow diet or a modified AIN-76 semipurified diet containing 2.5% corn oil and 2.5% menhaden fish oil (ICN Biochemicals, Inc.). The fatty acid composition of the refined fish oil in weight percent was as follows: 14:0, 9.5%; 16:0, 22.6%; 16:1, 17.5%; 18:0, 3.3%; 18:1, 14.0%; 18:2, 0.8%; 18:3(n-3), 3.1%; 18:4(n-3), 3.6%; 20:4(n-6), 1.6%; 20:5(n-3), 14.5%; 22:4(n-6), 1.2%; 22.5(n-3), 1.9%; and 22:6(n-3), 6.6%.

Platelet preparation. The animals were anaesthetized under ether, and blood was drawn by a cardiac puncture

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Abbreviations: 12-HHT, 12-Hydroxy-5,8,10-heptadecatrienoic acid; 12-HETE, 12-hydroxy-5,8,10,14-eicosatetraenoic acid; 12-HEPE, 12-hydroxy-5,8,10,14,17-eicosapentaenoic acid; DMA, dimethylacetal; ECL, equivalent chain length; EDTA, ethylenediamine tetraacetic acid; GLC, gas liquid chromatography; HPLC, high performance liquid chromatography; PC, choline phosphoglycerides; PE, ethanolamine phosphoglycerides; PI, inositol phosphoglycerides; PS, serine phosphoglycerides.

with a plastic syringe containing 77 mM disodium EDTA. Platelets were isolated and resuspended at a concentration of 3×10^8 cells/ml in 0.15M NaCl, 0.15M Tris (pH 7.4), and 77 mM disodium EDTA (90:8:2, v/v/v) (20).

Lipid extraction and analysis. Lipids were extracted from the suspended platelets in accordance with the Bligh and Dyer procedure (21). Total platelet lipids were fractionated by one-dimensional TLC on LK5 plates developed with chloroform/methanol/40% methylamine (60:20:5, v/v/v) (22). The phospholipids were visualized by spraying them with 0.1% 2′,7′-dichlorofluorescein in ethanol.

PI, PS, PC, and PE were scraped from the plate into screw cap tubes. Phospholipids were extracted twice with 5.0 ml of chloroform/methanol/water (5:5:1, v/v/v), followed by centrifugation. The 10 ml of extract was washed with the addition of 4.5 ml of chloroform and 2.0 ml of water, and then the upper dichlorofluorescein-containing phase was discarded. Phosphorus analysis was performed on aliquots from the total lipid extract and from each phospholipid by the method described by Rouser *et al.* (23).

Methyl esters were generated by heating the individual phospholipids at 80°C with 5% HCl in methanol in sealed tubes for 60 minutes. Analysis of the fatty acid methyl esters was performed on a Varian Vista 6000 gas chromatograph equipped with a glass column (10 feet × 2 mm, inner diameter) and packed with 10% SP-2330 on 100/120 mesh Supelcoport (Supelco, Bellefonte, PA). The carrier gas was He (30 ml/min) and 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 after injection and then rose 2°C/min to 190°C. The retention times and peak areas were quantitated with a Varian 4290 integrator. Fatty acid methyl esters were identified using appropriate standards.

Studies with stimulated platelets. Washed platelets were resuspended in 0.15 M NaCl, 0.15 M Tris (pH 7.4), 77 mM disodium EDTA (90:8:2, v/v/v) (3×10^8 cells/ml). Glucose (5 mM) was added and the cells were preincubated for 2 min at 37°C in siliconized tubes in a water bath. At that time 2 mM $CaCl_2$ was added and the cells were challenged with 2 μM ionophore A23187 (dissolved in dimethyl sulfoxide) for 30 min. Reactions were terminated by the addition of 0.15 ml of 2N formic acid per ml of platelet suspension, and the products were recovered by extracting the incubation mixture three times with three volumes of ethyl acetate. The recovery of the hydroxylated compounds, assessed by adding [^{3}H]-12-HETE to zero time samples run in parallel, was 95 ± 2%.

Reverse phase HPLC was carried out with a DuPont HPLC consisting of an 870 pump, 8800 series gradient controller, column oven, and a variable wave-length detector. The metabolites were separated by isocratic elution with 45% acetonitrile in water (pH 2.2), using a Zorbax 10 μ ODS column (0.46 × 25 cm) with a guard column (0.46 × 5 cm), and packed with Permaphase ODS (DuPont, Wilmington, DE). All samples were injected in 50 μl of methanol and the chromatographic separation was carried out at 35°C at a flow rate of 1.5 ml/min. The concentration of hydroxy acids in samples was calculated with reference to 12-HETE standard curves by monitoring at 235 nm.

The metabolites were isolated and converted to methyl esters by reaction with ethereal diazomethane. Methyl esters of hydroxy acids were converted to trimethylsilyl ether derivatives by reaction with 10 μl of N,0-*bis*(trimethylsilyl)trifluoro acetamide (Pierce Chemical Co.) and 10 μl of pyridine for 60 min at room temperature. Compounds were dissolved in isooctane for analysis by GLC.

GLC was carried out in a Varian Vista 6000 gas chromatograph equipped with a glass column (6 foot × 2 mm, inner diameter), and packed with 1% SP-2100 on 100/120 mesh Supelcoport (Supelco, Bellefonte, PA). The flow rate of He was 30 ml/min. The injector, oven, and detector temperatures were held at 250, 210, and 280°C, respectively. Equivalent chain lengths (ECL) were determined by comparison with a saturated series of methyl ester standards.

Mass spectrometry was performed with a Hewlett Packard 5970A mass selective detector and a 5790 gas chromatograph. Separations were carried out on a DB1 capillary column (25 m × 0.25 mm, inner diameter) obtained from Applied Science (State College, PA). Injections were made in the splitless mode with an initial temperature of 70°C and a valve time of 1 min. The temperature of the injector was 250°C and the transfer line was 280°C. One minute after injection the oven was programmed at 30°C/min to reach 220°C. The voltage of ionization was 70 eV.

RESULTS

The platelets from animals fed chow and fish oil contained 322 ± 68 and 432 ± 34 (n = 3) nmols of phospholipid phosphorous per 1×10^9 cells, respectively. The results in Table 1 show that dietary fat change did not alter the phospholipid composition.

The fatty acid composition of individual platelet phospholipids is shown in Table 2. Dietary fish oil did not markedly alter the level of either palmitic or stearic acid in any lipid except for PI. The levels of these two acids increased from 43 to 60% when fish oil was added to the diet. This compositional change is similar to that reported by Weiner and Sprecher (24) for rats fed ethyl linolenate. When fish oil was included in the diet there was the expected reduction of arachidonate in all phospholipids. The levels of arachidonate in PC, PE, PI, and PS were, respectively, 46, 60, 60, and 75% of that in the chow fed animals. This reduction in the arachidonate level was accompanied by an increase of 20:5(n-3) in all four phospholipids. When fish oil was added to the diet there was a reduction in the

TABLE 1

Phospholipid Composition of Rat Platelets

Phospholipid	mol % Phosphorous	
	Chow	Corn oil/Fish oil
Choline phosphoglycerides	42.6 ± 0.6	42.5 ± 1.0
Ethanolamine phosphoglycerides	30.2 ± 0.2	29.3 ± 0.9
Sphingomyelin	12.3 ± 0.4	11.5 ± 0.3
Inositol phosphoglycerides	4.3 ± 0.3	4.3 ± 0.1
Serine phosphoglycerides	10.6 ± 0.3	12.6 ± 0.4

Data are presented as mol % ± S.E.M. (n = 3).

TABLE 2

Fatty Acid Composition of Platelet Phospholipids from Rats Fed Chow or Corn Oil/Fish Oil Diet

	PC[a]		PE		PI		PS	
Fatty acid	Chow	Corn oil/Fish oil	Chow	Corn oil/Fish oil	Chow	Corn oil/Fish oil	Chow	Corn oil/Fish oil
16:0 DMA	—	—	5.4 ± 0.1	6.1 ± 1.5	—	—	—	—
16:0	54.1 ± 0.3	55.5 ± 1.2	8.1 ± 0.7	12.4 ± 2.6	11.7 ± 1.2	17.9 ± 2.5	10.6 ± 0.1	12.9 ± 4.6
16:1(n-7)	1.7 ± 0.1	2.4 ± 0.5	—	—	3.4 ± 1.0	1.7 ± 0.2	1.2 ± 0.4	0.6 ± 0.2
18:0 DMA	—	—	7.2 ± 0.0	6.4 ± 1.3	—	—	—	—
18:1 DMA	—	—	4.9 ± 0.1	4.5 ± 1.0	—	—	—	—
18:0	7.2 ± 0.1	11.7 ± 2.6	12.8 ± 0.3	17.4 ± 4.2	31.7 ± 3.1	42.3 ± 2.9	37.6 ± 2.2	40.8 ± 3.4
18:1(n-9)	8.1 ± 0.2	9.2 ± 1.1	8.4 ± 0.4	6.7 ± 0.8	7.4 ± 2.1	3.6 ± 0.8	8.3 ± 1.0	7.4 ± 1.6
18:2(n-6)	13.6 ± 0.5	10.3 ± 0.3	4.5 ± 0.1	4.0 ± 0.3	3.0 ± 1.1	1.5 ± 0.3	5.1 ± 0.9	6.1 ± 1.0
20:0(n-9)	—	—	0.2 ± 0.0	—	—	—	—	—
18:3(n-3)	0.5 ± 0.0	0.4 ± 0.2	0.9 ± 0.0	0.7 ± 0.3	—	—	1.2 ± 0.1	1.5 ± 0.5
20:3(n-6)	1.7 ± 0.3	1.2 ± 0.3	1.3 ± 0.3	1.3 ± 0.3	3.1 ± 0.3	5.0 ± 1.9	3.6 ± 0.3	3.6 ± 0.9
20:4(n-6)	8.1 ± 0.1	3.7 ± 0.6	34.7 ± 0.9	20.9 ± 2.7	32.0 ± 3.5	19.0 ± 5.8	27.9 ± 4.5	21.1 ± 3.5
20:5(n-3)	0.9 ± 0.5	2.9 ± 0.1	1.1 ± 0.1	10.4 ± 0.7	<0.2	5.8 ± 1.3	0.7 ± 0.1	2.4 ± 0.5
22:4(n-6)	1.2 ± 0.6	0.2 ± 0.0	6.2 ± 0.2	1.3 ± 0.0	2.1 ± 0.4	1.5 ± 0.5	4.1 ± 1.4	1.1 ± 0.4
22:5(n-6)	1.9 ± 0.8	0.1 ± 0.0	2.0 ± 0.0	0.9 ± 0.5	1.8 ± 0.7	0.9 ± 0.4	0.7 ± 0.3	0.3 ± 0.2
22:5(n-3)	0.4 ± 0.3	0.4 ± 0.1	1.5 ± 0.4	4.2 ± 0.4	—	1.2 ± 0.2	1.1 ± 0.3	2.7 ± 0.5
22:6(n-3)	0.5 ± 0.2	0.7 ± 0.4	0.7 ± 0.1	1.6 ± 0.6	—	0.4 ± 0.3	0.4 ± 0.1	1.2 ± 0.5

[a]Values represent the mean (mol %) ± S.E.M.

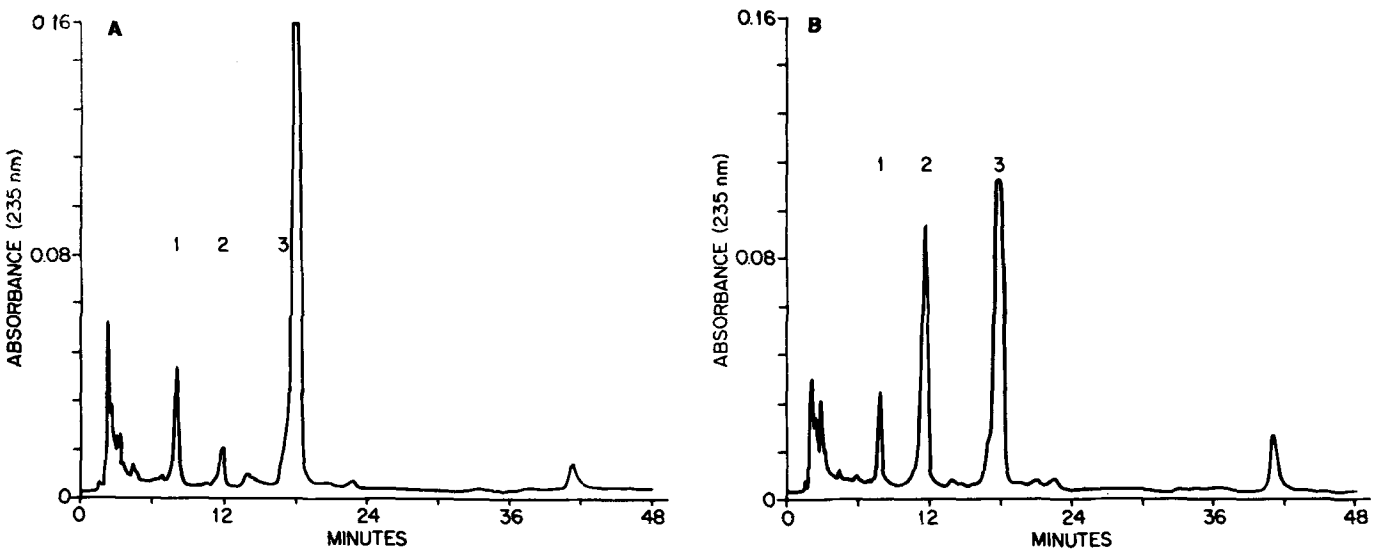

FIG. 1. Reverse phase HPLC chromatograms of metabolites obtained after stimulation of rat platelets (3×10^8 cells/ml) with A23187 (2 μM). (A) Platelets from a rat fed chow diet. (B) Platelets from a rat fed a corn oil/fish oil diet. Experimental conditions are described in Methods.

levels of 22-carbon (n-6) acids which was accompanied by an increase in 22-carbon (n-3) acids.

The reverse phase HPLC chromatograms in Figure 1 show that three major compounds were produced when platelets from both groups of animals were stimulated with ionophore A23187. In addition, several minor compounds were detected which we were unable to characterize. The amounts of these compounds were relatively independent of the dietary history of the rats and collectively they represented less than 3% of the integrated area as monitored at 235 nm.

The mass spectra of compounds 1, 2, and 3 were identical from both groups of rats. The mass spectrum of compound 1 (ECL = 19.2) had ions at m/z 366 (M^+; 10.7%), 351 (M − 15; 0.8%), 335 (M − 31; 2.8%), 295 (M − 71, loss of $^{\bullet}(CH_2)_4CH_3$; 34.2%), 276 (M − 90; 1.1%), 225 (M − 141, loss of $^{\bullet}CH_2CH{=}CH(CH_2)_3COOCH_3$; 65.7%) and 173 (10.9%). These results are similar to those reported for 12-HHT (2).

The mass spectrum of compound 2 (ECL = 21.4) had ions at m/z 295 (M − 109, loss of $C_2H_5(CH{=}CH\text{-}CH_2)^{\bullet}_2$; base peak), 205 (295 − 90; 10.5%) and 173 [probably a mix of 295 − (32 + 90) and M− (141 + 90)]. These are the characteristic ions reported by Hamberg (4) for 12-HEPE.

The mass spectrum of the methyl ester-trimethylsilyl

ether of compound 3 (ECL = 21.3) had ions at m/z 391 (M − 15; 1.3%), 375 (M − 31; 1.2%), 295 (M − 111, loss of $^{\bullet}CH_2CH{=}CH(CH_2)_4CH_3$; 70.5%), 229 (7.1%), 205 (295 − 90; 7%) and 173 (9.7%). Thus, this compound is 12-HETE.

Table 3 shows the nmol of 12-HHT, 12-HETE, and 12-HEPE produced from 1×10^9 cells, respectively, from the two groups of rats. Dietary fish oil depressed the synthesis of 12-HHT and 12-HETE by about 40% as compared with the chow fed controls. There was a concomitant fivefold increase in the synthesis of 12-HEPE. In theory, any unsaturated fatty acid has the potential of being released from membrane phospholipids for subsequent metabolism by platelet lipoxygenase(s) or cyclooxygenase. Table 4 compares the nmol of (n-6) and (n-3) fatty acids in 1×10^9 platelets from the two dietary groups. These values were calculated from the compositional data in Tables 1 and 2. When the results in Tables 3 and 4 are compared it can be calculated that 60% of the arachidonate in the chow fed rats was released and metabolized into 12-HHT and 12-HETE. When fish oil was included in the diet about 45% of the 20:5(n-3) was released and metabolized into 12-HEPE. When rats were fed fish oil there was an approximately twofold increase in the levels of both 22:5(n-3) and 22:6(n-3) in total platelet phospholipids. These two acids were both located primarily in PE. If these two acids were released and metabolized to the same extent as was arachidonate and 20:5(n-3), it can be calculated that 3-6 nmol of the hydroxy acids should have been produced by the platelets from the animals fed fish oil. The methodology used in these studies is sufficiently sensitive to detect metabolites made in these amounts.

TABLE 3

Effect of Diet on the Distribution of Metabolites Released from Ionophore Stimulated Platelets

Compound	Chow diet	Corn oil/Fish oil
	nmol/10^9 cells	
12-HHT	6.9 ± 1.3	4.2 ± 1.0
12-HEPE	3.5 ± 1.4	18.3 ± 1.9
12-HETE	64.4 ± 18.9	26.8 ± 0.7

Platelets were isolated from rats fed chow or corn oil/fish oil diet. Cells were challenged with ionophore A23187 for 30 min. Values are given as mean ± S.E.M.

TABLE 4

Effect of Diet on the Fatty Acid Composition of Individual Phospholipids

Fatty acid	PC	PE	PI	PS	Total
	nmol/1 × 10^9 platelets				
Chow diet					
20:4(n-6)	22.2	67.4	8.8	19.0	117
20:5(n-3)	2.4	2.1	—	0.5	5
22:5(n-3)	1.1	2.9	—	0.7	5
22:6(n-3)	1.4	1.4	—	0.3	3
Corn oil/Fish oil diet					
20:4(n-6)	13.6	52.9	7.1	23.0	97
20:5(n-3)	10.6	26.3	2.1	2.6	41
22:5(n-3)	1.5	10.6	0.4	2.9	15
22:6(n-3)	2.6	4.0	—	1.3	8

DISCUSSION

The fatty acid composition of individual platelet phospholipids from chow fed rats are in generally good agreement with previous findings (24). However, the level of palmitic acid in PS was higher than previously reported (24,25). The studies reported here show that modest changes in the composition of dietary fat alters the types of fatty acids found in platelet phospholipids. The semisynthetic diet contained 2.5% of each corn oil and fish oil but only about 30% of the fatty acids in fish oil were (n-3) acids. Adequate linoleate is therefore available for metabolism to arachidonate for subsequent acylation into phospholipids. When fish oil was included in the diet there was a reduction in arachidonate levels in all phospholipids which was accompanied by the acylation of 20:5(n-3). The ratio of 20:5(n-3)/20:4(n-6) in phospholipids has been used as an index of the availability of precursors for proaggregatory compounds (5,6). These ratios in PC, PE, PS, and PI in the fish oil fed rats were 0.78, 0.49, 0.11 and 0.30, respectively. These findings show that 20:5(n-3) does, in part, replace arachidonate in all phospholipids but that different specificities exist for incorporating the two fatty acids into phospholipids. Ishinaga *et al.* (25) and Nordoy and coworkers (26) also have reported that dietary fish oil supplements modify the fatty acid composition of individual phospholipids in different ways. When 20:5(n-3) is incubated with washed human platelets it is readily incorporated into PI (17). Conversely, when fish oils are fed to humans, only negligible amounts of 20:5(n-3) are found in this lipid (27,28). When ethyl linolenate (17) or fish oils were fed to weanling rats the PI contains significant amounts of 20:5(n-3). These findings show that *ex vivo* studies do not correlate with *in vivo* metabolism. Moreover, species differences may exist in defining the pathways for the synthesis of PI.

PI has long been recognized as an important source of arachidonate for subsequent metabolism by lipoxygenase and cyclooxygenase. Other studies show that both phospholipase C and A_2 contribute to the release of arachidonic acid from platelet phospholipids (29-33). Mahadevappa and Holub (34) recently reported that 46, 17, <5, and 33% of the thrombin induced release of arachidonate from human platelets came from PC, PE, PS, and PI, respectively. Conversely, over 95% of the 20:5(n-3) that was released came from PC and PE. These findings clearly show that the 20:5(n-3)/20:4(n-6) ratio by itself cannot be used as a sole criteria for defining the mass amounts of fatty acids that are released. In the studies reported here the PI and PE contained 5 and 64%, respectively, of the 20:5(n-3) that was potentially available for release. The types and amounts of fatty acids available for release are thus defined not only by molar phospholipid composition but the fatty acid content of individual phospholipids.

In these studies we used ionophore A23187 as an agonist for platelet activation. This unphysiological agent was used to maximize fatty acid release and the subsequent metabolism. Under these conditions, about 60% of the total platelet lipid arachidonate in chow fed rats was

metabolized into 12-HHT and 12-HETE. No attempt was made to quantitate thromboxane levels so this value is probably underestimated. In the fish oil supplemented rats about 32 and 42%, respectively, of the platelet arachidonate and 20:5(n-3) were metabolized. We were unable to detect the presence of any 12-hydroxy-5,8,10,13-nonadecatetraenoic acid, suggesting that 20:5(n-3) was only metabolized via the lipoxygenase pathway. It can be calculated from Tables 3 and 4 that some of the arachidonate and 20:5(n-3) must have come from PE. Dietary fish oil resulted primarily in an increase in the levels of 22:5(n-3) and 22:6(n-3) in this lipid. Previous studies have shown that platelets incorporate radioactive 22:6(n-3) into their phospholipids (17,18,34). This radioactive fatty acid was not, however, released when platelets wre activated (18,35). Under our experimental conditions, where we quantitate mass rather than radioactivity, it was not possible to detect oxygenated metabolites of either 22:5(n-3) or 22:6(n-3). Platelets from the fish oil supplemented animals contain 41, 15, and 8 nmol/10^9 platelets, respectively, of 20:5(n-3), 22:5(n-3), and 22:6(n-3). The amounts of 22-carbon (n-3) acids in platelet phospholipids are thus high enough that we should have been able to detect oxygenated metabolites if they were released and metabolized to the same extent as was 20:5(n-3). It is possible, however, that 22:5(n-3) and 22:6(n-3) were released but not metabolized. This is unlikely in view of our previous studies showing that exogenous 20:4(n-6) actually enhanced the metabolism of 22:5(n-3) (13).

It is possible that 22:6(n-3) might be released from phospholipids and metabolized if higher levels of fish oil had been fed to further increase the amount of this acid in the phospholipids. It is not clear whether the lipoxygenase products that are made from 22:6(n-3) modulate platelet aggregation. Rao *et al.* (36) reported that the addition of hydroperoxy acids made from 22:6(n-3) did not alter the conversion of exogenous arachidonic to thromboxane A_2. When 22:6(n-3) and arachidonate were added to platelets along with a lipoxygenase inhibitor these investigators reported that platelet aggregation was depressed. They concluded that 22:6(n-3) may inhibit platelet aggregation via a pathway that is independent of 22:6(n-3) lipoxygenation. This is indeed likely since 22:6(n-3) inhibits cyclo-oxygenase (15). Recently Croset *et al.* (37) reported that 14-hydroxy-4,7,10,12,16,19-docosahexaenoic acid inhibits platelet aggregation as induced by a stable endoperoxide analog. Our results suggest that neither 22:5(n-3) nor 22:6(n-3) is released from platelet phospholipids. It remains to be established whether these long chain (n-3) fatty acids alter platelet function by physical parameters such as viscosity or membrane fluidity (38).

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Cholelithiasis in Hamsters: Effects of Cholic Acid and Calcium on Gallstone Formation

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Dietary cholic acid (0.1%) and/or calcium (2.6% as calcium carbonate) were added to a semipurified diet containing cholesterol and ethynyl estradiol to determine whether the incidence of pigment and/or cholesterol gallstones would be changed. Male golden Syrian hamsters were fed the experimental diets for 96 days (Group 1, control; Group 3, cholic acid plus calcium) or only an average of 60 days (Group 2, 0.1% cholic acid). Animals in Group 2 became ill (weight loss, low food intake, diarrhea) possibly due to cholic acid (or deoxycholic acid) toxicity. Cholesterol gallstones and crystals were absent in all experimental groups. The incidence of pigment gallstones was: control, Group 1, 12/16; 0.1% cholic acid, Group 2, 3/13; and 0.1% cholic acid plus calcium, Group 3, 11/22. Cholic acid with or without calcium produced an elevation of both liver and plasma cholesterol: Group 2, 80.1 mg/g and 501 mg/dl; Group 3, 103.7 mg/g and 475 mg/dl vs Group 1, 65 mg/g and 209 mg/dl, respectively. The lithogenic indices of the bile were lower in Groups 2 and 3 compared to Group 1, controls, 0.45 and 0.58 vs 1.16, respectively. The extent of the portal tract pathology could not be correlated with the presence or absence of pigment gallstones or with the levels of lithocholic acid in the hamster bile. In summary, when semipurified diets were supplemented with ethynyl estradiol and cholic acid, with and without calcium supplementation, no cholesterol gallstones formed and the incidence of pigment gallstones was not altered.
Lipids 24, 482-487 (1989).

Animal models are important adjuncts to studies using human subjects. There exist several animal models to study either cholesterol and/or pigment cholelithiasis (1-5). With the exception of the work by Dam and Christensen who employed a nutritionally inadequate basal diet (1), it has not been possible to produce both cholesterol and pigment gallstones in the same model. Dam and co-workers showed that dietary changes can alter gallstone composition and morphology, but the mechanistic details of these changes remain unknown. We recently described a new model of pigment cholelithiasis in the hamster (6). This model used a nutritionally adequate diet supplemented with cholesterol (0.3%). The addition of cholesterol was necessary for the formation of pigment stones, but its role was not completely understood. Our model was based upon a hamster model of cholesterol cholelithiasis described by Pearlman *et al.* (2) except that a semipurified diet was substituted for rat chow, and the amount of ethynyl estradiol was increased from 0.000015 to 0.00015 g/100 g of food. Whereas the chow-based diet used by Pearlman *et al.* produced cholesterol gallstones, our modified semipurified diet led to the exclusive formation of pigment stones. The animals with pigment gallstones had increased levels of cholesterol in the bile and increased biliary total calcium concentration.

We recently reexamined the hamster model of pigment cholelithiasis. We added cholic acid to the semipurified experimental diet (6) used to produce pigment cholelithiasis with the expectation that this bile acid might increase intestinal cholesterol absorption, thus increasing biliary cholesterol and altering stone composition to form cholesterol stones rather than pigment stones. Further, we studied the effect of supplementing the cholic acid diet (6) with dietary calcium (calcium carbonate) hypothesizing that increased dietary calcium might alter biliary calcium levels and lead to the precipitation of calcium salts (7); these salts could then act as nucleating agents for either pigment and/or cholesterol gallstone formation.

MATERIALS AND METHODS

Cholesterol was obtained from Sigma Chemical Co. (St. Louis, MO). Ethynyl estradiol (Sigma) was found to be greater than 99% pure by gas-liquid chromatography (GLC) as the trimethylsilyl ether derivative. Cholic acid (Inolex, Park Forest South, IL) was found to be greater than 99% pure when analyzed by GLC.

Male golden Syrian hamsters (50 grams) were purchased from Harlan Sprague-Dawley, Indianapolis, IN. All animals were housed two per cage and were quarantined for 1 wk while being fed a chow diet and water ad libitum. The animals were then divided into three diet groups. Sixteen animals were fed a semipurified diet containing 0.3% cholesterol (defined as the lithogenic diet) plus ethynyl estradiol (15 μg/g of food, Group 1). This diet contained (g/kg): casein 200; dextrose 671; corn oil 50; cholesterol 3.0; cellulose 26; mineral mix 40; vitamin mix 10; and ethynyl estradiol 0.015, which was similar to the diet described earlier (6). The lithogenic diet was supplemented with 0.1% cholic acid (Group 2, 28 animals), and with 2.6% calcium carbonate (Group 3, 22 animals). All animals were maintained on an alternating 12-hr light and 12-hr dark cycle. The diets were prepared in pelleted form by Teklad (Madison, WI). Animals in Groups 1 and 3 gained weight and appeared healthy during the entire experimental period (96 days) and were then sacrificed by exsanguination under anesthesia with 10 mg of ketamine hydrochloride (Bristol Labs, Syracuse, NY) and ether.

The presence of gallstones and crystals was determined by microscopy as described previously (6). Blood, liver and bile were obtained to measure cholesterol levels, and biliary bile acid composition was determined according to the method described earlier (6,8). Total calcium in serum was measured colorimetrically using a Sigma Diagnostics kit no. 578 (Sigma Chemical Co., St. Louis, MO). All animals in Group 2 (0.1% cholic acid) failed to gain weight and almost 50% died (15 animals) during the

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Abbreviation: GLC, gas-liquid chromatograph(y).

experimental period. The surviving animals were sacrificed on different days (day 56, 68 and 75) by the procedure described above.

Thin slices of liver were taken from representative hamsters of each group (7 from Group 1, 5 of which had pigment gallstones; 5 from Group 2, 2 of which had gallstones; and 12 from Group 3, 8 of which had gallstones). The liver slices were fixed in Millonig's buffered formalin, embedded in paraffin, sectioned at 5μ, and stained with hematoxylin and eosin (6). Histopathologic changes were graded from 0 (no significant change) to 4 (severe alteration). The pathologist (R.J.S.) evaluated the microscopic sections without prior knowledge as to the dietary categories of the hamsters sampled.

Statistical differences among the groups were calculated using one-way analysis of variance followed by Dunnett's test for multiple comparisons. The significance of differences in gallstone incidence was determined using chi-square analysis (9,10).

RESULTS

Male Syrian hamsters (Groups 1 and 3) were fed the experimental diets for 96 days. Animals in Group 2 were fed the diet for an average of only 60 days, due to the unexpected toxicity of the cholic acid regimen. The average body weights for all animals were similar at the start of the experiment (89.8 to 93.5 g) (Table 1). Animals in Groups 1 and 3 gained similar amounts in weight, averaging 15–20 g. However, as described above, animals in Group 2 did not gain weight and 15 out of 28 animals died before the end of the experiment. The remaining animals became ill, as manifested by severe diarrhea and vomiting.

Gallstone incidence was determined at sacrifice. No animals developed cholesterol gallstones or biliary cholesterol crystals (Table 1). However, pigment gallstones were detected in Groups 1 and 3 (12/16 and 11/22 animals, respectively). These stones were small (less than 1 mm), dark, amorphous and showed no polarization by light microscopy. A lower incidence of pigment stones was observed in Group 2 (3/13) (Table 1).

Cholesterol levels were measured in liver, serum and bile (Table 2). Both liver and serum cholesterol levels were elevated in Groups 2 and 3 vs Group 1 (80.1 and 103.7 vs 65.2 mg/g; 501 and 475 vs 209 mg/dl, respectively). Animals in Group 2 had lower values for biliary cholesterol compared to Groups 1 and 3 (0.95 vs 1.45 and 1.20 mg/ml, respectively). Serum calcium levels were similar in all three experimental groups (0.103 ± 0.01 mg/ml, Group 1; 0.094 ± 0.01 mg/ml, Group 2; 0.095 ± 0.01 mg/ml, Group 3).

Biliary lipids were measured to determine the lithogenic index of the bile (Table 3). The total lipid concentration was similar for all groups. Mole percent of phospholipid was significantly elevated in Groups 2 and 3 compared

TABLE 1

Effect of Different Diets on Body Weight and Gallstone Formation in Hamsters[a]

Group	No. of animals	Diet	Initial body weight (g)	Final body weight (g)	Cholesterol stone/crystal		Pigment gallstones
1	16	Lithogenic diet + 15 ppm ethynyl estradiol	93.5 ± 2.0	109.4 ± 5.0	0/16	0/16	12/16
2	13	Lithogenic diet + 15 ppm ethynyl estradiol + 0.1% cholic acid[b]	89.8 ± 1.6	89.3 ± 4.3[c]	0/13	0/13	3/13[d]
3	22	Lithogenic diet + 15 ppm ethynyl estradiol + 0.1% cholic acid + 2.6% calcium carbonate	93.1 ± 1.6	107.5 ± 3.5	0/22	0/22	11/22

[a]Animals were treated using experimental conditions described under Materials and Methods. Numbers are average ± SEM; ppm, parts per million.

[b]Feeding for Group 2: 60 days.

[c]Different from Group 1, $p < 0.01$ by t-test.

[d]Different from Group 1, $p < 0.01$ by chi-square test.

TABLE 2

Effect of Different Diets on Tissue Cholesterol Levels in Hamsters[a]

Group	No. of animals	Diet	Liver cholesterol mg/g	Serum cholesterol mg/dl	Biliary cholesterol mg/ml
1	16	Lithogenic diet + 15 ppm ethynyl estradiol	65.2 ± 6.1	209 ± 16	1.45 ± 0.18
2	13	Lithogenic diet + 15 ppm ethynyl estradiol + 0.1% cholic acid	80.1 ± 5.4	501 ± 63[b]	0.95 ± 0.17[c]
3	22	Lithogenic diet + 15 ppm ethynyl estradiol + 0.1% cholic acid + 2.6% calcium carbonate	103.7 ± 6.6[b]	475 ± 40[b]	1.20 ± 0.24

[a]Animals were treated using experimental conditions described under Materials and Methods. Numbers are average ± SEM; ppm, parts per million.

[b]Different from Group 1, $p < 0.01$.

[c]Different from Group 1, $p < 0.05$.

to Group 1, but phospholipid levels in Group 3 were lower than in Group 2. Lithogenic indices were below 1.0 in Groups 2 and 3; the lithogenic index in Group 1 was above 1.0, but no cholesterol stones or crystals were detected in the gallbladder bile. In Group 3, animals with pigment stones (n = 11) had a statistically higher lithogenic index (LI = 0.68 ± 0.08) compared to animals in the same group with stones (LI = 0.47 ± 0.03) ($p < 0.02$).

Biliary bile acids were analyzed by GLC (Table 4). With the administration of cholic acid in Groups 2 and 3, the proportion of this bile acid in bile increased from 34.6% (Group 1) to 57.6 and 55.7% (Groups 2 and 3) with a concomitant increase in deoxycholic acid (Table 4). Lithocholic acid levels decreased in Groups 2 and 3 vs Group 1 (0.8 and 1.3 vs 7.3, respectively).

Table 5 provides a semiquantitative assessment of pathologic changes observed in the hamster livers sampled for light microscopy. All of the livers revealed substantial accumulations of a microvesicular lipid (Fig. 1). In four out of seven from Group 1 and in eight out of twelve from Group 3, the parenchymal steatosis was accompanied by the formation of periportal fatty cysts (Fig. 1). The livers of Group 2 animals did not contain fatty cysts. The portal tracts of the hamster livers from all dietary groups generally displayed mild to moderate bile duct proliferation, inflammatory infiltration, and fibrosis, but a few animals in dietary Groups 1 and 3 showed more advanced portal tract alterations (Fig. 2).

DISCUSSION

This laboratory has been concerned with the etiology of gallstone disease in animal models and the evaluation of naturally occurring bile acids as well as bile acid analogs in gallstone prevention and/or dissolution. To accomplish our objective, we felt that a single-animal model which consumes a small amount of food (about 10 g/day) would allow us to evaluate synthetic bile acid analogs available in limited quantities and compare them to the bile acids widely used for gallstone dissolution (11,12). Several studies have been reported on cholelithiasis in hamsters, but these experiments contained several anomalies (1,2). For example, Dam and Christensen reported that cholesterol gallstones could be produced in hamsters; however, the diets used were synthetic fat-free regimens containing large amounts of glucose (1). Since this diet was nutritionally deficient in essential fatty acids, many animals became ill and died prematurely. In addition, chenodeoxycholic acid failed to prevent the formation of gallstones in this model (13). Pigment stones were formed in the Dam hamsters by using supplements of soybean oil or cod liver oil, but the incidence was somewhat variable (1).

TABLE 3

Effect of Diet on Biliary Lipid Levels in Hamsters[a]

Group	Diet	Biliary lipids			Total lipid g/dl	Lithogenic index
		Bile acid mol %	Phospholipid mol %	Cholesterol mol %		
1	Lithogenic diet + 15 ppm ethynyl estradiol	95.5 ± 0.6	2.1 ± 0.6	2.4 ± 0.1	7.72 ± 0.84	1.16 ± 0.08
2	Lithogenic diet + 15 ppm ethynyl estradiol + 0.1% cholic acid	84.6 ± 2.4	13.4 ± 2.3[b]	2.0 ± 0.4	6.41 ± 1.4	0.45 ± 0.07[b]
3	Lithogenic diet + 15 ppm ethynyl estradiol + 0.1% cholic acid + 2.6% calcium carbonate	88.7 ± 0.9[b]	9.2 ± 0.7[c]	2.1 ± 0.2	6.96 ± 0.63	0.58 ± 0.05[b]

[a]Numbers are average ± SEM; ppm, parts per million.

[b]Differs significantly from Group 1, $p < 0.01$.

[c]Differs significantly from Group 2, $p < 0.05$.

TABLE 4

Effect of Different Diets on Biliary Bile Acids in Hamsters[a]

Group	Diet	Weight percent				
		Lithocholic acid	Deoxycholic acid	Chenodeoxycholic acid	Cholic acid	Others
1	Lithogenic diet + 15 ppm ethynyl estradiol	7.3 ± 0.8	13.4 ± 1.9	41.6 ± 3.6	34.6 ± 2.4	3.1 ± 0.5
2	Lithogenic diet + 15 ppm ethynyl estradiol + 0.1% cholic acid	0.8 ± 0.3[b]	28.1 ± 4.1[b]	10.1 ± 1.6[b]	57.6 ± 3.2[b]	3.4 ± 0.9
3	Lithogenic diet + 15 ppm ethynyl estradiol + 0.1% cholic acid + 2.6% calcium carbonate	1.3 ± 0.2[b]	25.6 ± 2.2[b]	14.6 ± 1.7[b]	55.7 ± 1.6[b]	2.8 ± 0.5

[a]Animals were treated using experimental conditions described under Materials and Methods.

[b]Differs from Group 1, $p < 0.01$.

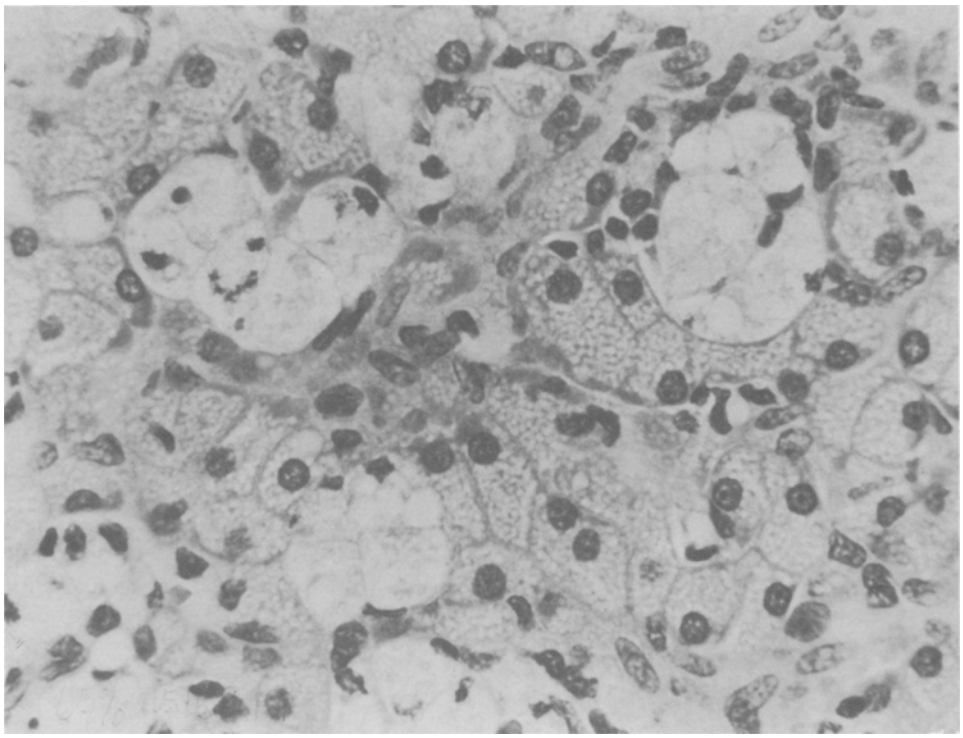

FIG. 1. Liver from a hamster fed the lithogenic diet + 15 ppm ethynyl estradiol (Group 1). The field illustrates periportal hepatocytes with a granular or bubbly appearance, reflecting marked microvesicular steatosis (grade 4). Also included are several fatty cysts, apparently the result of a confluence of contiguous, degenerating, lipid-laden hepatocytes. Nuclear fragments and pyknotic nuclei are evident in the cyst at the upper left. The cyst at the upper right has attracted an inflammatory response that includes multiple histiocytes. (H&E stain, ×420)

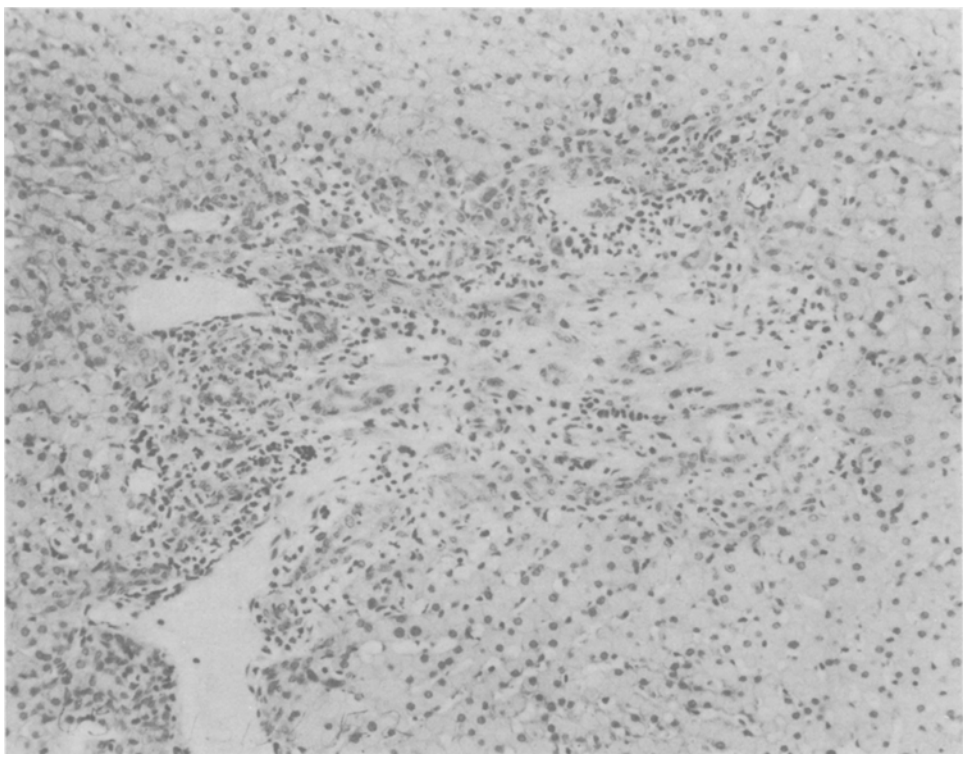

FIG. 2. Liver from a hamster fed the lithogenic diet + 15 ppm ethynyl estradiol (group 1). Across the center of the field is an enlarged portal tract displaying prominent bile duct proliferation, inflammatory infiltration, reactive fibrosis (grade 3). The faint granularity of the periportal hepatocytes is due to microvesicular steatosis. (H&E stain, ×105)

TABLE 5

Semiquantitative Assessment of the Pathologic Features of the Hamster Livers in Relation to Diet

Group	Diet	Parenchymal lipid					Portal inflammation and fibrosis					Bile duct proliferation				
		0	1	2	3	4	0	1	2	3	4	0	1	2	3	4
1	Lithogenic diet + 15 ppm ethynyl estradiol	—	—	—	2	5	2	3	—	2	—	—	3	2	2	—
2	Lithogenic diet + 15 ppm ethynyl estradiol + 0.1% cholic acid	—	—	—	2	3	—	1	4	—	—	—	1	4	—	—
3	Lithogenic diet + 15 ppm ethynyl estradiol + 0.1% cholic acid + 2.6% calcium carbonate	—	—	—	4	8	—	2	9	—	1	—	2	8	1	1

ppm, parts per million.

Another hamster model, described by Pearlman *et al.*, employed a chow diet supplemented with 0.24% cholesterol and ethynyl estradiol (15 μg/kg body weight/day) and produced a high incidence of cholesterol gallstones (2). This model is no longer reproducible presumably due to some apparent genetic variability of the hamsters (14).

We fed hamsters a semipurified diet containing both cholesterol (0.3%) and ethynyl estradiol (6). Our experiments employed a synthetic diet rather than rodent chow. By using a defined diet, we anticipated that any variability in dietary constituents (fat, carbohydrates, protein and fiber) which might alter the reproducibility of our model would be eliminated. In the present study, this diet was supplemented with cholic acid and further with calcium (as calcium carbonate).

The first hypothesis tested was whether addition of cholic acid altered the course or type of cholelithiasis. Hamsters which were fed the semisynthetic diet with ethynyl estradiol had a 75% incidence of pigment gallstones. This incidence was slightly higher than that obtained in an earlier study which also showed that an identical semisynthetic diet without cholesterol produced neither pigment nor cholesterol gallstones (6). The addition of cholic acid to the lithogenic diet failed to produce the desired response, namely a model of cholesterol cholelithiasis. Unexpectedly, this diet (Group 2) could not be fully evaluated in this experiment: Animals in this group (after about one month into the experiment) showed symptoms of gastrointestinal distress, such as diarrhea and vomiting. The surviving animals, all of which were sacrificed by day 75, had no cholesterol gallstones, even though liver and plasma cholesterol levels (but not biliary cholesterol) were elevated compared with controls (Group 1). Cholic acid is known to increase cholesterol absorption in rats (8,15) and in man (16). In an earlier study in hamsters, dietary cholic acid (0.1%) without added cholesterol increased serum and liver cholesterol concentrations significantly, although increased cholesterol absorption could not be detected during a 12-day experiment (17). In this earlier study, we felt that the chow diet, because of its fiber content, may have reduced the absorption of cholesterol in the presence of cholic acid (17). Consequently, we changed to a defined semipurified diet (low in fiber) rather than rodent chow.

Studies of hamsters which were fed essential fatty-acid deficient diets (EFAD) (the Dam diet [1]) showed increased levels of cholesterol secreted into bile with a concomitant increase in gallstone formation. In some cases, the level of biliary cholesterol increased two to three times (18). Presumably, the dietary insufficiency caused a selective enhancement in cholesterol secretion without a generalized increase in body cholesterol (19). The diets used in the present studies were nutritionally adequate, with the expectation of avoiding the weight loss and early death observed in studies using the Dam diet. We failed to observe an increase in biliary cholesterol (mol %) despite a marked increase in serum and liver cholesterol (Group 3 vs Group 1). This increase was attributed to increased cholesterol absorption. The failure to observe an increase in cholesterol secretion when phospholipid secretion was increased could be attributed to a change in the coupling between bile acid/cholesterol and/or phospholipid/bile acid secretion (20).

The distress observed in animals fed cholic acid was presumably due to the increased formation of deoxycholic acid. We observed that this bile acid, when administered intravenously to hamsters at a dose of 4 μmol/min/kg body weight, produced hemolysis, hemoglobinuria and finally death (21). However, intraduodenal administration of deoxycholic acid at the same dose produced little hemolysis (21). Cassidy and Tidball reported that intrarectal administration of sodium deoxycholate produced epithelial damage (22). The toxicity by deoxycholic acid may have appeared in our experiment because of the relatively prolonged administration of cholic acid; in earlier studies in which chow plus 0.1% cholic acid was fed for 2 wk the hamsters exhibited no toxicity (17). We suggest that long-term cholic acid feeding in hamsters produces increased levels of deoxycholic acid (28.1% of biliary bile acids) which apparently cause an undetermined kind of irreversible toxicity and finally death.

The second hypothesis of this study was that increased levels of dietary calcium altered the course of hamster cholelithiasis. One interesting finding of this experiment was that dietary calcium protected the hamsters from "cholic-acid toxicity," perhaps by binding calcium to the bile acid micelle or possibly by converting the bile acids to insoluble calcium salts (23,24). No animals in Group 3 became ill or died prematurely. Protective effects of calcium in rats given bile acids have been postulated (23,24). However, the addition of dietary calcium in our study failed to increase the incidence of pigment stones or produce cholesterol gallstones. The incidence of pigment stones in Group 3 was 50%; these stones were

similar in size and appearance to those formed in Group 1. Interestingly, the lithogenic indices of animals in Group 1 were above unity whereas those in Group 3 were below 1.0; nevertheless, we obtained only pigment gallstones in both cases. The pigment stones were found to contain 26.7% calcium phosphate, 12.8% calcium bilirubinate, 15.1% cholesterol, and 45.4% protein (6). It was suggested that the nature of cholesterol in bile (vesicles, liquid crystals) might play a role in the initial nucleation of calcium salts (6). Further, calcium precipitation in bile is a requisite for all pigment stones (7). The interaction between calcium, cholesterol and protein in supersaturated bile may determine, in a yet unexplained manner, the pathogenesis of gallstones. As stated above, the role of cholesterol in pigment gallstone formation in our model is uncertain; animals with gallstones in Group 3 have a significantly higher lithogenic index of bile than animals in the same group without stones. Studies using different concentrations of calcium as well as higher amounts of cholesterol might elucidate how cholesterol affects pigment cholelithiasis.

Liver histology was examined to find out whether the dietary regimens affected this organ in a deleterious manner. The mild to moderate portal tract alterations in the livers of most animals in all dietary groups and the occasional appearance of more severe portal tract changes in hamsters of Groups 1 and 3 also parallel our previous observations (6). The absence of severe portal tract alterations in hamsters of Group 2 probably can be explained by the shorter duration of treatment. In the previous study (6), supplementation of the diet with ethynyl estradiol appeared to ameliorate both the portal tract changes and the appearance of fatty cysts, but this was not evident in the present investigation. Finally, in the present study, the extent of the portal tract pathology could not be correlated with the presence or absence of gallstones or with the levels of lithocholic acid in the bile.

In summary, this study reports the reproducibility of the previously reported gallstone model of pigment cholelithiasis. Dietary cholic acid was unsuccessful in altering the course of this disease in hamsters, presumably due to its toxic effects. Dietary calcium protected hamsters from cholic acid toxicity but had no effect on the etiology of gallstone disease.

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Application of a GC-MS Method Using Deuterated Fatty Acids for Tracing *cis*-Vaccenic Acid Biosynthesis in Kaki Pulp

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A gas chromatographic-mass spectrometric method using [2,2-$^2H_2$]fatty acids has been developed to trace the biosynthesis of *cis*-vaccenic (*cis*-11-octadecenoic) acid in higher plants. The deuterated fatty acids and other unlabeled fatty acids in the biosynthetic reaction mixture were converted into bis(methylthio) derivatives and analyzed by mass chromatography. The principle of this method was based on the shift of key fragment ions (containing two deuterium atoms) due to the cleavage between the methylthio-substituted carbons. The labeled compounds were detected by the m/z values which shifted 2 mass units from those of the corresponding unlabeled compounds and estimated by a calibration curve based on the peak areas of the key fragment ions. For metabolic experiments, a homogenate fraction was prepared from the pulp part of maturing kaki (*Diospyros kaki*) fruit and incubated with ammonium [2,2-$^2H_2$]palmitoleate (*cis*-9-hexadecenoate) or [2,2-$^2H_2$]palmitoleoyl-CoA. The incubation resulted in the formation of detectable amounts of isotopically-labeled *cis*-vaccenic acid containing two deuterium atoms at the carbon chain between the double bond and the carboxyl group. This experimental evidence proved that *cis*-vaccenic acid was formed from palmitoleic acid by chain elongation.

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In the field of higher plant biochemistry, *cis*-vaccenic (*cis*-11-octadecenoic) acid has been found in milkweed (*Asclepias syriaca*) seeds, which was first reported by Chisholm and Hopkins (1). These investigators also postulated in a second paper on the same plant (2) that in the seed, palmitoleic (*cis*-9-hexadecenoic) acid was converted to *cis*-vaccenic acid. However, little attention has been directed to *cis*-vaccenic acid metabolism in higher plants, probably due to its low concentration in common vegetable oils. Some researchers regarded the route of *cis*-vaccenic acid biosynthesis as a chain elongation from palmitoleic acid based on radioisotope experiments of fatty acid metabolism in seedlings of barley (3,4), pericarp of ivy fruits (5), mitochondria of some vegetables (6) and cotyledon of *Sinapis alba* seeds (7). However, palmitoleic acid, which was thought of as an immediate precursor for *cis*-vaccenic acid biosynthesis by some researchers, was not used as the labeled substrate in these experiments (3-7). Moreover, the anaerobic pathway of *cis*-vaccenic acid biosynthesis in seedlings of barley presented previously (3,4) was not supported in recent reviews (8, 9). The biosynthetic pathway of *cis*-vaccenic acid has not yet been confirmed in higher plants (8-10).

In our ongoing study on *cis*-vaccenic acid in higher plants, we have found this acid to always occur with the varying contents in all the samples analyzed (11-13) and to be rich in pulp lipids of mango (14) and 19 other varieties of commonly available fruits (15). Among these fruits, the pulp lipids of kaki (*Diospyros kaki*, Ebenaceae family) fruits were the richest source of *cis*-vaccenic acid (95.1% of octadecenoic acids and 29.0% of total fatty acids) (15). Our further examinations on kaki fruits showed that the fruits from several cultivars of *Diospyros kaki* always contained *cis*-vaccenic acid as a major component (up to 90%) of octadecenoic acid isomers in their pulp lipids (Shibahara, A., et al., manuscript in preparation). These findings prompted us to use the kaki pulp as a convenient source of the enzyme system responsible for *cis*-vaccenic acid biosynthesis.

The method for analyzing fatty acids in biosynthetic experiments is based on our newly-developed procedure. In this paper, we report the newly-developed gas chromatographic-mass spectrometric method using [2,2-$^2H_2$]fatty acid tracers (16-19) and their DDA derivatives (12, 13) suitable for determining the labeled fatty acid isomers biosynthesized, and describe its application for tracing the pathway of *cis*-vaccenic acid biosynthesis with kaki pulp homogenate.

MATERIALS AND METHODS

Chemicals. Palmitoleic and *cis*-vaccenic acids were obtained from P.L. Biochemicals Inc. (Milwaukee, USA); oleic acid from Research Laboratory of Nippon Oil and Fats Co. (Amagasaki, Japan); palmitic acid (purity >99.0%) from Nakarai Chemicals Ltd. (Kyoto, Japan); and pentadecanoic acid (purity >98.0%) from Tokyo Kasei Kogyo Co. (Tokyo, Japan). The purity of monoenoic acids used was more than 99.5% by capillary gas chromatographic analysis of their methyl esters and mass chromatographic analysis as their DDA derivatives (13). CoA and NADPH were purchased from Oriental Yeast Co. (Tokyo, Japan); malonyl-CoA from Sigma Chemical Co. (St. Louis, USA); and deuterium oxide (purity 99.75%) from E. Merck (Darmstadt, Federal Republic of Germany). All other chemicals were analytical reagent grade, and all solvents were distilled in glassware before use. The [2,2-$^2H_2$]fatty acids were synthesized according to the methods given in the references (16, 17) with slight modifications. The [2,2-$^2H_2$]acyl-CoA was prepared by the acyl chloride method of Okuyama et al. (20) and its concentration was determined spectrophotometrically (21). Ammonium malonate and [2,2-$^2H_2$]palmitoleate were obtained from the corresponding acids by

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Abbreviations: DDA, dimethyl disulfide adduct; GC, gas chromatography; GC-MS, gas chromatography-mass spectrometry.

neutralization with methanolic ammonium hydroxide.

Preparation of standard mixtures for determining isotope content. Mass chromatography of the chemically-synthesized methyl [2,2-$^2H_2$]*cis*-vaccenate showed its isotope purity as 98.0% based on the peak areas of molecular ions at m/z 298 (labeled) and m/z 296 (unlabeled). The labeled methyl *cis*-vaccenate was dissolved in a definite volume of hexane, and its relative concentration to an internal standard (methyl palmitate) was determined by packed-column gas chromatography (GC). In the same manner, the relative concentration of unlabeled methyl *cis*-vaccenate in hexane was also determined. Known volumes of both of the ester solutions were mixed and subjected to the methylthiolation under the same conditions previously described (14). The resulting standard mixtures containing known amounts of DDAs of labeled and unlabeled methyl *cis*-vaccenates were analyzed by gas chromatography-mass spectrometry (GC-MS) to determine a calibration curve for estimating isotope content.

Homogenate preparation. Kaki fruits (ca. 150 g/fruit) grown in the experimental farm of Kyoto Prefectural University were harvested in October 1987 (just before maturation), when *cis*-vaccenic acid was accumulated in the pulp part (Shibahara, A., et al., manuscript in preparation). A kaki fruit was peeled and divided into pulp and seed parts. A piece of the pulp (10 g) was cut off and homogenized with 10 ml of 0.2 M sodium phosphate buffer (pH 6.5) in a Waring blender for 3 min. The homogenate was filtered through cotton cloth and used immediately for biosynthetic experiments. Protein was determined by the method of Lowry et al. (22).

Incubation conditions and lipid analysis. Typical incubation conditions were as follows: the filtered homogenate (1 ml) of kaki pulp was added to a solution (1.8 ml) containing ammonium [2,2-$^2H_2$]palmitoleate (10 μmol), ammonium malonate (20 μmol), $MgCl_2$ (20 μmol) and cofactors, such as ATP (3 μmol), NADPH (1 μmol) and CoA (1 μmol). The mixture was incubated at 30°C for 30 min with continuous shaking. In the cases where CoA esters were used as the substrate, [2,2-$^2H_2$]palmitoleoyl-CoA (7.74 μmol) and malonyl-CoA (1 μmol) were added in place of the corresponding ammonium salts. The reaction was stopped by the addition of 2 N HCl, and a known amount of pentadecanoic acid was added as an internal standard. The reaction mixture was extracted repeatedly with ether, and the combined extracts were evaporated to dryness. The lipids thus obtained were reacted with 14% BF_3/methanol at 80°C for 20 min. The formed fatty acid methyl esters were analyzed by capillary GC and then by GC-MS as their DDA derivatives.

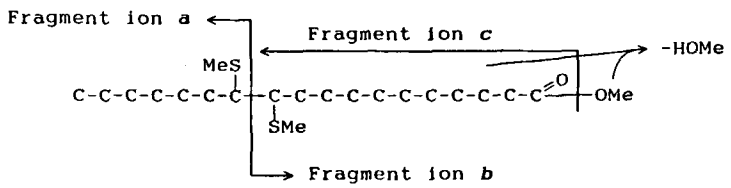
GC and GC-MS. Operating conditions of packed-column GC were the same as those in the previous report (14). Capillary GC was carried out with a Shimadzu GC-8A gas chromatograph equipped with a Shimadzu C-R2A Chromatopac integrator, a flame ionization detector, and a fused silica capillary column, ULBON HR-SS-10 (50 m × 0.24 mm i.d., chemically-bonded type, Shinwakako Co., Kyoto, Japan) operated at 190°C isothermally. Carrier gas was N_2 at a flow rate of 0.7 ml/min and a split ratio of 1/136. GC-MS analysis was performed on a Hitachi 663-30 gas chromatograph coupled to a Hitachi M-80A double-focusing mass spectrometer with an M-003 minicomputer on-line system. Column temperature was maintained at 200°C for fatty acid methyl esters and at 240°C for their DDA derivatives, and a scan range of 0-450 a.m.u. (2.8 sec/cycle) was used. Other operating conditions were the same as previously described (14).

RESULTS AND DISCUSSION

GC-MS analysis of deuterated compounds. Figure 1 shows mass spectra of the DDAs of methyl *cis*-vaccenate(A) and chemically-synthesized methyl [2,2-$^2H_2$]*cis*-vaccenate (B). The fragmentation of the derivatives by electron impact ionization can be expressed as a scheme represented there. The cleavage between the methylthio-substituted carbons of C-11 and C-12 gave the characteristic fragment ions *b* (m/z 245 for unlabeled and m/z 247 for labeled) and *c* (m/z 213 for unlabeled and m/z 215 for labeled). The ion *c* was yielded due to loss of methanol from the ion *b*. These assignments had been supported by our GC-high resolution MS observations previously reported (12). In contrast with the ions *b* and *c*, the peak of the fragment ion *a* in spectrum B did not shift from m/z 145 as having no stable-isotope label at the carbon chain. These spectra (Fig. 1) suggested that the conversion of labeled monoenoic acid methyl ester to its DDA derivative made it possible to identify simultaneously the carbon chain fragment to which two deuterium atoms attached and the original double-bond position in the aliphatic chain. Even when a mixture containing methyl esters of several varieties of monoenoic positional isomers ([2,2-$^2H_2$]acids and/or unlabeled acids) and also saturated and polyenoic acids was analyzed by GC-MS as their DDA derivatives, mass spectral data on their structures provided the accurate confirmation of the original double-bond positions in the molecules of the labeled and/or unlabeled isomeric monoenoates without confusion (data not shown).

Calibration curve for determining isotope content. For metabolic experiments, it was required to determine the isotope content of *cis*-vaccenic acid formed from the labeled substrate. In order to accomplish this, several standard mixtures having different proportions of methyl [2,2-$^2H_2$]*cis*-vaccenate and unlabeled methyl *cis*-vaccenate were analyzed by GC-MS as their DDA derivatives.

Table 1 shows a digital form of mass chromatogram obtained from GC-MS analysis of a standard mixture (DDA of methyl [2,2-$^2H_2$]*cis*-vaccenate, 37.9%) for example. Maximum intensity of the ion at m/z 247 originated from DDA of methyl [2,2-$^2H_2$]*cis*-vaccenate was recorded at scan number 74, whereas that of the ion at m/z 245 from unlabeled derivative was at scan number 75. The labeled compound thus eluted earlier than the corresponding unlabeled compound in a mass chromatographic peak due to the "isotope effect" similar to those in the references (17, 23, 24). Therefore, a mass spectrum taken at the top of a mass chromatographic peak did not show the proportion of labeled compound accurately. When the mass spectra such as in Figure 3 were required, relative intensity to record



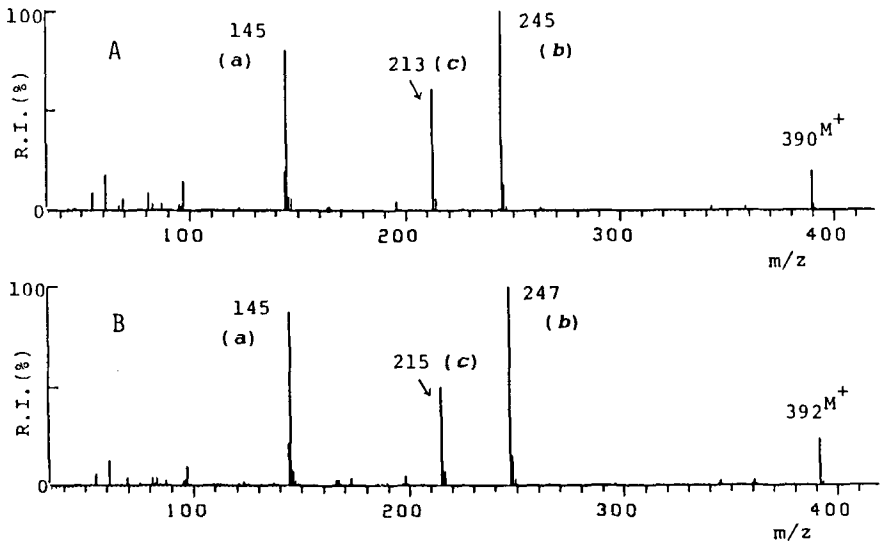


FIG. 1. Mass spectra of dimethyl disulfide adducts of methyl *cis*-vaccenate (A) and methyl [2,2-$^2H_2$]*cis*-vaccenate (B). Operating conditions are: column, 1 m × 3 mm i.d. glass column packed with 2% OV-101 on Chromosorb WHP; column temperature, 240°C; ionizing voltage, 20 eV; scan range, 0–450 a.m.u. (2.8 sec/cycle).

TABLE 1

Intensity of Key Fragment Ions in Mass Chromatogram of a Standard Mixture[a]

	Intensity m/z			
Scan no.	245	246	247	248
69	0	0	0	0
70	714	0	929	0
71	2130	550	2140	482
72	8445	1829	5893	1195
73	14203	3022	10417	1758
74	18800	5374	12793	2366
75	21490	4682	12570	2236
76	15436	2687	9986	1904
77	11267	2511	8911	1099
78	10142	2315	5318	960
79	6442	1724	3644	1011
80	4446	955	2115	502
81	3845	821	1638	199

[a]Composed of dimethyl disulfide adducts of methyl *cis*-vaccenate (62.1%) and methyl [2,2-$^2H_2$]*cis*-vaccenate (37.9%). Operating conditions are the same as in Fig. 1.

a mass spectrum was calculated by an integration program. The program's job was to integrate the intensities of a series of ions between a selected range of continuous scans (e.g., scan number 73 to 77 in Table 1), to divide the sum of intensities by the integration times (=5, in this case), and to output the result and its final mass spectrum. The data obtained in this way led to show an averaged relative intensity (Table 2) and an averaged mass spectrum (Fig. 3) at the top of a mass chromatographic peak.

As shown in Table 2, the natural abundance of stable-isotopes in the fragment ion *b* (cf. Fig. 1) of unlabeled compound amounted to 16.1% (m/z 246), 6.9% (m/z 247) and 1.3% (m/z 248) relative to the base peak ion at m/z 245, respectively, under the operating conditions used in this study. Consequently, the relative intensity (67.3%) of the key fragment ion at m/z 247 which originated from labeled standard mixture was due to itself plus naturally-occurring isotopes (6.9%). To avoid a tedious calculation for the existence of naturally-occurring isotopes (23, 24), the value *V* indicating the proportion of gross peak areas of the two key fragment ions was calculated simply by the following formula: V = peak area of m/z 247 / (peak area of m/z 247 + peak area m/z 245) × 100, where ions at m/z 247 and m/z 245 were representative of labeled and unlabeled compounds, respectively.

TABLE 2

Averaged Relative Intensity of Key Fragment Ions (m/z 245–248) in Mass Chromatographic Peaks of Standard Mixtures

Deuterated acid (%)[a]	Relative intensity (%) m/z			
	245	246	247	248
0.0	100.0	16.1	6.9	1.3
37.9[b]	100.0	22.5	67.3	11.5

[a]Content of dimethyl disulfide adduct of methyl [2,2-$^2H_2$]*cis*-vaccenate.
[b]Corresponding to that in Table 1. These data are given by the integration program. For details, see text.

Plotting the value V of several standard mixtures against their isotope contents gave the calibration curve shown in Figure 2. Each plot was an average of 3 to 7 determinations, in which no significant variation due to sample size (11) was observed. The increase in proportion was linear with increasing amounts of labeled derivatives in standard mixtures (0–98.0%). The calibration curve was constant in the experiments during several months, and no statistical treatment was employed. Microanalysis using this method showed that the limits of detection and quantification were at subpicogram and subnanogram levels, respectively.

These results lead to the conclusion that the newly-developed GC-MS method described here is suitable for both detecting and estimating the isotopically-labeled *cis*-vaccenic acid in distinction from the unlabeled monoenoic isomers. We applied this method for tracing the pathway of *cis*-vaccenic acid biosynthesis in fruit pulps.

Biosynthesis of cis-vaccenic acid. The pulp homogenate prepared from maturing kaki fruits was incubated with [2,2-$^2H_2$]palmitoleate or [2,2-$^2H_2$]palmitoleoyl-CoA in the absence or presence of some cofactors. Table 3 shows the formation of dideuterated *cis*-vaccenic acid from the labeled substrates. Only a trace amount of dideuterated *cis*-vaccenic acid was formed from incubation with [2,2-$^2H_2$]palmitoleate in the absence of a cofactor as shown at the line of experiment number 1 (Exp. 1) in Table 3. Repeated GC-MS runs of the same sample showed the presence of labeled *cis*-vaccenic acid; however, the value of labeling (0.1%) was somewhat doubtful. The addition of cofactors (ATP, NADPH and CoA) to the incubation medium stimulated the formation of labeled *cis*-vaccenic acid, determinable by GC-MS with reproducibility (Exp. 2).

When [2,2-$^2H_2$]palmitoleoyl-CoA was used as the substrate in place of the corresponding ammonium salt, the significant increment of labeled *cis*-vaccenic acid in the reaction products was observed (Exp. 3). These findings indicated that the enzymatic conversion of ammonium palmitoleate to its CoA or ACP thioester was the indispensable step to the *cis*-vaccenic acid-synthesizing reaction under the assay conditions used, and that the reaction proceeded by using endogenous cofactors in the pulp-raw homogenate. The addition of cofactors to the reaction mixture was effective.

In a series of metabolic experiments done in this study, the assay system of Experiment 5 (Table 3) using [2,2-$^2H_2$]palmitoleoyl-CoA and malonyl-CoA (in place of ammonium malonate) together with NADPH gave the very active formation of *cis*-vaccenic acid, the labeled *cis*-vaccenic acid was estimated as 45.4% of the

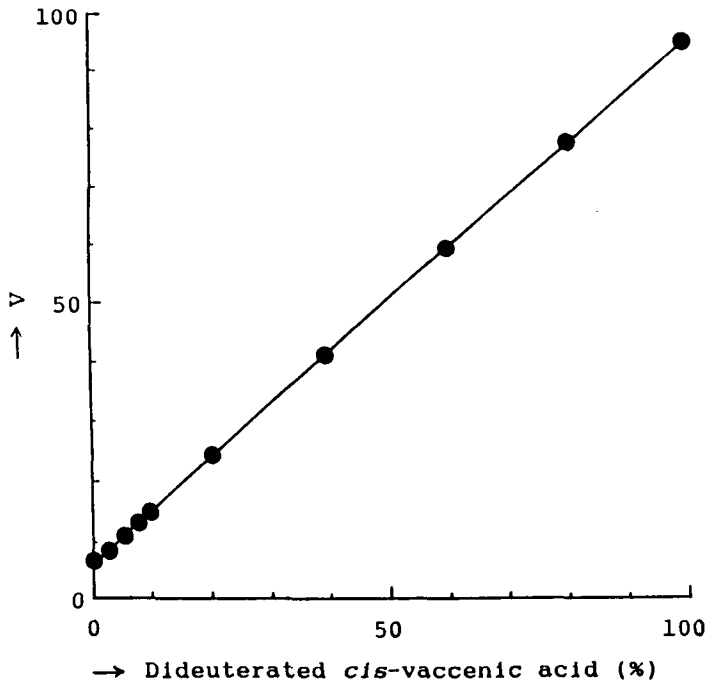


FIG. 2. Calibration curve for determination of dideuterated *cis*-vaccenic acid content. The concentration of dideuterated *cis*-vaccenic acid in standard mixtures is plotted on the *x*-axis, and the value V calculated by the following formula on the *y*-axis: V = peak area of m/z 247 / (peak area of m/z 247 + peak area of m/z 245) × 100. Each plot is an average of 3 to 7 determinations.

TABLE 3

Biosynthesis of *cis*-Vaccenic Acid from [2,2-$^2H_2$]Palmitoleate or [2,2,-$^2H_2$]Palmitoleoyl-CoA with Kaki Pulp Homogenate

Experiment no.	Substrate	Cofactor added	Dideuterated *cis*-vaccenic acid formed	
			% of total *cis*-vaccenic acid[a]	nmol/mg protein[b]
1	[2,2-$^2H_2$]Palmitoleate + Malonate	None	0.1	0.02
2	[2,2-$^2H_2$]Palmitoleate + Malonate	ATP,NADPH,CoA	0.8	0.24
3	[2,2-$^2H_2$]Palmitoleoyl-CoA + Malonate	NADPH	2.7	0.55
4	[2,2-$^2H_2$]Palmitoleoyl-CoA + Malonate	ATP	8.1	0.25
5	[2,2-$^2H_2$]Palmitoleoyl-CoA + Malonyl-CoA	NADPH	45.4	12.69

[a]Determined by use of the calibration curve shown in Fig. 2.
[b]After 30-min incubation at 30°C.
Each value is an average of duplicate assays.

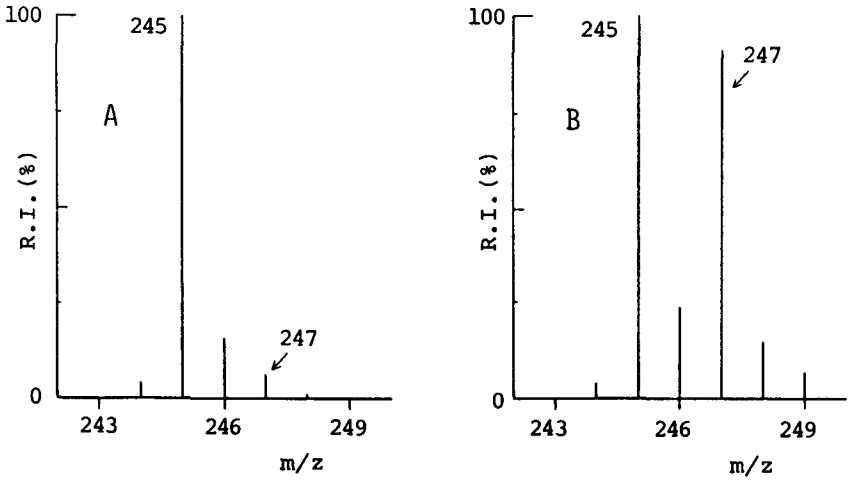


FIG. 3. Typical mass spectra showing the formation of labeled *cis*-vaccenic acid in (A), control experiment using a heat-inactivated homogenate; (B), experiment no. 5 in Table 3. Operating conditions are the same as in Fig. 1. These averaged spectra are given by the integration program. For details, see text.

total *cis*-vaccenic acid by the calibration curve in Figure 2. The synthetic activity for *cis*-vaccenic acid in Experiment 5 was 12.69 nmol/mg protein (an average of duplicate assays).

Figure 3 shows typical mass spectra which are evidential of the formation of labeled *cis*-vaccenic acid. No formation of labeled *cis*-vaccenic acid was observed in the control experiment with a heat-inactivated homogenate (spectrum A). Its averaged relative intensity (m/z 245–247) agreed closely with that of the authentic unlabeled compound shown in Table 2. On the other hand, there was a clear appearance of the key fragment ion at m/z 247 in spectrum B in Figure 3 that indicated the presence of dideuterated *cis*-vaccenic acid. In addition, a mass spectrum about the fragment ion *a* (cf. Fig. 1) showed no shifted ion in the range of m/z 145–149, indicating the absence of deuterium labeling at the carbon chain between the double bond and the terminal methyl group (data not shown).

From these results, we concluded that the enzymatically-formed *cis*-vaccenic acid contained two deuterium atoms at the carbon chain only between the double bond and the carboxyl group and that these two deuterium atoms were originated from the substrate, [2,2-$^2H_2$]palmitoleate or [2,2-$^2H_2$]palmitoleoyl-CoA.

If the labeled substrate ([2,2-$^2H_2$]palmitoleate or [2,2-$^2H_2$]palmitoleoyl-CoA) was β-oxidized, only one molecule in the resulting acetyl-CoAs from one molecule of the [2,2-$^2H_2$]hexadecenoyl-CoA must contain one deuterium atom. Therefore, it was practically impossible that the dideuterated *cis*-vaccenic acid with the specific labeling-pattern as above characterized was formed by de novo synthesis from the deuterated acetyl-CoAs each containing one deuterium atom.

Consequently, the experimental evidence shown in this study proved that *cis*-vaccenic acid was formed from palmitoleic acid by chain elongation.

Other possible pathways for cis-vaccenic acid biosynthesis. Since Chisholm and Hopkins (2) postulated the pathway of *cis*-vaccenic acid biosynthesis in higher plants as chain elongation from palmitoleic acid, the postulated pathway has been supported by some workers (25–30) merely based on their analytical data on the co-occurrence of palmitoleic and *cis*-vaccenic acids.

This chain elongation pathway has now directly been proved by our experiments.

Recently Kleiman and Payne-Wahl (31) reported the presence of *cis*-vaccenic acid in Meliaceae seed oils. In their paper, they pointed out the possibility that another pathway for *cis*-vaccenic acid biosynthesis also could be operational, such as desaturation of stearate by a Δ-11 desaturase. To their presentation (31), our preliminary experimental results with kaki pulp homogenate and ammonium [2,2-$^2H_2$]stearate seemed to give a negative support, because the two deuterium atoms which originated from [2,2-$^2H_2$]stearate were located mainly in oleic acid. Further examinations on the Δ-11 desaturation are underway in our laboratories and the detailed data will be reported elsewhere.

According to other hypothesized pathways, a direct dehydration of 11- or 12-hydroxy stearic acid due to the action of a dehydratase (like that of β-hydroxydecanoyl-ACP dehydratase) may occur to form *cis*-vaccenic acid, and a biohydrogenation of C_{18} polyenoic acids may result in the production of *cis*-vaccenic acid as reported in microorganisms (32, 33). Although neither oxygenated fatty acids nor C_{18} polyenoic acids relating to the structure of *cis*-vaccenic acid are found both in kaki pulp lipids (15) and in kaki pulp homogenate (present study), the formation of *cis*-vaccenic acid through these hypothesized pathways is not deniable entirely.

It is unclear whether these presumed processes (desaturation, dehydration and biohydrogenation) and other ones participate in the production of *cis*-vaccenic acid in higher plants, but we suppose that chain elongation from palmitoleic acid is one of the main pathways of *cis*-vaccenic acid biosynthesis, because a considerable amount of *cis*-vaccenic acid can be produced in the simple biosynthetic systems used in this study.

The chain elongation seems to proceed in the form of acyl-CoA or acyl-ACP and to be a condensing reaction depending on malonyl-CoA or malonyl-ACP. Further studies will be necessary to elucidate these synthetic mechanisms and to examine the properties of the enzyme system responsible for *cis*-vaccenic acid biosynthesis.

In the course of GC-MS analysis of the reaction products (Table 3), we observed the formation of labeled oleic acid containing two deuterium atoms at the carbon chain between the double bond and the carboxyl group. Its labeling-pattern was similar to that of labeled *cis*-vaccenic acid. The mechanism of incorporating two deuterium atoms from the substrate ([2,2-$^2H_2$]palmitoleate or [2,2-$^2H_2$]palmitoleoyl-CoA) into oleic acid was unclear. At least it was certain that the dideuterated oleic acid was not formed by de novo synthesis from the deuterated acetyl-CoAs which were produced by β-oxidation of the [2,2-$^2H_2$]hexadecenoyl-CoA as described above. Our report on the mechanism of this dideuterated oleic acid biosynthesis will be presented in the near future.

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Influence of Dietary Fat Composition on Intestinal Absorption in the Rat

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Omega-3 fatty acids influence the function of the intestinal brush border membrane. For example, the omega-3 fatty acid eicosapentaenoic acid (20:5ω3) has an antiabsorptive effect on jejunal uptake of glucose. This study was undertaken to determine whether the effect of feeding α-linolenic acid (18:3ω3) or EPA plus docosahexaenoic acid (22:6ω3) on intestinal absorption of nutrients was influenced by the major source of dietary lipid, hydrogenated beef tallow or safflower oil. The *in vitro* intestinal uptake of glucose, fatty acids and cholesterol was examined in rats fed isocaloric diets for 2 weeks: beef tallow, beef tallow + linolenic acid, beef tallow + eicosapentaenoic acid/docosahexaenoic acid, safflower oil, safflower oil + linolenic acid, or safflower oil + eicosapentaenoic acid/docosahexaenoic acid. Eicosapentaenoic acid/docosahexaenoic acid reduced jejunal uptake of 10 and 20 mM glucose only when fed with beef tallow, and not when fed with safflower oil. Linolenic acid had no effect on glucose uptake, regardless of whether it was fed with beef tallow or safflower oil. The jejunal uptake a long-chain fatty acids (18:0, 18:2ω6, 18:3ω3, 20:4ω6, 20:5ω3 and 22:6ω3) and cholesterol was lower in safflower oil than with beef tallow. When eicosapentaenoic acid/docosahexaenoic acid was given with beef tallow (but not with safflower oil), there was lower uptake of 18:0, 20:5ω3 and cholesterol. The demonstration of the inhibitory effect of linolenic acid or eicosapentaenoic acid/docosahexaenoic acid on cholesterol uptake required the feeding of a saturated fatty acid diet (beef tallow). These changes in uptake were not explained by differences in the animals' food intake, body weight gain or intestinal weight. Feeding safflower oil was associated with an approximately 25% increase in the jejunal and ileal mucosal surface area, but this increase was prevented by combining linolenic acid or eicosapentaenoic acid/docosahexaenoic acid with safflower oil. Different inhibitory patterns were observed when mixtures of fatty acids were present together in the incubation medium, rather than in the diet: for example, when 18:0 was in the incubation medium with 20:4ω6, the uptake of 20:4ω6 was reduced, whereas the uptake was unaffected by 18:2ω6 or 20:5ω3. Thus, (1) the inhibitory effect of eicosapentaenoic acid/docosahexaenoic acid on jejunal uptake of glucose, fatty acids and cholesterol was influenced by the major dietary lipid, saturated (beef tallow) or polyunsaturated fatty acid (safflower oil); and (2) different omega-3 fatty acids (linolenic acid versus eicosapentaenoic acid/docosahexaenoic acid) have a variable influence on the intestinal absorption of nutrients.
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Small changes in the percentage of total dietary lipids composed of essential and nonessential fatty acids, without concurrent alterations in dietary total fat, carbohydrate or protein, influence active and passive intestinal transport processes in the rat (1-4). These alterations in transport are not associated with changes in intestinal morphology or brush border membrane content of cholesterol or phospholipids (5, 6). In contrast, dietary manipulation is associated with variations in the fatty acid content of brush border membrane phosphatidylcholine and phosphatidylethanolamine. This change in membrane fatty acid composition has been used to advantage: feeding a polyunsaturated diet normalizes the enhanced intestinal uptake of glucose in diabetic rats (2). There is a spectrum of effects of dietary lipids on intestinal transport function (7), and feeding semisynthetic diets high in eicosapentaenoic acid (EPA, 20:5ω3) is associated with reduced jejunal uptake of glucose and reduced ileal uptake of galactose. Diets high in α-linolenic acid (LNA, 18:3ω3) are associated with reduced jejunal uptake of lauric acid, increased uptake of stearic acid, unchanged uptake of cholesterol and a 25% reduction in mucosal surface area (8). The altered transport could not be explained by the magnitude or direction of the morphological changes, nor could the alterations in transport be explained by differences in the animals' food intake or body weight gain. Although the feeding of EPA and/or docosahexaenoic acid (DHA) of marine origin are hypocholesterolemic (9-11), it is not clear whether this effect is influenced by other dietary constituents. Accordingly, this study was undertaken to establish the effect of feeding EPA/DHA or LNA on intestinal transport function when the major dietary lipid was saturated (beef tallow [BT]) or polyunsaturated (safflower oil [SO]) fat.

METHODS AND MATERIALS

Animals and diets. Male Sprague-Dawley rats weighing 50-60 g were used. Guiding principles in the care and use of laboratory animals, approved by the Canadian Federation of Biological Societies and by the Council of the American Physiological Society, were observed in the conduct of this study. Animals were allowed *ad libitum* access to water and food until the morning of the study. Animals were fed one of six diets (Table 1) for 4 weeks: a semipurified isocaloric diet containing BT, BT + LNA, BT + EPA/DHA, SO, SO + LNA, or SO + EPA/DHA.

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Abbreviations: AA, arachidonic acid; BT, beef tallow; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LNA, linolenic acid; SO, safflower oil; TDC, taurodeoxycholic acid; ANOVA, analysis of variance.

TABLE 1.

Diet composition

A. Macronutrients

Diet component	g/kg
Casein	270.00
Starch	200.00
Glucose	207.65
Cellulose	50.00
Vitamin mix	10.00
Mineral mix	50.85
Choline	2.75
Inositol	6.25
L-methionine	2.50
Fat[a] (see Table 1B)	200.00

[a]All diets were nutritionally adequate (National Research Council: Subcommittee on Laboratory Animal Nutrition (1978) Nutrient requirements of laboratory animals #10. Natl. Acad. Sci., Washington, D.C.).

B. The Fat Content of Each Diet was Prepared as Follows:

Diet	Beef tallow	Safflower oil	Linseed oil	Fish oil	Cholesterol[b]
Beef tallow					
No Suppl.	180 g	20 g	—	—	360 mg
+ LNA	140 g	10 g	50 g	—	280 mg
+ EPA	132 g	18 g	—	50 g	280 mg
Safflower oil					
No Suppl.	5 g	195 g	—	—	
+ LNA	—	150 g	50 g	—	
+ EPA	—	150 g	—	50 g	

[b]Beef tallow contains cholesterol (0.2%, w/w), therefore the other beef tallow-containing diets required cholesterol supplementation in order to keep the cholesterol content nearly constant in the diets at 0.03%.

C. Fatty Acid Composition of the Semisynthetic Diets

	Beef tallow (BT)			Safflower oil (SO)		
Fatty acid	—	+ LNA	+ EPA/DHA	—	+ LNA	+ EPA/DHA
14:0	4.2	2.7	5.0	0.3	0.1	2.0
16:0	28.9	20.4	23.8	7.6	6.2	7.6
16:1	0.5	0.4	3.5	0.1	0.1	3.2
17:0	2.1	1.4	0.5	0.1	0.1	0.6
18:0	51.2	38.2	39.3	5.8	3.4	9.6
18:1ω9	3.2	7.4	5.0	14.6	15.2	6.4
18:1ω7	0.2	0.3	0.9	0.2	0.1	0.4
18:2ω6	9.5	10.2	9.4	70.5	56.7	57.6
18:3ω3	0.1	18.8	0.8	0.6	17.9	0.9
20:4ω6			0.4			0.3
20:5ω3			7.5			7.8
22:5ω3			0.6			0.6
22:6ω3			2.6			2.4
ΣSFA	86.3	62.7	68.6	13.8	9.7	19.8
ΣMUFA	4.1	8.1	9.9	14.9	15.4	10.0
Σω6	9.5	10.2	9.8	70.5	56.7	57.9
Σω3	0.1	18.8	11.5	0.6	17.9	11.7

ΣSFA, sum of saturated fatty acids; ΣMUFA, sum of monounsaturated fatty acids; Σω6, sum of omega-6 polyunsaturated fatty acids; Σω3, sum of omega-3 polyunsaturated fatty acids.

Additional male Sprague-Dawley rats were raised on standard chow diet for 4 weeks and used for the interaction studies (see Materials and Methods, section 4). Animals were allowed *ad libitum* access to food and water until the morning of the study.

Probe and marker compounds. [^{3}H]-Inulin (mol wt ca. 5,000) was used as supplied by New England Nuclear (Boston, MA) to measure the adherent mucosal fluid volume. The [^{14}C]-labeled probes included stearic acid (FA 18:0) from ICN Biomedical Inc. (Montreal, Quebec); linoleic acid (FA 18:2) from Amersham Canada Ltd. (Oakville, Ontario); linolenic acid (FA 18:3) from New England Nuclear; arachidonic acid (FA 20:4), eicosapentaenoic acid (FA 20:5), cholesterol and docosahexaenoic acid (FA 22:6), L-glucose from Amersham Canada Ltd. and D-glucose from ICN Biomedical

Inc. Unlabeled probes were supplied by Sigma Chemical Company (St. Louis, MO). [^{3}H] and [^{14}C]-labeled probes were obtained from New England Nuclear, ICN Biomedical Inc. and Amersham Canada Ltd.

Individual experiments. The method used for preparation of micellar solutions of palmitic acid, stearic acid and cholesterol has been published (12). The long-chain fatty acids and cholesterol were dispersed in 20 mM taurodeoxycholic acid. The concentration of dispersed compounds was: cholesterol, 0.05 mM; long-chain saturated and unsaturated fatty acids, 0.1 mM; D-glucose, 5–40 mM and L-glucose, 1 mM.

Interaction studies. The uptake of radiolabeled 18:0, 18:2ω6, 18:3ω3, 20:4ω6, 20:5ω3 and 22:6ω3 was determined when these bile acids were present alone in taurodeoxycholic acid (TDC), as well as when they were present with other fatty acids. For example, the uptake of 20:4ω6 was determined alone, as well as in the presence of 18:0, 18:2ω6 and 20:5ω3 (Table 7).

Tissue preparation. Animals were sacrificed by overdose intraperitoneal injection of Euthanyl (sodium pentobarbital 450 mg/kg body wt). A 15 cm length of proximal jejunum and distal ileum was rapidly removed and rinsed gently with 50 ml of cold saline, as described (13–15). The intestine was opened along its mesenteric border and the mucosal surface was carefully washed with cold saline to remove visible mucus and debris. Pieces of intestine were cut from a segment and tissue was mounted as flat sheets in incubation chambers. Preincubation chambers contained oxygenated Krebs-bicarbonate buffer (pH 7.4) at 37°C; tissue discs were preincubated for 10 min to allow equilibration at this temperature. Then the transport chambers were transferred to other incubation beakers for specific experiments. The preincubation and incubation solutions were mixed at identical stirring rates with circular magnetic bars and the stirring rates were precisely adjusted by means of a strobe light. Stirring rates were reported as revolutions per minute at which time the stirring bar was driven. For most studies a stirring rate of 600 rpm was selected to achieve low effective resistance of the intestinal unstirred water layer (14).

Determination of uptake rates. After preincubation, the chambers were transferred to other beakers containing [^{3}H]-inulin and various [^{14}C]-probe molecules in oxygenated Krebs bicarbonate (pH 7.4 and 37°C). Validation studies demonstrated linear rates of fatty acid uptake between 4 and 10 min incubation, with constant volume of the adherent mucosal fluid as estimated from the ^{3}H-inulin space, and with extrapolation to zero uptake at zero time. Accordingly, a 6 min incubation period was used. After incubation of discs in labeled solutions for 6 min, the experiment was terminated by removing the chamber and quickly rinsing the tissue in cold saline for ca. 5 sec.

The exposed mucosal tissue was then cut out of the chamber with a circular steel punch and was gently blotted on filter paper. The tissue was dried overnight in an oven at 55°C. The dry weight of the tissue was determined, the sample was saponified with 0.75 N NaOH, scintillation fluid was added and radioactivity was determined by means of an external standardization technique to correct for variable quenching of the two isotopes.

Morphology. Previous reports (16–18) have described in detail the method used to assess the weight of the scraped mucosa and the remainder of the intestinal wall ("submucosa") and the villus and mucosal surface areas.

Expression of results. Uptake rate of the probes was calculated after correcting total tissue [^{14}C]-radioactivity for the mass of the probe molecule present in adherent mucosal fluid (14). Uptake rates were expressed as nmol of probe molecule taken up into the mucosa per 100 mg dry weight of tissue per minute (nmol/100 mg/min). Values obtained from different dietary groups are reported as mean ± SEM of results observed for nine animals in each group.

Analysis of variance (ANOVA) was used to test the significance of the difference between dietary groups. Where differences were significant, pair-wise tests of

TABLE 2

Effect of Dietary Omega-3 Fatty Acids on Acids on Animal Characteristics[a]

	Beef tallow			Safflower oil		
Characteristic	No supplement (9)*	+ LNA (9)	+ EPA/DHA (9)	No supplement (9)	+ LNA (9)	+ EPA/DHA (9)
Food intake g/rat/day	14.9 ± 1.1	15.1 ± 0.6	14.4 ± 0.8	16.3 ± 0.8	16.2 ± 0.4	16.7 ± 0.5
Average weight gain g/rat/day	5.5 ± 0.8	6.4 ± 0.5	6.2 ± 0.0	7.4 ± 0.2	7.3 ± 0.1	7.5 ± 0.2
Dry weight, mg/unit serosal surface area						
Jejunum	3.6 ± 0.3	3.3 ± 0.4	3.1 ± 0.2	2.9 ± 0.1[b]	3.6 ± 0.2[c]	2.8 ± 0.2[d]
Ileum	2.3 ± 0.2	2.4 ± 0.2	2.3 ± 0.2	5.3 ± 0.4[b]	2.2 ± 0.2[c]	2.2 ± 0.1[c]
% of intestinal wall comprised of mucosa						
Jejunum	77.8 ± 2.3	80.3 ± 1.9	74.1 ± 1.8	66.2 ± 2.4[b]	61.7 ± 3.2[b]	59.4 ± 3.4[b]
Ileum	62.0 ± 2.9	49.3 ± 3.2[c]	67.5 ± 2.5[d]	34.3 ± 1.6[b]	67.7 ± 1.4[b,c]	63.7 ± 2.3[c]

[a]After 4 weeks of feeding diets differing in linoleic acid to saturated fatty acid ratios.
[b]$p < 0.05$; S vs B, S + LNA vs B + LNA, S + EPA/DHA vs B + EPA/DHA.
[c]$p < 0.05$; B + LNA, vs B, B + EPA/DHA vs B, S + LNA vs S, S + EPA/DHA vs S.
[d]$p < 0.05$; B + EPA/DHA vs B + LNA, S + EPA/DHA vs S + LNA.
*No. animals per group.

statistical significance of the difference between any two means was determined using Student's t-test. The statistical significance of the difference between groups was assessed using Duncan's multiple range test, which confirmed the findings using ANOVA.

RESULTS

Animal characteristics. Food intake and body weight gain were similar in the six diet groups. Jejunal and ileal dry weight (mg/unit serosal surface area) were similar with BT, BT + LNA and BT + EPA/DHA, whereas LNA and EPA/DHA influence the jejunal and ileal weights when fed with SO (Table 2). For example, the dry weight was lower in the jejunum and higher in the ileum of animals fed SO versus BT, higher in the jejunum yet lower in the ileum of SO + LNA than in SO. The percentage of the jejunal wall comprised of mucosa was lower in animals fed SO than BT, although unaffected by supplementation with LNA or EPA/DHA. The percentage of the ileal wall comprised of mucosa was lower following LNA supplementation in animals fed BT.

The diets had variable effects on the villus height, width, thickness and surface area of the jejunum and ileum (Tables 3 and 4), but the mucosal surface area

TABLE 3

Effect of Feeding Omega-3 Fatty Acids[a] on Jejunal Morphology

Morphological parameter	Beef tallow			Safflower oil		
	No supplement	+ LNA	+ EPA/DHA	No supplement	+ LNA	+ EPA/DHA
Crypt depth, μm	76 ± 4	67 ± 4	67 ± 3	64 ± 4[c]	62 ± 3	73 ± 5
Villus height, μm	415 ±24	458 ±30	456 ± 9	429 ±21	329 ±14[b,c]	411 ±23[d]
Villus width at 1/2 height, μm	98 ± 2	123 ± 6[b]	122 ± 5[b]	118 ± 4[c]	120 ± 4	117 ± 4
Villus bottom width, μm	109 ± 6	140 ± 7[b]	130 ± 4[b]	104 ± 6	114 ± 6[c]	117 ± 5
Villus thickness, μm	452 ±29	403 ±18	386 ±23	361 ±26[c]	436 ±22[b]	348 ±15[d]
Villus surface area, μm²/villus	497 ±28	524 ±32	507 ±12	459 ±21	422 ±18[c]	424 ±26[c]
No. of villi/mm serosal length A	9.41± 0.50	7.30± 0.33[b]	7.75± 0.23[b]	9.86± 0.46	8.99± 0.49[c]	8.69± 0.44
No. of villi/mm serosal length B	2.32± 0.18	2.53± 0.11	2.68± 0.18	2.91± 0.21[c]	2.34± 0.10[b]	2.92± 0.12[c]
No. of villi/mm² serosa	20.83± 1.10	18.13± 0.83	20.08± 0.59	27.35± 1.28[c]	20.63± 1.12[b]	24.94± 1.26[d,c]
Mucosal surface area mm²/mm² serosa	10.23± 0.61	9.49± 0.73	10.19± 0.42	12.46± 0.64[c]	8.67± 0.52[b]	10.73± 1.07

[a]Diets high in saturated or polyunsaturated fatty acids.
[b]$p < 0.05$; B + LNA vs B, B + EPA/DHA vs B, S + LNA vs S, S + EDP/DHA vs S.
[c]$p < 0.05$; S vs B, S + LNA vs B + LNA, S + EPA/DHA vs B + EPA/DHA.
[d]$p < 0.05$; B + EPA/DHA vs B + LNA, S + EPA/DHA vs S + LNA.

TABLE 4

Effect of Feeding Omega-3 Fatty Acids[a] on Ileal Morphology

Morphological parameter	Beef tallow			Safflower oil		
	No supplement	+ LNA	+ EPA/DHA	No supplement	+ LNA	+ EPA/DHA
Crypt depth, μm	73 ± 5	64 ± 6	68 ± 4	74 ± 5	70 ± 5	54 ± 4[b,c]
Villus height, μm	235 ±11	210 ± 9	236 ± 9	284 ±13[d]	235 ±10[b]	228 ± 5[b]
Villus width at 1/2 height, μm	123 ± 7	109 ± 3	111 ± 5	93 ± 3[d]	116 ± 3[b]	96 ± 3[d,c]
Villus bottom width, μm	119 ± 6	115 ± 7	101 ± 4[b]	89 ± 4[d]	111 ± 5[b]	100 ± 3
Villus thickness, μm	214 ± 9	256 ± 7[b]	278 ±19[b]	236 ±11	231 ± 7[c]	213 ± 5[d]
Villus surface area, μm²/villus	185 ± 9	180 ± 7	217 ±9[b,c]	210 ± 9	190 ± 8	161 ± 4[b,c]
No. of villi/mm serosal length A	8.62± 0.47	8.98± 0.55	10.07± 0.43[b]	11.45± 0.61[d]	9.12± 0.33[b]	10.09± 0.34
No. of villi/mm serosal length B	4.75± 0.21	3.93± 0.11[b]	3.76± 0.26[b]	4.32± 0.21	4.37± 0.14[c]	4.73± 0.13[d]
No. of villi/mm² serosa	40.30± 2.21	35.06± 2.13	36.27± 1.54	48.45± 2.57[d]	39.51± 1.44[b]	47.41± 1.58[d,c]
Mucosal surface area mm²/mm² serosa	7.37± 0.42	6.32± 0.43	7.96± 0.61[c]	10.31± 0.90[d]	7.54± 0.44[b]	7.61± 0.30[b]

[a]Diets high in saturated or polyunsaturated fatty acids.
[b]$p < 0.05$; B + LNA vs B, B + EPA/DHA vs B, S + LNA vs S, S + EDP/DHA vs S.
[c]$p < 0.05$; B + EPA/DHA vs B + LNA, S + EPA/DHA vs S + LNA.
[d]$p < 0.05$; S vs B, S + LNA vs B + LNA, S + EPA/DHA vs B + EPA/DHA.

of both sites was about 25% higher in rats fed SO, as compared with BT. This greater mucosal surface area was not observed when the SO diet was supplemented with LNA or EPA/DHA.

Hexose uptake. LNA had no influence on glucose uptake when fed with BT or SO for both the jejunum and ileum (Tables 5 and 6). EPA/DHA reduced ($p < 0.05$) the uptake into the jejunum, but not into the ileum of BT fed animals, at 10 mM and 20 mM glucose. EPA/DHA had no effect on jejunal glucose uptake when fed with SO, but EPA/DHA increased ileal uptake of 10 mM glucose when fed with SO. Although the uptake of 10–40 mM glucose into the jejunum and the uptake of 5–40 mM glucose into the ileum was similar with feeding BT and SO, the uptake of 5–20 mM glucose into the jejunum was higher and the uptake of 40 mM glucose was lower when EPA/DHA was fed with SO than with BT; similar findings were noted in the ileum (Table 6). Thus, the effect of EPA/DHA on jejunal and ileal glucose uptake was influenced by whether the concurrent lipid was BT or SO.

Lipid uptake. The jejunal uptake of 18:0, 18:2ω6, 20:4ω6, 20:5ω3 and cholesterol was lower in animals fed SO than in those fed BT (Table 7). Adding LNA to BT had no influence on fatty acid uptake, but the uptake of cholesterol was reduced, and adding EPA/DHA to BT reduced only the uptake of 20:5 and cholesterol. The uptake of several fatty acids, as well as cholesterol, was lower in animals fed SO + LNA or SO + EPA/DHA, as compared with animals fed BT + LNA or BT + EPA/DHA, but adding LNA or EPA/DHA to the polyunsaturated fatty acids-SO diet did not accomplish further reduction in lipid uptake. In the ileum (Table 8), cholesterol uptake was lower in animals fed SO than in those fed BT, and this reduction persisted when SO was supplemented with LNA or EPA/DHA. Also, the uptake of 20:4ω6 was lower and 20:5ω3 was higher in animals fed SO + EPA/DHA BT + EPA/DHA. Thus, the demonstration of the inhibitory effect of LNA or EPA/DHA on cholesterol uptake required the feeding of a polyunsaturated fatty acid diet (SO).

Interaction studies. The uptake of 18:0, 18:3ω3 and 20:5ω3 was unaffected by the addition of other fatty acids to the incubation beakers (Table 9). In contrast, the uptake of 18:2ω6 was reduced by 18:3ω3 and

TABLE 5

Effect of Dietary Omega-3 Fatty Acids on Jejunal Glucose Uptake[a]

	Beef tallow			Safflower oil		
Substrate	No supplement	+ LNA	+ EPA/DHA	No supplement	+ LNA	+ EPA/DNA
D-glucose 5 mM	116 ± 21	134 ± 20	81 ± 11	174 ± 25[b]	169 ± 26	149 ± 23[b]
D-glucose 10 mM	230 ± 39	170 ± 29	117 ± 17[c]	199 ± 41	182 ± 19	211 ± 13[b]
D-glucose 20 mM	369 ± 63	328 ± 39	101 ± 16[c,d]	305 ± 32	357 ± 41	331 ± 47[b]
D-glucose 40 mM	496 ± 79	411 ± 57	420 ± 64	409 ± 60	364 ± 52	295 ± 27[b]
L-glucose 1 mM	5.8 ± 0.7	5.2 ± 0.7	6.5 ± 0.7	8.1 ± 1.1[b]	6.3 ± 0.8	6.1 ± 0.8

[a]In rats fed diets differing in linoleic acid to saturated fatty acid ratios (nmol/100 mg min[1]).
[b]$p < 0.05$; S vs B, S + LNA vs B + LNA, S + EPA/DHA vs B + EPA/DHA.
[c]$p < 0.05$; B + LNA vs B, B + EPA/DHA vs B, S + LNA vs S, S + EPA/DHA vs S.
[d]$p < 0.05$; B + EPA/DHA vs B + LNA, S + EPA/DHA vs S + LNA.

TABLE 6

Effect of Dietary Omega-3 Fatty Acids on Ileal Glucose Uptake[a]

	Beef tallow			Safflower oil		
Substrate	No supplement	+ LNA	+ EPA/DHA	No supplement	+ LNA	+ EPA/DNA
D-glucose 5 mM	164 ± 26	210 ± 48	122 ± 15	224 ± 53	130 ± 20	255 ± 46[b]
D-glucose 10 mM	179 ± 39	250 ± 57	172 ± 25	196 ± 34	255 ± 20	418 ± 31[c,d,b]
D-glucose 20 mM	297 ± 36	342 ± 50	305 ± 32	333 ± 56	323 ± 60	454 ± 27[b]
D-glucose 40 mM	364 ± 60	370 ± 57	282 ± 39	380 ± 76	434 ± 67	431 ± 66[b]
L-glucose 1 mM	4.2 ± 0.7	5.1 ± 0.7	4.4 ± 0.8	7.7 ± 1.6[b]	8.2 ± 1.3[b]	5.4 ± 0.9

[a]In rats fed diets differing in linoleic acid to saturated fatty acid ratios (nmol/100 mg min[1]).
[b]$p < 0.05$; S vs B, S + LNA vs B + LNA, S + EPA/DHA vs B + EPA/DHA.
[c]$p < 0.05$; B + LNA, vs B, B + EPA/DHA vs B, S + LNA vs S, S + EPA/DHA vs S.
[d]$p < 0.05$; B + EPA/DHA vs B + LNA, S + EPA/DHA vs S + LNA.

TABLE 7

Effect of Dietary Omega-3 Fatty Acids on Jejunal Lipid Uptake[a]

	Beef tallow			Safflower oil		
Substrate	No supplement	+ LNA	+ EPA/DHA	No supplement	+ LNA	+ EPA/DNA
FA 18:0 .1 mM	1.06 ± 0.24	1.72 ± 0.46	0.64 ± 0.16[b]	0.46 ± 0.10[c]	0.39 ± 0.11[c]	0.36 ± 0.07
FA 18:2 .1 mM	0.92 ± 0.15	0.95 ± 0.11	0.83 ± 0.17	0.46 ± 0.09[c]	0.70 ± 0.15	0.57 ± 0.15
FA 18:3 .1 mM	1.00 ± 0.22	0.81 ± 0.16	0.60 ± 0.09	0.81 ± 0.10	0.67 ± 0.20	0.66 ± 0.15
FA 20:4 .1 mM	1.13 ± 0.25	0.85 ± 0.16	0.91 ± 0.17	0.34 ± 0.06[c]	0.34 ± 0.08[c]	0.41 ± 0.11[c]
FA 20:5 .1 mM	0.94 ± 0.19	1.39 ± 0.23	0.63 ± 0.12[b]	0.55 ± 0.13[c]	0.49 ± 0.10[c]	0.48 ± 0.10
FA 22:6 .1 mM	0.64 ± 0.15	0.67 ± 0.14	0.99 ± 0.20	0.66 ± 0.14	0.57 ± 0.12	0.81 ± 0.19
Cholesterol 0.5 mM	1.36 ± 0.26	0.48 ± 0.08[d]	0.79 ± 0.20[d]	0.30 ± 0.07[c]	0.23 ± 0.05[c]	0.28 ± 0.04[c]

[a]In rats fed diets differing in linoleic acid to saturated fatty acid ratios (nmol/100 mg min[1]).
[b]$p < 0.05$; B + EPA/DHA vs B + LNA, S + EPA/DHA vs S + LNA.
[c]$p < 0.05$; S vs B, S + LNA vs B + LNA, S + EPA/DHA vs B + EPA/DHA.
[d]$p < 0.05$; B + LNA, vs B, B + EPA/DHA vs B, S + LNA vs S, S + EPA/DHA vs S.

TABLE 8

Effect of Dietary Omega-3 Fatty Acids on Ileal Lipid Uptake[a]

	Beef tallow			Safflower oil		
Substrate	No supplement	+ LNA	+ EPA/DHA	No supplement	+ LNA	+ EPA/DNA
FA 18:0 .1 mM	0.70 ± 0.18	0.42 ± 0.07	0.72 ± 0.22	0.42 ± 0.06	0.38 ± 0.12	0.58 ± 0.14
FA 18:2 .1 mM	0.65 ± 0.14	0.73 ± 0.12	0.89 ± 0.19	0.46 ± 0.15[b]	0.46 ± 0.08	0.57 ± 0.16
FA 18:3 .1 mM	0.78 ± 0.12	1.18 ± 0.21	1.17 ± 0.21	0.96 ± 0.14	0.76 ± 0.15	0.80 ± 0.18
FA 20:4 .1 mM	0.83 ± 0.08	0.69 ± 0.28	0.96 ± 0.14	0.30 ± 0.08[b]	0.34 ± 0.09	0.32 ± 0.10[b]
FA 20:5 .1 mM	0.72 ± 0.10	0.65 ± 0.09	0.41 ± 0.07[c,d]	0.54 ± 0.11	0.40 ± 0.08[b]	0.62 ± 0.09[b]
FA 22:6 .1 mM	0.62 ± 0.16	0.44 ± 0.08	0.53 ± 0.12	0.56 ± 0.16	0.53 ± 0.12	0.69 ± 0.16
Cholesterol 0.5 mM	0.82 ± 0.14	0.68 ± 0.12	0.47 ± 0.10	0.25 ± 0.05[b]	0.23 ± 0.05[b]	0.25 ± 0.04[b]

[a]In rats fed diets differing in linoleic acid to saturated fatty acid ratios (nmol/100 mg min[1]).
[b]$p < 0.05$; S vs B, S + LNA vs B + LNA, S + EPA/DHA vs B + EPA/DHA.
[c]$p < 0.05$; B + LNA, vs B, B + EPA/DHA vs B, S + LNA vs S, S + EPA/DHA vs S.
[d]$p < 0.05$; B + EPA/DHA vs B + LNA, S + EPA/DHA vs S + LNA.

TABLE 9

The Effect of Jejunal Fatty Acid Uptake in the Presence of Another Fatty Acid (nmol/100 mg min^{-1})

Fatty acid	Control	+ 18:0	+ 18:2	+ 18:3	+ 20:4	+ 20:5
18:0*	1.50 ± 0.31[a]		1.20 ± 0.22[a]	1.24 ± 0.17[a]	1.94 ± 0.33[a]	
18:2*	1.47 ± 0.2[a]	1.73 ± 0.25[a]		1.12 ± 0.15[b]	0.95 ± 0.12[c]	
18:3*	1.21 ± 0.13[a]	1.16 ± 0.15[a]	1.00 ± 0.1[a]		1.03 ± 0.12[a]	
20:4*	1.41 ± 0.19[a]	0.42 ± 0.06[b]	1.31 ± 0.17[a]			1.68 ± 0.18[a]
20:5*	1.80 ± 0.16[a,†]	1.51 ± 0.23[a]	1.23 ± 0.14[a]		1.32 ± 0.16[a]	
22:6*	1.34 ± 0.15[a]	0.85 ± 0.12[b]	1.34 ± 0.15[a]		0.66 ± 0.11[c]	

Different letters in the row represent a significant ($p < 0.05$) difference.
†$p < 0.05$; 20:5* vs 18:3*.
The fatty acids denoted by an asterisk (*) were radiolabeled. For example, when unlabeled 18:3 was added to the incubation medium (+ 18:3), the uptake of labeled 18:2 (18:2*) was reduced from 1.47 ± 0.2 to 1.12 ± 0.15 nmol/100 mg min^{-1} ($p < 0.05$).

20:4ω6 was reduced by 18:0, and uptake of 22:6ω3 was reduced by 18:0 and 20:4ω6. The uptake of 20:5ω3 into control jejunum was greater than the uptake of 18:3ω3.

DISCUSSION

The inhibitory effect of EPA/DHA on the uptake of 10 and 20 mM glucose was obvious in the presence of BT, but not with SO. Furthermore, glucose uptake is different when EPA/DHA is given with SO than with BT, with higher rates of uptake from 5–20 mM glucose, and lower rates of uptake at 40 mM glucose (Table 5). Thus, the inhibitory effect of EPA/DHA on glucose uptake occurs only when this omega-3 fatty acid is fed with a saturated fatty acid diet.

The influence of feeding EPA/DHA on lipid uptake was also affected by the saturation of the lipid in the diet: for example, reductions in the uptake of 18:0, 20:5ω3 and cholesterol with EPA/DHA were seen only when the major dietary lipid was saturated beef tallow (Table 7). It is important to stress that jejunal and ileal cholesterol uptake was lower in animals fed SO with or without LNA or EPA/DHA, than in those fed BT (Table 7). Lipid uptake was similarly low in animals fed the polyunsaturated SO, with or without the addition of omega-3 fatty acid when compared with animals fed the saturated BT. Thus, the addition of an omega-3 fatty acid from vegetable or marine oil sources had a different effect on the jejunal or ileal uptake of lipids when the major dietary lipid was saturated beef tallow vs polyunsaturated safflower oil.

The fish oil used in this study was essentially free of cholesterol, and the beef tallow contained 0.2% (w/w) cholesterol, giving the following cholesterol contents of the diets: beef tallow, 0.036%; beef tallow plus linseed oil, 0.026%; and beef tallow plus fish oil, 0.026%. These values are lower than the cholesterol content of standard laboratory chow, 0.06%. The differences noted between the diets are likely, therefore, to be on the basis of variations in the ratio of omega-3 to saturated fatty acids.

The mechanism of the effect of omega-3 fatty acids on active and passive intestinal function has not yet been established. Although there were modest changes in intestinal dry weight or the percentage of the intestinal wall comprised of mucosa in animals fed SO with or without LNA or EPA/DHA vs animals fed BT (Table 2), these changes were quantitatively insufficient or qualitatively in the inappropriate direction to explain the variations in intestinal transport. Also, supplementing SO with LNA or EPA/DHA prevented the increase in mucosal surface area, as compared with BT. Furthermore, the differences in mucosal surface area did not explain the direction or magnitude of the alterations in nutrient uptake. There were no differences in food intake or weight gain that could explain the transport effects of feeding BT or SO. When stearic acid was present in the incubation medium along with 18:2ω6, 18:3ω3 or 20:4ω6, uptake was unchanged (Table 9). A similar lack of interaction was noted for LNA and EPA, so that it is unlikely that the influence of dietary supplementation on lipid uptake reported in the feeding studies (Tables 7 and 8) was due to an effect occurring in the lumen of the intestine. However, several combinations of the fatty acids were inhibitory, such as the reduction in jejunal uptake of 18:2ω6 by 18:3ω3 and 20:4ω6, reduction in the uptake of 20:4ω6 by 18:0, and the reduction in jejunal uptake of 22:6ω3 by 18:0 and 20:4ω6 (Table 9). For these three polyunsaturated fatty acids (18:2ω6, 20:4ω3 and 22:6ω3) it is likely that their partitioning from the micelle into the brush border membrane was impaired by the presence of other long-chain fatty acids. The influence of these diets on microsomal lipid metabolizing enzyme activities or brush border membrane lipid composition remains unknown, but we speculate that the transport perturbations observed in the dietary studies have their explanation on the basis of altered membrane bulk phase and boundary lipids.

We recently reported on the relative lack of effect of feeding omega-3 fatty acids on the intestinal uptake of lipids (8). From this study, the effect of an EPA/DHA-containing diet on blood cholesterol levels would not likely appear to be influenced by any change in intestinal cholesterol absorption. However, such a conclusion must be made with caution, because the effect of a small change in dietary LNA or EPA/DHA may be influenced by other dietary factors, such as the polyunsaturated fatty acid/saturated fatty acid (P/S) ratio of the major lipid constituents of the diet. We recently have demonstrated that dietary EPA/DHA or LNA modulate serum and liver cholesterol and fatty acid metabolism in a different manner when fed with saturated fatty acids or with linoleic acid (unpublished observations, 1988). Omega-3 fatty acids appear to be more effective in decreasing cholesterol content and 20:4ω6 levels. More omega-3 fatty acids accumulate when fed in combination with saturated fat (19, 20). The major findings of the present study support the previous speculation: the omega-3 fatty acids, LNA and EPA, do influence the uptake of most fatty acids, as well as cholesterol, but this inhibitory effect of the omega-3 fatty acids is demonstrable only when these lipids are fed together with a saturated diet (beef tallow), rather than with an unsaturated fatty acid diet (safflower oil). Clearly, there is an influence of omega-3 fatty acids on intestinal active and passive transport processes, but the impact of dietary enrichment with omega-3 fatty acids depends on the presence and interaction of other dietary constituents.

The major metabolite of omega-6 fatty acids is arachidonic acid (AA), whereas the major metabolite of omega-3 fatty acids is DHA. The lipooxygenase pathway for EPA/DHA, similar to AA, produces 12-hydroperoxy-EPA/DHA (21). However, unlike AA, under normal conditions EPA/DHA is a poor substrate for the cyclooxygenase alternative pathway, and therefore prostaglandins are not formed to any appreciable extent (22, 23). Because EPA/DHA yields no appreciable prostaglandins and inhibits the conversion of linoleic acid to arachidonic acid (24–26), it is likely that the intestinal brush border membrane in animals fed beef tallow may become sufficiently enriched with the saturated fatty acids that the small addition of omega-3 fatty acids in the form of LNA or EPA/DHA may possibly reduce the membrane AA content.

Although EPA/DHA-containing diets may reduce jejunal glucose uptake (8) and might, on this basis,

appear to be useful to prevent the enhanced absorption of glucose that occurs in diabetes mellitus, such an inhibitory effect of EPA/DHA on glucose uptake occurs for only certain concentrations of glucose and when the major dietary lipid is beef tallow (Table 5). Thus, although it has become clear that omega-3 fatty acids may be useful to alter intestinal transport function, and that their cholesterol-lowering effect may partially be due to a reduction in fatty acid and cholesterol uptake, it is clear that the intestinal effects of the omega-3 fatty acids varies between those from a vegetable vs a marine source (LNA vs EPA/DHA). Furthermore, the optimal P/S ratio permitting this antiabsorptive effect of omega-3 fatty acids to be maximized needs to be established.

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Influence of Omega-3 and Omega-6 Fatty Acid Sources on Prostaglandin Levels in Mice

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Studies from this laboratory, employing a hairless mouse model, have indicated that a polyunsaturated fatty acid source rich in omega-3 (n-3) fatty acid (FA) inhibits ultraviolet (UV)-carcinogenic expression, when compared to that of diets containing predominantly n-6 fatty acids. Omega-3 FA is a poor substrate for cyclooxygenase, the rate-limiting step in prostaglandin (PG) synthesis—the latter, particularly PGE_2, are known to influence tumor biology. Based upon this rationale, plasma and cutaneous PGE_2 levels were determined from hairless mice fed diets containing either 4% or 12% corn or menhaden oil. After two weeks on the respective diets, plasma PGE_2 levels of corn oil-fed animals were approximately 6-fold greater than those of the menhaden oil-fed groups. A similar response was found in the dermis. Although the relationship to carcinogenic expression is unknown, dietary n-3 FA content can have a pronounced effect upon PGE_2 levels and possesses the potential for influencing other immunomodulators.
Lipids 24, 502-505 (1989).

Several epidemiological studies implicate dietary factors, especially dietary lipid, as potentiators of certain cancers in man (1-4). Although definitive evidence for lipid involvement in cancer expression in man has not been forthcoming, the rationale upon which lipid is suspect derives from an impressive literature describing the effects of lipid on cancer expression in several animal models (5-12). This influence of dietary lipid upon carcinogenesis was first reported by Watson and Mellanby (13) in 1930, who demonstrated enhanced incidence of coal tar-induced murine skin tumors. Since that time, studies exploring the effects of level and qualitative composition of dietary lipid on carcinogenesis have generally found that diets containing high levels of fat or rich in polyunsaturated fatty acids (PUFA) result in more facile tumor development. These effects have been most often demonstrated for chemically-induced tumors of the skin, mammae, and intestine (11), although UV-carcinogenesis of the skin is similarly effected (14-16). The latter observation suggests that the influence of lipid on carcinogenesis represents a generalized and important response, but the mechanism(s) of this response remains unclear.

Recently it was shown that diets rich in eicosapentaenoic acid (EPA), an n-3 fatty acid, mitigated the tumorigenic enhancement demonstrated by other PUFA in both chemically-induced rat mammary tumors and in UV-induced skin tumors in hairless mice (17, 18). Others have shown inhibition of chemically-induced pancreatic neoplasms (19) and lack of promotion of intestinal cancers in animals fed diets rich in n-3 fatty acids (20).

Both n-3 and n-6 fatty acids (FAs), or possibly their relative levels, can influence the flux of metabolites through the cyclooxygenase and lipoxygenase pathways, and several products of these pathways influence tumor biology. Prostaglandins (PG), particularly of the 2-series, appear to act as tumor promoters, down regulate macrophage tumoricidal activities, and inhibit IL-2 production (21-23). Increased PGE_2 levels have been associated with aggressive growth patterns of both basal and squamous cell skin carcinomas in humans (24).

Among the products of the lipoxygenase pathway, 12- and 15-hydroxyeicosatetranoic acid (12- and 15-HETE) inhibit tumor growth *in vitro* in one model (25), while the leukotrienes are chemotactic for macrophages (26) and may be involved in immunosurveillance. The n-3 fatty acids, by competing for binding sites on cyclooxygenase, may inhibit this enzyme or shunt potential PG precursors through the lipoxygenase path.

Based on the rationale that PGs play a role in potentiation of carcinogenic expression, and using a protocol identical to that in which an omega-3 FA source had been shown to mitigate UV-tumorigenesis, we sought to determine whether dietary sources of omega-3 and omega-6 FAs could influence plasma and cutaneous PGE_2 levels.

MATERIALS AND METHODS

Animals and diets. Female SKH-Hr-1 hairless mice, approximately 4-5 months of age, were employed in the study. The animals were bred from stock obtained from the Skin and Cancer Animal Colony, Temple University, Philadelphia, PA. Animals were fed a closed formula ration (Wayne Lab-Blox, Continental Grain Co., Chicago, IL) prior to initiation of the protocol. Two weeks prior to the start of irradiation, mice were randomized and placed on semisynthetic powdered diets containing as a fatty acid source either 4 or 12 percent corn oil, or 4 or 12 percent menhaden oil (Table 1). Menhaden oil was a gift from Zapata Haynie Corp., Reedville, VA. Due to rapid peroxidation of menhaden oil-containing diets, it is necessary to store them under N_2 or Argon at −20°C. Animals were fed *Ad libitum* and housed 4-6 animals/cage under 12 hour light-dark photoperiods at 21-23°C. All treatment procedures and care of animals were conducted in strict compliance with NIH guidelines in an AAALAC accredited facility.

Irradiation. Animals received irradiation 5 days per week from non-filtered General Electric UA-3 mercury arc lamps with principal emission lines at 254, 265, 280, 313, and 365 nm. The mice were irradiated unrestrained in their cages in an irradiation chamber

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Abbreviations: AAALAC, American Association for Accreditation of Laboratory Animal Care; PBS, phosphate buffered saline.

TABLE 1

Experimental Diets[a]

	1	2	3	4
Casein	27.4	27.4	27.4	27.4
Corn oil	4.0	—	12.0	—
Menhaden oil	—	4.0	—	12.0
Mineral mix[b]	6.0	6.0	6.0	6.0
Vitamin mix[c]	2.2	2.2	2.2	2.2
Corn starch	55.9	55.9	38.0	38.0
Celufil	4.5	4.5	14.4	14.4
Kcal/Gm	4.0	4.0	4.0	4.0

[a](% Weight). All dietary constituents, except menhaden oil, were obtained from United States Biochemical Corp., Cleveland, OH.
[b]United States Biochemical Corp.; Phillips and Hart salt mixture.
[c]United States Biochemical Corp.; Vitamin mix minus ascorbic acid and tocopherol.
Provides ≅20 units of Vitamin A, as the acetate, per gram of diet.

of special design (27). Total energy emitted from the lamps was mapped for five areas representing different sites within the 18.5 × 32.0 cm cages and the mean used to calculate dosage. The total energy was measured each week with a calibrated thermopile attached to a Keithley microvolt ampmeter. An initial suberythemic daily dose of 0.382 J/cm^2 was delivered. The dose was increased by 25% every two weeks to compensate for epidermal thickening.

Sample preparation. Animals were last irradiated 24-28 hours prior to sample collection, as it was felt that a measure of chronic, as opposed to acute UV-induced, changes in prostaglandin levels would be more representative of the tumor environment and would in addition be less sensitive to spurious fluctuations. Samples were collected at two-week intervals during the study.

Under halothane anesthesia, blood was drawn via cardiac puncture into a syringe containing approximately 0.1 volume of PBS, indomethacin (0.7 mg/ml), and EDTA (50 mM). The blood was centrifuged at 700*g* for 10 min. and the plasma stored in liquid nitrogen until radioimmunoassays were performed. After the blood was drawn, cervical dislocation was performed and a dorsal flap of skin removed. Epidermis and dermis were separated as previously described (28), and were either homogenized immediately (epidermis) in PBS containing indomethacin or were frozen at −20°C (dermis) and likewise homogenized at a later date. All homogenates were stored in liquid nitrogen until assayed. Aliquots of the homogenates were analyzed for protein content using the Lowry technique (29).

Radioimmunoassay and data analysis. Prostaglandin E_2 has a very short half-life *in vivo*, and hence direct measurements of its concentrations would be subject to transient fluctuations of a significant degree and require the measurement of very low levels. Bothwell *et al.* (30) have developed an assay of a stable bicyclic derivative of PGE_2 and its long-lived metabolites which circumvents these problems. Using a commercially available kit based upon this assay (TRK-800, Amersham, Arlington Heights, IL), the levels of this bicyclic derivative were measured in the plasma and cutaneous homogenates. Hereafter, it is this bicyclic derivative to which we refer as PGE_2. Standard curves were constructed from a weighted least-squares regression of the log-log transformed data. Comparisons of mean PGE_2 values were made using the two-tailed *t*-test, with significance based upon p-values of 0.05 or smaller.

RESULTS

Plasma PGE_2 levels. The concentrations of PGE_2 found in the plasma of mice fed the twelve percent corn oil and menhaden oil diets are shown in Figure 1. It can be seen that after two weeks on the diets, before irradiation was begun, there was an approximately 6-fold difference in the concentrations between these two groups. The levels found on the respective diets generally persisted over the six-week irradiation period, although due to small sample size the differences were not significant at eight weeks.

Results of PGE_2 analysis for the 4% and 12% corn and menhaden oil diets, with and without UV-irradiation and pooled across the eight-week feeding period, are shown in Figure 2. There were no increases in PGE_2 levels within a dietary group as a result of UV-exposure, nor were there differences between irradiated animals fed 4% or 12% diets within a dietary

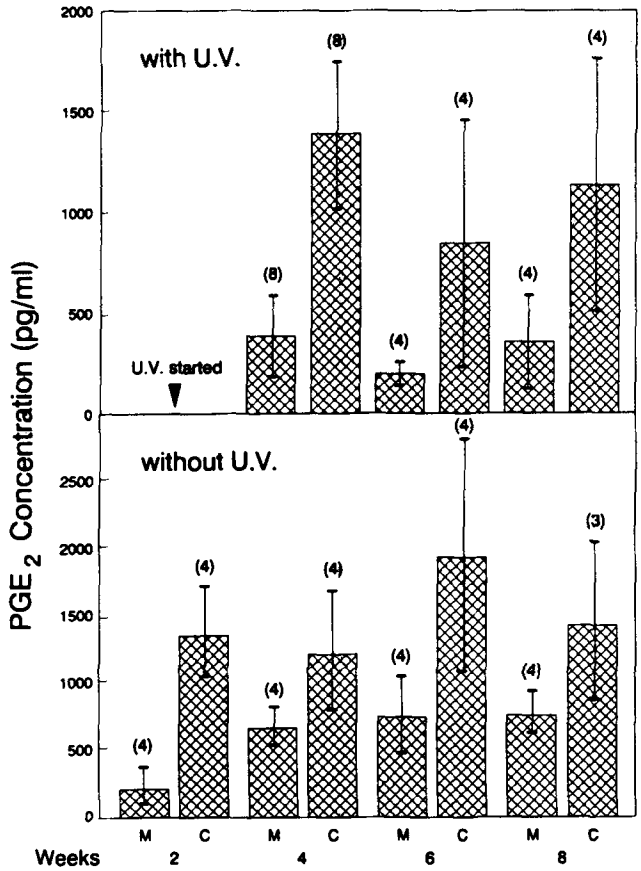

FIG. 1. Concentration of PGE_2 in the plasma of mice fed either 12% corn oil diets (C) or 12% menhaden oil diets (M), with and without UV-irradiation. The number of animals per treatment group is shown above the respective bar. Error bars represent the 95% confidence interval.

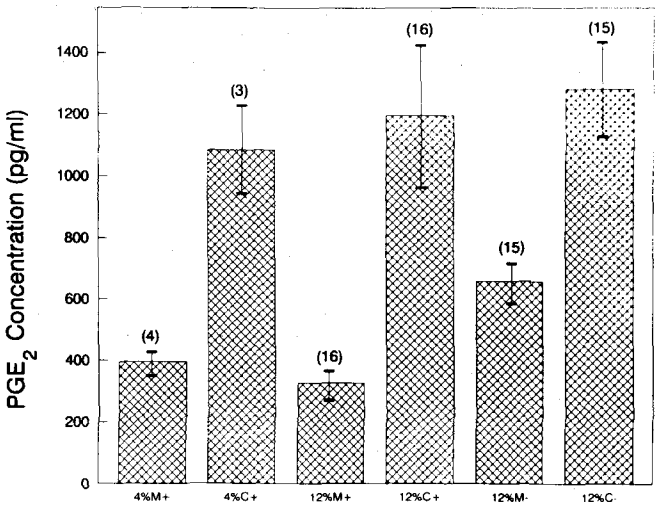

FIG. 2. Concentration of PGE_2 in the plasma of mice fed either 4 or 12% corn oil (C) or menhaden oil (M) diets, with (+) or without (−) UV-irradiation. The data have been pooled across the 8-week feeding period. The number of animals per treatment group is shown above the respective bar. Error bars represent the 95% confidence interval.

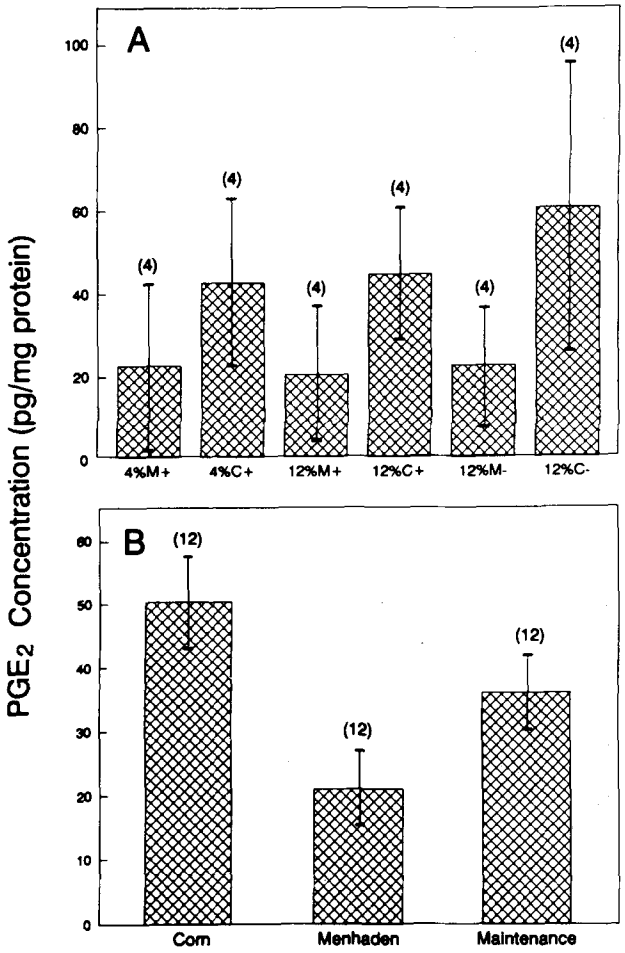

FIG. 3. A. Concentration of PGE_2 in the dermis of mice fed either 4 or 12% corn oil (C) or menhaden oil (M) diets, with (+) or without (−) UV-irradiation. The data have been pooled across the 8-week feeding period. B. Concentration of PGE_2 in the dermis. The data have been pooled within each dietary group. Maintenance diet represents a commercial closed-formula ration containing 4.5% soybean oil. The number of animals per treatment group is shown above the respective bar. Error bars represent the 95% confidence interval.

type. However, there are large differences, nearly 3-fold, between animals receiving corn oil versus animals receiving menhaden oil, regardless of irradiation or the dietary fat levels tested.

Dermal PGE_2 levels. The concentrations of PGE_2 found in the dermis are shown in Figure 3A,B. In Figure 3A there is a similar pattern to that shown in the plasma, namely menhaden oil-fed animals had lower PGE_2 concentrations than corn oil-fed animals, and showed no effect of UV-exposure. The 95% confidence intervals of these groups overlap, however. When results within a dietary type (Fig. 3B) are pooled, i.e., pooling 4% and 12% dietary lipid levels, a significant 2.5-fold increase in PGE_2 concentrations in corn oil- versus menhaden oil-fed animals is demonstrated.

Epidermal PGE_2 concentrations varied from 0.5 to 12 pg/mg-protein, but did not show any clear trends with respect to diet or UV-exposure.

DISCUSSION

In this study the effect of isocaloric diets of differing n-3 and n-6 fatty acid compositions on cutaneous and plasma PGE_2 levels was determined in a hairless mouse model in which mitigation of UV-carcinogenesis by diets rich in n-3 fatty acid had previously been demonstrated. We have shown marked decreases in PGE_2 levels, averaging nearly 3-fold, in the plasma from menhaden oil-fed animals versus those receiving diets containing corn oil. Similar (approximately 2.5-fold) decreases in dermal PGE_2 concentrations were also observed.

In addition to the effects on photocarcinogenesis, Orengo *et al.* (18) demonstrated that 12% menhaden oil diets inhibit UV-mediated inflammatory responses such as edema and erythema. As PGE_2 is known to be a mediator of these UV responses, this observation indirectly supports the contention of dietary modulation of PG levels. The present study did not show any PGE_2 increase in response to UV irradiation, suggesting that the inhibition of inflammatory response may have been due to a decreased PGE_2 level, per se, rather than a differential response to UV. Alternatively, any acute PGE_2 response to UVR may have eluded our sampling protocol, in which 24-28 hours elapsed between the last UV exposure and sample collection. Similarly, the decreased tumor multiplicity and increased tumor latency period reported earlier from animals on 4 and 12% menhaden oil diets may have been due to a decreased constitutive PGE_2 concentration rather than a differential PGE_2 response to UV. Such may be the case in those models showing lack of promotion of intestinal cancer from diets rich in EPA (20) or retardation of chemically-induced rat mammary cancer with indomethacin (31).

Only low levels of n-6 FAs are present in menhaden oil. Whether the observed effect on PGE_2 levels elicited by this dietary lipid source is related to essential fatty acid (EFA) deficiency, metabolic competition between n-6 and n-3 FAs, a specific effect of n-3 FA, or a combination of these possibilities, is unknown. However, inhibition of inflammatory responses to UV were reported to occur in animals fed diets containing 12% menhaden oil (18), a level in which the minimum required EFAs for mice are present. This, along with the current observation that plasma and dermal PGE_2 levels of animals fed diets containing either 4% or 12% menhaden oil did not significantly differ, suggests that dietary EFA deficiency cannot adequately explain our results.

These results on PGE_2 levels do not rule out the possible contribution of other products of prostanoid metabolism to tumor expression, and can perhaps best be considered as a marker of differential flux through these pathways as a result of dietary manipulation, the details of which are still being elucidated. Nevertheless, it is clear that dietary n-3 FA has a pronounced effect upon PGE_2 levels and possesses the potential for influencing a wide-range of other immunomodulators.

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The Combined Effects of Dietary Proteins and Fish Oil on Cholesterol Metabolism in Rats of Different Ages

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Male Sprague-Dawley rats at the ages of four weeks and nine months were fed purified diets containing 20% proteins either as casein (CAS), milk whey protein (WHY), or soybean protein (SOY) with 5% sardine oil for four weeks. The hypocholesterolemic effect of SOY was not statistically evident as compared to milk proteins at both ages, although serum cholesterol tended to be low in the SOY groups. A significant age-dependent increase in serum cholesterol was observed in all dietary groups. Liver cholesterol concentrations were comparable in young rats, whereas in adults they were significantly lower in the SOY than in the CAS or WHY groups. At both ages, the activity of liver 3-hydroxy-3-methylglutaryl coenzyme A reductase tended to be higher in the SOY than in the other groups. Fecal steroid excretion was significantly higher in rats fed SOY than those fed either CAS or WHY, especially in adult rats. Significant age- and dietary protein-effects were observed in fatty acid profiles of liver microsomal phospholipids. Thus, the effects of dietary proteins on various lipid parameters were essentially maintained even when fish oil served as the source of dietary fat.
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The importance of dietary components in regulating cholesterol metabolism is well known (1). Aging is an irreversible phenomenon which causes an increase in the serum cholesterol level. Consequently, the incidence of coronary heart disease is more frequent in aged than in young people (1-3).

It has been shown in experimental animals that soybean protein compared to casein has a potent serum cholesterol-lowering effect (4, 5). However, the effects of dietary proteins on cholesterol metabolism have been studied exclusively using n-6 polyunsaturated fatty acids (PUFA) as a fat source, irrespective of the observation that n-3 PUFA exert a considerable hypolipidemic action as compared to n-6 PUFA (6, 7). Sugano *et al.* (8) recently demonstrated the interaction of dietary proteins and fats on lipid metabolism in young rats, indicating the importance of fat type in determining the effect of dietary protein on cholesterol metabolism. Moreover, we previously observed that susceptibility of serum and liver cholesterol to dietary manipulation is modulated by age (7, 9-11). Since dietary fat influences the effect of dietary protein on serum cholesterol, this interaction may also be modulated by age. It is also known that fish oil, soybean protein, and age interfere with the desaturation of linoleate (7, 8). However, the combined effect of these factors on fatty acid metabolism has not been evaluated. In this context, we examined the influence of dietary proteins and fish oil on lipid metabolism in rats of different ages.

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Abbreviations: PUFA, polyunsaturated fatty acids; SOY, soybean protein; WHY, milk whey protein; CAS, casein; PC, phosphatidylcholine; HMG-CoA reductase; 3-hydroxy-3-methylglutaryl coenzyme A reductase.

MATERIALS AND METHODS

Animals. Young (three weeks old) and adult (nine months old) male Sprague-Dawley rats, obtained from Seiwa Experimental Animal Co., Fukuoka, Japan, were housed individually in a room with controlled temperature (20-23°C) and light (8 AM to 8 PM). After acclimation for one week, the rats were divided into three groups of six each and fed *ad libitum* one of the following three experimental diets containing either casein (CAS), milk whey protein (WHY) or soybean protein (SOY) as the protein source. After four weeks, the rats were killed at night (1 AM) by decapitation. Five days before killing, feces were collected for two days, and then lyophilized. The diets were prepared according to guidelines recommended by the American Institute of Nutrition (12) and contained by weight (%): dietary protein 20.0, sardine oil 5.0, mineral mixture 3.5, vitamin mixture 1.0, choline bitartrate 0.2, DL-methionine 0.3, cellulose 5.0, corn starch 15.0, and sucrose to 100. The fatty acid composition of the sardine oil, kindly provided by Nihon Fat and Oil Co., Tokyo, Japan, was by weight (%): 14:0, 5.6; 16:0, 16.4; 16:1, 7.5; 18:0, 3.2; 18:1, 15.9; 18:2n-6, 1.6; 18:4n-3, 3.1; 20:1, 7.7; 20:4n-6, 1.3; 20:5n-3, 14.8; 22:1, 6.7; 22:5n-3, 1.9; 22:6n-3, 10.3 and unknowns 4.0. The fish oil contained alpha-tocopherol (200 mg/kg) which was added as an antioxidant and 0.56 % cholesterol. The AIN vitamin and mineral mixtures were obtained from Nihon Nosan Kogyo Co., Kanagawa, Japan. Diets were prepared weekly and stored in the dark with oxygen absorbent at 5°C (7). The animals were provided with fresh food every day.

Analyses. The activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase of liver microsomes was measured as previously described (9). Serum, liver, and microsomal lipids were extracted, while cholesterol, triglyceride, and phospholipid were measured (13). Serum peroxide was determined by fluorometry according to the method of Yagi (14). Microsomal phosphatidylcholine (PC) was separated by thin-layer chromatography using chloroform/methanol/water (65/25/4, v/v/v) as a developing solvent. The fatty acids of PC were methylated in boron trifluoride/methanol, and analyzed by gas-liquid chromatography using a SILAR 10C column (15). Fecal neutral and acidic steroids were analyzed as previously reported (7). Microsomal protein was determined with the method used by Lowry *et al.* (16).

Data were analyzed using either the Student's t-test to examine the age-effect between the same dietary groups or by using a one-way analysis of variance

followed by the Duncan's multiple range test to examine all differences in the same age-group.

RESULTS

There was no significant difference in food intake, weight gain, and relative liver weight (g/100 g body weight) among age-matched rats fed different protein sources (data not shown).

Table 1 summarizes the concentration of serum lipids of young (four weeks old) and adult (nine months old) rats fed different proteins for four weeks. The concentration of serum cholesterol was comparable in age-matched rats, although it tended to be lower in the SOY than in the CAS and WHY groups. However, serum cholesterol increased significantly with age in all groups of rats. No significant protein-dependent differences were found in concentrations of triglyceride and phospholipid in age-matched rats, and a significant age-dependent increase was observed only in rats fed CAS. There were no significant diet- or age-dependent differences in concentrations of serum lipid peroxides.

Table 2 summarizes the concentration of liver lipids in young and adult rats fed different proteins. There was no significant difference in liver cholesterol concentrations in young rats, whereas in adult rats they were significantly higher in the CAS than in the SOY group, with the WHY group in the intermediate range. Thus, the age-related increase in liver cholesterol was observed in the two groups of rats fed animal proteins. The liver triglyceride level tended to be lower in the SOY than in the CAS and WHY groups, and a significant age-related increase was observed only in the latter two groups. The concentration of liver phospholipid remained unchanged regardless of diet or age.

Table 3 shows the activity of liver HMG-CoA reductase in young and adult rats fed different proteins. The reductase activity tended to be higher in the SOY than in the two animal protein groups. The activity was lower in the adult than in the young rats, although the difference was not significant.

Table 4 shows the fecal steroid excretion in young and adult rats fed different proteins. Feces were collected for two days, beginning five days before killing. The weight of dried feces was comparable in young rats, whereas in adult rats it was heavier in the SOY than in the CAS groups. The WHY group was intermediate in regards to fecal weight. Acidic steroid excretion was markedly higher in the SOY than in the two animal protein groups regardless of age, and it increased significantly with age. However, when daily acidic steroid excretion was expressed on the basis of unit body weight, no substantial increase in relation to age was observed (young–0.60 ± 0.11, 0.68 ± 0.11 and 1.35 ± 0.07; and adult–0.68 ± 0.11, 0.63 ± 0.10 and 1.03 ± 0.11 for CAS, WHY, and SOY mg/day/kg body weight, respectively). Neutral steroid excretion was comparable in young rats, whereas in adult rats it was significantly higher in the SOY than in the CAS

TABLE 1

Serum Lipid Concentrations in Young and Adult Rats Fed Different Proteins

		Cholesterol	Triglyceride	Phospholipid	Lipid peroxide
		(mg/100 ml)	(mg/100ml)	(mg/100ml)	(nmol/ml)
Young	CAS	84.7 ± 6.7	111 ± 6	183 ± 8	20.3 ± 4.7
	WHY	89.7 ± 10.2	95.8 ± 7.1	194 ± 18	13.5 ± 2.3
	SOY	72.4 ± 6.4	127 ± 16	185 ± 12	20.7 ± 3.4
Adult	CAS	137 ± 7*	168 ± 23*	251 ± 29*	13.8 ± 3.1
	WHY	131 ± 9*	123 ± 24	237 ± 9	15.3 ± 3.7
	SOY	105 ± 11*	128 ± 20	224 ± 27	13.5 ± 3.8

Values are mean ± SE of six rats.
*Significantly different ($p < 0.05$) from corresponding young rats.

TABLE 2

Liver Lipid Concentrations in Young and Adult Rats Fed Different Proteins

		Total cholesterol	Triglyceride	Phospholipid
			(mg/g)	
Young	CAS	3.09 ± 0.41	12.8 ± 2.9	38.2 ± 1.1
	WHY	2.81 ± 0.17	9.95 ± 1.56	37.7 ± 0.7
	SOY	2.80 ± 0.56	7.95 ± 2.70	37.9 ± 1.0
Adult	CAS	4.65 ± 0.38^{a}*	28.6 ± 2.8*	36.2 ± 0.8
	WHY	4.18 ± 0.48^{ab}*	34.2 ± 12.0*	36.2 ± 1.6
	SOY	2.85 ± 0.15^{a}	17.8 ± 5.6	36.9 ± 1.4

Values are mean ± SE of six rats.
^{ab}In age-matched rats, values in the same column without common superscript letters denote a significant difference ($p < 0.05$).
*Significantly different ($p < 0.05$) from the corresponding young rats.

TABLE 3

The Activity of Liver HMG-CoA Reductase in Young and Adult Rats Fed Different Proteins

Dietary N source	Enzyme activity	
	Young	Adult
	(pmol/min/mg microsomal protein)	
Casein	110 ± 18	78.7 ± 16.0
Whey protein	133 ± 36	80.4 ± 18.6
Soybean protein	192 ± 47	129 ± 35

Values are mean ± SE of six rats.

groups. The age-dependent decrease in neutral steroid excretion was observed in the CAS groups. From these results, total amounts of steroids excreted were significantly higher in the SOY than in the two animal protein groups, and the excretion increased significantly with age in the SOY group.

Table 5 shows the fatty acid composition of liver microsomal PC in young and adult rats fed different proteins. There was a significant age-effect in the fatty acid composition in all dietary groups. Generally, the percentage of n-6 fatty acids increased significantly whereas that of n-3 fatty acids decreased with age. In young rats, the percentage of 18:2n-6 was higher in the SOY than the CAS and WHY groups, whereas that of 20:4n-6 was comparable. In contrast, in adult rats the percentage of 18:2n-6 was comparable, whereas that of 20:4n-6 was higher in rats fed CAS or WHY than in those fed SOY. Therefore, the ratio of 20:3 plus 20:4 to 18:2 was higher in the two milk protein groups as compared to SOY, and it decreased with age in all groups. The percentage of 20:5n-3 was highest in the WHY in the young rats, whereas it was comparable in the adult rats. The percentage of 22:5n-3 and 22:6n-3 was not influenced by the type of dietary protein, although it decreased with age.

DISCUSSION

The responsiveness of cholesterol homeostasis in the liver to dietary cholesterol or cholestyramine appears to diminish with age, whereas the susceptibility to dietary fat type tends to increase with age (7, 9-11).

In rats, the hypocholesterolemic effect of SOY is evident especially when fed a cholesterolemic diet (4, 17, 18). In the present study, serum cholesterol did not

TABLE 4

Fecal Steroid Excretion in Young and Adult Rats Fed Different Proteins

		Fecal weight (g/day)	Acidic steroids (mg/day)	Neutral steroids (mg/day)	Total steroids (mg/day)
Young	CAS	1.69 ± 0.09	1.83 ± 0.20^{a}	8.13 ± 0.46	9.96 ± 0.44^{a}
	WHY	1.61 ± 0.10	2.00 ± 0.30^{a}	7.04 ± 0.65	9.04 ± 0.46^{a}
	SOY	1.73 ± 0.12	4.09 ± 0.26^{b}	8.47 ± 0.36	12.6 ± 0.4^{b}
Adult	CAS	1.62 ± 0.13^{a}	4.85 ± 0.84^{ab*}	5.15 ± 0.51^{a*}	10.0 ± 1.1^{a}
	WHY	2.11 ± 0.11^{ab*}	4.38 ± 0.64^{a*}	6.57 ± 0.69^{ab}	11.0 ± 1.1^{a}
	SOY	2.38 ± 0.24^{b*}	7.37 ± 0.72^{b*}	8.73 ± 0.76^{b}	16.1 ± 1.3^{b*}

Values are mean ± SE of six rats.

^{ab}In age-matched rats, values in the same column without common superscript letters denote significant difference ($p < 0.05$).

*Significantly different ($p < 0.05$) from the corresponding young rats.

TABLE 5

Fatty Acid Composition of Liver Microsomal Phosphatidylcholine in Young and Adult Rats Fed Different Proteins

	Young			Adult		
	CAS	WHY	SOY	CAS	WHY	SOY
	Weight (%)			Weight (%)		
16:0	25.6 ± 0.6	25.7 ± 0.6	25.0 ± 0.6	20.9 ± 0.7^{ab*}	20.5 ± 0.4^{a*}	22.5 ± 0.3^{b*}
16:1	3.3 ± 0.2	3.3 ± 0.2	3.3 ± 0.2	2.4 ± 0.2	2.5 ± 0.2	3.2 ± 0.3
18:0	19.6 ± 0.7	19.8 ± 0.5	19.3 ± 0.6	23.4 ± 0.7*	23.1 ± 1.0*	20.9 ± 0.8
18:1	10.2 ± 0.6	9.1 ± 0.3	9.5 ± 0.3	7.3 ± 0.2*	7.7 ± 0.3*	7.7 ± 0.3*
18:2	1.5 ± 0.1^{a}	1.2 ± 0.1^{b}	2.8 ± 0.1^{c}	7.3 ± 0.5*	6.9 ± 0.4*	7.0 ± 0.4*
20:3n-6	0.5 ± 0.0	0.5 ± 0.1	0.7 ± 0.0	1.7 ± 0.1*	1.5 ± 0.3*	1.2 ± 0.1*
20:4n-6	7.3 ± 0.4	7.3 ± 0.3	8.0 ± 0.3	17.5 ± 0.6^{a*}	16.9 ± 0.4^{a*}	14.5 ± 0.5^{b*}
20:5n-3	12.3 ± 0.4^{a}	14.1 ± 0.3^{b}	10.6 ± 0.4^{c}	7.1 ± 0.5*	8.2 ± 0.5*	8.7 ± 0.6*
22:5n-3	3.9 ± 0.2	4.2 ± 0.2	4.4 ± 0.2	1.7 ± 0.1^{a*}	1.7 ± 0.2^{a*}	2.5 ± 0.1^{b*}
22:6n-3	14.5 ± 0.6	13.7 ± 0.7	15.0 ± 0.5	9.8 ± 0.4*	9.6 ± 0.4*	10.6 ± 0.3*
20:3 + 20:4/18:2	5.2 ± 0.4^{a}	6.8 ± 0.4^{b}	3.1 ± 0.1^{c}	2.7 ± 0.1^{a*}	2.7 ± 0.2^{a*}	2.3 ± 0.1^{b*}

Values are mean ± SE of six rats. Fatty acids less than 1% are excluded.

^{abc}In age-matched rats, values in the same line without common superscript letters denote significant difference ($p < 0.05$).

*Significantly different ($p < 0.05$) from the corresponding young rats.

respond markedly to the type of dietary protein. In contrast, Sugano *et al.* (8) showed that when young rats were fed CAS, the serum cholesterol concentration was higher compared to those fed SOY, irrespective of the source of fats rich in n-6 PUFA. It is possible that the cholesterol-lowering effect of SOY is concealed to some extent by the potential hypocholesterolemic effect of fish oil in the present diets (6, 7). There was an age-related increase in the serum cholesterol concentration in all groups, whereas the age-effect in liver cholesterol was not observed in rats fed SOY. It is likely that when the rats were fed fish oil, that the SOY, as compared with the CAS, depresses the age-dependent increase in the liver cholesterol pool (7). Rats fed CAS and WHY had similar cholesterol concentrations in contrast to the finding reported by Sautier *et al.* (19).

Dietary protein did not influence the serum peroxide concentration in either age of rats. These results are in agreement with those reported by Nagata *et al.* (17) for young rats. However, the values observed in the present study were about ten times higher than those previously reported (17). In addition, an age-related increase in the concentration of lipid peroxide was not found in the present study, in contrast to observations made using various tissues including brain, liver, heart, kidney, and adrenals (14, 20). Thus, it is suggested that although the diets were handled carefully, the effect of fish oil on the increase in peroxide value is greater than that of aging. In this respect, fish oil diets require great care, as pointed out by Fritsche and Johnston (21). The increase in lipid peroxides in the blood may produce vascular damage (14).

There are contradictory data regarding the response of HMG-CoA reductase activity to dietary proteins (4, 22). In the present study, the reductase activity tended to be higher in rats fed SOY than in those fed two animal proteins at both ages. The increased cholesterol synthesis in rats fed SOY might be secondary to enhanced fecal steroid excretion (4). We have observed a lower reductase activity in young rats fed SOY than in those fed CAS. This trend was accompanied by a significant decrease in serum cholesterol concentration when corn oil was used as the dietary fat source. These proteins did not influence the reductase activity as well as the serum cholesterol concentration in adult rats. In another study, the reductase activity in rats fed SOY was higher than that in those fed CAS when the 1% corn oil was used (23). The discrepancy can be attributed to the difference in the quantity and the kind of dietary fat source. However, it is possible that the hypocholesterolemic effect of soybean protein is determined at least in part by the response of the cholesterol synthetic pathway.

A marked increase in fecal steroid excretion was observed in rats fed SOY as compared to CAS. A similar observation was reported by Park *et al.* (24). In addition, our results suggest that dietary manipulation to lower the cholesterol pool is more effective in adult than in young animals, since the magnitude of the reduction appeared to be greater in the former. It is possible that extensive modification of cholesterol metabolism by dietary means in adults may be related to degeneration of the homeostatic capacity with age. Previously, we showed that cholestyramine effectively decreased liver cholesterol in the adult but not in the young rats (10).

The fatty acid composition of microsomal PC reflected the fat ingested, especially in young rats. Interestingly, the percentage of n-3 PUFA was higher in the young rats, whereas that of n-6 PUFA was higher in the adults. This suggests an age-related change in turnover of tissue fatty acids (25). Although the eicosapentaenoate competes with linoleate for Δ6-desaturation (7, 26), the effect of dietary protein on the fatty acid desaturation system was not concealed by sardine oil. This indicates a remarkable effect of SOY on the metabolism of linoleate (8, 27). The combined use of SOY and fish oil in adult rats may accelerate the reduction of the desaturation of linoleate.

In summary, the effect of dietary protein on the serum cholesterol concentration was not evident when fish oil was fed as a fat source. However, SOY markedly lowered liver cholesterol in adult rats. The results suggest that a characteristic action of fish oil on cholesterol metabolism in relation to age may be modulated by dietary protein.

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Fatty Acid Composition of Umbilical Arteries and Veins: Possible Implications for the Fetal EFA-status

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Fatty acid compositions were determined of phospholipids, isolated from umbilical arteries and veins, obtained from Dutch neonates after vaginal delivery, terminating normal pregnancy. The fatty acid profiles of the cord vessels were characterized by the absence of eicosapentaenoic (timnodonic) acid, a low (2–3%) content of linoleic acid and reasonable amounts of arachidonic acid (10–15%) and docosahexaenoic (cervonic) acid (3–5%). Significant amounts of Mead acid (1–4%) and its direct elongation product (0.5–2%) were also observed. In each cord, the efferent blood vessels contained significantly more Mead acid and other fatty acids of the oleic acid (n-9) family and less fatty acids of the linoleic (n-6) and linolenic (n-3) families than the afferent blood vessel. This indicates that the essential fatty acid (EFA) status of 'downstream' neonatal tissue may be marginal. No signs of EFA-deficiency were observed in endothelial and smooth muscle cells in culture, or in blood vessels from adults. In all cords 22:5(n-6) was significantly higher in the artery compared to the vein, whereas for all other (n-6) fatty acids this difference was negative. Since the synthesis of 22:5(n-6) is known to be stimulated when the required amount of cervonic acid, 22:6(n-3), is too low, our observations also suggest that the cervonic acid status of the neonates investigated was not optimal. Further studies are in progress to relate these findings to maternal EFA status and complications of pregnancy.
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The essential fatty acid supply and return to and from the fetus is via the umbilical vein and arteries, respectively. The tissue of these blood vessels has no other way to obtain essential fatty acids than from the blood running through them. As a result, the vein and arteries of the umbilical vessels are in a situation where they may reflect the fatty acids present in the blood, and indicate the essential fatty acid status of the fetus.

We have compared the fatty acids of the phospholipids of umbilical arteries and veins of Dutch neonates, born after normal pregnancy, in order to assess the essential fatty acid status of the fetus; this information may be particularly relevant with regard to the production of eicosanoids from vascular tissue, which is dependent on the availability of precursor fatty acids (1, 2).

In order to be sure that any differences found did not just represent normal differences in the synthesis or degradation of fatty acids by the cell types of the arteries and veins, the fatty acid patterns of cultured arterial and venous endothelial and smooth muscle cells, cultured in a similar media, were evaluated. Moreover, data were collected for adult arteries and veins serving the same area.

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Abbreviations: EFA, essential fatty acid; TLC, thin layer chromatography; PUFA, polyunsaturated fatty acid.

MATERIALS AND METHODS

Vascular tissue and cells. Human umbilical cords were collected within one hour after normal delivery, terminating an uncomplicated pregnancy. The cords were rinsed with saline (NaCl, 0.9% w/v) and segments of about 5 cm were used to isolate both arteries and the vein. In some cases a sample of Wharton's jelly was collected also. The tissue was stored at −15°C until further treatment.

Surgical specimens of adult colonic arteries and veins, supplying the same area, were removed during partial colon resection performed in Maastricht. The tissue was rinsed with saline and stored at −15°C until further processing.

Arterial and venous endothelial and smooth muscle cells were isolated from umbilical cord vessels and cultured in RMPI 1640 medium (Gibco, Green Island, NY, USA) with 20% pooled human serum. After the cultures reached confluency, the monolayers were rinsed with phosphate-buffered saline. Subsequently, the cells were harvested using a rubber policeman, and washed several times with EDTA-containing saline (77mM EDTA + 45mM NaCl, pH 7.4). Mepacrine (Quinacrine, Sigma, St. Louis, MO, USA, Q-0250; 5mM) was added to prevent phospholipase activation during the collection and washing of the cells. Concentrated cell suspensions (35–50 × 10^6 cells) were stored at −15°C until analysis.

Lipid extraction. Immediately before lipid extraction, all vascular specimens and samples of Wharton's jelly were frozen in liquid nitrogen and pulverized in an aluminum mortar with a stainless steel pestle, previously cooled in liquid nitrogen. The pulverized samples were transferred into glass tubes precooled with liquid nitrogen, wetted with 2 ml methanol (Merck, Darmstadt, FRG, No. 6009), capped with a teflon-lined screw-cap and stored at −15°C until further analysis. All samples were allowed to warm up to room temperature, immediately before total lipid extraction, according to Bligh and Dyer (3). The lipid-containing fractions were taken up in a known volume of methanol/chloroform (Merck, 2445, mixture 1:1, v/v) and in some cases standard amounts were removed for measuring phospholipid class distribution (see below).

Isolation of phospholipids. Total phospholipids (PL) were isolated from the lipid extracts by thin layer chromatography (TLC) using TLC plates (20 × 20 cm) coated with a 0.5 mm thick layer of Silica gel 60 (Merck, 13894). Using a plate scriber, the plates were predivided into 6 lanes of 2 cm width and separated by 1 cm buffer zones to prevent contamination between adjacent lanes. The plates had been predeveloped, using a methanol/chloroform mixture 1:1, v/v). The lipid spots

were first developed with chloroform/methanol/water/acetic acid (Merck, 63) (10:10:1:1, v/v/v/v) until the solvent front had reached a level of 1.5–2 cm above the site of the application of the spot. After drying, petroleum ether (Merck, 1774)/diethylether (Merck, 923)/acetic acid (24:5:0.3, v/v/v) was used to develop the plate further. To separate the major PL classes—phosphatidyl choline (PC), phosphatidyl inositol + phosphatidyl serine (PI + PS), phosphatidyl ethanolamine (PE), sphingomyelin (SPH)— chloroform/methanol/acetic acid/H_2O (90:40:12:2, v/v/v/v) was used as the only developing solvent. The PL spots were visualized by spraying with Rodamine 6G (Merck 7600, 0.1% w/v in methanol), marked under UV-light (366 nm), scraped off, and collected into glass tubes.

Measurement of fatty acid composition of phospholipids. An amount of 1 ml boron trifluoride (BF_3, Sigma, B-1252, 14% w/v in methanol) was added to the gels and the tubes were screw-capped under a stream of nitrogen. Phospholipids were hydrolyzed and fatty acids methylated by placing the tightly closed tubes in a heating block at 100°C for 45 min (total PLs), 15 min (PE and PI + PS), 30 min (PC) or 90 min (SPH), as described by Morrison and Smith (4). After cooling, 1 ml of distilled water was added and the fatty acid methyl esters were extracted three times with 1 ml pentane (Merck 7288). The extracts were combined, evaporated under N_2 at 37°C, taken up in small amounts of iso-octane (Baker, 8206, J.T. Baker Chemicals, N.V., Deventer, the Netherlands), and analyzed by gas liquid chromatography (GLC) using an HP 5840A gas chromatograph, fitted with two glass columns (0.2 × 180 cm), 5% DEGS on Chromosorb® WHP 100/120 mesh (Chrompack, Middelburg, the Netherlands), and flame-ionisation detectors. The injection and detection temperatures were 250°C. The analyses were performed using temperature programming which had been optimized with a reference mixture containing most of the fatty acid methyl esters of interest. The standard for 22:5(n-6) was prepared from rat testis and that for 22:3(n-9) from blood platelets of EFA-deficient rats (1). The standard for 24:2(n-6) was obtained from blood platelets of rats that had been fed a high-linoleic acid diet for 10 weeks (1). All other standards were obtained from Chrompack and Unilever Research Vlaardingen, the Netherlands. The standard mixture was also used to identify the peaks (on the basis of retention times).

The starting temperature of the columns was 145°C. After 1 minute, the temperature gradually increased up to 190°C with a rate of 4°C/min. The flow rate of the carrier gas (N_2) was 20 ml/min. Using computer-assisted analysis, the fatty acid profiles were corrected for blank runs (1 for each series of 5 samples), originating from 'extraction' of saline, and were adjusted with respect to the internal standard, the methyl ester of 15:0. Results were expressed as area % of total fatty acids. Shortly after completing these measurements, a capillary gas chromatograph (PE 8320) was installed, fitted with a 50 m capillary column (WCOT fused silica, i.d. 0.25 mm, CP-Sil-88 0.20 μm, ex Chrompack). A subset of samples was rerun under these improved conditions. The results were essentially similar as those obtained with the packed columns. To prevent oxidation of the polyunsaturated fatty acids, the anti-oxidant BHT (butylated hydroxy toluene, 0.005% w/v, Sigma, B-1378) was added to all organic solvents.

Measurement of phospholipid class distribution. For the measurement of phospholipid class distribution, the various phospholipid classes were separated in the same way as described for the analysis of the fatty acid composition. The spots were visualized with iodine vapour, scraped off and collected into glass tubes. A solution of 0.5 ml $HClO_4$ 70% (w/v, Merck 519) was added and the phospholipids were destroyed by heating them to 180°C for 45 min to obtain inorganic phosphorus which was quantified according to the method described by Böttcher *et al.* (5). Phospholipid class distribution was calculated on the basis of the amounts of inorganic phosphorus determined.

Statistical analysis. Statistical analyses were performed using Student's t-test, paired and unpaired, as indicated when discussing the results.

RESULTS

Fatty acid compositions of total phospholipids of arteries, veins and Wharton's jelly, isolated from 5 Dutch cords, are given in Table 1. The jelly of Wharton contained only a relatively small amount of phospholipids and since the fatty acid composition compares very well with that of the vascular phospholipids, any residual jelly is unlikely to have affected the fatty acid composition of umbilical arteries and veins. Since in all studies both arteries from the same cords gave almost identical results, the values from each set of two measurements were averaged and treated as one figure. It should be noted that in these initial series no corrections for blank runs were made. A later series, however, demonstrated that this did not influence the results to any significant extent. From Table 1 it can be seen that striking differences exist between the umbilical vein, which is the afferent (supplying) fetal vessel, and the umbilical arteries, which are the efferent (= draining) fetal vessels, of the same cord. In general, the contents of (poly)unsaturated fatty acids of the (n-9) family are higher in artery than in vein phospholipids.

This particularly applies to Mead acid (20:3(n-9)) and dihomo Mead acid (22:3(n-9)), which are biochemical markers of essential fatty acid (EFA) deficiency (6,7). The levels of most polyunsaturated fatty acids of the (n-3) and (n-6) families are lower in the draining vessels (arteries) than in the supplying vessels (veins). There is one distinct exception to this later observation: 22:5(n-6). This ultimate desaturation and elongation product of the (n-6) family occurs significantly more in the efferent (arterial) than in the afferent (venous) phospholipids.

The respective percentages of phosphatidylinositol plus phosphatidyl serine were higher in arteries (9.6 ± 0.93%) than in veins (7.2 ± 1.13%) with an arterial-to-venous difference of 2.3 ± 0.24% (P_2 = 0.01, paired t-test). There were no significant differences in the percentages of phosphatidyl choline (artery = 41.6 ± 2.7%, vein = 46.2 ± 1.0%) phosphatidylethanolamine (artery = 24.5 ± 2.3%, vein = 23.6 ± 0.6%) or in sphingomyelin (artery = 24.4 ± 0.4%, vein = 22.9 ± 0.8%, all comparisons n = 3).

The various phospholipid classes contained the well-

TABLE 1

Fatty acid composition (%) of total phospholipids, isolated from vascular tissue and Wharton's jelly of umbilical cords (mean ± s.e.m.[a]; n = 5)

Fatty acid[b]	Artery (Efferent)	Vein (Afferent)	Wharton's jelly	E/A-difference[c] %	E/A-difference[c] P_2[d]
14:0	1.0 ± 0.07	0.9 ± 0.07	0.9 ± 0.08	0.1 ± 0.08	
15:0	0.7 ± 0.04	0.7 ± 0.06	1.4 ± 0.39	0.0 ± 0.03	
16:0 dma[e,f]	1.7 ± 0.32	2.2 ± 0.26	1.8 ± 0.43	−0.5 ± 0.43	
16:0	20.0 ± 0.62	21.6 ± 1.08	25.4 ± 1.49	−1.6 ± 0.48	0.029
16:1(n-7)	3.0 ± 0.27	2.4 ± 0.15	2.7 ± 0.10	0.6 ± 0.27	
?[g]	0.4 ± 0.02	0.4 ± 0.03	n.d.[h]	0.0 ± 0.03	
17:0	1.3 ± 0.05	1.3 ± 0.11	1.4 ± 0.23	0.0 ± 0.09	
18:0 dma[f] + ?	2.0 ± 0.25	2.4 ± 0.14	1.6 ± 0.22	−0.4 ± 0.28	
18:0[i]	14.8 ± 0.22	15.3 ± 0.69	13.2 ± 0.51	−0.5 ± 0.66	
18:1(n-9)	13.5 ± 0.63	9.9 ± 0.54	13.8 ± 0.68	3.6 ± 0.57	0.003
18:2(n-6)	1.7 ± 0.08	2.3 ± 0.16	2.9 ± 0.13	−0.6 ± 0.16	0.015
20:0	0.5 ± 0.05	0.5 ± 0.05	0.5 ± 0.05	0.0 ± 0.03	
20:1(n-9)	0.7 ± 0.07	0.5 ± 0.06	0.6 ± 0.07	0.2 ± 0.07	0.037
20:2(n-9)[f]	1.1 ± 0.09	0.8 ± 0.34	1.0 ± 0.20	0.3 ± 0.31	
20:3(n-9)	2.4 ± 0.20	0.5 ± 0.06	0.9 ± 0.23	1.9 ± 0.19	0.001
20:3(n-6)	1.3 ± 0.06	1.7 ± 0.19	1.3 ± 0.15	−0.4 ± 0.15	0.039
22:0	0.9 ± 0.04	0.8 ± 0.11	0.6 ± 0.06	0.1 ± 0.09	
20:4(n-6)[j]	12.0 ± 0.63	14.7 ± 0.54	12.2 ± 1.08	−2.7 ± 0.40	0.003
20:5(n-3)	0.05 ± 0.02	0.1 ± 0.01	n.d.	−0.05 ± 0.005	0.001
22:3(n-9)[f]	1.4 ± 0.20	0.4 ± 0.37	0.6 ± 0.09	1.0 ± 0.21	0.009
24:0	1.8 ± 0.17	1.6 ± 0.16	1.0 ± 0.13	0.2 ± 0.24	
22:4(n-6)	2.8 ± 0.06	4.9 ± 0.22	3.8 ± 0.41	−2.1 ± 0.27	0.001
24:1(n-9)	3.6 ± 0.28	3.4 ± 0.24	2.9 ± 0.39	0.2 ± 0.29	
22:5(n-6)	3.5 ± 0.51	2.5 ± 0.41	1.6 ± 0.43	1.0 ± 0.37	0.050
22:5(n-3)[k]	0.4 ± 0.02	0.9 ± 0.10	0.7 ± 0.10	−0.5 ± 0.08	0.004
22:6(n-3)	4.7 ± 0.22	4.6 ± 0.41	2.8 ± 0.32	0.1 ± 0.24	

[a]Standard error of the mean.
[b]Figure before colon indicates number of carbon atoms, figure after colon, the number of double bonds. The figure in parentheses refers to the position of the first double bond from the methyl end of the acyl chain.
[c]Difference between efferent (arteries) and afferent (veins) blood vessels calculated as artery − vein.
[d]Student's 2-sample test (paired).
[e]Dimethyl acetal.
[f]Tentative identification.
[g]Unidentified.
[h]Not detectable.
[i]+ 18:1 dma as minor component.
[j]+ 22:1(n-9), which is always a minor fraction only.
[k]+ 24:2(n-6) as minor component.

known class-specific fatty acid profiles, both for arteries as well as for veins. For all classes, the A/E differences were qualitatively similar to those observed for total phospholipids (Fig. 1; full data for phospholipid classes available on request). This also applied to 22:5(n-6), the relative content of which was always higher (although not always significantly, due to small numbers) in arteries than in veins, in contrast to the other (n-6) fatty acids.

The fatty acid composition of phospholipids isolated from adult blood vessels (Table 2) is quite different from that of fetal vessels, showing significantly higher proportions of linoleic acid, dihomo-γ-linolenic acid, arachidonic acid, timnodonic acid, 20:5 (n-3), and clupadonic acid, 22:5(n-3), both in arteries as well as in veins.

Interestingly, cervonic acid, 22:6(n-3) and its 'deficiency indicator' 22:5(n-6) are significantly lower in adult arteries and veins as compared with umbilical vessels. The same holds for most fatty acids of the n-9 family, Mead acid and dihomo Mead acid in particular. Consequently, no biochemical signs of EFA deficiency are observed in adult vessels, in contrast to the umbilical vasculature.

The fatty acid compositions of phospholipids, isolated from cultured endothelial and smooth muscle cells, are given in Table 3. Since Mead acid and its elongation product are not observed and the amount of 22:5(n-6) is very low, no biochemical signs of EFA deficiency are observed at all. No obvious differences were seen between arterial and venous cells, indicating that the 'metabolic make-up' of both types of cells is comparable.

DISCUSSION

This study demonstrates that the phospholipids of umbilical cord vessels contain considerable amounts of Mead acid, 20:3(n-9), especially the arteries. Ongari and coworkers observed the same fatty acid in the free fatty acid fraction of umbilical artery lipids (8). Mead acid, a desaturation and elongation product of oleic

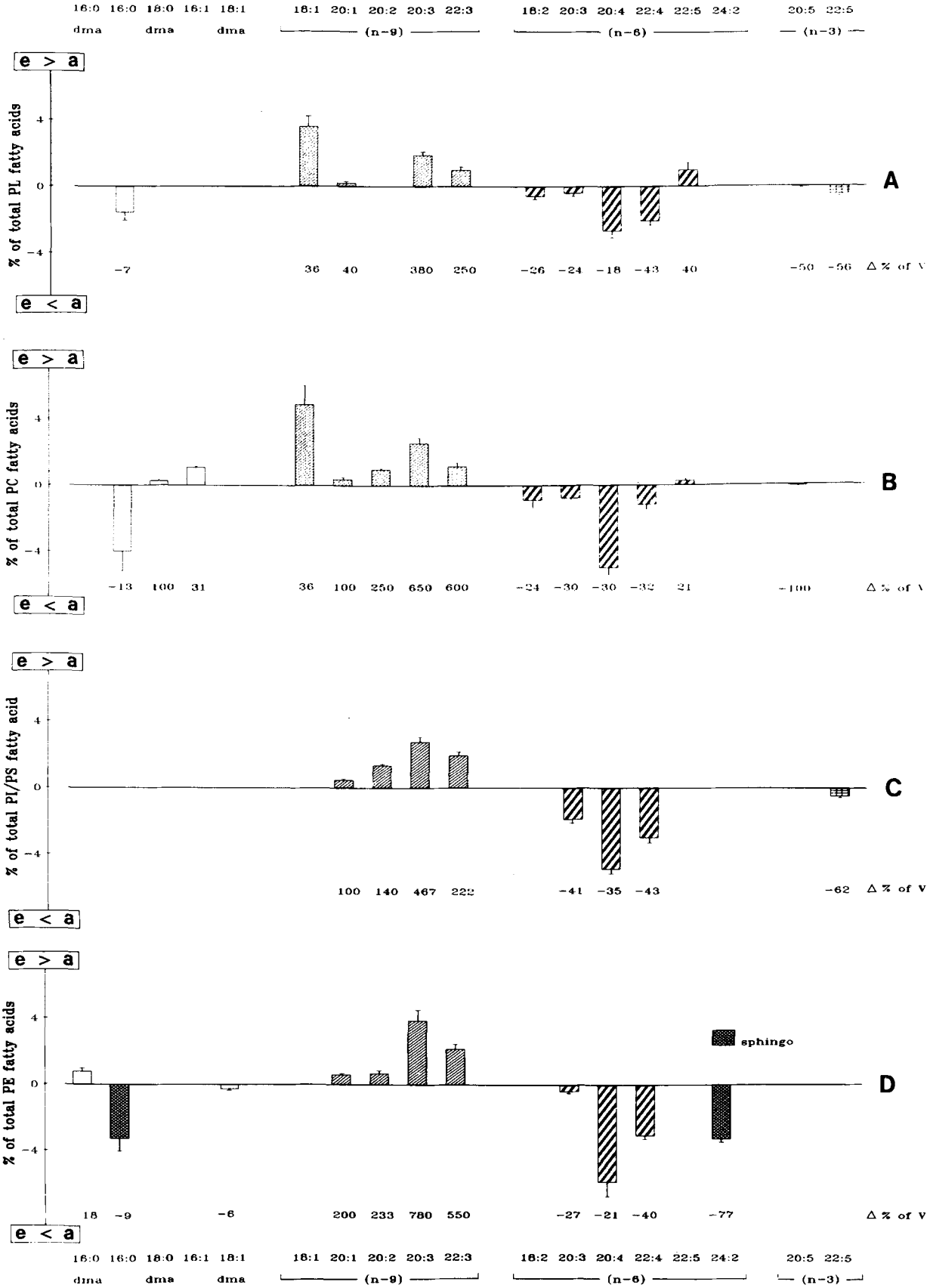

FIG. 1. Difference in fatty acid composition (%) of phospholipids, isolated from arteries (effert blood vessels, e) and veins (afferent vessels, a) of Dutch umbilical cords (mean ± s.e.m.). Significant differences are given only (P_2<0.10) □ saturated fatty acids, dimethyl acetals (dma) and 16:1 (n-7) ▨ (n-6) fatty acids ▩ (n-9) fatty acids ▦ (n-3) fatty acids A. Total phospholipids (n = 5), B. Phosphatidyl choline (n = 3), C. Phosphatidyl (inositol + serine) (n = 3), D. Phosphatidyl ethanolamine (n = 3); ■ sphingomyelin (n = 3).

TABLE 2

Fatty acid composition (%) of total phospholipids isolated from adult colonic arteries and veins serving the same area (mean ± s.e.m.; n = 6)

Fatty acid	Artery (Afferent)	Vein (Efferent)	E/A-difference[a]	
			%	P_2[b]
14:0	0.5 ± 0.08	0.5 ± 0.11	0.0 ± 0.04	
16:0 dma[c,d]	4.3 ± 0.15	2.5 ± 0.38	−1.7 ± 0.35	0.004
16:0	18.4 ± 0.59	18.7 ± 0.30	0.3 ± 0.52	
16:1(n-7)	1.9 ± 0.10	2.3 ± 0.20	0.4 ± 0.16	0.064
17:0	0.8 ± 0.07	0.8 ± 0.16	0.0 ± 0.19	
18:0 dma[d]	2.4 ± 0.20	2.3 ± 0.12	−0.1 ± 0.30	
18:0	15.7 ± 0.31	16.0 ± 0.33	0.3 ± 0.51	
18:1(n-9)	13.4 ± 0.85	16.4 ± 0.71	3.0 ± 0.66	0.006
?[e]	0.1 ± 0.06	0.4 ± 0.02	0.3 ± 0.07	0.017
18:2(n-6)	5.1 ± 0.30	7.0 ± 0.39	1.9 ± 0.40	0.005
20:0	0.3 ± 0.03	0.4 ± 0.05	0.1 ± 0.04	0.022
20:1(n-9)	0.4 ± 0.06	0.8 ± 0.08	0.4 ± 0.08	0.006
21:0	0.1 ± 0.04	0.1 ± 0.04	0.0 ± 0.03	
20:2(n-9)[d]	0.2 ± 0.04	0.3 ± 0.07	0.1 ± 0.10	
20:3(n-9)	0.1 ± 0.05	0.0 ± 0.03	−0.1 ± 0.04	0.050
20:3(n-6)	2.1 ± 0.17	2.2 ± 0.26	0.1 ± 0.16	
22:0	0.8 ± 0.13	0.7 ± 0.09	−0.1 ± 0.08	
20:4(n-6)	20.3 ± 0.57	18.5 ± 0.83	−1.8 ± 0.83	0.085
22:1(n-9)	0.5 ± 0.03	0.5 ± 0.03	−0.0 ± 0.02	
20:5(n-3)	0.3 ± 0.03	0.3 ± 0.04	0.0 ± 0.02	
24:0	0.7 ± 0.15	0.3 ± 0.04	−0.4 ± 0.14	0.027
22:4(n-6)	3.8 ± 0.37	2.8 ± 0.16	−1.0 ± 0.31	0.026
24:1(n-9)	1.7 ± 0.13	1.4 ± 0.09	−0.3 ± 0.16	
22:5(n-6)	0.7 ± 0.07	0.4 ± 0.05	−0.3 ± 0.11	0.056
22:5(n-3)[k]	1.8 ± 0.15	1.8 ± 0.14	0.0 ± 0.86	
22:6(n-3)	3.2 ± 0.23	2.4 ± 0.28	−0.8 ± 0.21	0.014
?	0.2 ± 0.02	0.1 ± 0.05	−0.1 ± 0.06	0.090

[a]Difference between efferent (veins) and afferent (arteries) blood vessels calculated as artery − vein.
[b]Student's 2-sample test (paried).
[c]Dimethyl acetal.
[d]Tentative identification.
[e]Unidentified.

acid, only accumulates in tissues if insufficient amounts of linoleic and α-linolenic acid are available (6) to occupy the Δ6-desaturase, the key enzyme in desaturation and elongation of (poly)unsaturated fatty acids having a double bond in the Δ9,10 position (9). Consequently, Mead acid is considered a reliable marker of EFA-deficiency (7). Cord vessels also contain dihomo Mead acid, 22:3(n-9), the direct elongation product of Mead acid.

The suggestion of a fetal linoleic acid shortage is supported by the very low linoleic acid content of umbilical blood vessels as compared with adult vascular tissue. The amounts of most other (n-6) PUFAs are also considerably lower in fetal than in adult blood vessels (except for 22:5(n-6), see later). The superior EFA-status of adult vs fetal blood vessels is emphasized by the absence of Mead acid and dihomo Mead acid in phospholipids of adult blood vessels (Table 2).

The presence of (dihomo) Mead acid in fetal vascular tissue does not necessarily reflect a poor EFA-status but can also be caused by the fact that the Δ6-desaturase in fetal blood vessels is so active that it converts oleic acid even if sufficient linoleic acid and longer-chain exist, more unsaturated derivatives are available to cover the local requirement. Studies to test this possibility are presently in progress but from the observation that human umbilical endothelial and smooth muscle cells in culture do hardly contain any Mead acid in their phospholipids (10, 11, see also Table 3), it is highly probable that young vascular cells have no inherent 'over-active' Δ6-desaturase which would enable the production of Mead acid at an adequate EFA-supply. Consequently, it is likely that the EFA-status of umbilical blood vessels, and of the arteries in particular, is poor indeed.

Umbilical blood vessels do not have a vasa vasorum and, consequently, their fatty acid profile is likely to reflect the average EFA status of the fetal blood over a prolonged period of time. In all cases investigated, the umbilical arteries (the efferent fetal blood vessels) contain significantly less (n-6) fatty acids (except 22:5(n-6), see later) and more (dihomo) Mead acid than the umbilical vein (the afferent fetal blood vessel). This could imply that the requirement of fetal 'downstream' tissue for EFAs, and for linoleic acid in particular, is not adequately covered under normal conditions.

It is now well established that an isolated deficiency of cervonic acid stimulates the Δ4 desaturase and, consequently, causes the enhanced synthesis of 22:5(n-6) from 22:4(n-6), adrenic acid (12, 13). In Table 4 the ratio between 22:5(n-6) and 22:4(n-6) is given as a reflection of the Δ4 desaturase activity in adult and fetal arteries and veins. From this table it appears that in adult blood vessels there is hardly any difference between arteries and veins, whereas in umbilical vessels this ratio not only is much higher than in adult

TABLE 3

Fatty acid composition (%) of total phospholipids, isolated from human umbilical arterial and venous endothelial and smooth muscle cells, cultured *in vitro*

	Endothelial cells		Smooth muscle cells	
Fatty acid	Arterial	Venous[a]	Arterial	Venous
14:0	0.9	1.1	0.7	0.7
16:0 dma[b,c]	2.9	2.2	1.9	2.0
16:0	20.9	23.7	20.5	21.7
16:1(n-7)	3.1	3.2	3.1	3.0
17:0	1.5	2.3	1.6	0.6
18:0 dma[c]	2.1	1.3	1.5	1.7
18:1 dma[c]	1.0	0.8	0.9	1.0
18:0	12.8	13.2	13.0	12.8
18:1(n-9)	15.8	17.3	19.6	20.3
18:2(n-6)	10.6	12.3	14.1	12.4
18:3(n-6)	0.3	n.d.	0.3	0.4
20:1(n-9)	0.7	0.5	0.2	0.3
?[d]	1.3	1.0	0.7	0.7
20:3(n-9)	n.d.[e]	n.d.	n.d.	n.d.
20:3(n-6)	1.7	1.5	2.2	2.5
20:4(n-6)	14.9	8.8	9.2	9.2
22:1(n-9)	n.d.	n.d.	0.4	0.4
23:0	0.4	1.1	0.2	0.1
20:5(n-3)	1.1	0.4	0.2	0.2
24:0	n.d.	n.d.	0.5	0.3
22:4(n-6)	5.1	4.7	4.0	4.0
22:5(n-6)	n.d.	n.d.	0.2	0.2
22:5(n-3)	1.5	2.0	2.1	2.1
22:6(n-3)	2.0	2.1	2.7	3.3

[a]Mean of two different cell cultures.
[b]Dimethyl acetal.
[c]Tentative identification.
[d]Unidentified.
[e]Not detectable.

TABLE 4

Ratio between 22:5(n-6) and 22:4(n-6) in phospholipids of adult colonic blood vessels (n = 6) and fetal vessels from the umbilical cord (n = 5, mean ± s.e.m.)

Blood vessels	Arteries	Veins	P_2
Adult	0.18 ± 0.02	0.14 ± 0.02	NS
Fetal	1.29 ± 0.18	0.51 ± 0.08	0.004
P_2	<0.001	0.001	—

vessels but, moreover, is 2.5 times higher in arteries as compared with veins. This indicates that the cervonic acid requirement of 'downstream' fetal tissue is not adequately covered in Dutch neonates, which may have negative consequences for the central nervous system and the visual system, the development and proper functioning of which requires a sufficient supply of cervonic acid (14).

The low levels of linoleic and arachidonic acid in umbilical vessels (and in the umbilical arteries in particular) may have patho-physiological implications. Mead acid is a good substrate for a platelet lipoxygenase and the resulting hydroxy fatty acid has been shown to stimulate thrombin-induced platelet aggregation (15). If transferred to placental blood vessels, this metabolite of Mead acid could, therefore, promote a prothrombotic condition.

Because of the low arachidonic acid content of umbilical phospholipids, the formation of antithrombotic prostacyclin by umbilical vessels may be expected to be rather low. Indeed, Ongari and coworkers (8) demonstrated that prostacyclin production of umbilical arteries is lower, the higher the ratio between Mead acid and arachidonic acid in tissue free fatty acids, thus linking prostanoid formation to EFA-status. Since the phospholipids of fetal blood platelets are rich in arachidonic acid (23.9% ± 1.02, n = 5, unpublished results), the potential production of fetal prothrombotic thromboxane A_2 can be expected to be high (1, 2). This unfavourable prostacyclin/thromboxane balance may result in an enhanced thrombotic tendency which has been connected to conditions like hypertensive pregnancy (16–18).

It was rather surprising to find hardly any timnodonic acid in the umbilical phospholipids. This implies that the vascular formation of prostanoids of the 3-series will be very low in these vessels.

It should be realized that the cords were obtained after normal deliveries, and, therefore, an effect of labour on the fatty acid composition of the newborn-tissue cannot be excluded. However, the fatty acid profile of umbilical cord vessels obtained after caesarian section is essentially similar to the picture reported here (unpublished observation).

Our results, indicating a poor EFA status of normal neonates, warrant further research with regard to fetal EFA-supply in relation to certain complications of pregnancy.

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METHODS

A Novel Spectrophotometric Assay for Lipase Activity Utilizing *cis*-Parinaric Acid

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A new spectrophotometric assay for determining the activity of acylglycerol hydrolases (lipases, E.C. 3.1.1.3) was developed and optimized for yeast lipase *(Candida cylindracea)*. Studies with porcine pancreatic lipase were also conducted and the influence of various detergents and divalent cations on the assay was evaluated. The assay uses *cis*-parinaric acid (PnA), a naturally occurring fatty acid that has unique spectroscopic properties, and takes advantage of the reversible binding of fatty acids to bovine serum albumin (BSA). Free PnA has an ultraviolet absorption peak at 321.2 nm. When PnA is bound to BSA, however, the peak shifts to 324.2 nm. The assay mixture contains 6 μM PnA, 1 μM BSA, 75 μM triolein, and 0.3 mM taurocholate in a 50 mM tris-HCl buffer with 1 μM EDTA. The release of oleic acid from triolein is monitored over time by measuring the ratio of optical densities (OD) at 319.0 and 329.0 nm. Initially, there is maximum binding of PnA to BSA, and the OD ratio is approximately 1.0. Upon addition of lipase, PnA is displaced from the BSA by oleic acid released from triolein, and the OD ratio increases to a maximum of about 1.8. However, when calcium is present in the reaction mixture an insoluble calcium-PnA complex forms, resulting in a progressive decrease in OD at both 319.0 and 329.0 nm. The kinetic assay described here is simple, rapid, sensitive, reproducible, inexpensive, and it can be adapted to measure the activity of a variety of calcium-independent lipases. Under similar assay conditions, activities for *Candida cylindracea* lipase obtained with this assay are similar to those obtained with ^{14}C-labelled triolein. *Lipids 24,* 518-525 (1989).

Various lipases play major roles in the regulation of fat metabolism. Thus, the control of lipase activity may be important in the etiology of obesity and atherosclerosis. Until recently the measurement of lipase activity was tedious and time-consuming. The available methods for detection and determination of lipase activity have been reviewed by Jensen (1). A classical method for measuring lipase activity involves the use of radioactively-labelled fatty acids. It also requires chemical separation of the substrate from released fatty acids prior to scintillation counting (2). Continuous monitoring of enzyme activity is not possible using this method. A titrimetric assay for determining liberated fatty acids is also commonly used to measure lipase activity. It can provide continuous kinetic data if a pH stat is available (3).

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Abbreviations: BHT, butylated hydroxytoluene; BSA, bovine serum albumin; CoA, Coenzyme A; EDTA, ethylenediaminetetraacetate; OD, optical density; PnA, *cis*-parinaric acid; TLC, thin layer chromatography; U, units.

Recently, a number of new enzymatic and colorimetric lipase assays have been developed which attempt to simplify the study of lipase biochemistry. Hosaka *et al.* (4) described an enzymatic colorimetric method for phospholipase A in which CoA esters of free fatty acids were formed and oxidized to H_2O_2. In the presence of peroxidase, a colored adduct was then formed. Woollett *et al.* (5) described a similar method for lipoprotein lipase in which free fatty acids were quantified spectrophotometrically by the oxidation of NADH after a series of coupled enzymatic reactions beginning with acyl CoA synthetase.

Roberts *et al.* (6) described a convenient spectrophotometric assay using a pH indicator. When butyric acid is released from tributyrin, the pH of the reaction mixture drops and the absorbance at 227 nm is decreased. In addition, Renard *et al.* (7) described a colorimetric lipase method which uses long-chain fatty acid-substituted thioesters. Other colorimetric lipase assays use synthetic fluorescent substrates such as p-substituted phenols (8), but these assays may not give an accurate reflection of physiological enzyme activities (1). When studying lipases which normally act on long-chain fatty acid-containing lipids, the use of substrates containing these long-chain fatty acids is preferred.

The spectrophotometric assay described here for triacylglycerol lipase (E.C. 3.1.1.3) utilizes triolein as a substrate and uses a fluorescent, naturally occurring 18-carbon fatty acid, *cis*-parinaric acid (9,11,13,15-*cis,trans,trans,cis*-octadecatetraenoic acid) for monitoring oleic acid release. Parinaric acid (PnA) is isolated from the seed kernels of Fijian *Parinari laurinum.* Because of its unique ultraviolet and fluorescent properties it has gained considerable importance as a probe for studying physical and biochemical properties of membrane lipids. PnA has major ultraviolet absorption peaks at 304.2 nm and 321.2 nm. It can be readily incorporated into lipids such as triglycerides, phospholipids, and cholesterol esters and apparently behaves like the polyunsaturated fatty acids normally found in mammalian lipids (9-12).

Binding of fatty acids to albumin is a well-recognized phenomenon and an important component of the mammalian lipid transport system (13). PnA binds reversibly to human and bovine serum albumins with a binding affinity similar to that of other long chain fatty acids (14). This binding causes a shift in the PnA absorbance spectrum. In 1980, Berde *et al.* (14) described a method for monitoring serum and plasma fatty acid concentrations which was based on the competitive binding of PnA and blood fatty acids with albumin. We have utilized this competitive binding concept to develop an *in vitro* assay for rapidly and conveniently measuring lipase activity.

Acylglycerol lipases from different sources vary in their specificities, pH optima, and thermostabilities. Their

activities can be altered by a number of factors due to the action of lipases at the oil-water interface of emulsified lipid substrates. The presence or absence of proteins, phospholipids, or bile acids, as well as physical factors such as micelle size can all contribute to enzyme stabilization and inactivation (1,15–18). Therefore, two different commercial lipases, from porcine pancreas and yeast *(Candida cylindracea),* were chosen to evaluate the present assay. The pancreatic enzyme attacks the primary ester groups (1- and 3-) of triglycerides and has little action on the remaining 2-monoglycerides. The yeast enzyme, on the other hand, lacks positional specificity (17).

MATERIALS AND METHODS

Yeast lipase (from *Candida cylindracea*), bovine serum albumin (BSA, essentially fatty acid-free), sodium taurocholate, 1-monoolein, 1,3-diolein, triolein, and oleic acid were purchased from Sigma Chemical Co. (St. Louis, MO). Porcine pancreatic lipase was purchased from Serdary Research Laboratories (London, Ontario, Canada).

The specific activity reported by the supplier for the yeast *(Candida cylindracea)* lipase was 3800 U/mg protein or 690 U/mg solid, where 1 U = 1 μmol fatty acid released per mg protein per hour (measured at pH 7.7, 37°C, for 30 minutes). The activity reported for the pancreatic enzyme was 20–25 U/mg solid.

Cis-parinaric acid was purchased from Molecular Probes, Inc. (Eugene, OR). Purity was determined by measurement of ultraviolet absorption and extinction coefficients, as well as by thin-layer chromatography (TLC) and gas liquid chromatography. The ultraviolet absorption showed no triene absorption in the 250 nm region and the extinction coefficients in methanol for peak 1 (306.1 nm) and peak 2 (320.4 nm) were 80,000 and 74,000 $M^{-1}cm^{-1}$, respectively, in agreement with published values (19). TLC using a benzene/ethyl ether/ethyl acetate/acetic acid (70:10:10:0.3, v/v/v/v) developing solvent and iodine vapor visualization showed a single fatty acid spot. The methyl ester of PnA prepared with N′,N′ dimethylformamide dimethyl acetal (20) was injected into a Varian Model 3700 gas chromatograph (Varian Instrument Group, Houston, TX) equipped with a flame ionization detector. A 6 ft × 1/8 inch glass column packed with 10% SP-2330 (100/120 Supelcoport) was used at isothermal oven temperature (180°C), and the injector and detector ports were maintained at 210°C and 230°C, respectively. A single peak for *cis*-PnA was observed indicating that there was no contamination with the *trans* isomer. *Cis*-PnA obtained from Serdary Research Laboratories was also analyzed and found to be as pure as the sample from Molecular Probes, Inc.

The parinaric acid lipase assay was tested under a variety of conditions. The effects of various triglyceride substrate preparations and various levels of calcium, magnesium, and taurocholate on standard curves and enzyme activities are described in the results and discussion section. The parameters summarized in this section are those which gave the highest specific activities for the enzymes chosen.

Once the assay conditions were optimized for the yeast lipase, the reliability of the assay was determined by testing the repeatability with varying levels of enzyme protein and on different occasions with identical conditions. In addition, the specific activity of the yeast lipase was compared using the parinaric acid lipase method described here and a standard radioactive lipase assay procedure using the same conditions.

For the radioactive lipase assay, tri-[1-^{14}C]olein (56 μCi/μmol) and [1-^{14}C]oleic acid (58 μCi/μmol) were purchased from Amersham (Arlington Hts., IL), and potassium carbonate-potassium borate buffer (0.05 M, pH 10) was purchased in capsule form from Fisher Scientific Co.

Reagents and stock solutions for lipase assay. A 0.3 mM solution of taurocholate in 50 mM tris-HCl buffer containing 1 μM EDTA was prepared fresh daily. The optimum buffer pH for each lipase was selected from published data. A pH 7.7 buffer was used for the yeast enzyme (as recommended by the supplier) and a pH 8.0 buffer was used for the porcine pancreatic lipase (21).

A 50 μM solution of bovine serum albumin (BSA) was prepared daily and the actual molarity was determined by measuring the OD of this solution at 280 nm using an extinction coefficient of 46,230 $M^{-1}cm^{-1}$.

The 1.0 mM stock solution of *cis*-parinaric acid (PnA) was prepared in absolute ethanol as described by Berde *et al.* (14). The molarity was checked by reading the OD at the peak near 304.2 nm and using the extinction coefficient in ethanol of 78,000 $M^{-1}cm^{-1}$ (14). To prevent peroxidative and light damage, 0.05% BHT was added to the stock solution, and aliquots were stored at −20°C in foil-covered, 3 ml vials. PnA stored in this manner was stable for at least 6 months.

The glycerol-dispersed triglyceride substrate was prepared using the method of Severson *et al.* (22). A 1.0 ml aliquot of triolein (10 mg/ml in chloroform/methanol, 2:1, v/v) was transferred to a small beaker and evaporated to dryness under a stream of nitrogen. Glycerol (5.0 g or 4.0 ml) and 80 μl of absolute ethanol were added, and the mixture was homogenized with two 20-second pulses at 30% maximum speed using a Kinematica polytron (Brinkmann Instruments, Westbury, NY). The resulting suspension was then centrifuged at approximately 1000 rpm for 10 minutes, or until all bubbles were dispersed. The triglyceride substrate was prepared daily.

A 0.30 mM stock solution of oleic acid in ethanol was prepared for use as a standard.

Assay procedure—parinaric acid method. A 3.0 ml aliquot of buffer containing 0.3 mM taurocholate was pipetted into each cuvette. BSA (60 μl) and triglyceride substrate (80 μl) were added, along with the calculated amount of PnA required to attain a 6:1 molar ratio of PnA to BSA (approximately 20 μl). Absolute ethanol was used in place of PnA for the blank. Samples were thoroughly mixed and equilibrated at 37°C for at least 10 minutes. The assay commenced with the addition of an appropriate amount of enzyme prepared in buffer. Final concentrations in each cuvette were 0.3 mM taurocholate, 6 μM PnA, 1 μM BSA and 75 μM triolein. Variable, positive-displacement pipettes were used for all volumetric procedures.

The lipase activity was monitored on a Bausch and Lomb Spectronic 1001 spectrophotometer equipped with circulating water bath (Milton Roy Co., Rochester, NY) as the change in ratio of OD readings at 319.0 and 329.0 nm. The Spectronic 1001 is a programmable spectrophotometer which can automatically read absorbancies in up to 6 cuvettes sequentially at several different

wavelengths and at specific time intervals. When sample readings were taken at one-minute intervals, only 3 cuvettes could be read simultaneously. Therefore, the 3 cuvettes for the next run were held in the remaining positions for temperature equilibration. The enzyme was added at 10 second intervals to match the time required for the spectrophotometer to complete the reading of each cuvette.

It was found that the initial equilibration period of ten minutes was critical not only for temperature adjustment, but also for acquiring a stable initial reading. There was a small, but gradual increase in OD ratio during the first 6–8 minutes after addition of PnA to each cuvette.

As oleic acid was released by the action of lipase, the turbidity of blank and sample cuvettes decreased. During a typical run, loss of turbidity resulted in the reduction of blank OD readings at both 319.0 and 329.0 nm from 0.000 to −0.025. Blank OD readings were therefore subtracted from the sample readings at each time point before calculating OD ratios.

To obtain standard curves, 10 μl increments (3 nmol) of stock oleic acid were added to each cuvette in place of the enzyme. The linear portion of the standard curve was used to calculate equivalents of oleic acid released during lipase runs. Plots of oleic acid released vs time were prepared and the slope of the linear portion was used to calculate specific activity (1 U = 1 μmol oleic acid released/mg lipase protein/hr).

The protein concentration of each lipase was determined using the Lowry method (23).

Assay procedure—radioactive lipase method. The parinaric acid lipase method was validated by determining the specific activity of yeast *(Candida cylindracea)* lipase with radioactively-labelled triolein. Assay conditions were kept as close as possible to the conditions used for the PnA method, except that PnA was deleted and the triolein substrate was prepared with 1.5% ^{14}C-labelled triolein.

Three ml of buffer (50 mM tris-HCl with 1 μM EDTA, pH 7.7) containing 0.3 mM taurocholate, 50 μM BSA, and 75 μM triolein was equilibrated to 37°C. The enzyme was added (0.145 μg enzyme protein per tube), and at 5 or 10 minute intervals 200 μl aliquots of the reaction mixture were removed and placed immediately into tubes containing 1.05 ml potassium carbonate-potassium borate buffer (pH 10) and 3.25 ml methanol/chloroform/heptane (1.41:1.25:1, v/v/v), as described by Belfrage and Vaughan (24). The tubes were immediately capped and mixed vigorously. Phase separation was facilitated by centrifugation at 500 × *g* for 15 minutes at 4°C. One ml of the upper aqueous layer was removed from each tube and mixed with 3.5 ml Scintiverse II counting cocktail (Fisher Scientific Co.). Counts were measured with a Beckman model LS7500 Scintillation Counter (Beckman Instruments, Columbia, MD). Although the enzyme reaction was allowed to continue for one hour, the reaction was no longer linear at 15 minutes. Therefore, only the 5 and 10 minute time points were used to calculate enzyme activities.

Two trials using the radioactive lipase assay were conducted, giving a total of 8 replicates. In the first trial, data was collected at 10 minute time points, and in the second, data was collected at 5 minute time points. A t-test showed no significant differences between the specific activities calculated for the 5 and 10 minute time points of the second trial. The specific activities calculated from the 10 minute time points were used for comparison with the parinaric lipase method.

A standard curve for the radioactive method was generated using [1-^{14}C]oleic acid. Labelled oleic acid was added to a stock oleic acid solution (0.3 mM in ethanol), and varying amounts of this standard were added to tubes identical to those used for the enzyme assay. Aliquots (200 μl) from each tube were removed and treated as described above, and radioactive counts in 1 ml of the upper phase were determined by scintillation counting. Standard curves were linear for all levels of oleic acid added (up to 300 nmol per 3 ml), and the presence or absence of labelled triolein had no effect on the standard curve.

For determining statistical significance at $p < 0.05$, Student t-tests and analysis of variance were performed using a statistical package for personal computers developed by StatSoft, Inc. (Tulsa, OK).

RESULTS AND DISCUSSION

Assay conditions for parinaric acid method.

1. *Factors influencing the standard curve.* When pancreatic lipase (which has primary ester specificity) hydrolyzes triglycerides, 1,2(2,3) diglycerides and 2-monoglycerides are formed. When a non-specific lipase (such as that from *Candida cylindracea*) acts on triglycerides, 1,2(2,3) and 1,3 diglycerides and 1(3) and 2-monoglycerides are formed (17).

Figure 1 shows a typical standard curve produced by adding oleic acid to an assay medium containing 6 μM PnA and 1 μM BSA in 50 mM tris-HCl buffer without added taurocholate or triglyceride substrate. It also shows the effects of adding 1-monoolein and 1,3-diolein. Neither of the later two ligands appear to displace PnA from BSA in this assay and have negligible effects on the standard curve. The same response is expected for 2-monoolein and 1,2(2,3) diolein.

Under the conditions of Figure 1, the starting OD ratio (319.0/329.0 nm) is generally close to 1.0. There are slight variations in the initial OD ratios from one run to the next, but the change in OD ratio upon addition of a given

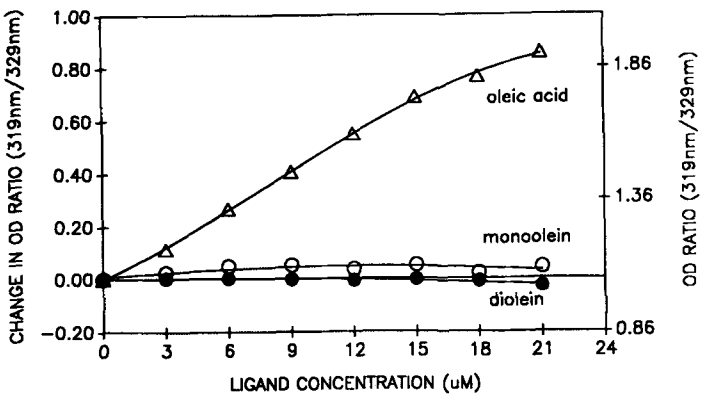

FIG. 1. Changes in OD ratio (319.0/329.0 nm) with the addition of oleic acid, 1-monoolein and 1,3-diolein. The assay preparation contained 0.3 mM taurocholate in a pH 7.7 tris-HCl buffer (50 mM) with 1 μM EDTA. BSA and PnA were present at 1 μM and 6 μM, respectively. Each value is a mean of duplicates.

level of oleic acid is very consistent when the assay conditions are held constant. With the addition of oleic acid, the OD ratio increases linearly until most of the PnA is displaced from BSA and is present in the unbound form. At this point, the standard curve levels off and further addition of oleic acid has little or no observable effect on the OD ratio. In Figure 1, the maximum OD ratio was approximately 1.8. This ratio is highly dependent upon the composition of the reaction mixture. As shown below, it can be influenced by the presence of various detergents and cations.

2. *Optimal levels of PnA and BSA.* Use of this assay is most successful when 6 μM PnA and 1 μM BSA are used. Changing the concentrations of PnA and BSA while holding their ratios constant leads to a change in the initial OD readings at 319.0 and 329.0 nm. At both wavelengths, the OD readings are increased by higher levels of PnA and BSA and decreased by lower concentrations. The incremental change in OD ratio with addition of oleic acid is attenuated, however, as PnA levels are increased above 6 μM. With lower levels of PnA, the reaction curve levels off more rapidly even though changes in OD ratios are higher for each data point. This leaves fewer points in the linear region of the curve.

3. *Optimal level of triolein.* A concentration of 75 μM of triolein gives maximum enzyme activity for both the pancreatic and the yeast lipase with 0.3 mM taurocholate, 6 μM PnA, and 1 μM BSA. Additional triolein does not give higher activities, and may result in an attenuation of the OD ratio change.

4. *Use of detergents.* Since the assay is run in an aqueous medium, it is necessary to completely suspend the triolein substrate in buffer. Several methods were tried for suspending the triolein, with differing effects on the standard curve and yeast lipase activity.

The glycerol-dispersed triolein of Severson *et al.* (22) appears to be the most favorable triglyceride substrate for this assay since glycerol has no adverse effects on the standard curve, and large amounts of triolein can easily be dispersed in glycerol. Therefore, although the preparation of triglyceride substrate can influence the activity of an enzyme at different pH levels (22), the preservation of the standard curve remains a primary determinant in the choice of substrate emulsion for this assay.

When the detergent, Tween 20, was added to the buffer at a level sufficient to completely suspend 25 μM triolein, the OD ratio dropped from 1.0 in the absence of added oleic acid to 0.75. This ratio remained unchanged when oleic acid was added. The standard curve was similarly obliterated by levels of Tween 20 which were insufficient to completely suspend the triolein in the buffer.

Triton X-100 has been used both to solubilize lipases and enhance their specific activities (3), or to prepare triglyceride substrate emulsions. But it has also been shown to inhibit the activities of certain lipases (25). Brooks and Weinhold (26), who studied rat lung acidic triacylglycerol lipase, used Triton X-100 with triolein substrate at a final concentration of 0.1% (v/v) Triton X-100 in the reaction vial. In the present assay, standard curves were maintained when 0.0005 and 0.001% Triton X-100 (v/v) was added, but the addition of 0.025% Triton X-100 caused a progressive and dramatic reduction in slope of the standard curve. At the levels of Triton X-100 necessary to completely emulsify the triolein, no standard curve could be achieved. Furthermore, the presence of Triton X-100 at levels as low as 0.0005% completely inhibited the activity of yeast lipase.

Vegetable gums are often used to suspend triolein for lipase assays. Schneeman (27) used 10% gum acacia while others have used gum arabic (2,28). The 5% preparation of gum arabic (Sigma Chemical Co.) used with the present assay was relatively ineffective for suspending the desired concentration of triolein even with sonication. Consequently, the lipase activities were low. Gum arabic added to the assay medium in the presence of a glycerol-dispersed triolein (described in the materials and methods section) resulted in reduced OD readings at 319.0 and 329.0 nm and somewhat depressed standard curves, but there was no substantial loss of enzyme activity.

The effect of taurocholate on *Candida cylindracea* lipase actvity is shown in Figure 2. The results are presented as relative activities and represent the means of 6 replicates for each level of taurocholate. With the addition of either 0.3 mM or 0.9 mM taurocholate, yeast enzyme activity was increased relative to the taurocholate-free control, but with the addition of 0.6 mM taurocholate, the activity was slightly decreased. No definitive explanation can be given for this observation, yet the sigmoidal response observed in this experiment with the addition of taurocholate was replicated in three additional experiments done under similar conditions (pH 7.7 tris-HCl buffer, 6 μM PnA, 1 μM BSA, and 25 μM triolein).

However, these results are in concert with reports that the effect of taurocholate on lipase activity is variable. In 1966, Tomizuka *et al.* (29) showed distinctly different effects of taurocholate on *Candida cylindracea* lipase activity under two different sets of assay conditions. When they used a 0.1 M phosphate buffer (pH 7.0) and a substrate of polyvinylalcohol-emulsified olive oil, they observed a decrease in activity between 0 and 0.1% (1.8 mM) taurocholate, followed by a marked increase in activity which climaxed at 0.3% (5.6 mM) taurocholate. Enzyme activity was essentially doubled at this point. When the substrate was prepared by another method

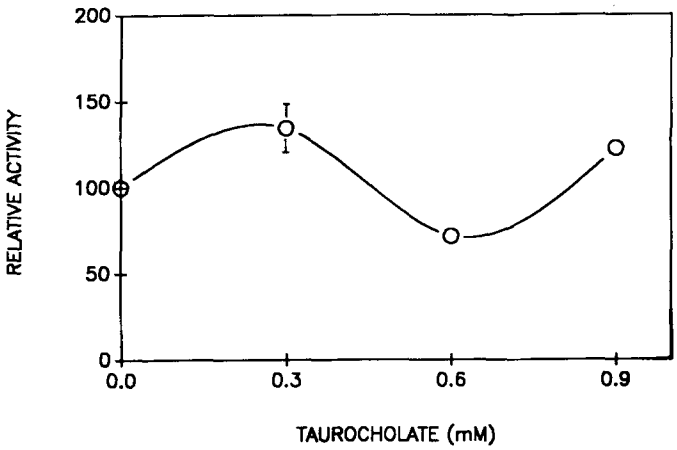

FIG. 2. Effect of taurocholate level on yeast enzyme activity. Assay conditions were similar to those described for Table 1 except that 25 μM triolein was used. Each point is the mean ± SEM for 6 replicates.

without polyvinylalcohol, the activities, though variable, were depressed for all levels of added taurocholate.

More recently, Borgstrom and Erlanson (18) showed interactions between BSA and taurodeoxycholate in the activation and inhibition of porcine pancreatic lipase. Additional studies are needed to ascertain whether such interactions are occurring here.

5. *Sonication.* When a 25 μM triolein substrate was used for this assay, a preparation of buffer, taurocholate, and triolein substrate could be prepared in 50 ml batches. This mixture was sonicated with a Fisher Sonic Dismembrator, Model 300 (Fisher Chemical Co.) for various time periods of up to 3 minutes. Similar reproducible results were obtained for enzyme assays when the mixture was sonicated either on ice or at ambient temperature. However, the enzyme activity was not optimized with 25 μM triolein. When 50 ml preparations containing 50 to 100 μM of triolein were used, the triglyceride-taurocholate emulsion quickly settled out. For each successive assay using 3.0 ml aliquots of solution pipetted from the bottom of the beaker, enzyme activity was decreased. Sonication of the mixture for up to 5 minutes before the addition of BSA did not improve the results. In the presence of 75 μM triolein, more consistent results were observed when the triolein substrate was added directly to the cuvette and sonication was omitted.

6. *Addition of calcium and magnesium.* The binding between free fatty acids and cations to form insoluble soaps is a well recognized phenomenon. Since some lipases require calcium or magnesium for activation, we sought to determine the maximum levels of these cations which could be supported by this assay system. Figure 3 shows the effect of varying the calcium concentrations between 0 and 5 mM on the OD readings at 319.0 and 329.0 nm when oleic acid was added to buffer containing BSA and PnA. It also shows the effects of calcium on the shape of the standard curve. Figure 4 shows the effects of adding 0 to 10 mM magnesium to the mixture. In the absence of added cations, there is an increase in OD at 319.0 nm and a concomitant decrease in OD at 329.0 nm with added oleic acid. For levels of calcium less than 0.01 mM (not shown), there was essentially no effect on the standard curve, but with higher concentrations of calcium (Figure 3), OD readings at both wavelengths were decreased. This was apparently due to the formation of a precipitable complex between PnA and calcium. However, only minimal decreases in OD were seen when magnesium was added at levels as high as 10 mM (Figure 4), indicating that magnesium does not readily complex with PnA.

As expected, calcium and magnesium altered the lipase reaction curves in the same way that they altered the standard curves. At high calcium concentrations, OD readings and OD ratios (319.0 nm/329.0 nm) dropped off before maximum release of oleic acid from triolein was achieved (Figure 5). Activity of the pancreatic lipase was enhanced by calcium, but levels of calcium above 0.01 mM interfered with the measurement of lipase activity (Figure 5). Only minor changes in the shape of the lipase curves were observed when magnesium was added (Figure 6).

Reliability of parinaric acid method.

1. *Linearity of yeast lipase activity at different concentrations of enzyme.* As seen in Table 1, the assay is reproducible under a given set of conditions, and linearity is achieved when different levels of enzyme were added. In Table 1, no significant differences in specific activities were observed when five concentrations of the yeast enzyme were added. For this experiment, the enzyme solution was prepared in advance (one week maximum) and frozen in small aliquots which were thawed just before use.

2. *Repeatability of assay.* The specific activity of the yeast lipase was determined on four separate occasions. On each occasion, the enzyme solution was prepared and frozen in 1 ml aliquots during the morning for use in the afternoon. For each of the four trials, the same stock

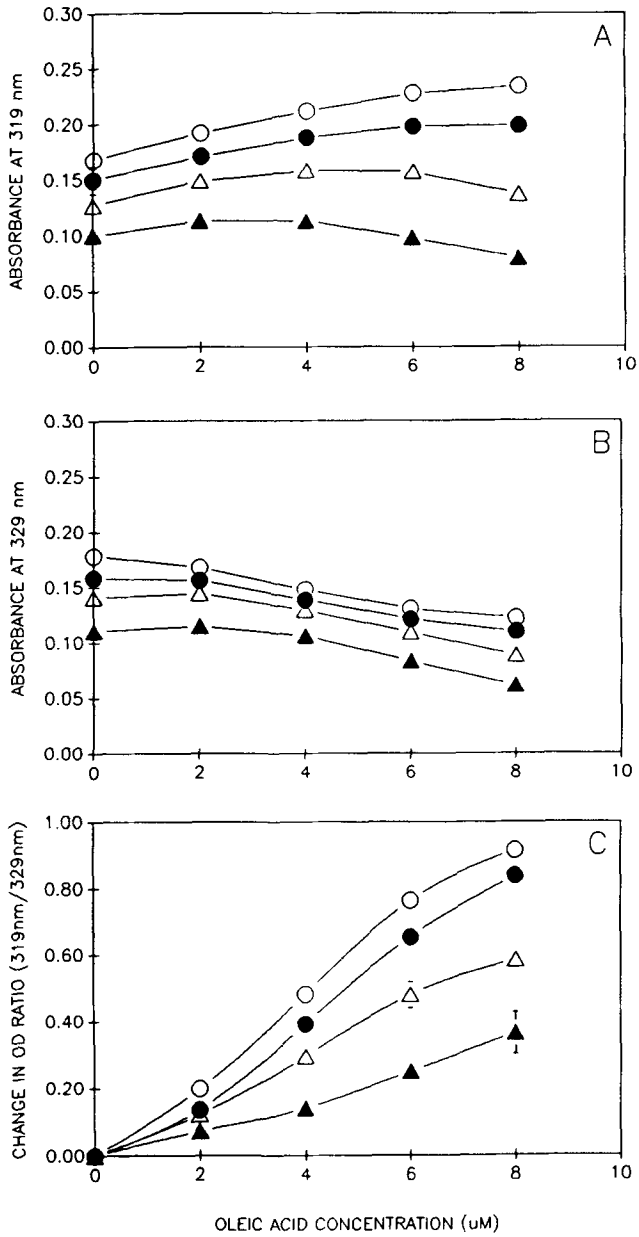

FIG. 3. The effect of calcium concentration on standard curves. Assay preparations were similar to those described for Figure 2 except that 0.6 mM taurocholate was used. Each point represents the mean ± SEM for four replicates (standard errors for most data points are smaller than the symbols). Open circles represent 0 mM Ca; closed circles, 0.1 mM Ca; open triangles, 0.5 mM Ca; and closed triangles, 5.0 mM Ca. Calcium was added as calcium chloride.

solutions of PnA and oleic acid standard (stored at −20°C) were used. The BSA solution, 0.3 mM taurocholate solution in pH 7.7 tris-HCl buffer, and triglyceride substrate were prepared fresh daily, according to the general procedure.

The results from this study are shown in Table 2. Analysis of variance indicates that there were no significant differences between trials.

3. *Comparison with radioactive lipase assay.* The specific activity of yeast lipase determined by the radioactive method was 2775.0 ± 98.5 U (mean ± SEM, n = 8). Data from all four trials in Table 2 were combined to get a mean specific activity for the parinaric acid method of 2624.7 ± 100.5 U (n = 20). Both assays were conducted during the same month and assay conditions were similar. A two-tailed t-test indicates that there were

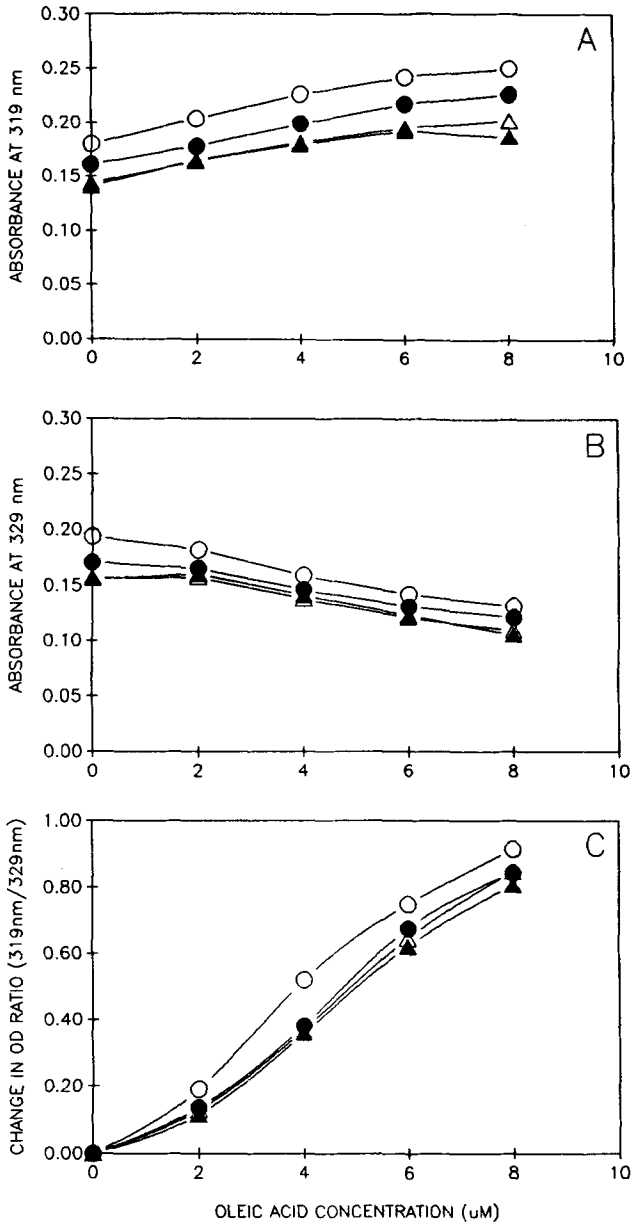

FIG. 4. The effect of magnesium concentration on standard curves. Assay preparations were identical to those described for Figure 3. Each point is the mean for duplicate assays. Open circles represent 0 mM Mg; closed circles, 0.5 mM Mg; open triangles, 5 mM Mg; and closed triangles, 10 mM Mg. Magnesium was added as magnesium sulfate.

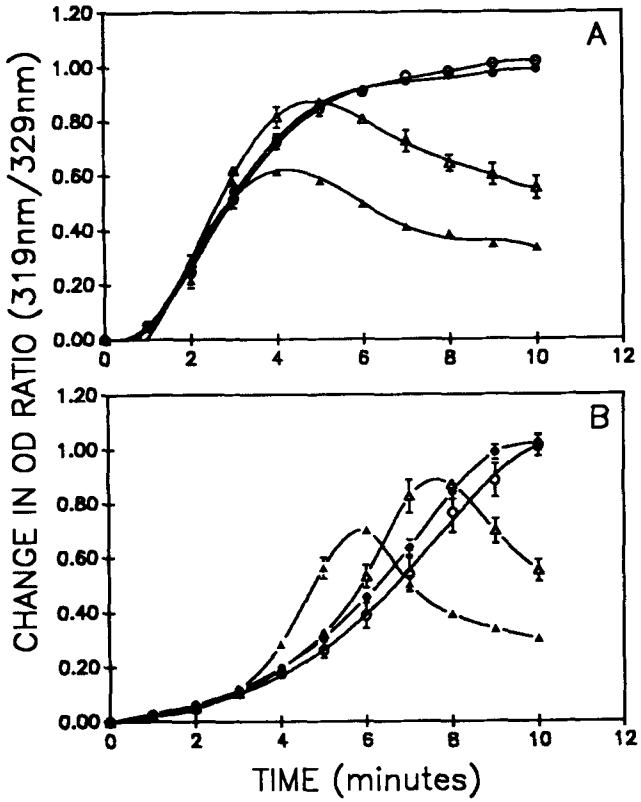

FIG. 5. Effect of calcium on measurement of lipase activity. Assay conditions were similar to those described in Figure 2 except that a pH 7.7 buffer was used for the yeast lipase and a pH 8.0 buffer was used for the porcine pancreatic lipase. The levels of enzyme protein added were 0.58 μg per 3 ml cuvette for the yeast lipase and 3.26 μg per 3 ml cuvette for the pancreatic lipase. Each point represents mean ± SEM for four replicates. Open circles, 0 mM Ca; closed circles, 0.01 mM Ca; open triangles, 0.1 mM Ca; and closed triangles, 0.5 mM Ca. A = yeast lipase and B = pancreatic lipase.

TABLE 1

Specific Activities for Yeast *(Candida cylindracea)* Lipase Calculated at Five Different Levels of Enzyme Addition

Enzyme protein (μg/cuvette)	Specific activity (μmol oleic acid released/ mg protein/hour)
0.290	2033 ± 59.2
0.145	2042 ± 169.5
0.072	2216 ± 184.7
0.048	2023 ± 51.8
0.036	2264 ± 95.2

The assay preparation contained 0.3 mM taurocholate in a pH 7.7 tris-HCl buffer (50 mM) with 1 μM EDTA. BSA and PnA were present at 1 μM and 6 μM, respectively, and triolein in glycerol suspension was present at 75 μM. Each value represents mean ± SEM for 6 replicate assays.

no significant differences between the two assay methods ($t = 0.849$, $p = 0.408$).

The specific activities of *Candida cylindracea* lipase determined in this trial were about 70% that of the activities reported by the supplier. The lower activities in our studies are probably primarily due to losses in activity with freezing and storage which we have observed throughout this study. In one experiment, for example, when the same yeast enzyme solution was assayed by the PnA method before and after freezing, the losses in activity which occurred were approximately 35%. Although we were aware that freezing and thawing could reduce lipase activity, losses also occurred when the preparations were held on ice for several hours. The enzyme preparations were therefore frozen and thawed just prior to use to ensure repeatability of assays over time.

In summary, the present assay appears to be a reliable and simple one for measuring yeast lipase activity *in vitro*. It is repeatable, sensitive, and compares well with radioactive lipase methods when assay conditions are kept constant. The present method is simpler than the radioactive assay, it is much less expensive, and it allows one to observe the course of the enzyme reaction over time. The method used to prepare the triglyceride substrate is critical, however, and because of the interaction between calcium and PnA, the assay cannot be used in the presence of calcium at levels of 0.01 mM or higher. Magnesium has a lesser effect and 10 mM magnesium can be used with no untoward difficulties. Currently we are evaluating the use of this assay for different lipases and are attempting to improve the sensitivity of the assay by using a spectrofluorometer instead of a UV spectrophotometer.

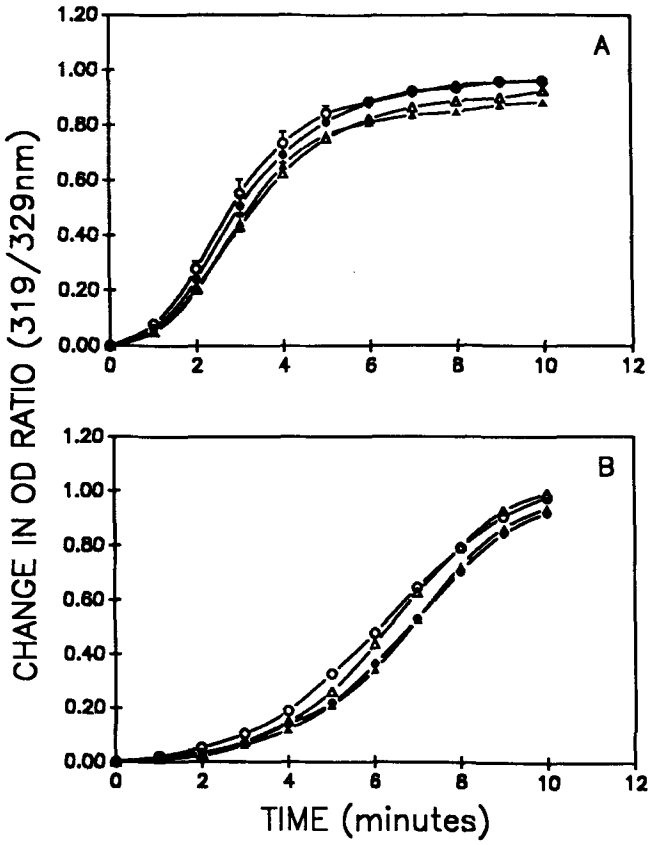

FIG. 6. Effect of magnesium on measurement of lipase activity. Assay conditions were similar to those described for Figure 5. Each point represents the mean ± SEM for four replicates. Open circles represent 0 mM Mg; closed circles, 0.5 mM Mg, open triangles, 5 mM Mg; and closed triangles, 10 mM Mg. A = yeast lipase and B = pancreatic lipase.

TABLE 2

Specific Activities of Yeast *(Candida cylindracea)* Lipase Determined on Four Separate Occasions Using the Parinaric Acid Method

Trial no.	Specific activity (μmol oleic acid released/ mg protein/hour)
1	2347.2 ± 66.5 (4)
2	2358.3 ± 222.7 (5)
3	2709.9 ± 93.0 (6)
4	3011.1 ± 252.9 (5)

The assay conditions were identical to that described in Table 1. Enzyme was added at a level of 0.145 μg enzyme protein/3 ml cuvette. Each value represents the mean ± SEM, and numbers in parentheses are the number of replicates for each trial.

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Isolation and Purification of Perfluorodecanoic and Perfluorooctanoic Acids From Rat Tissues

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A procedure for the extraction, separation, and isolation of perfluorodecanoic and perfluorooctanoic acids from biological samples is described. The use of conventional lipid extraction procedures leads to substantial loss of the perfluorinated fatty acids added to tissue. The presence of sulfuric acid in aqueous saline during phase partitioning is essential for the recovery of perfluorodecanoic and perfluorooctanoic acids in the organic phase following their extraction from tissue. The perfluorinated fatty acids are co-eluted with simple lipids from silica gel columns using diethyl ether/trifluoroacetic acid (100:1, v/v). Simple lipids are separated by thin layer chromatography. By substituting trifluoroacetic acid for acetic acid in the developing solvents, perfluorodecanoic and perfluorooctanoic acids migrate with other free fatty acids. *Lipids 24,* 526–531 (1989).

The complete substitution of fluorine for hydrogen of a hydrocarbon imparts greater chemical and thermal stability, as well as surfactant properties to the molecule when compared to its nonperfluorinated analog (1). These properties are exploited in industrial applications of perfluorocarboxylic and perfluorosulfonic acid derivatives, which are used as lubricants, plasticizers, corrosion inhibitors, and surfactants (2,3). Perfluorooctanoic (PFOA) and perfluorodecanoic (PFDA) acids are representative of the perfluorinated carboxylic acids. While their toxicity (4–6) and their effects on hepatic lipid metabolism (5–8) have been studied extensively, only limited information on their metabolic fate is available. In studies of its disposition and excretion in rodents (9,10), PFOA was quantitated by measuring inorganic fluorine before and after combustion of biological samples. Although this procedure eliminates the problems of quantitative extraction of perfluorinated fatty acids from small tissue samples (6), it provides little or no information on the possible metabolism of these compounds. In order to elucidate the metabolic fate and disposition of PFDA and PFOA we must know their behavior during extraction, separation, and isolation from tissue. The addition of acid enhances the recovery of medium-chain fatty acids (e.g., octanoic and decanoic acids) extracted from tissues (11,12). The electron-withdrawing fluorine atoms in the aliphatic chain promote the dissociation of the carboxylic hydrogen in PFDA and PFOA. Therefore, pH considerations are more critical here than with their nonperfluorinated counterparts (13). The proposed procedure for the isolation and purification of PFDA and PFOA from rat tissues, which was developed with recently synthesized ^{14}C-labeled material (14), takes into account the unique properties of perfluorinated fatty acids.

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Abbreviations: PFDA, perfluorodecanoic acid; PFOA, perfluorooctanoic acid; FFA, free fatty acid; PL, phospholipid; TLC, thin layer chromatography.

MATERIALS AND METHODS

Chemicals. [1-^{14}C]PFDA and [1-^{14}C]PFOA (6 mCi/mmol) were synthesized as previously described (14). [9,10-^{3}H]-Palmitic acid (28.5 Ci/mmol) and L-α-dipalmitoyl-[2-palmitoyl-9,10-^{3}H(N)]phosphatidylcholine (58 Ci/mmol) were purchased from NEN Research Products (Boston, MA). The decanoic acid, octanoic acid, and trifluoroacetic acids were obtained from Aldrich Chemical Co. (Milwaukee, WI). Palmitic acid, L-α-dipalmitoyl-phosphatidylcholine, cholesterol, and cholesteryl palmitate were obtained from Sigma Chemical Co. (St. Louis, MO). Tripalmitin was purchased from Supelco Inc. (Bellefonte, PA). Disposable spe® silica gel columns (500 mg sorbent) and 2′,7′-dichlorofluorscein were from J.T. Baker Chemical Company (Phillipsburg, NJ). Silica gel 60 analyatical TLC plates, layer thickness 0.25 mm, without fluorescent indicator were from EM Science (Darmstadt, West Germany). Bovine Fraction V albumin was obtained from Miles Laboratories Inc. (Elkhart, IN), and free fatty acids were removed using activated charcoal (15). Hionic-Fluor and Soluene-350 were purchased from Packard Instrument Co. (Downers Grove, IL). All other chemicals and solvents were of reagent grade and used without further purification.

Animals. Male Sprague-Dawley rats (275–300 g), were obtained from Harlan Sprague-Dawley (Indianapolis, IN) and allowed *ad libitum* access to feed (Purina Rat Chow, Ralston Purina Co., St. Louis, MO) and tap water. Rats were anesthetized with pentobarbital sodium (60 mg/kg intraperitoneally). Lightly heparinized blood was obtained from the inferior vena cava and the plasma was separated. The gastrocnemius, liver, kidney, epididymal fat pad, testis, and heart were excised, frozen by freeze-stop technique (16), and then ground to the consistency of fine sand in a pre-cooled mortar and pestle.

Preparation of radioactive material. Complexes of [1-^{14}C]PFDA and [1-^{14}C]PFOA to albumin were prepared (17). With each perfluorinated fatty acid, [9,10-^{3}H]palmitic acid was also complexed to albumin and used to label the endogenous tissue pool of FFA. Pooled tissue was divided into two aliquots (1–1.5 g) which comprised the two experiments. One aliquot of pooled tissue was labeled with [1-^{14}C]PFDA representing Experiment I, while the other aliquot was labeled with [1-^{14}C]PFOA for Experiment II. The albumin-complexes were added (2–3 μCi/g of both ^{3}H and ^{14}C) to frozen tissue powder and ground in a mortar and pestle that had been pre-cooled with liquid nitrogen. With plasma, FFA-albumin was added to thawed samples (0.3 μCi/ml of both ^{3}H and ^{14}C).

For determination of the radioactivity added, approximately 100 mg of tissue was solubilized in 2 ml of Soluene-350 at 60°C for at least 6 hr. Solubilized tissue and all other samples were counted in 10 ml of Hionic-Fluor scintillation cocktail. The ^{14}C and ^{3}H radioactivity was determined in a Packard Liquid Scintillation Analyzer (Model 2000CA). The quench was evaluated by channels ratio and dpm calculated with a Packard DPM 1-2-3 software program.

Proposed procedure. The isolation of lipids, including PFDA and PFOA, from tissues was adapted from a procedure described initially by Frost and Wells (18). Frozen tissue was homogenized in chloroform/methanol/1N H_2SO_4 (12:12:1, v/v) in a ratio of ten volumes of extracting solvent to one volume tissue. All tissues were assumed to have a density of 1 g/ml. After one hour the homogenate was centrifuged (12,000 × *g* for 20 min) at 4°C, and the first crude extract was saved. The pellet was homogenized in chloroform/methanol/1N H_2SO_4 (36:12:1, v/v) in the same ratio of solvent to tissue as in the initial homogenization. Following standing for one hour and centrifugation as described above, the second crude extract was combined with the first. An aqueous solution of 0.36 M H_2SO_4 and 15 mM NaCl was added to this total crude extract in a ratio of 5 volumes to 1 volume tissue, vortexed, and centrifuged (400 × *g* for 20 min) at 20–25°C. The upper phase was removed with a fine needle attached to a water aspirator, and the final lower phase was stored at 4°C.

Lipids were extracted from the plasma using a modification (18) of a technique reported initially by Bligh and Dyer (19). Plasma was extracted (at 20–25°C for 1 hr) with chloroform/methanol/1N H_2SO_4 (2:1:0.1, v/v) in a ratio of 6.2 volumes to one of plasma. Two phases were produced with the addition of chloroform and the aqueous solution of 0.36 M H_2SO_4 and 15 mM NaCl, each in a ratio of 2 volumes to 1 volume plasma. After centrifugation (400 × *g* for 20 min) at 20–25°C, the lipid containing lower phase was collected.

Lipid content of the final lower phase was determined by a micromethod for the estimation of nonvolatile organic matter (20). Tripalmitin and phosphatidylcholine yielded equivalent standard curves. The simple lipids, including the perfluorinated fatty acids, were separated from the complex lipids using disposable spe® silica gel columns (500 mg sorbent). The lipid samples to be separated were dried under a stream of nitrogen and dissolved in 0.1 ml chloroform/methanol (2:1, v/v). These lipid mixtures (7–10 mg lipid per column), were quantitatively transferred to a column using three times 0.1 ml chloroform/methanol (2:1, v/v). The mixture was allowed to adsorb to the silica gel for 5 min. Elution of simple lipids, including perfluorinated fatty acids, was carried out with 3 ml diethyl ether/trifluoroacetic acid (100:1, v/v) under slight negative pressure. The simple lipid eluate was dried under nitrogen at 20–25°C and dissolved in 0.5 ml chloroform/methanol (2:1, v/v).

A unidimensional two-solvent TLC system was adapted from the one originally described by Kelley (21). The major simple lipids: cholesterol, cholesteryl esters, triacylglycerols, and the free fatty acids—including PFDA and PFOA—were separated. Silica Gel 60 plates were activated in a 110°C oven for at least 12 hr. Standards were prepared in chloroform/methanol (2:1, v/v) to give a final concentration of 1 mg/ml. Tissue samples were applied 3 cm from the bottom of the plate and then developed to a height of 16 cm in a solvent system of petroleum ether/diethyl ether/trifluoroacetic acid (70:30:1, v/v). The plates were air-dried and developed to a height of 11 cm in the same direction in diethyl ether/petroleum ether/trifluoroacetic acid (70:30:1, v/v). Lipids were visualized by spraying the plates with 2′,7′-dichlorofluorscein (0.1%, w/v) in methanol, followed by examination under uv light. The spots were then scraped and eluted in 1 ml diethyl ether/trifluoracetic acid (100:1, v/v) following centrifugation (400 × *g* for 20 min) at 20–25°C, and finally were transferred using a pasteur pipette.

In vivo administration of [1-^{14}C]PFDA and [1-^{14}C]-PFOA. Radiolabeled PFDA and PFOA (4.62 mCi/mmol and 5.44 mCi/mmol, respectively) were dissolved in propylene glycol/water (1:1, v/v) and administered via i.p. injection. The rats received 9.4 μmol/kg body weight of either [1-^{14}C]PFDA or [1-^{14}C]PFOA and were killed four days later. The liver was then excised and stored as previously discussed. The extraction, separation, and isolation of PFDA- and PFOA-derived radioactivity from the liver was performed as discussed in the proposed procedure of Materials and Methods.

RESULTS AND DISCUSSION

The initial procedure employed to extract tissue lipids along with the perfluorinated fatty acids was an adaptation of the method described by Folch *et al.* (22). Extraction with chloroform/methanol (2:1, v/v) of palmitic acid and both the perfluorinated acids from liver tissue was nearly complete (Table 1). The subsequent addition of 50 mM NaCl solution, which is necessary for phase separation, gave 83% recovery of palmitic acid from the crude extract in the lower organic phase. In contrast, only 18 and 20% of PFDA and PFOA, respectively, were recovered in this lower phase. The perfluorinated fatty acids were distributed predominantly in the upper aqueous phase. An additional wash with chloroform/methanol/water (3:48:47, v/v) improved the recovery of palmitic acid in the final lower phase, whereas that of both the perfluorinated acids worsened (Table 1). The partitioning of PFDA and PFOA into the more polar upper phase is not unexpected. Recovery of more than 70% of medium-chain fatty acids from aqueous solutions is often difficult (12,23). Additionally, the electron-withdrawing ability of the fluorine atoms in PFDA and PFOA stabilizes the anion which is formed by the dissociation of the carboxyl hydrogen (24). This results in the more favored partitioning of the perfluorinated acids into the aqueous phase.

As PFDA and PFOA were not recovered to the same extent as palmitic acid with the Folch extraction procedure, an alternative method for their isolation was examined. A two-stage procedure described by Frost and Wells (18) for the extraction of decanoic acid from rat tissues utilizes sulfuric acid to decrease the pH of the solvent system, thereby enhancing the movement of FFA into the less polar organic phase. With this method, extraction from liver of all FFA (including the perfluorinated fatty acids) was complete (Table 2). Phase separation effected by the addition of NaCl as described by Frost and Wells (18) resulted in an incomplete recovery of PFDA and PFOA (data not shown). Perfluorinated fatty acids are strong organic acids with pK_a's in the range of 0.5–0.8 (25). The proposed acidification (0.36 M H_2SO_4) of the saline solution aided in the partitioning of these fatty acids into the lower organic phase (Table 2). The loss of PFDA and PFOA was reduced from 84% and 79% in the Folch *et al.* procedure (Table 1) to 8% and 16%, respectively, in the proposed method (Table 2). Further increases in the concentration of sulfuric acid in the aqueous NaCl solution did not improve recovery of PFDA

TABLE 1

Distribution of PFDA and PFOA in a Chloroform/Methanol Extract of Rat Liver[a]

	Experiment I[b]		Experiment II[b]	
	[^{3}H]Palmitic acid	[^{14}C]PFDA	[^{3}H]Palmitic acid	[^{14}C]PFOA
	(dpm/mg wet weight)			
Total radioactivity	3640 ± 124	4050 ± 95	3090 ± 230	3130 ± 200
Crude extract	4000 ± 493	4070 ± 52	3410 ± 466	3150 ± 430
Pellet	192 ± 85	743 ± 133	153 ± 78	633 ± 137
First upper phase	120 ± 34	3400 ± 523	79 ± 20	2470 ± 303
First lower phase	3380 ± 387	342 ± 44	2810 ± 406	108 ± 14
Second upper phase	9 ± 3	106 ± 28	8 ± 2	52 ± 9
Final lower phase	3640 ± 422	291 ± 34	3050 ± 349	74 ± 10

[a] Performed with a Folch *et al.* (22) isolation procedure adapted to small samples. Tissue was homogenized in 19 volumes chloroform/methanol (2:1, v/v) and centrifuged at 1000 × *g* for 10 min at 20–25°C. A volume of 50 mM NaCl equal to 0.2 times the volume of the crude extract was added, vortexed, and centrifuged as above. The first upper phase was removed and a washing solution of chloroform/methanol/water (3:48:47, v/v) was added in a volume 0.15 times the crude extract. The second upper phase was removed. Values are means ± SD for five aliquots of the same tissue sample.

[b] Preparation of radioactive tissue is described in Materials and Methods.

TABLE 2

The Effect of Addition of Acid on the Distribution of PFOA and PFDA in a Chloroform/Methanol Extract of Rat Liver[a]

	Experiment I[b]		Experiment II[b]	
	[^{3}H]Palmitic acid	[^{14}C]PFDA	[^{3}H]Palmitic acid	[^{14}C]PFOA
	(dpm/mg wet weight)			
Total radioactivity	6450 ± 311	7260 ± 400	4750 ± 221	3890 ± 285
First crude extract	5460 ± 526	5570 ± 516	4370 ± 214	3130 ± 272
Total crude extract	5890 ± 291	6650 ± 293	4360 ± 366	3590 ± 308
Pellet	117 ± 23	280 ± 166	80 ± 20	135 ± 46
Upper phase	59 ± 17	581 ± 103	42 ± 13	618 ± 95
Final lower phase	6420 ± 283	6470 ± 283	4890 ± 442	3100 ± 337

[a] As given in the proposed procedure of Materials and Methods. Values are mean ± SD for five aliquots of the same tissue sample.

[b] Preparation of radioactive tissue is described in Materials and Methods.

and PFOA in the organic phase due to a great decrease in the volume of this lower phase. Trifluoroacetic acid, which is used in subsequent eluting and developing solvents, was not useful in this step. Substitution of sulfuric acid with trifluoroacetic acid in the proposed extraction procedure resulted in the formation of a tri-phasic system, further complicating the isolation of the tissue lipids. Thus sulfuric acid was used preferentially in systems with aqueous components (e.g., extraction of PFDA and PFOA from tissue), whereas trifluoroacetic acid was more useful in steps utilizing organic solvents (e.g., elution of perfluorinated acids from silica gel). The applicability of the proposed procedure to a number of rat tissues was examined (Table 3). The overall average recovery of [9,10-^{3}H]palmitic acid from all tissues was 98%, whereas that of [1-^{14}C]PFDA and [1-^{14}C]PFOA was 87 and 84%, respectively.

Adsorption chromatography on silica gel columns has been used extensively to separate simple lipids—including FFA—from complex lipids in organic extracts. A method described initially by Roemen and Van Der Vusse (26) eluted heart tissue FFA from silica gel columns using chloroform/methanol (39:1, v/v). The perfluorinated fatty acids were bound with greater affinity than palmitic acid to the silica gel and were not eluted with this mixture (Table 4). Based on eluting strength, solvent mixtures can be employed to selectively separate lipid classes (27,28). Diethyl ether has a greater eluting strength than chloroform and can remove the neutral lipids that bind more tightly to the column. Acidic simple lipids are known to be strongly adsorbed to silica gel and can be removed using diethyl ether (27). However, diethyl ether alone did not improve the elution of the perfluorinated fatty acids from the columns (Table 4). The addition to diethyl ether of trifluoroacetic acid (100:1, v/v), a strong organic acid (pK_a of 0.3), resulted in complete recovery of radioactivity in the eluate. [^{3}H]Phosphatidylcholine was added to a liver organic extract to label the endogenous pool of PL and was shown to remain bound to the column under these conditions (96%).

TABLE 3

Recovery of PFDA and PFOA from Various Rat Tissues with an Acidified Chloroform/Methanol Extract[a]

	Experiment I[b]		Experiment II[b]	
	[^{3}H]Palmitic acid	[^{14}C]PFDA	[^{3}H]Palmitic acid	[^{14}C]PFOA
	(% Recovery[c])			
Liver	99 ± 4	89 ± 4	103 ± 9	80 ± 9
Heart	105 ± 9	91 ± 8	91 ± 12	80 ± 10
Gastrocnemius	92 ± 5	81 ± 5	92 ± 8	84 ± 7
Testis	95 ± 8	85 ± 9	96 ± 7	93 ± 8
Epididymal fat pad	99 ± 11	91 ± 9	87 ± 9	76 ± 7
Kidney	105 ± 8	91 ± 6	105 ± 8	96 ± 6
Plasma	102 ± 6	83 ± 3	97 ± 3	77 ± 4

[a] As given in the proposed procedure of Materials and Methods.

[b] Preparation of radioactive tissue is described in Materials and Methods.

[c] Recovery expressed as a percent of radioactivity determined in a solubilized tissue sample. Values are mean ± SD for five aliquots of the same tissue sample.

TABLE 4

Recovery of PFDA and PFOA from spe® Silica Gel Columns

	FFA		
Eluting solvent mixture	[^{3}H]Palmitic acid	[^{14}C]PFDA	[^{14}C]PFOA
	(% Recovery[a])		
Chloroform/methanol (39:1, v/v)	77[b]	<1	<1
Diethyl ether	69	2	2
Diethyl ether/trifluoroacetic acid (100:1, v/v)	103 ± 8[c]	102 ± 3	96 ± 7

[a] Recovery expressed as percent of radioactivity in FFA added to the column with a lipid extract of liver.

[b] The value of a single determination is indicated for each FFA with both chloroform/methanol and diethyl ether elutions.

[c] Given as mean ± SD for five replicates.

Glacial acetic acid is used in thin layer chromatography of lipids to ensure the presence of fatty acids in the unionized form and also to facilitate their development (migration) on the TLC plate. A unidimensional, two-solvent system (21) utilizing acetic acid was unable to move the PFDA and PFOA from the origin, whereas their nonperfluorinated analogs migrated to the same extent as palmitic acid (Fig. 1). Replacement of acetic acid with trifluoroacetic acid caused PFDA and PFOA to behave similarly to decanoic and octanoic acids (Fig. 2). The major lipid classes were also easily distinguishable in this system. The typical R_f values for cholesteryl palmitate (0.78), tripalmitin (0.58), palmitic acid (0.48), and cholesterol (0.34) that were relative to the first solvent front were also very similar to those found in the acetic acid system. The final recovery after elution from the TLC plate, which is expressed as a percent of radioactivity in the solubilized liver sample was 86, 74, and 94% for PFDA, PFOA, and palmitic acid, respectively.

The presence of sulfuric acid in the aqueous saline solution and trifluoroacetic acid in eluting and developing solvents allows PFDA and PFOA to be studied concurrently with nonperfluorinated FFA. Thus, the inclusion of strong acids at appropriate steps in extraction, separation, and isolation of perfluorinated fatty acids from tissue samples is imperative.

The proposed procedure for the extraction, separation, and isolation of the perfluorinated acids was validated following an *in vivo* administration of either PFDA or PFOA to rats (Table 5). The amount of PFDA- or PFOA-derived radioactivity found within the liver four days after i.p. administration accounted for approximately 50 and 20% of the administered dose, respectively. The acidified chloroform/methanol extraction procedure resulted in recovery of over 80% of the total radioactivity from the livers of both PFDA-and PFOA-treated animals. When placed on the silica gel columns, the majority of the [1-^{14}C] labeled material in the extract was eluted with the simple lipids using diethyl ether/trifluoroacetic acid (100:1, v/v). The complex lipids, such as phospholipids, were removed from the column using chloroform /methanol/water (5:5:1, v/v). This eluate contained less than 3% of the total tissue radioactivity. The final recovery of liver [^{14}C] in the simple lipid eluate was 74% for PFDA-treated rats and 78% for PFOA-treated rats. The behavior of ^{14}C-derived radioactivity in the liver of

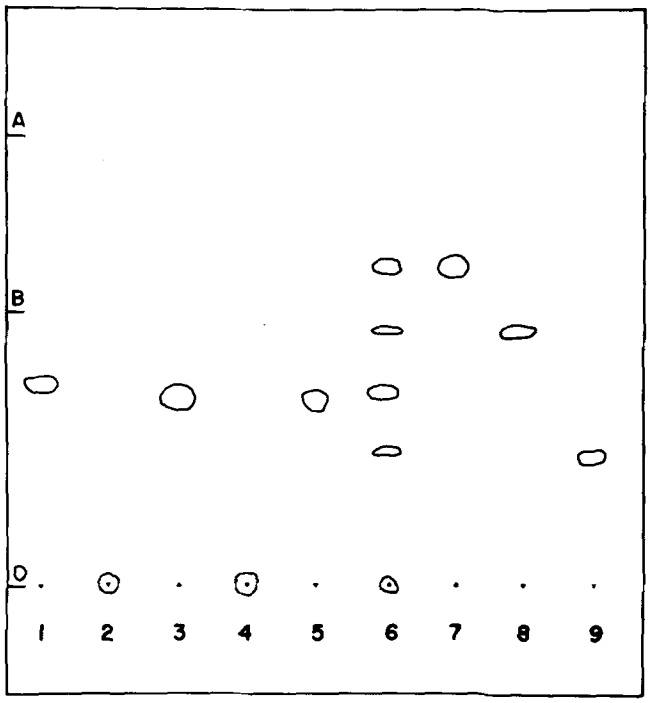

FIG. 1. TLC separation of fatty acid and simple lipid standards, using glacial acetic acid in the developing solvents. Lipids were spotted 3 cm (O) from the bottom of the TLC plate and developed to a height of 16 cm (A) in a solvent system of petroleum ether/diethyl ether/glacial acetic acid (70:30:1, v/v). The plate was air-dried and developed in the same direction in diethyl ether/petroleum ether/glacial acetic acid (70:30:1, v/v) to a height of 11 cm (B). Standards were dissolved in chloroform/methanol (2:1, v/v) and spotted as follows: 1, palmitic acid (20 μg); 2, PFDA (50 μg); 3, decanoic acid (50 μg); 4, PFOA (50 μg); 5, octanoic acid (50 μg); 6, mixture of palmitic acid, PFDA, cholesteryl palmitate, tripalmitin, and cholesterol; 7, cholesteryl palmitate (20 μg); 8, tripalmitin (20 μg); 9, cholesterol (20 μg).

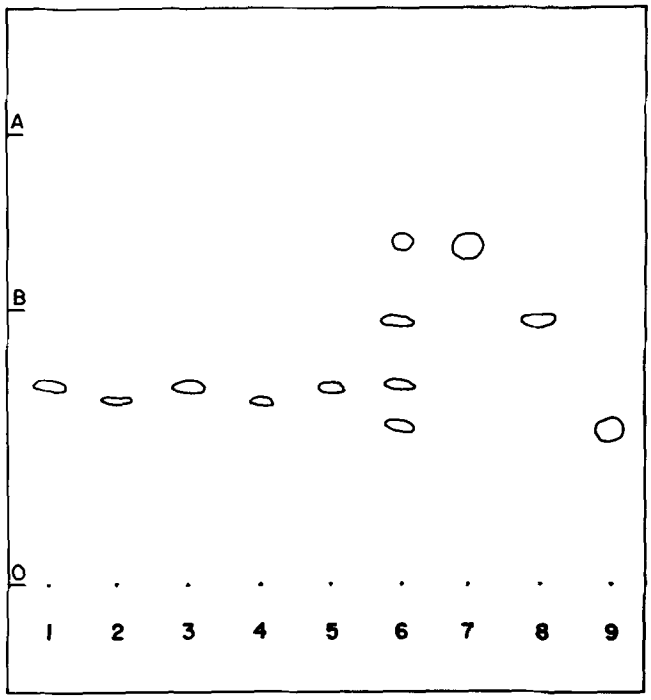

FIG. 2. TLC separation of fatty acid and simple lipid standards, using trifluoroacetic acid in the developing solvents. All conditions were the same as described in the legend to Fig. 1, except that glacial acetic acid was replaced by trifluoroacetic acid in the TLC developing system.

TABLE 5

Extraction and Separation of Hepatic [^{14}C]PFDA- and PFOA-derived Radioactivity Four Days Following *In Vivo* Administration to the Rat[a]

	[^{14}C]PFDA- and PFOA-derived radioactivity	
	PFDA	PFOA
	(dpm/mg wet tissue)[b]	
Total radioactivity	1080 ± 76	429 ± 68
Chloroform/methanol extract	889 ± 43	364 ± 55
Complex lipids	28 ± 10	12 ± 2
Simple lipids	804 ± 5	333 ± 37

[a]Male Sprague-Dawley rats (200 ± 20 g) were given a single i.p. injection of either [1-^{14}C]PFDA (9.4 μmol/kg, 4.62 mCi/mmol) or [1-^{14}C]PFOA (9.4 μmol/kg, 5.44 mCi/mmol) and killed four days later. The liver was excised and stored as stated in Materials and Methods. Extraction and separation techniques are given in the proposed procedures of Materials and Methods.

[b]Values are the mean ± SD for four animals.

the *in vivo* treated animals resembled that seen for the extraction (Table 2) and separation (Table 4) of tissue labeled with the parent compounds *in vitro*. In addition, utilization of simple lipid TLC methods, as described in the proposed procedure, resulted in essentially all the radioactivity being recovered in the FFA-spot. Thus, four days following a single i.p. dose (9.4 μmol/kg) of either PFDA or PFOA, [^{14}C]perfluorinated acid-derived radioactivity was found as the parent compound and was not incorporated into triacylglycerols or cholesterol esters.

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Determination of HDL_2 Cholesterol by Precipitation with Dextran Sulfate and Magnesium Chloride: Establishing Optimal Conditions for Rat Plasma

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Optimal conditions for analyzing HDL_2 cholesterol in small amounts of rat plasma have been studied using different concentrations of dextran sulfate and $MgCl_2$ to precipitate lipoproteins containing apolipoprotein B and/or apo E. When the $MgCl_2$ level was 91 mM, the supernate cholesterol was rather constant at a level of about 50–60% of the total plasma cholesterol concentration. Immunochemical determination of the apo A-I content indicated that no major losses of the HDL_2 fraction took place under these conditions. The recovery of about 96% of HDL_2 lipoproteins after the precipitation of rat plasma and the almost complete absence of lipoproteins belonging to the VLDL, LDL and HDL_1 fractions was demonstrated by agarose gel electrophoresis. Thus, the method should be suitable for screening the HDL_2 cholesterol content in small volumes of rat plasma.
Lipids 24, 532–534 (1989).

During recent years much interest has been focused on the function of plasma HDL in the regulation of total body cholesterol metabolism and its possible role as a protective factor against the development of atherosclerosis and ischemic heart disease in man (1,2). Despite the widely held view that nutritional inadequacies may promote the development of hypercholesterolemia and atherosclerosis, little is known of the effects of dietary factors on the concentration and properties of plasma high density lipoprotein (HDL). Not only the level of dietary fat and cholesterol, but also the nature of the dietary protein has been shown to exert a marked effect on the plasma cholesterol concentration in both experimental animals and humans (3). In studies at our laboratory, we have observed significant effects of the type of dietary protein on plasma triacylglycerol and cholesterol levels, and on the plasma contents of very low density lipoprotein (VLDL) and low density lipoprotein (LDL) in both female and male rats (4,5).

So far, very little is known of possible influences of the nature of the dietary protein on blood HDL levels. Reliable screening methods, involving precipitation of VLDL and LDL by polyanions, e.g., heparin or dextran sulfate in combination with divalent cations, e.g., Mn^{2+}, Mg^{2+} or Ca^{2+}, have been developed for the HDL cholesterol analysis of human plasma or serum (6). However, the indiscriminate use of such methods in rat studies may give erroneous results because the optimal precipitation conditions in rat plasma may be different from those established in human plasma (7). The situation is also more complex in rats, compared with humans, because rat plasma contain an apo E-rich HDL fraction, HDL_1, which is not normally present in human plasma. Therefore, the aim of the present investigation was to study if methods used for HDL_2 cholesterol analysis in human plasma (6) are suitable for use also with rat plasma. Comparisons were also made with a similar method based on precipitation with phosphotungstate and Mg^{2+} ions (8). A third widely used precipitation method based on heparin and Mn^{2+} ions was not considered since Mn^{2+} ions interfer with the presently employed method for enzymatic determination of cholesterol (9,10).

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Abbreviations: Apo, apolipoprotein; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; EDTA, ethylenediamine tetraacetic acid; HDL, high density lipoprotein; LDL, low density lipoprotein; Tris,tris(hydroxymethyl)aminomethane; VLDL, very low density lipoprotein.

MATERIALS AND METHODS

Preparation of plasma. Male outbred Sprague-Dawley strain rats, weighing about 300–350 g, were used as blood donors. The rats had been maintained on a commercial chow diet (R3:brood stock feed for laboratory rats and mice, Ewos, Södertälje, "normocholesterolemic rats"), or an atherogenic casein diet (5) containing 0.5% cholesterol ("hypercholesterolemic rat") for 4 weeks. Prior to sampling, the rats were fasted for 4 hr and then anesthetized with diethyl ether and killed by terminal exsanguination. The blood was collected from the abdominal aorta in centrifuge tubes containing 40 μmol EDTA, 1 nmol DTNB (inhibitor of the enzyme lecithin-cholesterol acyltransferase, EC 2.3.1.43) and 2000 KIE Trasylol (aprotinin, proteinase inhibitor [Bayer, Leverkusen]) in 410 μl 0.15 M sodium chloride. Plasma was separated at 500 × *g* for 20 min.

Precipitation of plasma. Dextran sulfate of a weight average molecular weight of about 500 000 (Pharmacia Fine Chemicals AB, Uppsala, Sweden) was used to prepare solutions in distilled water ranging in concentrations from 10 to 60 g/liter. The concentration of the magnesium chloride solutions used ranged from 0.5 to 4 mol/liter.

Fifty μl of fresh plasma were precipitated by adding 5 μl of a mixture of equal volumes of dextran sulfate solution and $MgCl_2$ solution at different specified concentrations in 1.5 ml micro-centrifuge polypropylene test tubes (Treff AG, Degerheim, Switzerland). The mixture was incubated at +4°C for 1 hr, then centrifuged at 2500 × *g* for 15 min. The supernate was separated and used for cholesterol and apo A-I analysis and agarose gel electrophoresis. All precipitations were performed in duplicates. The concentration values given for dextran sulfate and $MgCl_2$ in the results section are the final concentrations reached in the incubation mixture.

In addition, precipitation was also carried out with sodium phosphotungstate and $MgCl_2$ as described by Siegler and Wu (8).

Cholesterol analysis. A commercial enzymatic colorimetric test (Prod. No. 237574, Test-Combination, Boehringer, Mannheim) was used to determine total cholesterol contents (free and esterified cholesterol) of whole

plasma and the supernates obtained by centrifugation of precipitated plasma. All analyses were performed in duplicate.

Determination of apo A-I. Apo A-I was analyzed by electroimmuno assay (11). Monospecific antibodies were raised in rabbits by immunization with apo A-I purified as described elsewhere (12). The following conditions were employed for the apo A-I assay. The gel was prepared in 0.02 M Tris/0.005 M Tricine (N-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine) buffer, pH 8.6, containing 0.3 mM calcium lactate, 1% agarose (Indubiose A37, IBF) and 5% Dextran T-10 (Pharmacia Fine Chemicals, Uppsala, Sweden). The Tris-Tricine buffer served as the electrode buffer as well as sample diluent. Electrophoresis was performed at +5°C, 6 hr at 10 V/cm.

Agarose gel electrophoresis. The agarose gel electrophoresis of plasma lipoproteins was carried out in phosphate buffer at pH 7.0 as previously described (13). The gels were stained with Sudan Black B and evaluated by densitometric analysis on an Ultra Scan XL, enhanced laser densitometer (LKB, Bromma). The data was processed using a LKB 2400 Gel Scan XL program.

RESULTS AND DISCUSSION

Supernate cholesterol levels observed after dextran sulfate/$MgCl_2$ precipitation of plasma. Table 1 shows the amount of cholesterol recovered in the supernates after precipitation of plasma with specific combinations of dextran sulfate and $MgCl_2$. Precipitation with a final concentration of 91 mM $MgCl_2$ at each of the dextran sulfate concentrations used in the experiment resulted in a minimum level corresponding to about 0.93 to 1.06 mM cholesterol, representing about 50–60% of the cholesterol content of whole plasma. Using a lower concentration of $MgCl_2$, as is commonly the case in "human" methods, resulted in supernate cholesterol contents significantly higher than this minimum level. This indicates insufficient precipitation of one or more of the lipoprotein fractions of the VLDL, LDL and/or HDL_1 density intervals. That this was the case was verified by the presence of VLDL and LDL and/or HDL_1 bands on agarose gel electrophoresis (results not shown). Precipitation of part of the HDL_2 fraction seemed to occur at the two highest $MgCl_2$ levels. Partial precipitation of rat plasma HDL fraction with increasing amount of polyanions and cations has also been reported by Gustavsson and Nordlund (7).

These findings underline that methods used for HDL cholesterol determinations may give incorrect results with rat plasma, unless the $MgCl_2$ concentration used in the precipitation is increased compared with that commonly used in procedures adapted for human samples. At any rate, this is true when commercially available dextran sulfate preparations of a molecular weight of 500,000 is used as the polyanionic component. As shown more conclusively below the proportion of whole plasma cholesterol found in the supernate under optimal precipitation conditions, i.e., using an $MgCl_2$ concentration of 91 mM actually represents the plasma HDL_2 cholesterol fraction.

The lipoprotein and apo A-I contents of supernates. The lipoprotein composition of supernates was qualitatively studied by means of agarose gel electrophoresis at pH 7.0. We have previously shown, by using fractions isolated by preparative ultracentrifugation and analyzed by SDS-polyacrylamide gel electrophoresis of apolipoproteins, that the leading band represent the HDL_2 fraction, and that VLDL and LDL plus HDL_1 fractions are positioned as indicated in Figure 1 (13).

In the supernates representing optimal precipitation conditions (see above), only distinct HDL_2 bands

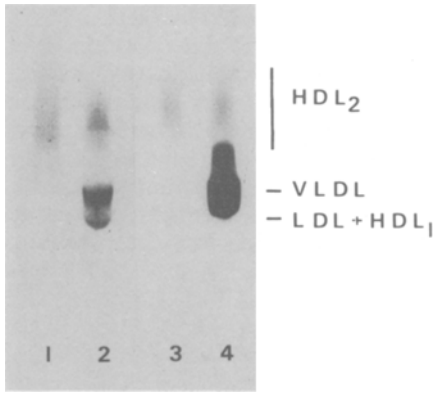

FIG. 1. Agarose gel electrophoresis of precipitated normal plasma (lane 1) and whole normal rat plasma (lane 2), precipitated hypercholesterolemic plasma (lane 3) and hypercholesterolemic whole plasma (lane 4). Stained with Sudan Black B. Precipitation conditions were 91.0 mmol/l of $MgCl_2$ and 2.73 g/l of dextran sulfate.

TABLE 1

Cholesterol and Apolipoprotein A-I Contents of Supernates Obtained After the Precipitation of Plasma With Dextran Sulfate and $MgCl_2$ at Various Specified Combinations[a]

$MgCl_2$ concentration (mmol/l)	n	Dextran sulfate concentration (g/l) 0.45 Cholesterol (mmol/l)	0.45 apo A-I (g/l)	0.91 Cholesterol (mmol/l)	0.91 apo A-I (g/l)	1.82 Cholesterol (mmol/l)	1.82 apo A-I (g/l)	2.73 Cholesterol (mmol/l)	2.73 apo A-I (g/l)
22.5	6	1.68 ± 0.11	0.29 ± 0.04	1.48 ± 0.20	0.24 ± 0.03	1.56 ± 0.26	0.31 ± 0.01	1.74 ± 0.24	0.31 ± 0.04
45.0	6	1.34 ± 0.32	0.32 ± 0.02	1.17 ± 0.11	0.29 ± 0.02	1.17 ± 0.15	0.29 ± 0.03	1.37 ± 0.20	0.32 ± 0.03
91.0	6	1.05 ± 0.15	0.29 ± 0.04	0.99 ± 0.04	0.29 ± 0.01	0.93 ± 0.13	0.28 ± 0.05	0.97 ± 0.15	0.30 ± 0.05
136.0	3	0.90 ± 0.13	0.24 ± 0.01	0.86 ± 0.22	0.14 ± 0.04	0.77 ± 0.18	0.17 ± 0.04	0.86 ± 0.22	0.23 ± 0.08
182.0	3	0.86 ± 0.13	0.23 ± 0.02	0.86 ± 0.16	0.15 ± 0.02	0.82 ± 0.26	0.16 ± 0.08	0.99 ± 0.15	0.29 ± 0.02

[a]The values represent mean value and standard deviation of n = 6 or n = 3 different experimental series, using plasma from normocholesterolemic rats. Each point was run in duplicate in each individual series.

appeared and there were no signs of any lipoproteins belonging to VLDL, LDL or HDL_1. The staining intensity of the HDL_2 band was virtually the same in precipitated and unprecipitated plasma, indicating that there was no major loss of this lipoprotein fraction during precipitation. In a separate study, six different rat plasmas were precipitated with $MgCl_2$ (91 mM) and dextran sulfate (at each of the three highest concentration levels shown in Table 1). The whole plasma as well as the precipitated plasma was subjected to agarose gel electrophoresis and the dye uptake in the different lipoprotein fractions was evaluated by densitometric analysis. The amount of HDL_2 recovered after precipitation constituted 96% (SD = 5%) of the HDL_2 content of whole plasma, irrespective of the dextran sulfate concentration used. That there was quite a low loss of HDL_2 during precipitation was further substantiated by the results of the apo A-I analyses (Table 1). The apo A-I concentration did not differ between whole plasma and precipitated plasma supernates. However, at the two highest $MgCl_2$ concentrations used, a fall in apo A-I content of supernates was observed indicating partial precipitation of HDL_2. Results showing a complete precipitation of VLDL, LDL and HDL_1, with no loss of HDL_2, were achieved under optimal precipitation conditions with normal as well as hypercholesterolemic (Figure 1) rat plasma. In comparison, the phosphotungstate/Mg^{2+} method gave about 5-10% lower values for HDL_2 cholesterol than were observed with the dextran sulfate method (results not shown). This discrepancy may be due to some differential interference with the enzymatic cholesterol determination, or to a small degree to coprecipitation of HDL_2 (6) when using the phosphotungstate method.

In conclusion, the dextran sulfate method used under optimal precipitation conditions is suitable to use for screening the HDL_2 cholesterol content in small volumes of normal as well as hypercholesterolemic rat plasma. It is also a convenient method for achieving a rapid isolation of a HDL_2 fraction for apolipoprotein, lipid class and/or fatty acid analysis from a small volume of plasma.

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Application of Selective Ion Monitoring to the Analysis of Molecular Species of Vegetable Oil Triacylglycerols Separated by Open-Tubular Column GLC on a Methylphenylsilicone Phase at High Temperature

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Analyses of the molecular species of authentic triacylglycerols and of soybean triacylglycerols, which were separated on a fused silica open-tubular column coated with a methylphenylsilicone gum stationary phase, were carried out by selective ion monitoring mass spectrometry. It was determined that peak assignments could be made by selecting certain characteristic ions with the same retention time on the SIM profile, that is, three ions of the type RCO^+ corresponding to the fatty acyl residues (R_1CO^+, R_2CO^+ and R_3CO^+) and the corresponding three M-acyl ions ($[M-OCOR_1]^+$, $[M-OCOR_2]^+$ and $[M-OCOR_3]^+$) instead of the molecular ion. The yield of $[RCO-1]^+$ and $[RCO-2]^+$ and relating these to the unsaturated fatty acyl residues is inconvenient for an exact peak assignment. The SIM method was further applied to the peak assignment of the triacylglycerols from palm, cottonseed and safflower oils and certain new fatty acid combinations are proposed.
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The analysis of the molecular species of naturally occurring triacylglycerols by high performance liquid chromatography (HPLC) on a reversed phase column has been successfully applied (1-5). Recently, HPLC coupled to a mass spectrometer has been used for the identification of the molecular species of triacylglycerols (6-9). Gas liquid chromatography (GLC) has also been used for this purpose, using apolar columns such as JXR or SE-30. These will separate triacylglycerols on the basis of total acyl carbon number (9-11). With recent progress in fused silica open-tubular columns, triacylglycerol separations have been achieved on the basis of the number of double bonds, as well as on total number of acyl carbons. Both "polar" columns, usually methylphenylsilicone gums, and apolar liquid phases may be used (12-14). There have already been several applications of the "polar" columns in the separation of triacylglycerols from vegetable oils (15-17), egg yolk, and chicken and butter fats (18). In these, the identification of peak components was based on well-documented literature data, and on the interpretation of triacylglycerol profiles obtained from other analytical techniques, e.g., reversed phase HPLC and GLC of the sample spiked with standards. The final assignments were made in these cases by the chromatographic retention order obtained with several authentic standards (15,18).

The effective technique of GC/MS has been applied to the analyses of triacylglycerols separated by GLC with both packed (19,20) and open-tubular (13) apolar columns. In the case of the "polar" open-tubular columns, however, our early work showed that a high level of background noise, caused by bleeding from the column, prevented satisfactory analyses of the electron impact mass spectra. When the sample was increased to improve the signal to noise ratio, column sample overload occurred, and the resolution between peaks became poor. An alternative highly sensitive and selective method, selective ion monitoring (SIM), has therefore been applied to the peak assignments. By monitoring several characteristic fragment ions derived from the electron impacted triacylglycerol, satisfactory peak identifications for soybean triacylglycerols were achieved, and extended to other vegetable oil triacylglycerols.

MATERIALS AND METHODS

Materials. A triacylglycerol mixture of SSS, OOO, LLL and LnLnLn, of >99% purity, was purchased from Gaschro Kogyo Inc. (Tokyo, Japan). SSS and LLL of >99% purity were obtained from Sigma Chemical Co. (St. Louis, MO) and anhydrous sodium methoxide from Aldrich Chemical Co. (Milwaukee, WI). Other chemicals were of reagent grade.

Interesterification. Triacylglycerols of the mixed type were synthesized by interesterification between authentic SSS and LLL using sodium methoxide as a catalyst (21). The reaction was performed at 120°C for 20 min in a vacuum with continuous stirring.

Isolation of vegetable oil triacylglycerols. Triacylglycerols of commercial retail vegetable oils were isolated by silicic acid preparative thin-layer chromatography (22).

Gas chromatography (GC) of fatty acid methyl esters (FAME). The fatty acid composition of the triacylglycerols was determined by GLC on a Supelcowax-10 fused silica open-tubular column (0.25 mm i.d. × 30 m, 0.25 μm in film thickness, Supelco Japan, Tokyo) at 195°C using helium as the carrier gas.

GLC and SIM-GC/MS of triacylglycerols. Triacylglycerols were analyzed by GLC using a Shimadzu GC 12A instrument (Kyoto, Japan), with flame ionization detection (FID), equipped with a MP65HT fused silica open tubular capillary column (65% methylphenylsilicone,

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Abbreviations: L, linoleic acid (C18:2 n-6); LLL, trilinolein; Ln, linolenic acid (C18:3 n-3); LnLnLn, trilinolenin; M. myristic acid; O, oleic acid (C18:1 n-9); OOO, triolein; P, palmitic acid; S, stearic acid; SSS, tristearin; DB, the number of double bonds in fatty acyl chains; FAME, fatty acid methyl esters; FID, flame ionization detector; GC, gas chromatography; GC/MS, gas chromatography/mass spectrometry; GLC, gas-liquid chromatography; HPLC, high performance liquid chromatography; Rt, retention time; SIM, selective ion monitoring; TC, the number of total acyl carbons.

0.1 μm thickness, 0.25 mm i.d. × 25 m, Quadrex, New Haven, CT) and a Shimadzu movable solventless on-column injector. The injector and column oven were held isothermally at 370°C and 345°C, respectively. Helium was used as the carrier gas at a constant inlet pressure of 1.75 Kg/cm^2. Usually 1-2 μl of 0.02% chloroform solutions of triacylglycerols were injected.

SIM-Mass spectrometric analysis of triacylglycerols was carried out on a Shimadzu QP 1000 quadrupole mass spectrometer fitted with an EI source to which the outlet of the MP65HT column (0.25 mm i.d. × 15 m) was connected directly. The conditions were 70eV electron beam energy, 3 KV accelerating energy and a source temperature of 330°C.

RESULTS AND DISCUSSION

SIM of simple acid triacylglycerols. The most intense fragment ions were obtained at 70 eV and an ion source temperature of 330°C, but under these conditions most of the triacylglycerols yielded a weak molecular ion with an intensity less than 1% of the base peak (23). The intense ions found were usually $[M-OCOR]^+$, $[M-CH_2OCOR]^+$, $[M-RCOOH]^+$, RCO^+, $[RCO+74]^+$ and $[RCO+128]^+$. The first three ions produce useful information on the molecular weight of the triacylglycerol, and the last three ions reflect the nature of the acyl chains. Under our experimental conditions, the overall profiles of SIM chromatograms of RCO^+, $[RCO+74]^+$ and $[RCO+128]^+$ were very similar. Therefore, we selected the most intense ion RCO^+, from the first three fragment ions. For similar reasons, $[M-OCOR]^+$ was selected as the appropriate ion yielding information on the molecular weight of the triacylglycerol.

Intact SSS, OOO, LLL and LnLnLn, which were mixed in equal amounts, were separated by GLC and monitored by both FID and SIM. The FID trace (not shown) gave a definite LnLnLn peak eluting last and relatively small compared to the first three. The SIM profiles obtained are shown in Figure 1. When the fragment ions monitored were m/z 267, m/z 265 and m/z 607, the Rt of peak No. 1 was 7.4 min. The fragment ion of m/z 267 corresponded to the RCO^+ of a stearic acid component. The ion of m/z 607 is equivalent to the difference in mass number between the molecular weight (890) of SSS and a stearic acid anion. Since all of the triacylglycerols analyzed here were composed of one type of acyl group, this peak could be identified with confidence as SSS. At Rt 8.2 min, peaks of fragment ions at m/z 265, m/z 267, m/z 263, m/z 603 and m/z 607 were detected. A triacylglycerol having unsaturated fatty acyl groups usually yields $[RCO+2]^+$ and $[RCO+1]^+$ as well as RCO^+. These ions are relatively more intense than those from saturated triacylglycerols (23). Thus, the characteristic ions resulting from the unsaturated triacylglycerol OOO are m/z 265, which corresponds to RCO^+ of an oleic acid residue, and m/z 267 and m/z 263, to $[RCO+2]^+$ and $[RCO-2]^+$, respectively. The ion of m/z 603 is due to cleavage of an oleic acid anion from OOO. It was concluded from these results that the triacylglycerol of peak No. 2 is definitely OOO.

A triacylglycerol having more unsaturated fatty acyl groups yields more intense $[RCO+1]^+$ and $[RCO+2]^+$.

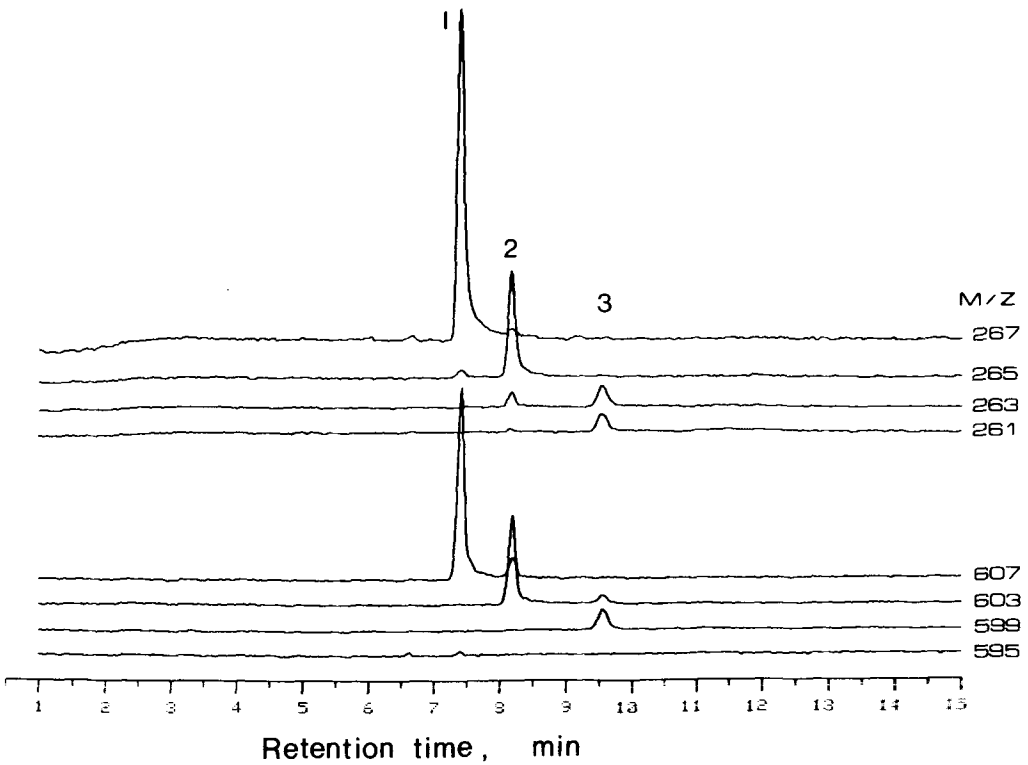

FIG. 1. Selective ion monitoring profiles arising from a standard mixture of SSS, OOO, LLL and LnLnLn. 1, SSS; 2, OOO; 3, LLL. The fragment ions of m/z 261 and m/z 595 from LnLnLn were not detected (see text). Column, MP65HT (0.25 mm i.d. × 15 m) at 345°C; ion source, 70 eV at 330°C.

When monitored with m/z 263 and m/z 261, the ion peaks appeared at Rt 9.5 min. Ions of m/z 599 and m/z 603 (the latter at 44% of the former in ion intensity) were also detected at the same Rt. The ions of m/z 261 and m/z 599 were due to the RCO^+ of a linoleic acid residue and $[M-OCOR]^+$ of LLL, respectively.

Although the standard triacylglycerol mixture used in the analysis also contained LnLnLn as shown by the FID analysis, no fragment ion peak corresponding to LnLnLn was found in the SIM profile of Figure 1. This is probably due to the instability of the linolenic acid component during electron impact ionization (23). Our results suggest that the SIM of RCO^+ and $[M-OCOR]^+$ is useful for the identification of triacylglycerols with saturated, monounsaturated and diunsaturated acid groups, but may not be suitable for those with more highly unsaturated acyl chains.

SIM of mixed acid triacylglycerols. The ester-interchange reaction between SSS and LLL in equal molar ratios theoretically produces SSS, SSL, SLL and LLL in the molar ratios of 1:3:3:1 (where the respective positional isomers of SSL and SLL such as SLS and LSS, and LSL and LLS are not resolved [24]). The formation of two new triacylglycerols was confirmed by GLC-FID, athough the late-eluting peak corresponding to LLL was quite small (the gas chromatogram is not shown). These four synthesized triacylglycerols would be expected to yield individual fragment ions, RCO^+ and $[M-OCOR]^+$, as calculated and summarized in Table 1. For example in the case of SSL, the fragment ion peaks, when monitored with m/z 267 and m/z 263 for RCO^+ as well as with m/z 603 and m/z 607 for $[M-OCOR]^+$, appeared individually at the same GLC retention time as peak No. 2 shown in Figure 2. At the same Rt, unfortunately, we could also find several peaks of fragment ions m/z 265, m/z 261, m/z 605 and m/z 601, beside the essential RCO^+ and $[M-OCOR]^+$. These fragment ions are due to $[RCO-1]^+$ and $[RCO-2]^+$ as well as $[M-(OCOR+1)]^+$ corresponding to unsaturated fatty acyl residues of triacylglycerols (linoleic acid in the case of SLL) as already mentioned above. The formation of such fragment ions usually complicates the process of peak assignment. For example, when oleic and linolenic acid residues are thought to be the origins of the fragment ions of m/z 265 and m/z 261, respectively, the triacylglycerol in peak No. 3 could be identified erroneously as SLnO, although the true component is SLL. However, if the m/z 263 ion corresponds to $[RCO-2]^+$ of an oleic acid residue of SLnO, then the intensity of the m/z 263 ion is expected to be less than that for m/z 265. The result presented for peak No. 3, therefore, suggests that the triacylglycerol of peak No. 3 can be considered to be SLL rather than SLnO.

TABLE 1

Calculated m/z of Fragment Ions From Different Molecular Species of Triacylglycerols

Molecular species of triacylglycerol	Fatty acid residue	m/z of fragment ion		
		M^+	RCO^+	$[M-OCOR]^+$
	S		267	607
SSS	S	890	267	607
	S		267	607
	S		267	603
SSL	S	886	267	603
	L		263	607
	S		267	599
SLL	L	882	263	603
	L		263	603
	L		263	599
LLL	L	878	263	599
	L		263	599
	O		265	603
OOO	O	884	265	603
	O		265	603
	S		267	599
SLnO	Ln	882	261	605
	O		265	601

As already mentioned (15–18), triacylglycerols having the same total acyl carbon number and the same number of double bonds seem to co-elute on an open-tubular column used in the present study because of their close retention times. The problem here is that an unresolved peak including triacylglycerols such as SLL and SLnO leads to confusion in the peak assignment when using SIM profiles. If SLL and SLnO could be resolved by using a more efficient GLC column, then the above mentioned problem could be resolved, as mentioned below. For example, OOO has been separated from SLO on a methylphenylsilicone column (15) although their TC and DB are the same. Thus, their corresponding fragment ion peaks could be expected to be separated slightly from each other on the SIM profile.

SIM of soybean triacylglycerols. Soybean oil triacylglycerols, shown in Figure 3, are resolvable into about 14 peaks (15,18). Figure 4 shows the fragment ion peaks in the SIM profiles of the 14 peaks having individual retention times. The same liquid phase was used in the FID and GC/MS columns, and the peaks with corresponding numbers in the FID chromatogram in Figure 3 and the SIM profile in Figure 4 are identical in molecular species.

The consequence of the analyses of authentic triacylglycerols indicates that peak assignments can be accomplished by locating certain fragment ions having the same retention times on the SIM profile, that is, three kinds of RCO^+ corresponding to the fatty acyl residues on the glycerol moiety, and three kinds of $[M-OCOR]^+$. The soybean oil triacylglycerols used in the present study consisted primarily of 18:2(n-6), 18:1(n-9), 16:0, 18:3(n-3) and 18:0 fatty acids, and the sum of these fatty acids accounted for about 98.9% of total fatty acids (Table 2). Therefore, only triacylglycerols which consist of combinations of the above prominent fatty acids were considered. Their corresponding fragment ions were calculated and summarized in Table 3. By using the data of this table, peak assignments were carried out for the SIM profile in Figure 4.

The component of peak No. 1 had two kinds of RCO^+ of m/z 239 and m/z 265 and two kinds of $[M-OCOR]^+$ of m/z 551 and m/z 577. The only triacylglycerol having this characteristic profile was regarded as POP (see combinations of characteristic ions in Table 3).

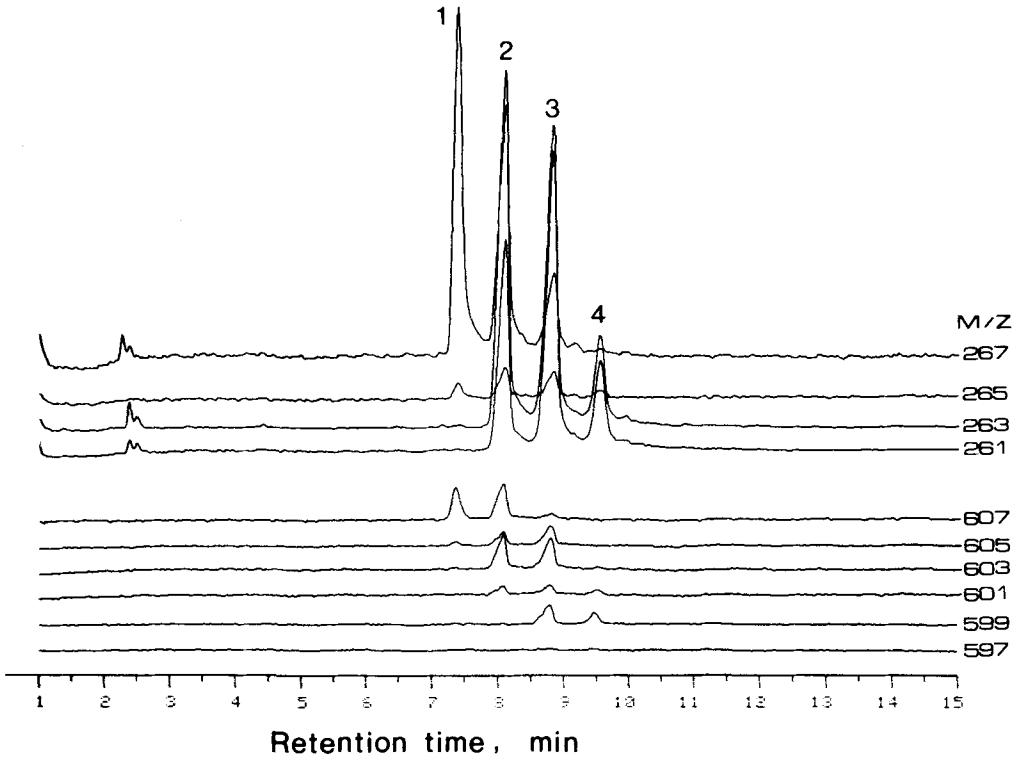

FIG. 2. Selective ion monitoring profiles arising from the interesterified products between SSS and LLL. 1, SSS; 2, SSL; 3, SLL; 4, LLL. GLC and SIM-GC/MS conditions were similar to those of Fig. 1.

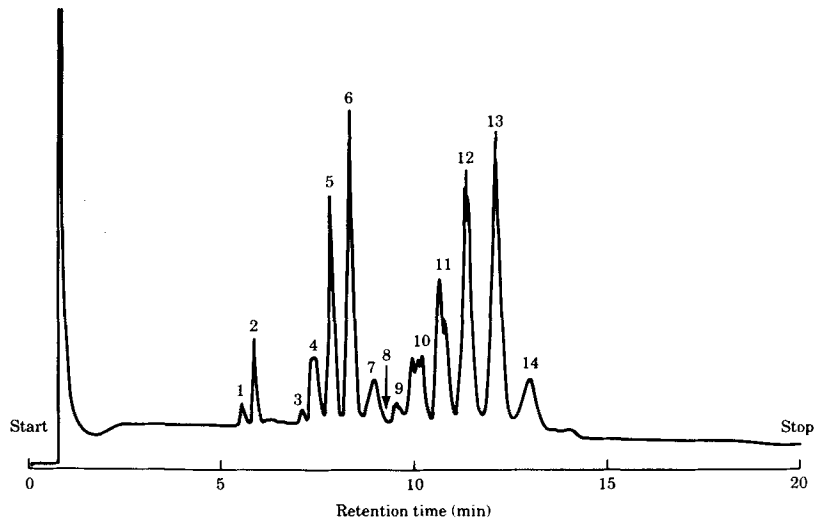

FIG. 3. FID open-tubular gas chromatogram of soybean oil triacylglycerols. Column, MP65HT (0.25 mm i.d. × 25 m) at 345°C; injector, a solventless movable injector at 370°C; carrier gas, helium at 1.75 Kg/cm2.

The fragment ions of m/z 239 and m/z 263 for peak No. 2 corresponded to a palmitic acid and a linoleic acid residue, respectively. The fragment ion of m/z 261 is considered to be the RCO^+ of a linolenic acid residue and/or the $[RCO-2]^+$ of a linoleic acid residue. Two other ion peaks, $[M-OCOR]^+$ at m/z 575 and m/z 551, appeared at the same Rt. Therefore, from the m/z in Table 3, the triacylglycerol in peak No. 2 which could account for all the ions mentioned above was assigned the structure PLP; m/z 261 was thus identified as $[RCO-2]^+$ corresponding to a linoleic acid residue.

Peak No. 3 showed ion peaks at m/z 239, m/z 265 and m/z 267 having the same Rt. There could be two positional monounsaturated isomers of 18:1 acids, respectively

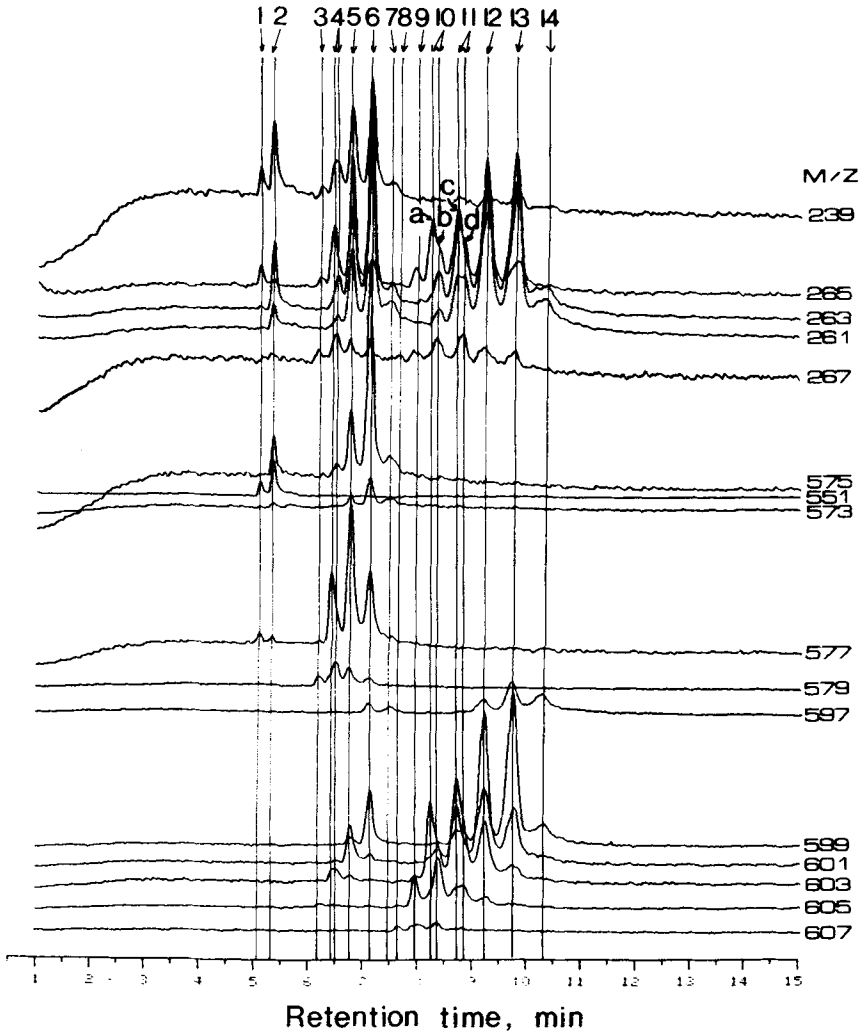

FIG. 4. Selective ion monitoring profiles arising from soybean oil triacylglycerols. The conditions were those of Fig. 1.

TABLE 2

Fatty Acid Composition of Soybean Oil Triacylglycerols

Fatty acid	Mean ± S.D. (%) (n = 6)	Fatty acid	Mean ± S.D. (%) (n = 6)
14:0	0.07 ± 0.00	18:1 n-7	1.26 ± 0.06
15:0	0.04 ± 0.00	18:2 n-6	53.50 ± 0.28
16:0	10.62 ± 0.42	18:2[a]	0.33 ± 0.01
16:1 n-7	0.08 ± 0.00	18:3 n-3	6.85 ± 0.06
17:0	0.11 ± 0.00	18:3[b]	1.46 ± 0.03
17:1 n-8	0.06 ± 0.00	20:0	0.33 ± 0.06
18:0	3.61 ± 0.09	20:1 n-9	0.15 ± 0.04
18:1 n-9	21.06 ± 0.29	22:0	0.30 ± 0.04

[a]Sum of two kinds of mono-*trans* geometrical isomers (25).

[b]Sum of three kinds of *cis-trans* geometrical isomers (25).

18:1 (n-9) and 18:1(n-7) as indicated by Table 2. Therefore, the components of this peak could include mixture of two triacylglycerols: 16:0-18:1(n-9)-18:0 and 16:0-18:1(n-7)-18:0.

In the broad peak No. 4 (Fig. 3), the triacylglycerol yielding the m/z 265 ion was eluted slightly faster than the triacylglycerol yielding m/z 263 and m/z 267. The ion peak monitored at m/z 239 was also broad. From these profiles, peak No. 4 was determined to be two unresolved species of triacylglycerols; one consisted of only palmitic and oleic acids, and the other of one each of palmitic, linoleic and stearic acids. Besides the above fragment ions, the ion peaks of m/z 577 and m/z 603 had the same Rt as those of m/z 265 and m/z 239, and m/z 579, m/z 575 and m/z 603 had the same Rt as m/z 239, m/z 263 and m/z 267. The triacylglycerols yielding the former characteristic ions were regarded as POO and the latter as PLS.

The prominent triacylglycerol of peak No. 5 was identified as PLO since its ion peaks of RCO^+ were found at m/z 239, m/z 265 and m/z 263 and those of $[M-OCOR]^+$ at m/z 601, m/z 577 and m/z 575 (Table 3). However, the ion of m/z 261 posed another possibility; if the m/z 261 ion was derived from a linolenic acid residue on the glycerol moiety, PLnS might be included in peak No. 5.

When the fragment ions of m/z 239 and m/z 263 as RCO^+ and those of m/z 575 and m/z 599 as $[M-OCOR]^+$ were monitored, four well-resolved ion peaks appeared at

TABLE 3

Proposed Molecular Species of Vegetable Oil Triacylglycerols and the Calculated m/z of Fragment Ions

TC:DB[a]	Molecular species	Fatty acid residue	m/z of fragment ions			TC:DB[a]	Molecular species	Fatty acid residue	m/z of fragment ions		
			M^+	RCO^+	$[M-OCOR]^+$				M^+	RCO^+	$[M-OCOR]^+$
		M		211	551			P		239	597
46:0	MPP	P	778	239	523	52:5	PLnL	Ln	852	262	575
		P		239	523			L		263	573
		P		239	551			S		267	605
48:0	PPP	P	806	239	551	54:1	SOS	O	888	265	607
		P		239	551			S		267	605
		M		211	577			S		267	603
48:1	MOP	O	804	265	523		SLS	L	886	263	607
		P		239	549	54:2		S		267	603
		M		211	575			S		267	603
48:2	MLP	L	802	263	523		SOO	O	886	265	605
		P		239	547			O		265	605
		P		239	579			O		265	603
50:0	PPS	P	834	239	579		OOO	O	884	265	603
		S		267	551			O		265	603
		P		239	577			S		267	601
50:1	POP	O	832	265	551	54:3	SLO	L	884	263	605
		P		239	577			O		265	603
		P		239	575			S		267	601
	PLP	L	830	263	551		SLnS	Ln	884	261	607
		P		239	575			S		267	601
50:2											
		M		211	603			O		265	601
	MOO	O	830	265	549		OLO	L	882	263	603
		O		265	549			O		265	601
		M		211	601			S		267	599
50:3	MLO	L	828	263	555	54:4	SLL	L	882	263	603
		O		265	553			L		263	603
		M		211	599			S		267	599
50:4	MLL	L	826	263	547		SLnO	Ln	882	261	605
		L		263	547			O		265	601
		P		239	605			O		265	599
52:1	POS	O	860	265	579		OLL	L	880	263	601
		S		267	577			L		263	601
		P		239	603			O		265	599
	POO	O	858	265	577	54:5	OLnO	Ln	880	261	603
52:2		O		265	577			O		265	599
		P		239	603			S		267	597
	PLS	L	858	263	579		SLnL	Ln	880	261	603
		S		267	575			L		263	601
		P		239	601			L		263	599
	PLO	L	856	263	577		LLL	L	878	263	599
52:3		O		265	575			L		263	599
		P		239	601			S		267	595
	PLnS	Ln	856	261	579	54:6	SLnLn	Ln	878	261	601
		S		267	573			Ln		261	601
		P		239	599			O		265	597
	PLL	L	854	263	575		OLnL	Ln	878	261	601
52:4		L		263	575			L		263	599
		P		239	599			L		263	597
	PLnO	Ln	854	261	577	54:7	LLnL	Ln	876	261	599
		O		265	573			L		263	597

[a]TC, the number of total acyl carbons; DB, the number of double bonds.

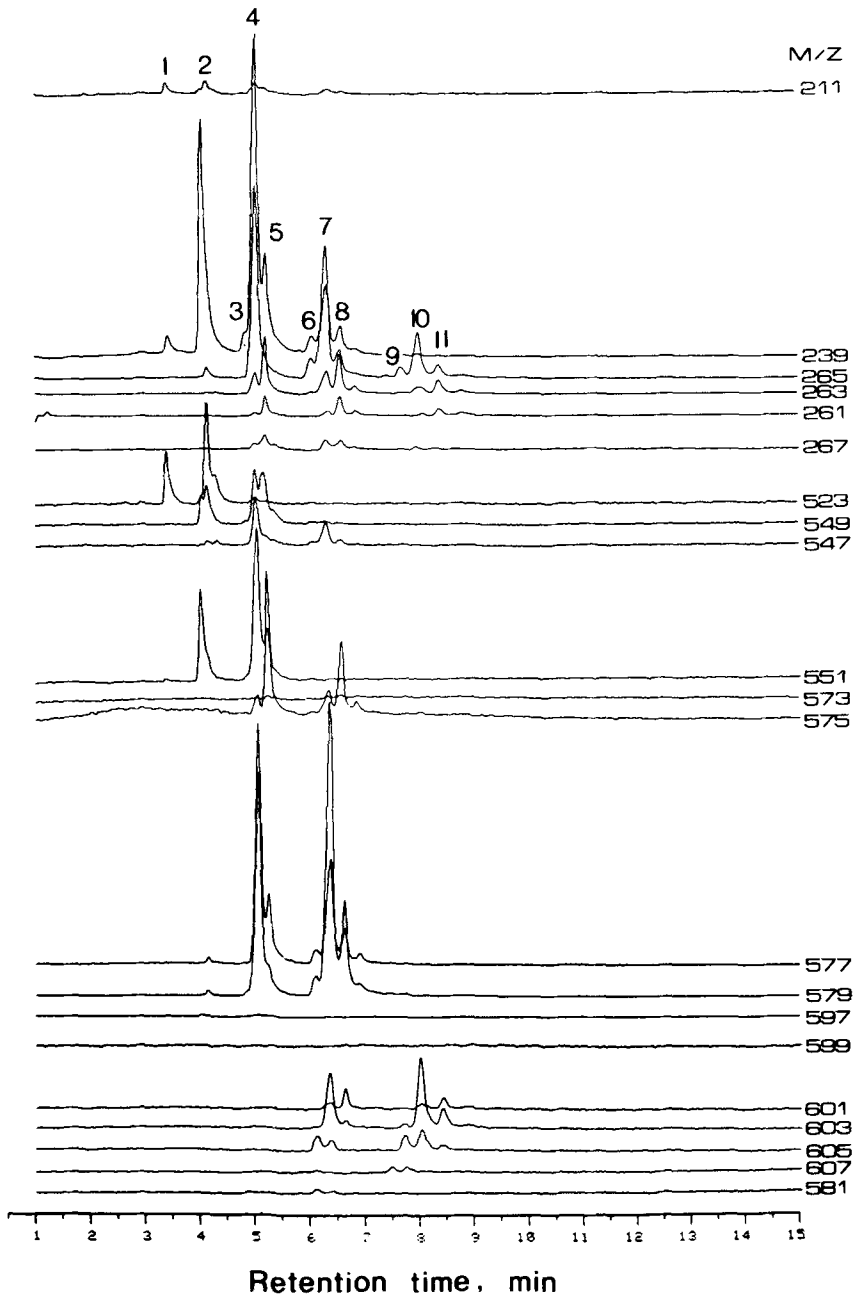

FIG. 5. Selective ion monitoring profile of palm oil triacylglycerol. The italicized triacylglycerols were not reported in earlier work (see text). 1, MPP; 2, PPP, MOP and MLP; 3, PPS; 4, POP and PLP; 5, MLO; 6, POS; 7, POO and PLS; 8, PLO and *PLnS*; 9, SOO and *SLS*; 10, OOO and SLO; 11, *SLL* and OLO. The conditions are given in the caption of Fig. 1.

the same Rt (peak No. 6) on the SIM profile. This strongly suggests that the prominent triacylglycerol is PLL. If a part of the m/z 261 ion could be derived from a linolenic acid residue, PLnO could be proposed as another component in peak No. 6. Peak No. 7 must be considered briefly. The only triacylglycerol tentatively identified from the characteristic fragment ions of Figure 4 was PLnL. Similarly, peak No. 8 was simply identified as SOS.

Peak No. 9 was determined to be an unresolved peak containing SOO and SLS, since a part monitored with the fragment ion of m/z 265 corresponding to an oleic acid residue was eluted faster than that monitored with m/z 263 corresponding to a linoleic acid residue. From these results, it is suggested that SOO elutes faster than SLS on this particular liquid phase.

The prominent triacylglycerol of peak No. 10 ("a" in Fig. 4) had two fragment ion peaks of m/z 265 corresponding to RCO^+ and m/z 603 to $[M-OCOR]^+$. From these results, the prominent triacylglycerol "a" in peak No. 10 was identified as OOO. The SIM profile (Fig. 4) of m/z 265 showed a shoulder peak at 8.4 min marked "b" in peak No. 10. As shown in the fatty acid composition of

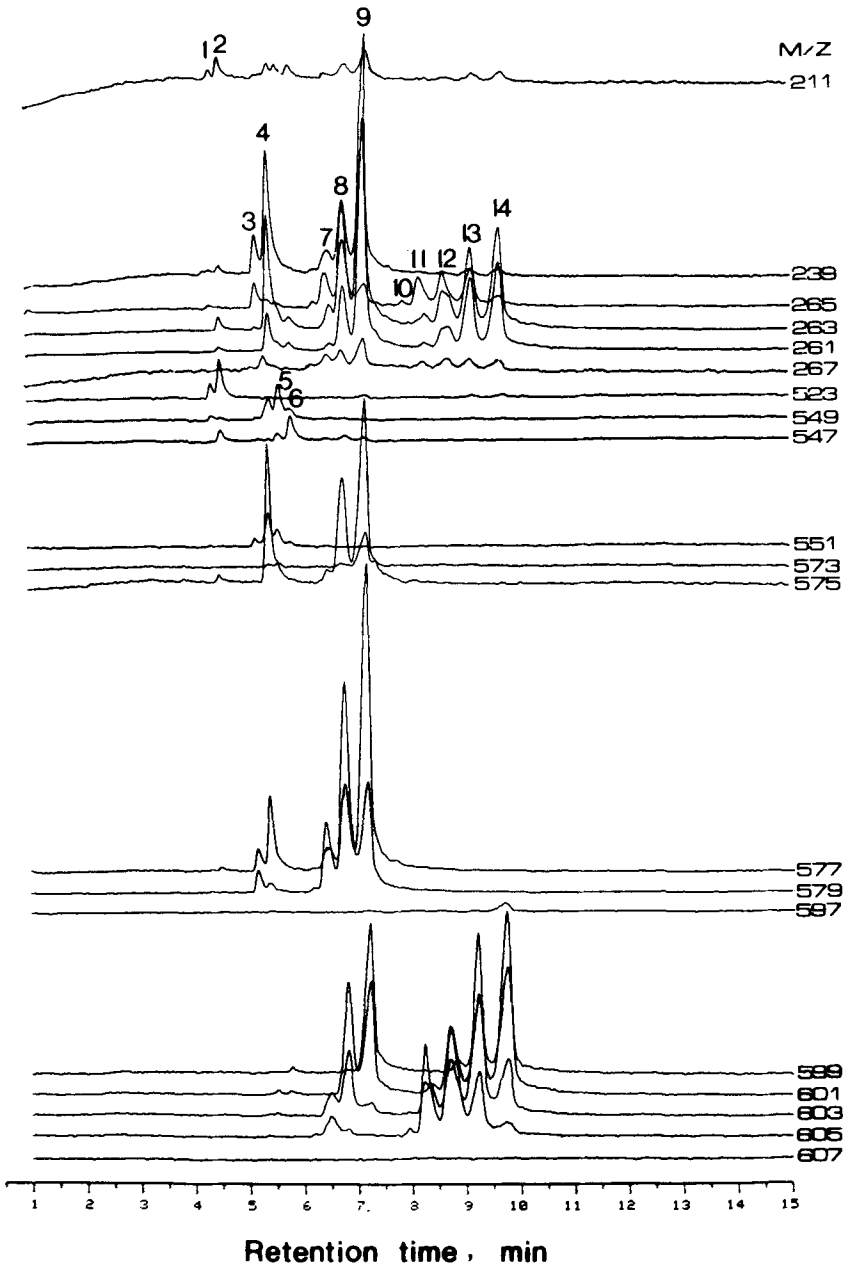

FIG. 6. Selective ion monitoring profile of cottonseed oil triacylglycerol. 1, MOP; 2, MLP; 3, POP; 4, PLP and *MOO*; 5, PLP and MLO; 6, MLL; 7, POO and PLS; 8, PLO and *PLnS*; 9, PLL and *PLnO*; 10, SOS; 11, OOO and SLO; 12, OLO, SLL and *SLnO*; 13, OLL, *OLnO* and *SLnL*; 14, LLL and *OLnL*. For italicized triacylglycerols see the caption of Fig. 5.

triacylglycerols present, 18:1(n-7) acid accounted for about 5% of 18:1 acids (Table 2). The shoulder component "b" of peak No. 10 can, therefore, be tentatively identified as a trimonounsaturated triacylglycerol which has 18:1(n-7) as a part of the fatty acyl residues in the glycerol moiety. Alternatively, the shoulder component can be considered a mixture of SLO and SLnS. The elution order of these triacylglycerols would, therefore, be OOO followed by SLO and/or SLnS.

Two slightly resolved ion peaks appeared on the SIM profile monitored at m/z 263 and m/z 261 for peak No. 11 of Figure 4 (marked "c" and "d"); the peak seemed to contain at least two molecular species of triacylglycerol. By combining the fragment ions having the same Rt on the SIM profile as shown in Table 3, the faster eluting triacylglycerol "c" was identified as OLO and the slower eluting one "d" as SLL and/or SLnO.

From a characteristic combination of m/z 265, m/z 263, m/z 599 and m/z 601, and a combination of m/z 265, m/z 261, m/z 599 and m/z 603 in peak No. 12, OLL and OLnO are proposed as the respective peak components. Beside these triacylglycerol species, the existence of the minor component SLnL was demonstrated in peak No. 12, since the peak apexes of m/z 597, m/z 601 and m/z 603 showed

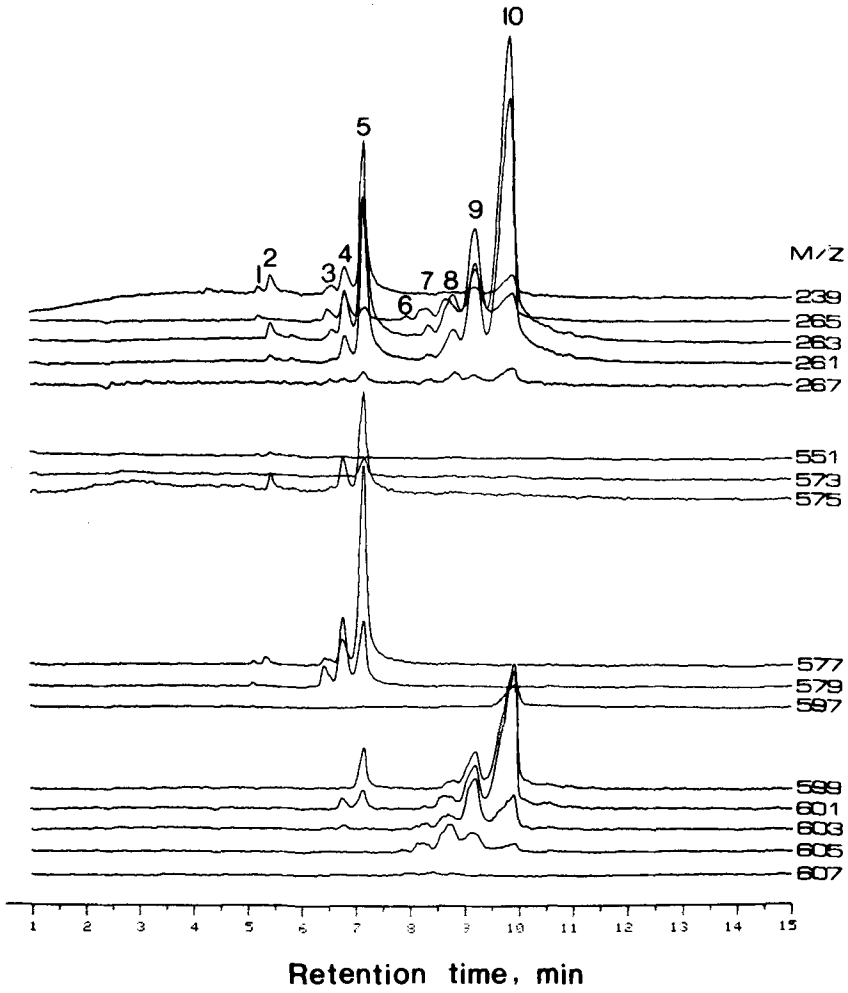

FIG. 7. Selective ion monitoring profile of safflower oil triacylglycerol. 1, POP; 2, PLP; 3, POO and PLS; 4, PLO; 5, PLL and PLnO; 6, SOO; 7, OOO and SLO; 8, OOL, SLL and *SLnO*; 9, OLL, *OLnO* and *SLnL*; 10, LLL, *SLnLn* and *OLnL*. For italicized triacylglycerols see the caption of Fig. 5.

the same retention time and eluted slightly behind the other ions monitored.

The ion peak monitored at m/z 265, peak No. 13 in Figure 4, apparently eluted slower than those of m/z 263 and m/z 261 and had the same Rt as those of m/z 597, m/z 599 and m/z 601. From these results, this slower-eluting triacylglycerol was identified as OLnL from the characteristics of the fragment ions (Table 3). LLL was also identified from the ion peaks of m/z 263 and m/z 599. Additionally, the existence of a triacylglycerol having a stearic acid residue, SLnLn, is possible in peak No. 13, because two peaks appeared at the same Rt on the SIM profile when monitored with m/z 267, m/z 261 and m/z 601. However, confirmatory evidence is lacking, because monitoring m/z 595 corresponding to $[M-OCOR]^+$ of SLnLn was not carried out in this study.

The acyl assignments to peak No. 14 were relatively simple. From the ion peaks of m/z 263 and m/z 261 corresponding to RCO^+ and m/z 599 and m/z 597 to $[M-OCOR]^+$, LLnL can be proposed as the peak composition.

The following seven molecular triacylglycerol species, PLnS, PLnO, SLnS, SLnO, OLnO, SLnS and OLnL, proposed from this work, have not been found in soybean oil triacylglycerols in the previous studies in which triacylglycerols were separated into molecular species by a GLC technique similar to that of the present study. However, these triacylglycerol species were reported in soybean oil which was analyzed by HPLC (8,26,27).

Further application. Further applications to other triacylglycerols from palm, cottonseed and safflower oils are shown in Figures 5–7. In the figures some novel triacylglycerol species which have not been reported in previous studies (15,18) were identified by the present SIM-GC/MS technique and are italicized in the figure captions.

One of the strong points of this method seems to be that the peak assignment is possible under the worst conditions such as a high background level caused by column bleeding. Therefore, the peak assignment of high-boiling compounds such as wax esters, which can be resolved not only by an apolar open-tubular column (28) but also by a polar open-tubular column such as SP 2340 (29), may become possible with SIM. Further application

to the peak assignment of the diacylglycerol derivatives which separate on a polar open-tubular column such as SP 2330 (30) is expected.

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Instability of Malondialdehyde in the Presence of H_2O_2: Implications for the Thiobarbituric Acid Test

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The content of thiobarbituric acid-reactive material (primarily malondialdehyde) is frequently used to estimate the extent of lipid peroxide formation. However, malondialdehyde is unstable in the presence of millimolar concentrations of hydrogen peroxide. This observation considerably limits the applicability of the thiobarbituric acid test, as hydrogen peroxide is known to be formed in a number of lipid peroxidation-promoting systems. The instability of malondialdehyde in the presence of hydrogen peroxide seems to account for the inconsistent outcomes in studies relating the manipulations of intermediate H_2O_2 levels to the initiation of lipid peroxidation.
Lipids 24, 545-549 (1989).

The spectrophotometric measurement of an adduct formed between malondialdehyde (MDA) and thiobarbituric acid (TBA) is commonly used to estimate the relative levels of lipid peroxidation (1). The implicit assumption of this procedure is that MDA is a relatively stable product which does not undergo further metabolic transformations; either in the course of its generation, or during the development of the MDA-TBA chromophore. As reported in this study, such an assumption is not valid under conditions which favor the accumulation of hydrogen peroxide in reaction mixtures. MDA is readily degraded in the presence of millimolar concentrations of H_2O_2, resulting in the disappearance of TBA reactivity.

Previous studies have demonstrated the generation of hydrogen peroxide and/or hydroxyl radicals in certain lipid peroxidation-promoting systems (2-4). The metal ion-catalyzed conversion of hydrogen peroxide to hydroxyl radicals has been implicated in the reaction mechanisms involved in the initiation of lipid peroxidation (5). However, the support for this concept was equivocal, because in certain systems the amount of TBA-reactive MDA remained either unchanged or decreased in the presence of increased H_2O_2 levels (6, 7). As shown in this study, hydrogen peroxide can either stimulate or inhibit the accumulation of MDA in the course of lipid peroxide formation, the latter effect being primarily attributed to the degradation of MDA.

MATERIALS AND METHODS

MDA was prepared by heat-assisted acid hydrolysis of 1,1,3,3-tetraethoxypropane (8).

The effect of H_2O_2 on MDA was investigated after the samples were incubated at room temperature for 10 minutes in a final volume of 0.5 ml. Changing the reaction time to both 5 and 15 minutes did not have any effect on the observed changes in the amount of MDA-TBA complex. The incubation was terminated by the addition of 1 ml of TBA reagent to yield final concentrations of 0.5% TBA, 10% trichloroacetic acid, 0.63 M HCl, and 2 mM EDTA in a final volume of 1.5 ml (9). The development of the MDA-TBA complex was carried out by heating the samples for 15 minutes at 95°C. The amount of MDA-TBA complex was determined from the absorbance at 532 nm (molar absorption coefficient = 1.56×10^5 $M^{-1} \cdot cm^{-1}$). The spectrophotometric measurements of samples containing membrane-generated MDA were preceded by a centrifugation (3,000 g × 10 min) to remove precipitated proteins.

In experiments which were designed to minimize the amount of hydrogen peroxide after the addition of TBA reagent, the incubation of MDA with H_2O_2 was carried out in a final volume of 0.125 ml. After the incubation, the samples were diluted 10-fold with distilled water. Thirty μl aliquots of diluted samples were then transferred into test tubes containing 1 ml of TBA reagent and 470 μl of distilled water. This resulted in 500-fold dilution of the original samples for the development of MDA-TBA complex.

For the measurements of pH dependency of the H_2O_2 effect, the concentrated stock solution of MDA was neutralized by NaOH and then diluted 10-fold into either 1 M acetate-acetic acid buffer pH 3.5, or 1.5 M TRIS-HCl buffer pH 7.4. After the incubation with H_2O_2, the samples were diluted 1,000-fold for the development of MDA-TBA complex.

The microsomal fraction from rat liver was prepared by a two-step centrifugation of tissue homogenate. The removal of nuclei and cell debris by low-speed centrifugation (800 g × 10 min) was followed by centrifugation at 100,000 g for 60 minutes. 10 mM imidazole-HCl pH 7.4 was used as a vehicle for both the preparation of tissue homogenate and the resuspension of final pellet. The protein content in microsomal fraction was determined according to Lowry *et al.* (10).

The lipid peroxidation of liver microsomes was determined after the incubation of membranes (200-300 μg of protein) for 60 minutes at 37°C in the presence of 150 mM Tris-HCl pH 7.4, 1 mM KCl, and amounts of hydrogen peroxide and/or ascorbate/$FeSO_4$, as indicated in the text in the final volume of 0.5 ml.

Statistical Evaluation of Data. Unless indicated, the results are presented as mean ± standard error of three replicates for individual points obtained in a typical experiment. In the graph presentations, the magnitude of standard error is within the size of symbols used to illustrate the mean values.

TEP and TBA were obtained from Sigma Chemical Co. Other reagents were of analytical grade and were purchased from standard commercial sources.

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Abbreviations: A, absorbance; MDA, malondialdehyde; TBA, 2-thiobarbituric acid; TEP, 1,1,3,3-tetraethoxypropane

RESULTS AND DISCUSSION

The incubation of MDA standard with various amounts of H_2O_2 at room temperature for 10 minutes resulted in a concentration-dependent decline in the amount of MDA-TBA chromophore (Fig. 1). The relative values of H_2O_2-induced decline in the absorbance at 532 nm were similar over a wide range of initial MDA concentrations. The relative decrease in A_{532} was unaffected by the degree of the dilution of samples for the development of MDA-TBA chromophore. This indicates that the observed changes in the amount of chromophore resulted from the reaction between MDA and H_2O_2 during the incubation and not during the heating with TBA.

It has been previously noted that the acid hydrolysis of TEP generates several other products besides MDA which are converted to MDA upon heating with TBA (11). These observations raised the possibility that H_2O_2-induced changes are due to its reactivity with substances other than MDA. To address this question, we examined the effect of H_2O_2 on MDA generated by peroxidation of membrane lipids. In these experiments we observed a similar pattern of H_2O_2-induced decline in the amount of MDA-TBA complex as compared to that seen with MDA standard (Fig. 1).

The H_2O_2-induced decline in the amount of MDA-TBA complex could not be accounted for by the bleaching effect of the H_2O_2 on the MDA-TBA chromophore. The addition of exogenous H_2O_2 (at concentrations up to 5 mM) to the MDA-TBA adduct resulted only in marginal changes in absorbance at 532 nm (Table 1), and concentration of H_2O_2 as high as 40 mM was required to induce a 10% decline in the amount of chromophore. In our experiments, the concentrations of H_2O_2 during the development of MDA-TBA complex did not exceed 4 mM.

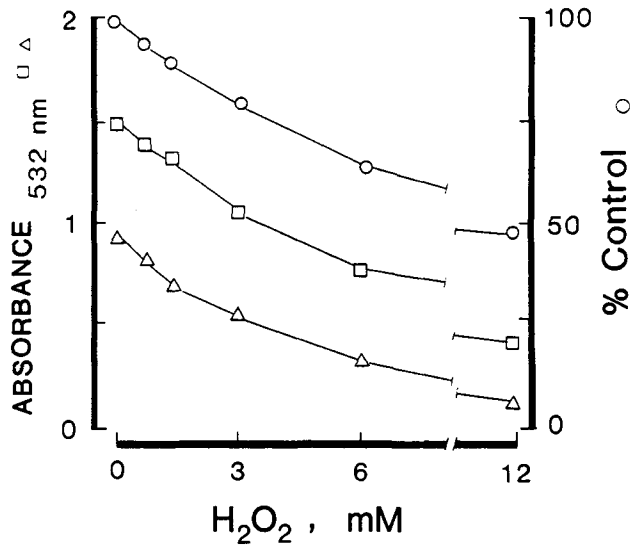

FIG. 1. Effect of H_2O_2 on the amount of MDA-TBA complex. (□), MDA standard (27 μM) was incubated with various amounts of H_2O_2 for 10 min at room temperature. The samples were diluted 3-fold in the assay for MDA-TBA complex. (△), the incubation mixtures contained 2.8 mM MDA standard and the amounts of H_2O_2 as indicated. The samples were diluted 500-fold for the development of MDA-TBA complex. (○), the liver microsomes were incubated for 60 min at 37°C in the presence of 100 μM ascorbic acid, 100 μM $FeSO_4$, 150 mM Tris-HCl pH 7.4, and 1 mM KCl. At t=60 min the samples were supplemented by sodium azide (5 mM) and various amounts of H_2O_2 as indicated. The incubation continued for an additional 5 min. The reaction was terminated by addition of 1 ml of TBA reagent and the samples were assayed for the amount of MDA-TBA chromophore. The amounts of MDA-TBA complex are expressed as % of control value (t=60 min).

The H_2O_2-induced decline in A_{532} was accompanied by an apparent accumulation of yellow chromophore in the reaction mixtures, which was proportional to the concentration of H_2O_2 during the development of MDA-TBA complex. It should be pointed out that the TBA solution alone showed a significant absorption in the spectral region below 420 nm with continuously increasing absorbance as the wavelengths were approaching the UV region (Fig. 2a). The accumulation of yellow chromophore in the presence of H_2O_2 could be accounted for by the formation of an additional peak at 383 nm, superimposed on the absorption spectrum of TBA solution (Fig. 2b). The increase in absorbance at 383 nm was found to be a linear function of H_2O_2 concentration (up to 5 mM), and was observed both in the presence and absence of MDA (Fig. 2c). In all likelihood, this peak may represent an oxidation product of TBA formed independently of the reaction between TBA and MDA. In the context of this study, the appearance of yellow chromophore in samples tested for MDA-TBA complex may serve as an indicator of the accumulation of hydrogen peroxide in reaction mixtures.

The observed interaction between H_2O_2 and TBA originally suggested the possibility that the changes in the amount of MDA-TBA complex in the presence of H_2O_2 may be partly attributed to the depletion of TBA. However, this was not the case, as the decline in the absorbance at 532 nm remained unaffected when the development of MDA-TBA complex was carried out after a 500-fold dilution of reaction mixtures containing MDA and H_2O_2 (Fig. 1; see Methods for details). Under such conditions, the levels of H_2O_2 were too low to have any significant effect on the absorbance of TBA solution at 383 nm.

We also examined the pH dependency of H_2O_2-induced degradation of MDA. Since MDA was pre-

TABLE 1

The effect of H_2O_2 on MDA-TBA complex.

H_2O_2 (mM)	A_{532}	% of control
0	0.707 ± 0.002	100
5	0.697 ± 0.003	98.5
10	0.690 ± 0.003*	97.6
20	0.667 ± 0.002*	94.3
30	0.651 ± 0.004*	92.1
40	0.633 ± 0.003*	89.5
50	0.608 ± 0.002*	85.7

*Statistically significant differences from the value in the absence of H_2O_2 ($p < 0.05$).

The MDA-TBA complex was prepared by heating the MDA standard (original concentration 9 μM) with TBA in the absence of H_2O_2. The MDA-TBA complex was then diluted 2-fold into media containing different concentrations of H_2O_2. The measurements were done after 10 min. incubation at room temperature.

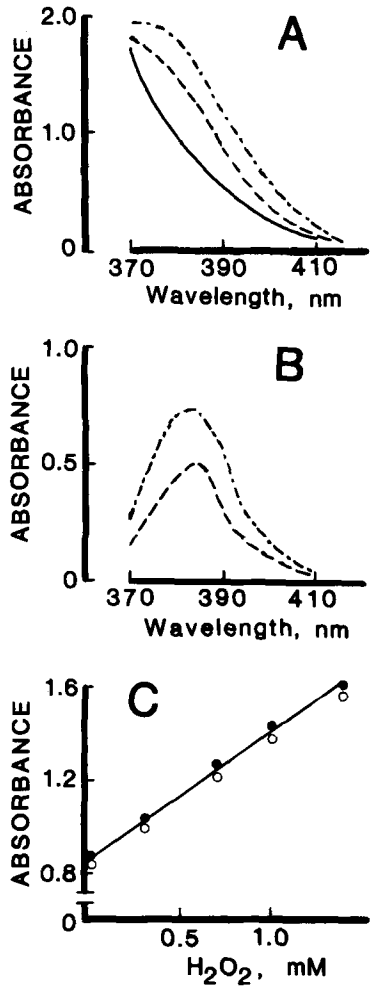

FIG. 2. A. The absorption spectra of TBA reagent at the concentration used for the development of MDA-TBA complex in the absence (——), and presence of 0.67 mM (– – –), and 1.33 mM (–•–•) H_2O_2. B. The absorption spectra of TBA reagent in the presence of 0.67 mM (– – –), and 1.33 mM (–•–•), H_2O_2 corrected for the absorbance in the absence of H_2O_2. C. The absorbance of TBA reagent at 383 nm in the presence of increasing concentrations of H_2O_2 either in the absence (•), or presence (○), of 4.5 μM MDA. The measurements were preceded by the incubation of the samples at 95°C for 15 min.

pared by acid hydrolysis of TEP, and the development of MDA-TBA complex required the acidification of samples when using MDA generated by lipid peroxidation, we considered the possibility that H_2O_2-induced degradation of MDA might be confined only to the strongly acidic region of pH. In fact, Ohkawa *et al.* (12) have reported that when the TBA test was used to analyze the MDA content in biological samples, substantially higher readings were obtained when the development of MDA-TBA complex was carried out at pH 3.5 as compared to more acidic buffers.

The measurements of pH effect were carried out using a 1000-fold dilution of samples containing MDA and H_2O_2 for the development of MDA-TBA complex, thus preventing any additional interaction between H_2O_2 and MDA after the acidification. This was checked by running parallel controls containing MDA and H_2O_2 at concentrations corresponding to those present during the development of the MDA-TBA complex. The results are summarized in Table 2. The increase of pH from 0.5 to 3.5 and 7.4, respectively, reduced the degree of H_2O_2-induced MDA degradation as compared to that seen at pH 0.5. However, millimolar concentrations of H_2O_2 were sufficient to induce a significant decline in the amount of MDA-TBA complex at pH 7.4. Therefore, the actual extent of H_2O_2-induced degradation of MDA will depend upon both the pH of the reaction mixtures in the course of lipid peroxide formation, and on the degree of acidification during the development of MDA-TBA complex.

TABLE 2

pH-dependence of H_2O_2-induced degradation of MDA.

pH	H_2O_2 concentration: In incubation (mM)	H_2O_2 concentration: In assay (μM)	A_{532}	% of control
0.5	0	0	0.470 ± 0.002	100
	0	6	0.474 ± 0.004	100
	0	12	0.469 ± 0.002	100
	6	6	0.258 ± 0.003*	55
	12	12	0.150 ± 0.002*	32
3.5	0	0	0.475 ± 0.003	100
	0	6	0.472 ± 0.002	100
	0	12	0.474 ± 0.004	100
	6	6	0.406 ± 0.002*†	86
	12	12	0.379 ± 0.003*†	80
7.4	0	0	0.469 ± 0.004	100
	0	6	0.472 ± 0.003	100
	0	12	0.474 ± 0.003	100
	6	6	0.434 ± 0.002*†	92
	12	12	0.409 ± 0.002*†	86

*Statistically significant differences ($p < 0.05$) from the corresponding values obtained in the absence of hydrogen peroxide in the incubation.

†Statistically significant differences ($p < 0.05$) from the corresponding values obtained at pH 0.5.

MDA (3 mM) was incubated in the presence of either 6 mM or 12 mM H_2O_2 for 10 min at room temperature. The development of MDA-TBA complex was carried out after 1000-fold dilution of samples. Parallel controls containing 6 and 12 μM H_2O_2 during the development of chromophore were run to ascertain that no additional decomposition of MDA occurred after the acidification. Results are mean ± S.E.M. from three replicates for each point. The data has been evaluated by two-way analysis of variance.

The findings of this report may prove to be of particular relevance to studies where the TBA test is used to identify the radical species involved in the initiation and propagation of lipid peroxidation. It is well established that the initial step in the production of free radicals in biological systems is the formation of an oxygen singlet. The branching of the radical chain usually involves the conversion of an oxygen singlet to hydroxyl radicals by a reaction sequence involving the transient formation of hydrogen peroxide (5). If these reaction mechanisms are valid, then in the light of our findings the hydrogen peroxide would have either a stimulatory or inhibitory effect on the level of MDA detected by the TBA test. Under conditions when the intermediate levels of H_2O_2 in the lipid peroxidation-promoting systems are below the threshold of MDA degradation, the experimental manipula-

tions which stimulate the H_2O_2 levels would enhance the accumulation of MDA. In turn, excessive levels of H_2O_2 will result in an apparent inhibition of lipid peroxidation due to reaction of H_2O_2 with MDA.

We have examined this pattern of interactions using a microsomal fraction isolated from rat liver. As is shown in Fig. 3a, the incubation of membranes in the presence of increasing concentrations of H_2O_2 resulted in a net increase of MDA content. Under conditions when endogenous catalase activity was inhibited by sodium azide, the stimulatory effect of H_2O_2 was changed into a biphasic pattern consisting of an initial increase (<1 mM), and a subsequent decrease (> 1mM), of MDA content. The transition between the stimulatory and inhibitory effects of H_2O_2 occurred at a concentration corresponding to the threshold of H_2O_2-induced degradation of MDA at pH 7.4.

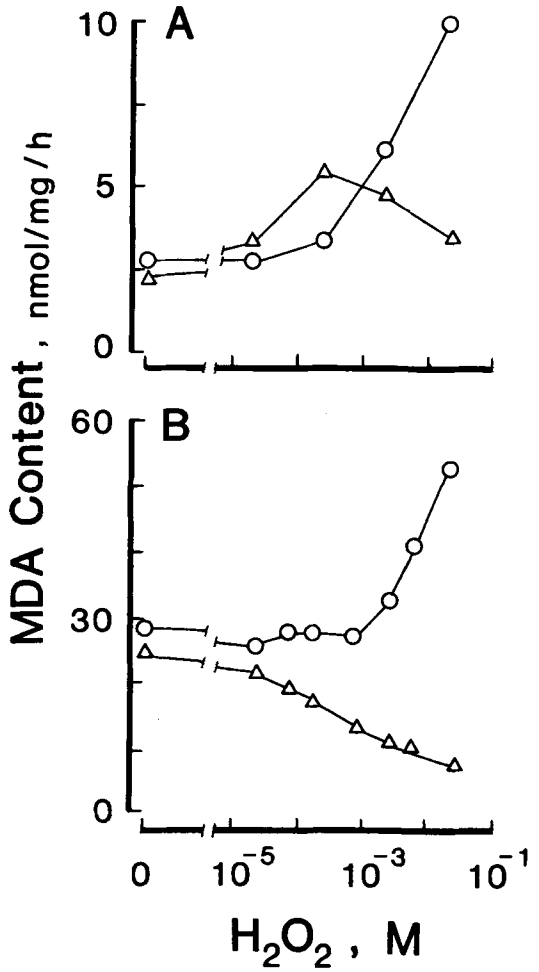

FIG. 3. Effect of exogenous H_2O_2 on the generation of MDA by rat liver particulate membrane fraction in the absence (A), and presence (B), of 100 μM ascorbic acid/100 μM $FeSO_4$. The membranes (250 μg protein) were incubated for 60 min at 37°C in a medium containing 150 mM Tris-HCl pH 7.4 and 1 mM KCl either in the absence (○), or presence (△), of 5 mM sodium azide. The results are expressed as means of three replicates from the parallel measurements on the same membrane preparation in order to eliminate the variations in MDA formation from preparation to preparation. The results were confirmed in two additional independent experiments.

A qualitatively similar pattern of interactions was also observed when liver microsomes were supplemented by 100 μM Fe^{2+} and 100 μM ascorbic acid (Fig. 3b). In the absence of azide, the addition of exogenous H_2O_2 enhanced the amount of MDA detected by TBA test. The suppression of endogenous catalase activity by sodium azide was sufficient enough to cause a significant decline in the amount of MDA (to 84 ± 3% of control values, $p < 0.05$), even in the absence of exogenous H_2O_2. This decline became more pronounced in the presence of exogenously added H_2O_2.

In summary, our findings indicate that the measurement of lipid peroxidation by the TBA test is of limited value under conditions which favor the accumulation of hydrogen peroxide in reaction mixtures. It has been noted previously that in some systems the experimental manipulations which tend to increase the intermediate levels of H_2O_2 were accompanied by a decline in the amount of MDA-TBA complex (6, 7). Such observations are often interpreted as a lack of involvement of H_2O_2 and/or hydroxyl radicals in the initiation of lipid peroxidation. In light of the present study, such conclusions are unwarranted.

Our findings also offer a plausible explanation for numerous controversial observations relating the role of superoxide dismutase, catalase and glutathione peroxidase to the initiation of lipid peroxidation. In different systems, superoxide dismutase and catalase were reported to either stimulate (13, 14) or inhibit (15-18) the accumulation of MDA. Similarly, glutathione peroxidase was shown to inhibit the MDA content in some systems (19), but not in others (20, 21). Since these enzymes are involved in the metabolism of hydrogen peroxide, the magnitude and direction of their effects on the MDA content will be determined by the actual levels of H_2O_2.

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COMMUNICATIONS

Sialylation of Lacto-*N*-neotetraosyl Ceramide by a Solubilized Sialyltransferase(s) from Chicken Skeletal Muscle: Effect of Phosphatidylcholine and Sphingomyelin

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The sialytransferase(s) that transfers sialic acid to lacto-*N*-neotetraosylceramide and other glycosphingolipids with a galactose nonreducing terminus has been successfully solubilized from embryonic chicken skeletal muscle. The enzyme can be stored in 50 mM HEPES (pH 6.8), 1% Triton CF-54, and 20% glycerol at −70°C for as long as six months. Addition of phosphatidylcholine or sphingomyelin (0.167%) readily reactivates the stored inactive enzymes and such activity persists for about two weeks at 0°-4°C with the peak activity occurring at 1 to 2 days. Sphingomyelin from chicken muscle, which contains mainly C16:0 and C18:0, is 2.1-fold more effective than bovine brain sphingomyelin at the same concentration (0.4%).
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One possible function of glycoconjugates is the regulation of cell-cell interactions via masking terminal carbohydrates (antirecognition) (1,2). In recent years, structural analysis of numerous animal glycoconjugates has frequently revealed a terminal NeuAc $\alpha 2 \rightarrow 3$ Gal-GlcNAc structure (3). At least three different enzymes that transfer sialic acid to form NeuAc $\alpha 2 \rightarrow 3$ Gal have been distinguished on the basis of preferred acceptor (4-7). Wide variations in sialosyl-galactosyl transferase according to tissue and species have been established using purified enzymes (8-11). Our previous studies of an enzyme preparation from chicken skeletal muscle indicated that the sialyltransferase that transfers the sialic acid from CMP-sialic acid to $nLcOse_6Cer$ differed from that of chicken brain in kinetic characteristics (12,13). In addition to NeuAc-$nLcOse_6Cer$, a disialo compound with another sialic acid attached by $\alpha 2 \rightarrow 8$ linkage has also been characterized (14). Different transferases may mediate the addition of sialic acid to $nLcOse_6Cer$ and NeuAc-$nLcOse_6Cer$, but this is not yet clear. In order to resolve this and also to explore the role of sialoglycoconjugates in cell-cell recognition and tissue development, the relevant enzyme(s) must be purified.

In this study we have established conditions for solubilization and stabilization for up to six months of skeletal muscle sialyltransferases activity and reported the biosynthesis of NeuAc-$nLcOse_4Cer$ from $nLcOse_4Cer$ employing the solubilized enzyme.

EXPERIMENTAL

Eggs were obtained from the Animal Genetic Laboratory, University of Connecticut, and incubated in our laboratory. CMP-sialic acid was purchased from New England Nuclear. Biosil A was obtained from Bio-Rad, and the detergents; DEAE Sephadex A50 (120-150 mesh), CMP-NeuAc, bovine brain phosphatidylserine, phosphatidylethanolamine, standard sphingomyelin, bovine liver phosphatidylinositol, and phosphatidylcholine were obtained from Sigma Chemical Company. Precoated TLC plates from E. Merck were obtained from A. H. Thomas. All the glycolipids and sphingomyelin from bovine brain and chicken muscle were isolated and purified in our laboratory, and were homogenous by TLC using different solvent systems.

*Preparation of asialo-GM_1, lacto-*N*-neotetraosylceramide and lacto-*N*-neohexaosylceramide.* GM_1 was purified from bovine brain, while sialosyl (*N*-acetyl and *N*-glycolyl) lacto-*N*-neotetraosylceramide, and sialosyl lacto-*N*-neohexaosylceramide were isolated and purified from bovine erythrocytes (15). Asialo GM_1, lacto-*N*-neotetraosylceramide and lacto-*N*-neohexaosylceramide were prepared from GM_1, sialosyl-lacto-*N*-neotetraosylceramide and sialosyl-lacto-*N*-neohexaosylceramide, respectively, by acid hydrolysis and then further purified by column chromatography as previously described (13).

Solubilization of sialyltransferase. Skeletal muscle from 12-day-old chicken embryos (E12) was carefully removed from the skeleton and kept frozen at −70°C until used. All procedures were carried out at 0-4°C. Muscle was homogenized with ice-cold 50 mM HEPES buffer (pH 6.8) containing 20 mM $MnCl_2$ or $MgCl_2$ (4 vol/gm tissue) in a polytron homogenizer. The homogenate was centrifuged at 100,000 × *g* for 30 minutes in a Beckman L5-50 ultracentrifuge. The pellet was homogenized again with the same buffer, and Triton CF-54 was added to bring the concentration to 1.5% (w/v) and stirred at 4°C for 1 hr. The extract was centrifuged at 100,000 × *g* for 30 min. The clear supernatant was decanted and a second extraction was carried out with overnight stirring. The two 100,000 × *g* supernatants were pooled and glycerol was added to bring the concentration to 15-20% (v/v). The protein was precipitated with 60% ammonium sulfate and centrifuged at 15,000 × *g* for 30 minutes in a Sorvall RC 5B centrifuge. The supernatant was carefully aspirated and the pellet was suspended in 50 mM HEPES (pH 6.8),

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Abbreviations: GM_3, GM_1, GD_{1a}, GD_{1b}, GT_{1b} were designated according to Svennerholm, L. (1963) *J. Neurochem. 10*, 613-623. $GgOse_4Cer$ and $nLcOse_4Cer$ are short designations recommended by the IUPAC-IUB Commission on Lipid Nomenclature (1977) *Eur. J. Biochem. 79*, 11-21. Other abbreviations used are IV^3 NeuAc-$nLcOse_4Cer$; $nLcOse_6Cer$, V^4Gal, IV^3 GlcNAc-$nLcOse_4Cer$; NeuAc-$nLcOse_6Cer$, VI^3 NeuAc, V^4 Gal, IV^3 GlcNAc-$nLcOse_4Cer$; PC, phosphatidylcholine; SM, sphingomyelin, SAT, sialyltransferase; Hepes, 4-2(hydroxyethyl)1-piperazineethane sulfanic acid. Structures of glycolipids: $nLcOse_4Cer$, Gal($\beta 1 \rightarrow 4$)GlcNAc($\beta 1 \rightarrow 3$)Gal($\beta 1 \rightarrow 4$)-GlcCer; $GgOse_4Cer$, Gal($\beta 1 \rightarrow 3$)GalNAc($\beta 1 \rightarrow 4$)Gal$\beta(1 \rightarrow 4)$GlcCer.

1% Triton CF-54 (w/v), 20% glycerol (v/v) solution, and dialyzed overnight. The retentate was centrifuged at 15,000 × *g* for 30 min and the clear supernatant used as an enzyme source. The protein was assayed as described by Bradford (16).

Assay system. Except where stated otherwise, the incubation system contained the following components (in μmol): glycolipid substrate, 0.025; CMP-^{14}[C]NeuAc, 0.016 (2 × 10^6 cpm/μmol); $MgCl_2$, 0.25; Triton CF-54, 80 μg; and the enzyme preparation 65–75 μg of protein in an final volume of 55 μl. Unless mentioned otherwise, the phospholipids were added directly to the enzyme solution at a concentration of 0.25% (gm/100 ml). The mixtures were incubated at 37°C for 3 hr and assayed by double chromatography (17) (descending chromatography with 1% borax followed by ascending chromatography with chloroform:methanol:water [60:40:9, v/v/v]).

Stability studies. Solubilized sialyltransferase was assayed after storage at −70°C at different time periods. The enzyme solution was kept at 0°–4°C for a period of 16 days and assayed periodically for activity after the addition of sphingomyelin (0.25%) with various amounts of (a) glycerol (0–50%); (b) buffer (pH 5.0–8.0), and (c) Triton CF-54 (0.1–1%).

Isolation, purification, and characterization of radioactive product. The radioactive product was purified from a large (100 tubes) batch by DEAE-Sephadex A50 column and Biosil-A column chromatography as described previously (13). Approximately 5,000 cpm of the purified radioactive product was mixed with authentic NeuAc-lacto-*N*-neotetraosylceramide (10 μg) from chicken muscle and spotted on a precoated TLC plate with standard NeuAc-nLcOse$_4$Cer and a standard ganglioside mixture from human brain. After developing with chloroform:methanol:0.25% $CaCl_2$ (60:40:9, v/v/v), the plate was surveyed by autoradiography and gangliosides were visualized with resorcinol-HCl (18). Approximately 100 μg of the product was methylated and separated on a LH20 column (19,20). The combined glycolipid fraction after hydrolysis and acetylation was analyzed in a Perkin-Elmer gas chromatogram as previously described (13).

RESULTS AND DISCUSSION

Chicken muscle sialyltransferase(s) is membrane bound and requires detergent and cation (Mg^{+2} or Mn^{+2}) for optimum activity. The enzymes go into solution (at least 90%) in the presence of 1.5% Triton CF-54. This is facilitated by Mg^{+2}/Mn^{+2}. The solubilized enzyme is very labile and virtually all activity disappears over the course of 24–48 hours at 0°–4°C, and even at −70°C. Addition of the phospholipids, phosphatidylcholine, phosphatidylserine, and sphingomyelin readily reactivates the enzyme for longer than four days (Table 1) with decreasing activity thereafter, and loss of all activity by 16 days. In the majority of the experiments the activity is greatest at 24–48 hours and the reaction velocity is linear for as long as 5 hr. HEPES buffer at pH 6.5–7.0 (Table 2), 1% Triton CF-54, and 12.5 to 25% glycerol (Table 3) permitted lengthy preservation. Once stored, the activity of

TABLE 1

Effect of Phospholipid on Soluble Enzyme

Tube no.	Phospholipids (0.167%)	Enzyme activity (pmol/mg/hr) 0 hr	24 hr	48 hr	96 hr	144 hr
1	No phospholipids present	4 ± 1	2	7 ± 1	0	0
2	Phosphatidylethanolamine	5 ± 1	3	6 ± 1	1	0
3	Phosphatidylcholine	705 ± 12	721 ± 17	795 ± 20	781 ± 21	638 ± 9
4	Phosphatidylserine	374 ± 15	398 ± 12	410 ± 15	356 ± 13	175 ± 7
5	Phosphatidylinositol	16 ± 1	11 ± 1	8 ± 1	3	0
6	Sphingomyelin	736 ± 17	757 ± 15	820 ± 20	787 ± 16	645 ± 11

Substrate used: nLcOse$_4$Cer (0.025 μmol).

TABLE 2

Effect of Different Buffers and pH

Buffer 0.1M	pH	Enzyme activity (pmol/mg/hr) 0 hr	24 hr	48 hr	96 hr
Cacodylate	5.0	377 ± 12	437 ± 14	570 ± 16	320 ± 11
	5.5	451 ± 15	543 ± 16	663 ± 18	573 ± 16
	6.0	556 ± 15	642 ± 17	710 ± 20	615 ± 16
HEPES	6.0	560 ± 15	695 ± 18	785 ± 21	613 ± 16
	6.5	636 ± 17	763 ± 20	805 ± 19	643 ± 15
	7.0	700 ± 17	816 ± 18	903 ± 23	750 ± 17
Phosphate	7.0	635 ± 17	807 ± 18	622 ± 16	490 ± 14
	7.5	607 ± 16	823 ± 19	656 ± 17	413 ± 10
	8.0	586 ± 15	756 ± 18	628 ± 17	405 ± 10

Substrate used: nLcOse$_4$Cer (0.025 μmol).

TABLE 3

Effect of Concentration of Detergent and Glycerol

Tube no.	Reagent	Concentration (%)	Enzyme activity (pmol/mg/hr) 0 hr	24 hr	48 hr	96 hr
1[a]	—	—	19 ± 1	24 ± 1	11 ± 1	7 ± 1
2	Triton CF-54	0.25	331 ± 8	397 ± 12	403 ± 12	166 ± 8
3		0.50	342 ± 10	429 ± 13	550 ± 16	297 ± 9
4		0.75	357 ± 10	648 ± 17	688 ± 18	334 ± 12
5		1.0	401 ± 12	601 ± 16	884 ± 22	537 ± 15
6[b]		—	661 ± 16	667 ± 15	111 ± 6	0
7	Glycerol	12.5	730 ± 17	660 ± 15	842 ± 21	619 ± 16
8		25.0	618 ± 15	604 ± 14	768 ± 17	459 ± 14
9		37.5	114 ± 6	219 ± 8	231 ± 8	22 ± 1
10		50.0	36 ± 2	48 ± 2	70 ± 2	176 ± 8

[a]The enzyme was suspended in buffer/glycerol in absence of detergent.

[b]The enzyme was suspended in buffer/1% Triton CF-54 in absence of glycerol.

Substrate used: $nLcOse_4Cer$ (0.025 μmol).

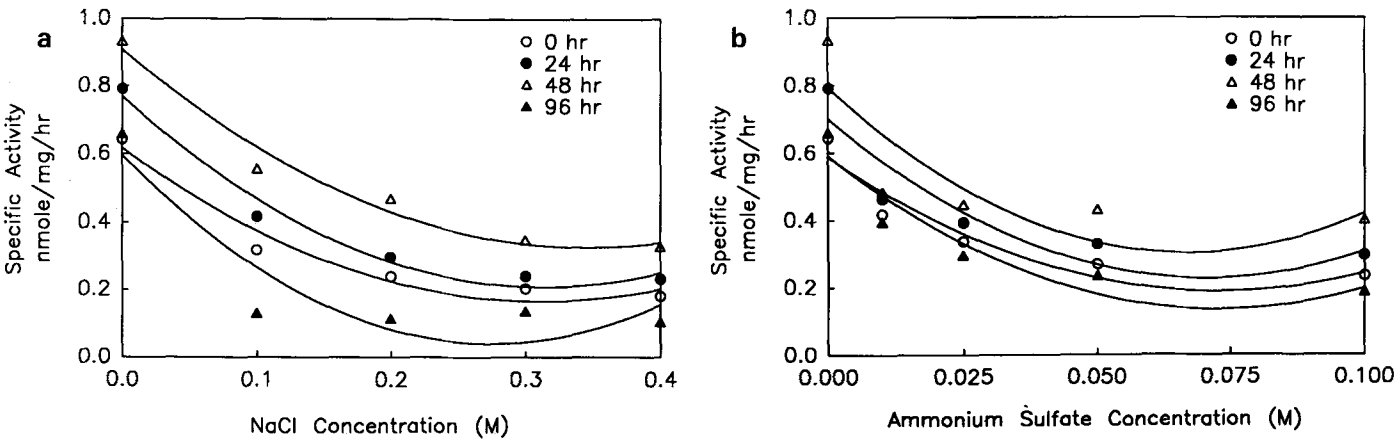

FIG. 1. (a) Effect of sodium chloride upon sialyltransferase. (b) Effect of ammonium sulfate concentration on sialyltransferase.

the enzyme disappeared quickly in the presence of a detergent concentration >1.0%. The enzyme was precipitated by ammonium sulfate and this was useful for both detergent removal and concentration. Since NaCl is used to elute the enzyme from the affinity column (9–11), the effect of both salts upon transferase activity was examined. As shown in Figure 1, both are inhibitory and ammonium sulfate is even more so on a molar basis. The salt inhibitions are reversible, but approximately 33% of the solubilized enzyme activity is lost upon complete removal of ammonium sulfate. Glycolipids with a nonreducing terminal β-galactose at the end are active acceptors, whereas those with terminal α-sialic acid linkage are not. The order of acceptor efficiency is $GgOse_4Cer$, GM_1, $nLcOse_6Cer$, and $nLcOse_4Cer$. Substrate competition studies indicate that the same enzyme mediates sialic acid transfer to all of these as reported in Golgi vesicles from rat liver (21).

The purified radioactive product was homogenous and co-migrated with standard NeuAc-$nLcOse_4Cer$ (Lane 2, Fig. 2) prepared from chicken muscle. In the absence of detergent the product is hydrolysed by neuraminidase (Type VIII) from *Clostridium perfringens,* indicating that the sialic acid is attached to the terminal galactose of $nLcOse_4Cer$. The partially methylated alditol acetate of the radioactive product has two peaks identified as 2,4,6 tri-*O*-methyl galactitol and 2,3,5 tri-*O*-methyl glucitol, as compared to authentic standard NeuAc-$nLcOse_4Cer$. The methylated aminosugar is identified as 3,6-di-*O*-methyl-2-deoxy-2-N-methyl acetamidoglucitol. The absence of 2,3,4,6-tetra-*O*-methyl galactitol confirms that the sialic acid is linked to the nonreducing end of the oligosaccharide by α2→3 linkage.

The activity of membrane-bound enzymes is known to be affected by phospholipids (22–29). Westcott *et al.* (30) showed that purified porcine submaxillary gland sialyltransferase activity stimulation by phosphatidylcholine is related to the length of the aliphatic acyl-derivative such that the C_{16}-C_{18} acyl-derivative was more effective than the C_{14}, and the C_{12} acyl-derivative had no effect. The inactive skeletal muscle transferase in 50 mM HEPES (pH 6.8), 1% Triton CF-54, and 20% glycerol is readily reactivated by the addition of phosphatidylcholine and sphingomyelin (0.167%). The stimulation increases

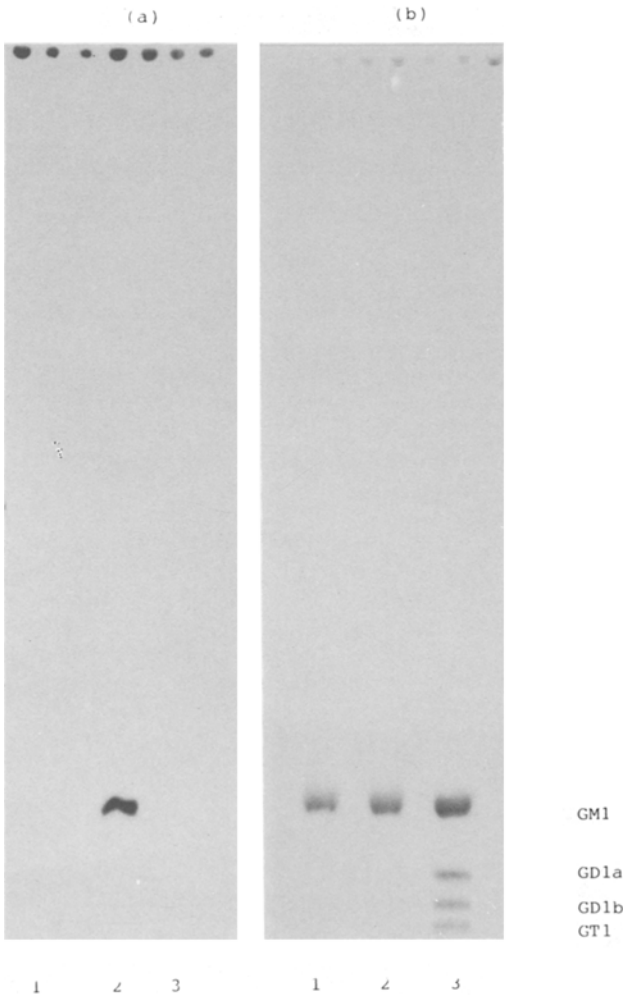

FIG. 2. (a) Autoradiogram of the radioactive product. (b) Same plate after visualization with resorcinol spray. Lane 1: authentic $NeuAcnLcOse_4Cer$. Lane 2: radioactive product with authentic $NeuAcnLcOse_4Cer$. Lane 3: ganglioside mixture from human brain. The plate was developed with $CHCl_3$/MeOH/0.25% $CaCl_2$ (60/40/9, v/v/v).

as the concentration of sphingomyelin is increased with an optimum concentration at 0.6–0.8% (Fig. 3). Sphingomyelin from chicken muscle (0.4%) was 2.1-fold more effective than bovine brain sphingomyelin. The major fatty acids of chicken muscle sphingomyelin are C_{16} (60%) and C_{18} (30%), in contrast to those of bovine brain sphingomyelin, which has more C_{24} (69%) as compared to C_{18} (24%) and C_{16} (3%). Hence, this finding is consistent with that of Westcott *et al.* (30), who found that the C_{16}-C_{18} acyl-derivative of phosphatidylcholine stimulated activity maximally. We propose that acyl-derivatives longer than C_{18} tend to either inhibit the activity or be ineffective.

We found 95% of the transferase activity in the membrane-bound form (the pellet after centrifugation at 100,000 × *g*) and 5% in the supernatant. This raises the possibility that membrane-bound and soluble forms of the skeletal muscle SAT exist that differ by the presence of a lipophilic domain as has been proposed for the A and B forms of porcine submaxillary SAT depending upon

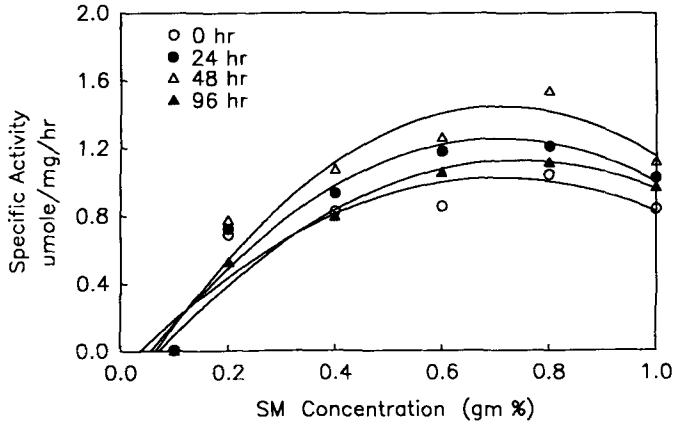

FIG. 3. Effect of concentration of sphingomyelin on sialyltransferase.

whether they can be incorporated into liposomes or not (31). Studies are under way in our laboratory to purify the SAT to homogeneity and to examine in a definitive way the reactivation of muscle transferase activity by sphingomyelin and phosphatidylcholine.

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Lack of Effects of *Trans* Fatty Acids on Eicosanoid Biosynthesis with Adequate Intakes of Linoleic Acid

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The minimum requirement of linoleic acid to prevent effects of dietary C18 *trans* fatty acids on eicosanoid biosynthesis in rats was assessed. In a first experiment, six groups of animals were fed diets with a high content of *trans* fatty acids [20% of energy (en%)], and increasing amounts of linoleic acid (0.4 to 7.1 en%). In a second experiment, four groups of rats were fed diets designed to compare *trans* fatty acids with saturated and *cis*-monounsaturated fatty acids of the same chain length at the 2 en% linoleic acid level. After 9–14 weeks the biosynthesis of prostacyclin by pieces of aorta and the biosynthesis of hydroxy-heptadecatrienoic acid and 12-hydroxy-eicosatetraenoic acid by platelets were measured. The fatty acid compositions of aorta phospholipid and platelet lipid were also determined. Both the prostacyclin-production by aorta pieces and the production of hydroxy-heptadecatrienoic acid and 12-hydroxy-eicosatetraenoic acid by platelets appeared to be a linear function of the arachidonic acid level in aorta phospholipid and platelet lipid, irrespective of the *trans* fatty acid content in the diet. This indicates that *trans* fatty acids do not directly influence enzymes involved in eicosanoid biosynthesis. In a direct comparison with *cis*-monounsaturated or saturated fatty acids with 2 en% linoleic acid in the diet, only a moderate reduction in arachidonic acid level in aorta phospholipids in the group fed *trans* fatty acids was observed. The geometry of the double bond did not influence the arachidonic acid level in platelet lipid, although the diet rich in saturated fatty acids increased arachidonic acid levels significantly compared with all other diets. Neither prostacyclin-production nor hydroxy-heptadecatrienoic acid or 12-hydroxy-eicosatetraenoic acid-production were significantly affected by *trans* fatty acids when 2 en% linoleic acid was present in the diet. Our study indicates that in rats 2 en% linoleic acid is sufficient to prevent effects of dietary *trans* fatty acids on eicosanoid synthesis.
***Lipids* 24, 555–563 (1989).**

Trans fatty acids from partially hydrogenated oils and ruminant fats from part of the human diet. Throughout the years there has been a marked interest in the biological effects of *trans* fatty acids. It is now recognized that they are well absorbed by both man and experimental animals and can be found in most tissues after ingestion (1-5). *Trans* fatty acids are incorporated into phospholipids of biological membranes, altering their fatty acid composition (1, 3, 6, 7). They also might influence the linoleic acid metabolism, resulting in a decreased formation of eicosanoid precursors (7, 8). Either by this decrease in precursor level or by direct effects on the eicosanoid-synthesizing enzymes, *trans* fatty acids could possibly diminish the production of prostaglandins, thromboxanes and hydroxy fatty acids.

During the last few years there has been a growing interest in the effect of dietary *trans* fatty acids on the eicosanoid metabolism. Kinsella (8) reported the inhibition of prostaglandin production in rats fed linolaidic acid (9*t*,12*t*-18:2). This inhibition, deduced from decreased serum prostaglandin E_1 (PGE_1), PGE_2 and $PGF_{2\alpha}$ concentrations, could be partly attributed to a decreased amount of prostaglandin precursors in the tissues, e.g. di-homo-γ-linolenic acid and arachidonic acid. But also a direct inhibitory effect of 9*t*,12*t*-18:2 on prostaglandin synthesis was considered. Hwang *et al.* (9) found that at a dietary dose equal to or higher than that of linoleic acid, *t*,*t*-18:2 decreased the arachidonic acid level in platelets and diminished (although not significantly) the production by platelets of thromboxane B_2 (TXB_2), $PGF_{2\alpha}$ and 12-hydroxy-eicosatetraenoic acid (12-HETE). The serum concentration of TXB_2 and $PGF_{2\alpha}$ also decreased. *t*,*t*-18:2 however, is not a substantial component of the human diet (2,4). *t* Monoenoic acids (*t*-18:1) are quantitatively much more important components in our diet.

Presently the generally accepted view is that *trans* fatty acids formed during the partial hydrogenation of vegetable oils (mainly monoenoic isomers) do not exert undesirable effects when compared with saturated or *cis*-monounsaturated fatty acids, provided sufficient linoleic acid is present in the diet (2-4,6,10). Recently, it has been reported that high dietary levels of *trans* isomers of monoenoic acids do not interfere with platelet cyclooxygenase or lipoxygenase, provided sufficient amounts of linoleic acid are available (11).

It was our aim to investigate the relationship between dietary *trans* fatty acids and linoleic acid and the eicosanoid synthesis in the rat in a quantitative way by determining the amount of linoleic acid necessary to prevent any effect of *trans* fatty acids on the eicosanoid synthesis. To investigate possible effects of *trans* fatty acids on the eicosanoid metabolism, two tissues that are known to be actively involved in eicosanoid production, aorta and platelet, were chosen for our studies. In the first experiment we investigated the effects of diets having a constant amount of C18 *trans* fatty acids and an increasing amount of linoleic acid on the prostacyclin (PGI_2) synthesis by pieces of aorta, and on the syntheses of hydroxy-heptadecatrienoic acid (HHT), a cyclooxygenase product, and 12-

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Abbreviations: ADP, adenosine diphosphate; A_gNO_3, silver-nitrate; BHT, di-*tert*-butyl-*p*-cresol; CB, cocoa butter; DEGS, diethylene glycol; EFA, essential fatty acid; GC, gas chromatography; GLC, gas-liquid chromatography; HCNO, hydrogenated coconut oil; 12-HETE, 12-hydroxy-eicosatetraenoic acid; HHT, hydroxy-heptadecatrienoic acid; OV, olive oil; OV-LL, low-linoleic acid olive oil; PGI_2, prostacyclin; PHSO, partially hydrogenated soybean oil; PPP, platelet-poor plasma; PRP, platelet-rich plasma; SSO, sunflower seed oil; TLC, thin-layer chromatography; TX, thromboxane.

HETE, a lipoxygenase product, by blood platelets of the rat. In the second we compared the effects of *trans* fatty acids with those of long-chain (C18) saturated or *cis*-monounsaturated fatty acids at a linoleic acid level of 2 en%, on the same parameters.

EXPERIMENTAL

Materials. The fats used in the two experiments were: a special, partially hydrogenated soybean oil (PHSO), hydrogenated coconut oil (HCNO), sunflower seed oil (SSO), cocoabutter (CB), olive oil (OV), and a low-linoleic acid olive oil (OV-LL). The origin of the experimental fats is described by Zevenbergen *et al.* (7).

Diethylether and hexane were obtained from Baker Chemicals (Deventer, The Netherlands). BHT (2,6-di-*tert*-butyl-p-cresol) was obtained from Fluka AG (Buchs, Switzerland). 15-Hydroxy-11,13-eicosadienoic acid (C20:2-150H) was prepared as described by Claeys *et al.* (12). Collagen, HPTLC-plates (Silicagel F254), Uvasol solvents and all other reagents and chemicals were obtained from Merck (Darmstadt, FRG).

Animals and diets. Weanling male SPF-Wistar rats (CPB/WU, Central Breeding Station TNO Zeist, The Netherlands) were used in both experiments. The animals were housed individually in a climatized room. The mean temperature was 23.0 ± 1.0°C, the relative humidity 45–70%, and there was a day/night cycle of 12/12 h. The animals had free access to water and food. This food, a semisynthetic diet with 40 en% fat was composed of (in $g.MJ^{-1}$ of the total diet) casein 14.8; vitamin mixture 0.2; salt mixture 1.3; cellulose 3.8; maize starch 25.2; experimental fat 10.3 (for compositions of vitamin and salt mixture, see Ref. 13). The animals were weighed and examined weekly. In the first experiment, 8 groups (7 of 12 animals each and one of 24 animals: group 3) were fed diets on the basis of the PHSO, HCNO, OV and SSO. The oils were mixed in such a way as to yield 6 diets having an increasing linoleic acid content (0.4–7.1 en%), but a constant *trans* fatty acid level of 20 en% (for detailed composition of experimental fats see Ref. 7). Two fats served as references, one consisting of HCNO and OV (2 en% linoleic acid), another mainly of OV (5 en% linoleic acid). Table 1 shows the calculated fatty acid compositions of the experimental fats. The feeding period lasted 13–14 weeks.

In the second experiment 4 groups of 24 rats each (randomly selected from 24 litters of 4 rats) were fed diets containing either 20 en% *trans* fatty acids (PHSO), mainly saturated fatty acids (CB), *cis*-monounsaturated fatty acids (OV-LL), or a mixture of PHSO, CB and OV (PHSOMix, 10 en% *trans* fatty acids; see Ref. 7 for composition). All diets contained 2 en% linoleic acid. The fatty acid compositions are given in Table 2. These diets were fed for 9–11 weeks.

Fatty acid composition of dietary fats. The composition of PHSO was determined by a combination of silver nitrate — thin layer chromatography ($AgNO_3$-TLC) separation and capillary GC as described before (7). The compositions of the other fats and oils were determined by GLC (5% DEGS packed column) after transmethylation with methanolic HCl.

Aggregation experiments with platelets. At the

TABLE 1

Calculated Fatty Acid Composition (%) of the Experimental Fats (Groups), First Experiment.

Type of fatty acid[a]	Group 1	2	3	4	5	6	7[b]	8[b]
6:0	0.1	—	—	—	—	—	0.2	—
8:0	1.2	0.8	0.8	0.8	0.5	—	3.8	—
10:0	1.0	0.7	0.7	0.7	0.4	—	3.2	—
12:0	8.9	6.1	6.1	6.1	3.7	0.1	27.0	—
14:0	3.6	2.5	2.5	2.5	1.6	0.2	10.3	—
16:0	10.3	10.4	10.1	9.9	9.6	9.4	10.5	12.2
16:1	0.3	0.3	0.3	0.2	0.2	0.2	0.4	1.0
17:0	0.1	0.1	0.1	0.1	0.1	0.2	—	0.1
17:1	—	—	—	—	—	0.1	0.1	0.2
18:0	8.5	7.9	8.1	8.1	7.8	7.2	8.0	2.9
18:1 *c*	16.5	20.3	17.5	15.3	14.8	16.1	30.7	69.7
18:1 *t*	39.1	39.1	39.1	39.1	39.1	39.1	—	—
18:2 9*c*,12*c*	1.0	2.0	5.0	7.5	12.5	17.7	5.0	12.5
18:2 *ct*,*tc*9,12	0.6	0.6	0.6	0.6	0.6	0.6	—	—
18:2 9*t*,12*t*	0.5	0.5	0.5	0.5	0.5	0.5	—	—
18:2 other	7.7	7.7	7.7	7.7	7.7	7.7	—	—
18:3	—	—	—	—	—	0.1	0.2	0.5
20:0	0.3	0.3	0.3	0.3	0.4	0.4	0.2	0.2
20:1 *c*	0.3	0.3	0.3	0.3	0.3	0.3	0.1	0.3
22:0	0.3	0.4	0.3	0.3	0.4	0.5	0.1	0.3
actual LA-level[d] (%)	1.1	n.d.	5.4	n.d.	n.d.	n.d.	5.2	n.d.

[a]The notation for the fatty acid indicates chain length and number of double bonds *c*=*cis*-isomers, *t*=*trans*-isomers, other=non-9,12-18:2 isomers (non-conjugatable).
[b]Reference-fats.
[c]Also contains conjugated 18:2 isomers.
[d]Linoleic acid level determined by GLC, n.d.=not determined.

TABLE 2

Fatty Acid Compositions (%) of the Experimental Fats (Groups), Second Experiment.

Type of fatty acids[a]	Group			
	CB	OV	PHSO	PHSOMix
6:0	—	—	—	—
8:0	—	—	0.8	—
10:0	—	—	0.7	—
12:0	—	0.1	5.6	—
14:0	0.2	—	2.4	0.1
16:0	23.5	11.9	10.0	15.3
16:1	—	—	0.3	—
17:0	0.3	—	0.1	0.1
17:1	—	0.1	0.1	0.1
18:0	31.9	2.0	8.0	12.1
18:1 *c*	36.9	78.7	17.5	39.9
18:1 *t*	—	—	39.1	19.5
18:2 9*c*,12*c*	4.5	5.5	5.3	4.7
18:2 *ct*,*tc*9,12	—	—	0.6	0.3
18:2 9*t*,12*t*	—	—	0.5	0.2
18:2 other	—	—	7.7	3.9
18:3	0.2	0.6	—	—
20:0	1.3	0.4	0.5	0.6
20:1	—	0.4	0.5[b]	0.4[b]
22:0	0.3	0.2	0.4	0.3

[a]The notation for the fatty acid indicates chain length and number of double bonds *c*=*cis*-isomers, *t*=*trans* -isomers, other-non-9,12-18:2 isomers (non-conjugatable).
[b]Also contains conjugated 18:2 isomers.

end of the feeding period the animals were kept overnight without food, weighed, and then sacrificed by aorta cannulation under ether anesthesia. Blood was collected and mixed gently with Na-citrate (3.8%; 9:1, v/v). Platelet-rich plasma (PRP) was prepared by centrifuging the blood at 140 × g at room temperature for 20 min. After taking off the PRP, the blood was centrifuged a second time (600 × g; 10 min) to prepare platelet-poor plasma (PPP). The platelet count of the PRP was adjusted to $1*10^6$ platelets/μl with autologous PPP. Aggregation of the platelets was induced in 0.5 ml PRP in a Chronolog aggregometer with four different dosages of collagen (18, 30, 50, and 150 μl; concentration 0.29 mg collagen/ml on nitrogen-basis). The delay and the slope (tg α) of the aggregation curves were monitored and 5 min after the addition of the collagen, the reaction was stopped by adding 0.5 ml of Uvasol methanol. To these samples and samples of nonstimulated PRP, we added a drop of butyrylhydroxytoluene solution (BHT in methanol; 4 g/l) and the samples were frozen and stored at −25°C for lipid and hydroxy fatty acid analysis.

Bioassay of PGI_2-production by aorta pieces. Immediately after the collection of blood, the aorta was removed and pieces (⌀ 3 mm) were punched out of the thoracic part of the aorta. These pieces were incubated in 0.2 ml of buffered saline (17 g NaCl; 0.4 g KCl; 0.4 g KH_2PO_4; 2.9 g $Na_2HPO_4 \cdot H_2O$) for 4 minutes. The 50 μl of the incubate was then added to PRP of control rats and the effect of the incubate on the ADP-induced aggregation of this PRP was measured according to Hornstra *et al.* (14). The prostacyclin-like activity was quantified by using a calibration curve of purified prostacyclin (PGI_2). The abdominal part of the aorta was brought in 0.2 ml of buffered saline with one drop of BHT and stored at −25°C for lipid analysis.

Determination of hydroxy fatty acids with HPLC. To the aggregated PRP samples 15-hydroxy-11*cis*,13*trans*-eicosadienoic acid (C20:2-150H) was added as internal standard. The lipids were extracted according to Folch *et al.* (15), with Uvasol-quality solvents. Hydroxy fatty acids were separated from prostaglandins and other lipids on small silica gel columns (16). The hydroxy fatty acids were separated on a Varian HPLC with a Lichrosorb RP-18 column (5 μm, 250 × 4.6 mm; detection by U.V. at 234 nm) and quantified.

Fatty acid analysis. Platelets and aorta pieces were extracted according to Bligh and Dyer (17). The phospholipid fraction of the aorta was isolated by preparative TLC, according to Christie (18). This fraction and the total lipids of the platelets were *trans*-esterified with methanolic HCl. The methyl esters were analyzed by GLC on packed columns (5% DEGS or Apiezon) or glass capillary columns (Silar 88) by standard methods.

Statistics. All data except for the fatty acid compositions were subjected to an analysis of variance according to the randomized complete blocks design. The days of sacrifice served as blocks in the first experiment, litters in the second. In the first experiment, Dunnett's procedure was applied to find out which groups differed systematically from the standard group (group 3, 20 en% *trans* fatty acids and 2 en% linoleic acid). In the second experiment, the Student-Newman-Keuls multiple-range test was applied to locate possible systematic differences between the dietary treatments.

RESULTS

General condition of the animals. All animals seemed to be in good health in both experiments, showing no overt signs of EFA-deficiency. In neither experiment did systematic differences in food consumption or body weight related to dietary treatment occur. At the time of sacrifice the weight of the animals ranged from 370-400 g in the first experiment, and from 350-400 g in the second. The results of histo-pathological examination, haematology, and clinical chemistry will be reported separately (Zevenbergen and Verschuren, submitted for publication).

Fatty acid composition of the aorta phospholipids. The dietary linoleic acid level had hardly any influence on the 18:2 level of aorta phospholipid in the first experiment, while the arachidonic acid incorporation was increased by raising the linoleic acid intake (Table 3). Other prostaglandin precursors were present in low amount (20:3 n-6) or were not detected (20:5 n-3). The total sum of saturated fatty acids remained rather constant, whereas the sum of all n-6 fatty acids, including 18:2, increased.

Dietary *trans* fatty acids clearly increased the level of linoleic acid. The arachidonic acid level was highest in reference group 7 (with 2 en% linoleic acid). The other reference diet did not induce an arachidonic acid level different from that induced by the corresponding *trans* fatty acid containing diet (compare groups 5 and 8). Other polyunsaturated fatty acids were hardly influenced by *trans* fatty acids, except for 22:6 n-3, of which the level in the *trans* fatty acids fed groups was

TABLE 3

Fatty Acid Composition (%) of Aorta Phospholipids, First Experiment[a]

Type of fatty acid[b]	Group							
	1	2	3[c]	4	5	6	7[d]	8[d]
12:0	0.1	0.1	—	—	0.1	0.1	—	—
14:0	0.5	0.5	0.4	0.4	0.3	0.3	0.9	0.5
16 al.	1.7	1.3	1.4	1.6	1.6	1.4	2.2	1.4
16:0	18.2	18.9	18.1	18.0	18.1	18.4	19.0	18.9
16:1 n-7	2.7	2.6	2.3	2.2	2.2	2.0	1.3	1.4
18 al.	2.1	2.2	2.1	2.1	2.3	2.5	2.6	1.9
18:0	14.7	14.9	15.3	15.8	16.1	15.8	18.6	16.4
18:1[e]	18.6	18.0	16.2	15.3	14.0	14.0	11.9	14.0
18:2[e]	7.0	6.1	6.9	7.1	7.0	7.4	3.5	4.7
18:3 n-6	0.2	0.1	0.2	—	0.1	0.2	0.1	0.1
20:0[f]	0.7	0.9	0.9	0.7	0.8	0.9	0.9	0.9
20:1 n-9	0.6	0.6	0.6	0.6	0.4	0.4	0.4	0.4
20.9[g]	0.5	0.4	0.3	0.3	0.3	0.2	0.1	—
20:3 n-9	0.4	0.3	0.5	0.5	0.6	0.6	0.3	0.4
21.5[g]	1.8	1.4	0.6	0.5	0.3	0.3	0.6	0.5
20:3 n-6	1.1	1.1	1.0	0.9	0.8	0.7	0.9	0.7
22:0[f]	0.8	0.9	0.9	0.9	0.9	0.9	0.9	0.9
20:4 n-6	17.5	18.5	21.2	22.0	22.4	22.5	23.5	22.5
23.4[g]	0.8	0.6	0.2	—	—	0.1	0.5	0.3
22:4 n-6	4.1	4.3	5.2	5.4	6.0	6.1	5.0	4.9
22:5 n-6	3.7	3.9	4.1	3.8	3.9	3.9	4.1	4.2
22:5 n-3	0.3	0.3	0.1	—	0.2	0.2	0.1	0.4
22:6 n-3	1.4	1.7	1.1	0.9	0.8	0.7	1.7	1.9
24:0[f]	1.3	1.1	1.2	1.2	1.1	1.4	1.4	1.4
sum	97.5	97.4	97.5	97.4	97.3	97.4	97.5	95.0
total n-6[h]	33.6	34.0	38.6	39.3	40.3	40.7	37.1	37.0
total sat[h]	36.0	36.7	36.4	36.8	37.2	37.5	41.1	38.5

[a]Each value represents the mean of two pools of 6 animals. Analysis is performed on packed column (5% DEGS). — = Not detected.
[b]The notation indicates chain length and number of double bonds al. = dimethylace tal.
[c]Mean of 4 pools of 6 animals.
[d]Reference-groups.
[e]Both *cis* and *trans* isomers.
[f]Quantitated with apiezon-column.
[g]Carbon-number of un-identified fatty acids.
[h]Total n-6 and total sat = the sum of the relative amount of n-6 fatty acids and 18:2 and saturated fatty acids, respectively.

lower than that in the groups fed reference diets. This could be due to the somewhat higher level of 18:3 n-3 in the diets of the latter groups. *Trans* fatty acids did not reduce the total amount of n-6 fatty acids. In the second experiment (Table 4) *trans* fatty acids increased the linoleic acid level. Compared to the groups fed either of the other two diets, the arachidonic acid level in the groups fed *trans* fatty acids was diminished in a dose-dependent manner (difference between the PHSO-group—20 en% *trans* fatty acids, and the PHSOMix-group—10 en% *trans* fatty acids. The total amount of n-6 fatty acids of all groups was similar. Both monoenoic and dienoic *trans* fatty acids were incorporated into aorta phospholipids at low levels (respectively, 3.2 and 1.2% with a dietary level of total *trans* fatty acids of 20 en%), as determined by capillary GLC. *Trans* fatty acids seemed to be incorporated mainly at the expense of stearic acid. The total saturated fatty acids level was therefore somewhat reduced in the groups fed *trans* fatty acids. The capillary GLC technique also revealed that the increase in 18:2 level caused by *trans* fatty acids was largely the result of an increase in the amount of 9*cis*,12*cis*-18:2 (linoleic acid), as the amount of other dienoic C18 fatty acids was low.

PGI_2-production of aorta pieces. The PGI_2-production from aorta pieces shows a strong linear relationship (confidence > 99%) with the log-dose of linoleic acid in the diet (first experiment; Fig. 1). The reference group fed 2 en% linoleic acid (diet consisting of HCNO and OV) displayed a remarkably high PGI_2-production. Not only was it higher than the corresponding *trans* group (group 3), but it was also higher than the reference group fed 5 en% linoleic acid. With 5 en% linoleic acid in the diet, no difference could be found between the *trans* fatty acids fed group and the reference group. Also, when the PGI_2-production of aorta pieces of the *trans* groups was plotted against the arachidonic acid level in aorta phospholipids, a strong linear relationship appeared (Fig. 2). When extrapolated to higher arachidonic acid levels, the two reference groups did not deviate significantly from this relationship. The PGI_2-production of aorta pieces in the second experiment did not reveal significant differences between the groups (mean values ± s.e.m. of 24 animals for the CB, OV, PHSO and PHSOMix-group, respectively: 1.20 ± 0.051, 1.19 ± 0.049, 1.15 ± 0.066 and 1.15 ± 0.049 ng/ml).

Fatty acid composition of platelets. The fatty acid

TABLE 4

Fatty Acid Composition of Aorta Phospholipids as Found by Packed Column GLC (A) and by Capillary Column GLC (B). Second experiment[a]

Type of fatty acids[a]	Group			
	CB	OV	PHSO	PHSOMix
		—A—		
14:0	0.2	0.3	0.5	0.3
16:0	18.4	18.5	17.3	18.0
16:1	0.8	1.0	2.1	1.7
18:0	20.5	18.0	15.0	17.5
18:1[c]	12.3	16.2	17.5	16.1
18:2[c]	3.2	2.9	6.5	4.5
18:3 n-6	0.3	0.1	—	0.1
20:0[d]	1.0	0.9	0.8	0.9
20:1	0.4	0.6	0.5	0.5
20:3 n-9	1.0	0.9	0.9	1.0
20:3 n-6	0.7	0.7	0.9	0.8
20:4 n-6	22.9	21.1	20.3	21.4
22:0[d]	1.0	0.7	0.8	0.9
22:3	0.7	0.7	0.3	0.5
22:4 n-6	3.5	3.1	3.6	3.5
24:0[d]	1.3	0.9	0.9	1.0
22:5 n-6	3.5	3.4	3.5	3.4
22:5 n-3	—	0.4	0.2	0.3
22:6 n-3	1.8	2.5	1.3	1.8
24:1[d]	1.4	2.1	1.6	1.6
sum	94.8	95.0	94.3	95.4
total n-6[e]	34.1	31.3	34.8	33.7
total sat[e]	42.4	39.3	35.3	38.6
		—B—		
18:1 *t*			3.2	2.0
18:1 *c*			13.1	13.4
18:1 total			16.1	15.4
18:2 *t*			1.2	0.1
18:2 9*c*,12*c*			5.0	3.7
18:2 total			6.2	3.8

[a]Each value represents the mean value of 4 pools of 6 animals.
[b]The notation indicates chain length and number of double bonds; *c*=*cis* isomer, *t*=*trans* isomer, —=not detected.
[c]Both *cis* and *trans* isomers.
[d]Quantitated with apiezon-column.
[e]Total n-6 and total sat=the sum of the relative amount of n-6 fatty acids and 18:2 and saturated fatty acids, respectively.

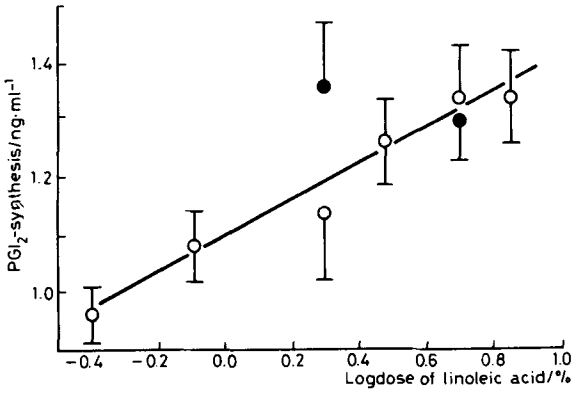

FIG. 1. PGI_2-synthesis (ng/ml) of aorta pieces as a function of the log-dose of dietary linoleic acid (in en%) in the first experiment. - represents the two reference groups (groups 7 and 8); ○ represents the *trans* fatty acid fed groups. Data given are means ± s.e.m. of 11 animals. The regression line was calculated by the least square method, excluding the two reference groups.

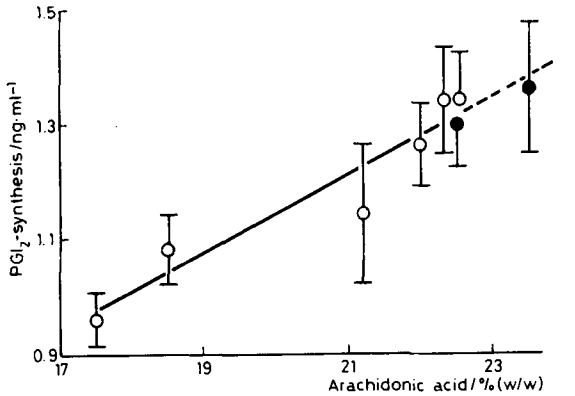

FIG. 2. PGI_2-synthesis (ng/ml) of aorta pieces as a function of the arachidonic acid level (wt%) in aorta phospholipid in the first experiment. ● represents the two reference groups (groups 7 and 8); ○ represents the *trans* fatty acids fed groups. Data given are means ± s.e.m. of 11 animals. The regression line was calculated by the least square method, excluding the two reference groups.

composition of platelets was influenced by the dietary level of linoleic acid in a similar way as that of aorta phospholipids (Table 5). *Trans* fatty acids clearly increased the amount of linoleic acid incorporated in platelet lipids. The arachidonic acid level in the reference group fed 2 en% linoleic acid was higher than that in all other groups. There was no difference between the reference group fed 5 en% linoleic acid and the corresponding *trans* group. The levels of other prostaglandin precursors (20:3 n-6 and 20:5 n-3) were low—they were not influenced by *trans* fatty acids. Capillary GLC showed a slight decrease in the amount of *trans*-18:2 isomers with increasing dietary linoleic acid; the level of *trans* monoenes was not decreased. A clear effect of *trans* fatty acids on the levels of 9*cis*,12*cis*-18:2 was demonstrated. In the second experiment a significant difference in arachidonic acid incorporation between the OV-group and the CB-group was evident (Table 6). The *trans* fatty acids containing diets caused arachidonic acid levels in between those of the OV- and CB-groups. As in aorta phospholipid, platelet lipid in the OV-group contained the lowest level of total n-6 fatty acids. The incorporation of *trans* fatty acids in platelet lipids was somewhat higher than that in aorta phospholipids.

Platelet aggregation experiments. In the first experiment platelet aggregation was induced with 4 doses of collagen. The lowest dose (5.2 μg) failed in triggering all PRP samples and was therefore not included in the statistical analysis. The delays (in s) of the aggregation curves of PRP induced by 8.7, 14.5 or 43.5 μg collagen are presented in Fig. 3. With the low dose of collagen (8.7 μg) there seems to be a dose-response relationship between the dietary linoleic acid levels and the delay. No significantdifferences could be found between group 3 and the other groups for each dose of collagen. In the second experiment some systematic differences were detected in the delay as well as the

TABLE 5

Fatty Acid Composition (%) of Platelet Lipids as Found by Packed Column GLC (A) and by Capillary Column GLC (B), First Experiment[a]

Type of fatty acid[a]	Groups							
	1	2	3[c]	4	5	6	7[d]	8[d]
			—A—					
12:0	—	—	—	0.2	0.1	—	—	—
14:0	0.7	0.7	0.7	0.7	0.5	0.4	1.8	0.4
16 al.	1.1	1.1	1.0	0.9	0.9	1.0	1.7	1.4
16:0	22.3	23.0	23.7	23.6	23.3	23.3	26.4	29.2
16:1 n-7	4.5	4.1	4.3	4.4	4.1	4.4	1.4	1.6
18 al.	2.0	2.4	2.4	2.4	2.3	2.1	2.5	1.8
18:0	12.2	12.8	12.5	12.7	12.7	12.8	16.4	15.4
18:1[e]	19.5	18.1	16.8	16.3	15.6	15.4	10.2	13.3
18:2[e]	5.6	5.1	5.4	5.9	6.5	7.0	2.8	3.0
18:3 n-6	0.2	0.1	0.1	0.1	0.2	0.1	0.1	0.1
20:0[f]	0.6	0.8	0.8	0.6	0.7	0.6	0.6	0.6
20:1 n-9	1.3	1.3	1.2	0.9	1.1	1.3	0.9	1.2
20:3 n-9	3.3	2.1	1.4	1.1	1.1	1.0	0.9	0.7
20:3 n-6	1.2	0.9	0.7	0.5	0.5	0.4	0.5	0.3
22:0[f]	1.1	1.1	1.3	1.2	1.2	1.2	1.2	1.0
20:4 n-6	14.8	16.4	17.3	18.2	18.6	18.3	22.2	18.4
22:1 n-9	1.3	1.3	1.3	1.1	1.3	1.3	0.5	0.8
20:5 n-3	0.3	0.1	0.1	—	—	—	—	—
22:4 n-6	1.6	2.6	3.7	4.5	4.8	5.0	4.1	4.6
22:5 n-6	3.6	3.3	3.1	2.9	2.9	2.7	4.2	4.7
22:5 n-3	—	—	0.1	0.7	0.8	1.1	0.3	—
22:6 n-3	—	—	—	—	—	—	—	—
24:0[f]	1.2	0.8	1.2	1.1	1.6	1.2	1.4	1.1
sum	95.5	95.4	95.8	97.1	97.3	97.6	96.7	96.9
total n-6[g]	25.8	27.5	29.6	31.6	33.0	33.1	33.4	30.8
total sat[g]	38.1	39.2	40.2	40.1	40.1	39.5	47.8	47.7
			—B—					
18:1 *t*	4.9	5.4	5.7	7.2	7.7	5.6	0.1	0.3
18:1 *c*	14.4	13.0	10.7	9.3	8.5	9.8	9.5	12.9
18:1 total	19.3	18.4	16.4	16.5	16.2	16.4	9.6	13.2
18:2 *t*	1.1	0.9	0.9	0.8	0.5	0.7	—	—
18:2 9*c*,12*c*	4.1	3.7	4.2	4.9	5.5	5.4	2.9	3.2
18:2 total	5.2	4.6	5.1	5.7	6.0	6.1	2.9	3.2

[a]Each value represents the mean of 2 pools of 6 animals. Analysis is performed on packed column (5% DEGS).
[b]The notation indicates chain length and number of double bonds al.=dimethylacetal, —=not detected, *c*=*cis*-isomers, *t*=*trans*-isomers.
[c]Mean of 4 pools of 6 animals.
[d]Reference-groups.
[e]Both *cis* and *trans* isomers.
[f]Quantitated with apiezon-column.
[g]Total n-6 and total sat=the sum of the relative amount of n-6 fatty acids and 18:2 and saturated fatty acids, respectively.

tangent α of the aggregation curves (Table 7). The CB-diet induced the highest delay and the lowest tg α compared to the other diets. The *trans* fatty acids fed groups had values between those of the CB-group and the OV-group when 11.6 μg collagen was applied. When 29 μg collagen was used, the PHSO-diet caused the same delay as the CB-diet but a significantly higher tg α.

Hydroxy fatty acid production of platelets. Hydroxy fatty acid production was measured after stimulation of PRP. Both maximum (43.5 μg collagen) and submaximal (14.5 μg collagen) triggering were used. The concentrations of the monohydroxy fatty acids 12-hydroxy-5*cis*, 8*cis*,10*trans*-hepta-decatetraenoic acid (HHT) and 12-hydroxy-5*cis*,8*cis*,10*trans*,14*cis*-eicosatetraenoic acid (12-HETE) in the supernatans of the stimulated platelets, after maximal triggering, as determined by HPLC are given in Fig. 4 as a function of the dietary linoleic acid level. Both the HHT and 12-HETE values of group 1 (0.4 en% linoleic acid; 20 en% *trans* fatty acids) were significantly lower than those of group 3 (2 en % linoleic acid, (conf. > 99%.) The HETE-concentration of group 6 (7.1 en% linoleic acid) was significantly higher than that of group 3 (conf. > 95%). The reference groups (7 and 8) with 2 and 5 en% linoleic acid, respectively, were not significantly different from the standard *trans* group (group 3). Submaximal triggering of the platelets produced less 12-HETE and HHT, but the effects of the dietary fats were very similar as with maximal triggering, i.e.,

TABLE 6

Fatty Acid Composition (%) of Platelet Lipids as Found by Packed Column GLC (A) and by Capillary Column GLC (B), Second experiment[a]

Type of fatty acids[b]	Group			
	CB	OV	PHSO	PHSOMix
		—A—		
14:0	0.3	0.5	0.7	0.3
16:0	26.2	29.7	24.0	25.4
16:1	0.9	1.6	4.0	2.3
18:0	17.4	14.0	10.9	14.1
18:1[c]	9.4	15.6	17.2	14.5
18:2[c]	2.7	2.2	6.0	4.0
18:3 n-6	0.3	0.2	—	0.2
20:0[d]	0.7	0.6	0.6	0.7
20:1	0.7	1.4	1.1	1.2
20:3 n-9	1.2	1.0	0.3	1.2
20:3 n-6	0.4	0.4	0.7	0.6
20:4 n-6	23.6	18.3	18.4	20.0
22:0[d]	1.2	0.6	0.9	0.9
22:3	0.8	0.7	0.3	0.6
22:4 n-6	3.9	3.4	3.3	3.6
24:0[d]	0.7	0.5	0.4	0.5
22:5 n-6	2.5	3.0	2.0	2.4
22:6 n-3	0.4	0.5	0.2	0.4
24:1 [d]	1.6	2.6	1.6	1.8
sum	94.9	96.5	92.6	94.7
total n-6[e]	33.4	27.5	30.4	30.8
total sat[e]	46.5	45.9	37.5	41.9
		—B—		
18:1 *t*			5.9	2.6
18:1 *c*			11.5	11.8
18:1 total			17.4	14.4
18:2 *t*			1.2	0.6
18:2 9*c*,12*c*			4.5	3.2
18:2 total			5.7	3.8

[a]Each value represents the mean value of 4 pools of 6 animals.
[b]The notation indicates chain length and number of double bonds; *c*=*cis* isomer, *t*=*trans* isomer, —=not detected.
[c]Both *cis* and *trans* isomers.
[d]Quantitated with apiezon-column.
[e]Total n-6 and total sat=the sum of the relative amount of n-6 fatty acids and 18:2 and saturated fatty acids, respectively.

a significantly decreased HHT and 12-HETE in group 1 compared to group 3 (conf. > 99%, data not shown). No significant differences in HHT-production between the groups were found in the second experiment (Table 8). The 12-HETE-production in the CB-group was significantly higher than that in the other groups.

DISCUSSION

The influence of dietary *trans* fatty acids on tissue fatty acid composition in rats has been the subject of many investigations. All tissues investigated have been reported to incorporate (monoenoic) *trans* fatty acids, although to a different degree (1, 19). In this report emphasis is laid on the changes in the tissue levels of polyunsaturated fatty acids brought about by *trans* fatty acids, as polyunsaturated fatty acids are precursors of the biosynthesis of eicosanoids.

HHT and malondialdehyde are formed during the biosynthesis of the potent pro-aggregatory compound

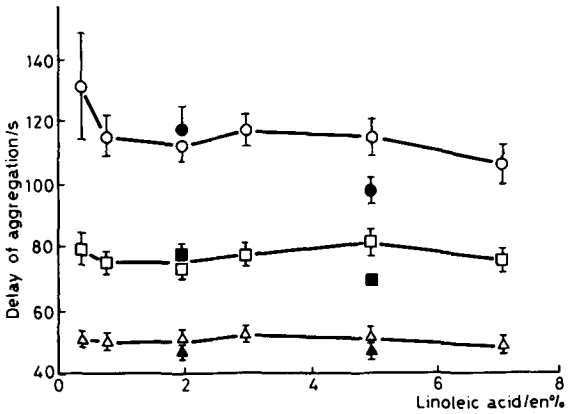

FIG. 3. Delay of aggregation (s) of PRP, stimulated with three doses of collagen as a function of the level of dietary linoleic acid (3n%) in the first experiment. ○ ● = 8.7μg; □ ■ = 14.5 μg and △ ▲ = 43.5 μg collagen. ● ■ ▲ represents the two reference groups (groups 7 and 8); ○□△ represents the *trans* fatty acids fed groups. Data given are means ± s.e.m. of 11 animals.

thromboxane A_2 (TXA_2) (20). TXA_2 is a product of the cyclooxygenase pathway. This biologically active compound is rapidly converted into TXB_2, a stable, inactive product. Concomittantly with the synthesis of every TXA_2 molecule, approximately one molecule of HHT and one of MDA are formed. The synthesis of TXA_2 can be followed via the analysis of the hydroxy fatty acid HHT, which can be quantitated via HPLC (16). PGI_2, also a product of the cyclooxygenase pathway, formed by the vascular tissue (21) and TXA_2, formed by platelets, are thought to play an important role in platelet aggregation, and hence in thrombotic processes (22). So both the anti-aggregatory PGI_2 and the pro-aggregatory TXA_2 originate from the same precursor arachidonic acid, and have the same endoperoxide as intermediate. In a second pathway in platelets, arachidonic acid is converted by a lipoxygenase into a hydroperoxyde fatty acid, which in turn is reduced to the hydroxy fatty acid 12-HETE. The role of hydroxy fatty acids in thrombotic processes is not clear; it has been suggested that they support the clotting process (23) and promote adhesion of leucocytes to the vascular wall (24).

Trans fatty acids are often reported to reduce tissue arachidonic acid levels (25). This could be due to their effect on arachidonic acid biosynthesis (26–29), however, other mechanisms are also possible (30, Zevenbergen and Houtsmuller, in press). In this experiment the arachidonic acid level in the groups fed *trans* fatty acids is diminished when compared to that in groups fed diets high in saturated fatty acids (groups 7 and CB). However, when effects of dietary *trans* fatty acids and *cis*-monounsaturated fatty acids (groups 8 and OV) are compared, the situation is less clear. In the second experiment, in aorta phospholipids the level of arachidonic acid in the animals fed OV is somewhat higher than that in the animals fed *trans* fatty acids, while there is no difference between those groups with respect to the platelet arachidonic acid content. For both linoleic and arachidonic acid the effects of *trans*fatty acids seems to be dose-dependent as is evi-

TABLE 7

Aggregation of PRP Induced with Two Doses of Collagen, Second Experiment[a]

Group	11.6 μg collagen		29 μg collagen	
	Delay	tg α	Delay	tg α
CB	163 ± 9.2[2]	4.30 ± 0.233[2,3,4]	78 ± 1.9[2]	5.91 ± 0.122[3]
OV	133 ± 5.2[1]	4.99 ± 0.155[1]	70 ± 1.9[1,3]	6.13 ± 0.097
PHSO	149 ± 7.2	4.84 ± 0.185[1]	78 ± 2.6[2]	6.23 ± 0.111[1]
PHSOMix	147 ± 6.7	4.84 ± 0.168[1]	73 ± 2.4	6.16 ± 0.111

[a]Each value represents the mean value and s.e.m of the delay (in s) and tg α of the aggregation curves for the four groups (n=24).

1,2,3,4. Values with superscript 1,2,3, or 4 are significantly different from those of group CB, OV, PHSO or PHSOMix, respectively, for $p < 0.05$.

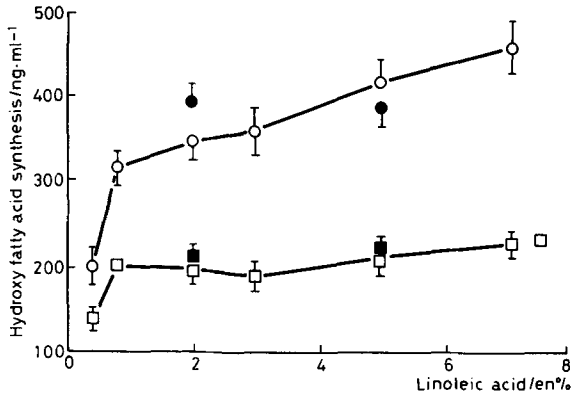

FIG. 4. Hydroxy fatty acid synthesis (ng/ml) of platelets after stimulation with 43.5 μg collagen (maximal triggering), as a function of the level of dietary linoleic acid (en%) in the first experiment. ○● = HETE; □■ = HHT. ●■ represents the two reference groups (groups 7 and 8); ○□ represents the *trans* fatty acids fed groups. Data given are means ± s.e.m. of 11 animals.

TABLE 8

Production of Hydroxy Fatty Acids (in ng) by 10^9 Platelets Stimulated with 29 μg Collagen, Second Experiment[a]

Group	HHT	HETE
CB	323 ± 13.0	631 ± 26[2,3,4]
OV	337 ± 15.8	573 ± 23[1]
PHSO	306 ± 11.4	537 ± 15[1]
PHSOMix	321 ± 12.0	568 ± 28[1]

[a]The values represent the mean ± s.e.m. of 24 animals per group.

1,2,3,4. Values with superscript 1,2,3, or 4 are significantly different from those of group CB, OV, PHSO or PHSOMix, respectively, for $p < 0.05$.

dent from a comparison of the PHSOMix group (10 en% *trans* fatty acids), the PHSO group (20 en% *trans*fatty acids), and the other two groups (no *trans* fatty acids). The relative effect of these isomeric fatty acids on tissue arachidonic acid level is certainly not universal and may depend on tissue and phospholipid class (7) as well as on the diet used for comparison. In both our experiments, the diets rich in saturated fatty acids (the HCNO/OV-diet—group 7—and the CB-diet) induced much higher arachidonic acid levels than the OV-diets (groups 8 and OV-diet). As is evident from our experiments and those of others (11), the lowering of arachidonic acid induced by *trans* fatty acids compared to *cis*-mono-unsaturated fatty acids is small or non-existing in platelets.

Many feeding studies to investigate biological effects of *trans* fatty acids have been performed using isolated fatty acids (monoenoic and/or dienoic isomers). In our experiment we used PHSO, a special, partially hydrogenated soybean oil, containing a high amount of various monoenoic *trans* isomers next to a relatively high amount of dienoic *trans* fatty acids—isomers of linoleic acid. The diets were composed in such a way as to contain 40% *trans* monoenes and almost 8% *trans* dienes (ca. 0.5% 9*t*,12*t*-18:2 and 0.6% other 9,12-18:2 isomers), thus by far exceeding the *trans* content of human diets (31, 32). The advantage of this approach is that the distribution of positional isomers of both monoenoic and polyenoic *trans*fatty acids in the experimental diet is similar to that in the human diet. Because of this, more realistic conclusions can be reached with regard to the nutritive value of *trans* fatty acids containing food products.

Many investigators have found that significant changes in eicosanoid synthesis can be brought about by dietary fat (33-36). These changes are at least partly caused by reduced eicosanoid precursor levels in tissues. A linear relationship between PGI_2-synthesis of aorta pieces and phospholipid arachidonic acid level in rabbits has been demonstrated (34). Similarly, HHT- and HETE-formation by rat platelets is well correlated with platelet arachidonic acid content (11, 34). In our first experiment, where a wide range of dietary linoleic acid levels was applied, these relationships were confirmed. PGI_2-synthesis showed a strong linear relationship with the arachidonic acid level in aorta phospholipid. The two reference groups, devoid of *trans* fatty acids, fitted the regression line calculated from the data of the *trans* fatty acids fed groups perfectly. Similar relationships were observed for the HHT- and HETE-production by platelets (data not shown). We therefore conclude that *trans* fatty acids do not directly influence the enzymes involved in eicosanoid synthesis, but exert their effect on eicosanoid production by influencing the tissue precursor level, i.e., arachidonic acid. This is in line with the findings of Nugteren (37), who indicated that the cyclooxygenase activity of sheep seminal vesicles was not inhibited substantially by

isomeric fatty acids, unless a *cis,trans* mono-conjugated system was present. Levels of *cis,trans* conjugated fatty acids are negligible in our experimental diets.

In the seond experiment no significant differences in PGI_2- and HHT-synthesis were found between the groups. This is in accordance with a study where the 6-keto-$PGF_{1\alpha}$ production (the stable PGI_2-metabolite) from aorta pieces did not differ between rats fed olive oil or a partially hydrogenated low-erucic-acid-rapeseed oil (both with 5 en% linoleic acid) (11). Royce *et al.* found that in swine, too, a diet rich in *trans* fatty acids and a diet containing lard (both with 5 en% linoleic acid) caused a similar release of PGI_2, measured as 6-keto-$PGF_{1\alpha}$, from coronary arteries (38).

Neither Blomstrand et al. (11) nor Royce et al. (38) found any effects of *trans* fatty acids on TXA_2- production of platelets, either measured via a radio-immuno assay for TXB_2 (38) or via HHT-release (11), which is in agreement with our experiment. Blomstrand *et al.* (11) also reported that *trans* fatty acids rich diets, unlike oleic acid rich diets, do not influence the HETE-synthesis by platelets. This again agrees with our study. We thus confirm the conclusions of others that high amounts of *trans* fatty acids in hydrogenated vegetable oils do not interfere with the cyclooxygenase or lipoxygenase pathways if sufficient linoleic acid is present in the diet. Our study indicates that in rats 2 en% linoleic acid is sufficient to prevent effects of dietary *trans* fatty acids on eicosanoid production.

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ERRATUM

"Analysis of Seed Oils Containing Cyclopentenyl Fatty Acids by Combined Chromatographic Procedures," by W.W. Christie, E.Y. Brechany and V.K.S. Shukla, *Lipids, 24,* 116-120 (1989).

The second to the last paragraph of the "Results" section should read—"Permanganate-periodate oxidation of the diene from *H. anthelmintica* gave a compound with a mass spectrum interpretable as that expected from the methyl ester derivative of a C_{14} tribasic acid, i.e., an apparent molecular ion at *m/z* = 299 (30%), presumably representing loss of an $-OCH_3$ ion from the internal ester group. There are also substantial ions at *m/z* = 128 (73%), 152 (35%), 160 (66%), 184 (45%), 207 (20%), 225 (20%) and 239 (20%). The spectrum of the corresponding compound from the cyclic diene of *T. kurzii* has an apparent molecular ion at *m/z* = 271 (26%), and substantial ions also at 128 and 160 among others. The dibasic acid fragments, which should also be formed during the oxidation, were apparently lost on work-up and were not detected."

Effect of Essential Fatty Acid Depletion on Tissue Phospholipid Fatty Acids in Spontaneously Hypertensive and Normotensive Rats

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Weanling male spontaneously hypertensive (SHR) and normotensive (WKY) rats were maintained on a fat-free semisynthetic diet and killed at various intervals. The effects of fat-depletion on the appearance of essential fatty acid (EFA) deficiency symptoms, the progressive changes of major fatty acids in plasma, liver, heart, and kidney phospholipids (PL), and in skin total lipids were compared between these two strains. After five weeks on the diet, the slower growth and the appearance of EFA deficiency symptoms became evident in SHR. In general, fat-depletion reduced the levels of n-6 fatty acids, whereas it increased those of 20:3n-9. However, the fat-depletion induced reduction of 18:2n-6 in heart PL and 20:4n-6 in kidney, while the elevation of 20:3n-9 in plasma, heart, and kidney PL were greater in WKY than in SHR. As a result, the elevation of biochemical EFA deficiency index—20:3n-9/20:4n-6 ratio—was greater in WKY than in SHR. In comparison with WKY, the concentrations of liver triacylglycerols and the weights of adipose tissues in SHR were reduced to a greater extent, indicating an active catabolism of triacylglycerols in SHR. This study suggests that the earlier appearance of morphological symptoms of EFA deficiency in SHR was not associated with the reducing n-6 EFA levels or with an elevation of triene/tetraene ratio, but possibly to a reduced supply of n-6 EFA for skin prostaglandin synthesis.
Lipids 24, 565–571 (1989).

The spontaneously hypertensive rat (SHR), which was derived from the normotensive Wistar-Kyoto rat (WKY), has been frequently used as an animal model for the study of experimental hypertension. This strain exhibits high blood pressure and develops vascular pathology similar to that seen in humans (1–3).

Earlier, Church *et al.* (4) demonstrated that essential fatty acid (EFA) deficiency exacerbated the development of hypertension in SHR. Singer *et al.* (5) have found the presence of abnormal plasma lipid patterns in this strain: decreased percentages of 18:2n-6, and low polyunsaturate/saturate (P/S) ratio in serum as compared to normotensive controls in all age groups. Furthermore, the development of hypertension in SHR has been shown to be modulated by dietary n-3 and n-6 polyunsaturated fatty acids (PUFAs) (6–12). Hence, it is possible that the hypertension observed in SHR as opposed to WKY is related to fundamental differences between the strains in lipid metabolism. The differences might be related to the composition of long chain PUFAs in cell membranes, since PUFA composition of membranes modulates functions such as fluidity and permeability. Membrane PUFAs also serve as the precursors for synthesis of prostaglandins (PGs) and other eicosanoids. In kidneys, PGs have been implicated as modulators in regulating blood pressure. The availability of PUFAs for the synthesis of antihypertensive products (i.e., PGI_2) in SHR could also dictate the progress of hypertenson.

In a preliminary study, we observed an earlier appearance of essential fatty acid (EFA) deficiency symptoms in SHR when compared to WKY. This could be due to a higher requirement or a lower availability of EFAs in SHR than in WKY. To examine such a possibility, we have compared the depletion rate of tissue EFA levels in SHR and in WKY when EFAs are withheld from the diet.

MATERIALS AND METHODS

Forty weanling SHR and WKY male rats (3 wk old) that were purchased from Taconic Farms (Germantown, NY) were used in this study. All the animals were group-housed, four per cage, in a temperature (21 °C) and humidity (40%) controlled room with a light/dark (14/10) cycle. All animals were maintained on a fat-free semisynthetic diet (Teklad Test Diets, Madison, WI) for a period of 12 weeks. The detailed composition of the diet has been previously described (13). Briefly, it is comprised of 20% protein, 70.2% sucrose, 3.5% mineral mix (AIN-76), 5% cellulose, 1% vitamin mix and 0.3% DL-methionine.

Body weight and EFA deficiency symptoms were examined weekly. The symptoms were essentially scored according to the method used by Holman (14). The appearances of EFA deficiency symptoms were scored seprately. A scale of 0–2.0 was used for the feet and tail, and a scale of 0–1.0 was used for the eye/nose; for a maximum value of 5.0. The scale rose in increments of half units (0.5).

Feet. (a) Slight scaliness of skin on the toes of the hind feet (score = 0.5); (b) cracked appearance on dorsal surface of the hind feet (score = 1.0); (c) scaly appearance on both front and hind feet (score = 1.5); (d) cracked appearance and rough hair over the entire surface of all four feet (score = 2.0).

Tail. (a) Presence of scaliness at tip of tail (score = 0.5); (b) scaliness of one quarter of tail (score = 1.0); (c) scaliness of one half of tail (score = 1.5); (d) scaliness over entire tail (score = 2.0).

Nose/eye. (a) Presence of lesion (score = 0.5); (b) severe lesion around the eye and nose (score = 1.0).

Groups of four animals from either the SHR or the WKY group were killed (exsanguination via cardiac puncture under anesthesia with Halothane) at various intervals (0, 1, 2, 3, 4, 5, 6, 8, 10, and 12 weeks). Blood was collected into a test tube containing EDTA (1 mg/ml blood). Plasma was then separated by centrifugation. The liver, heart, and kidneys were excised rapidly, rinsed with ice-cold saline, and blotted and frozen until analyzed. A

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Abbreviations: EDTA, ethylenediamine tetraacetic acid; EFA, essential fatty acids, PG, prostaglandin; PGI_2, prostacyclin; PL, phospholipids; P/S, polyunsaturate/saturate; PUFAs, polyunsaturated fatty acids; SHR, spontaneously hypertensive rats; TG, triacylglycerols; TLC, thin layer chromatography; WKY, normotensive Wistar-Kyoto rats.

piece of shaved skin (2 × 8 cm) from the scapular region was removed and the subcutaneous fat depot cleaned. Lipids in various tissues (except skin) were extracted by the method used by Folch *et al.* (15). Total lipids were separated by thin layer chromatography (TLC) into cholesteryl esters, triacylglycerols (TG), and total phospholipids (PL). Fatty acids in the PL fraction were methylated with BF_3/methanol. Total fatty acids in the skin, which were extracted into hexane after saponification, were also methylated. The fatty acid methyl esters were analyzed on a Hewlett-Packard gas chromatograph (model 5880A) equipped with a flame ionization detector, and a glass column (2.3 mm i.d. × 180 cm) packed with 10% Silar 10C on 100/120 Gas Chrom Q (Applied Science, State College, PA). Helium was used as the carrier. The conditions for the analyses have been previously described (16). Fatty acid methyl esters wre identified by comparing their retention times either using a packed column (10% Silar 10C) or a 30-meter flexible fused silica capillary column (Supelcowax 10, Supelco Canada), with those of commercial standards (Nu Chek Prep, Elysian, MN, and Supelco, Bellefonte, PA). 22:5n-6 was identified by comparing it with the retention time of 22:5n-6 in the PUFA-2 standard (Supelco), and also with that which was extracted and partially purified from rat testicular phosphatidylethanolamine. Aliquots of liver lipid extracts were analyzed for TG concentration using triheptadecanoin (Nu Chek) as an internal standard. After TLC separation and methylation, the fatty acid methyl esters were analyzed by gas liquid chromatography as described earlier. Calibration of liver TG concentrations were based on the relative area ratios of 17:0 and other fatty acids on gas chromatogram and converting factors.

Data are presented as the mean ± SD of four animals. One-way ANOVA was used to assess the changes during the course of the feeding. Student's t-test was also performed to assess the significance of the differences between the matched groups.

RESULTS

Body weight and liver lipids. There were no significant differences in body weight between SHR and WKY prior to the feedings. After four weeks on the fat-free diet the SHR grew significantly more slowly than the WKY (Fig. 1). The EFA deficiency symptoms began to appear after five weeks on the diet and were more severe in the SHR than in the WKY (score 4 vs. 2 in a maximum score of 5). Statistically, the concentrations of liver TG were not significantly different between the two strains at day 0 (3.0 ± 1.1 mg/g in WKY vs. 3.7 ± 0.8 mg/g in SHR), but they were significantly higher ($p < 0.01$) in WKY than in SHR (183 ± 40 vs. 23 ± 4 mg/g) after five weeks on the diet. Thus, EFA deficiency-induced fatty liver appeared to be more severe in WKY than SHR. The relative weights (% body wt) of adipose tissues (epididymal fat and perirenal adipose tissues) were also significantly reduced by more than twofold in SHR compared to WKY (data not shown).

Iritani and Narita (17) have demonstrated in their study that tissue PL concentrations were not changed by the dietary manipulation; the concentrations of each fatty acid of the PLs in the tissues were parallel to the percentages of fatty acid compositions. Therefore, the composition of PL PUFAs are shown as percentages in the figures and in Table 1.

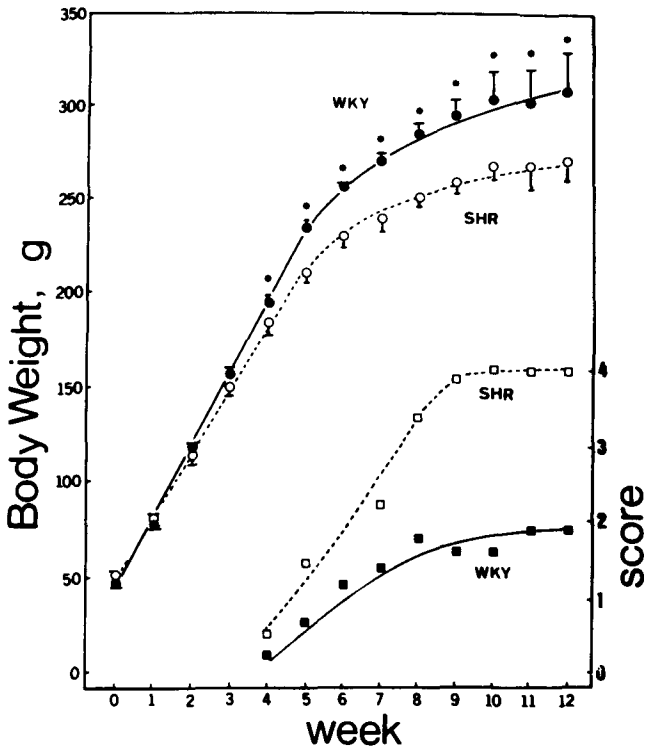

FIG. 1. Body weights (○, ●) and scores of essential fatty acid deficiency symptoms (□, ■) in normotensive rats (WKY, solid symbols) and in spontaneously hypertensive rats (SHR, open symbols) fed on a fat-free diet for 12 weeks.

Plasma phospholipids. The effect of fat-depletion on major fatty acids in plasma PL is shown in Figure 2. The initial levels of 20:4n-6 were higher in the SHR than in the WKY, but those of 18:2n-6 were not significantly different between the two strains. During fat-depletion both 18:2n-6 and 20:4n-6 fell progressively (Fig. 2A). The patterns of change were not significantly different between the two strains. The levels of 20:3n-9 rose rapidly during the first five weeks, but then slowly plateaued (Fig. 2B). The elevation of 20:3n-9 was significantly less in SHR than in WKY during the first eight weeks.

Liver phospholipids. Figure 3 shows the effects of fat-depletion on major fatty acids in liver PL. In general, the levels of 18:2n-6 and 20:4n-6 reduced rapidly during the first two weeks on the diet, and then continuously, but at a slower rate, thereafter (Fig. 3A). On the other hand, the levels of 20:3n-9 rose rapidly during the first five weeks, then slowly leveled off (Fig. 3B). There were no significant differences observed between the SHR and the WKY in the patterns or the contents of any fatty acids, including C22 n-6 long chain acids (22:4n-6 and 22:5n-6).

Heart phospholipids. The changes of major fatty acids in heart PL from WKY and SHR are shown in Figure 4. Fat-depletion continuously reduced the levels of 18:2n-6 and 20:4n-6 throughout the duration of the feeding period (Fig. 4A). At all of the time points examined, there were no significant differences between WKY and SHR in either the contents or the reduction patterns of 20:4n-6. The contents of the long chain C22 n-6 fatty acids in WKY

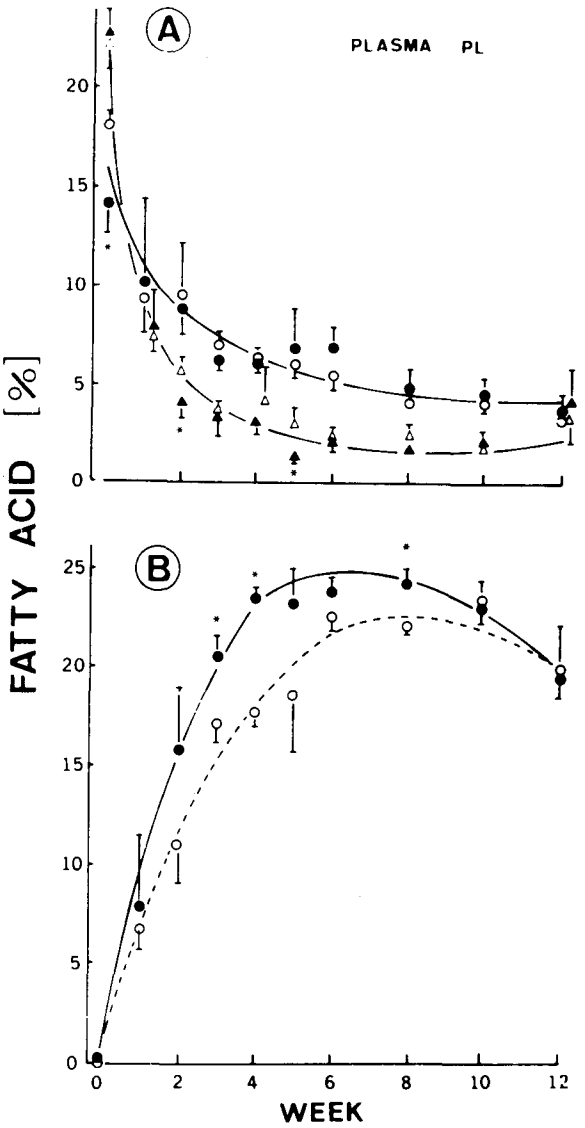

FIG. 2. Changes of 18:2n-6 (△,▲) and 20:4n-6 (○,●) (A) and of 20:3n-9 (○,●) levels (B) in plasma phospholipids from SHR (open symbols) and WKY (solid symbols) after feeding a fat-free diet for a period of 12 weeks. Each data point represents mean ± SD of four animals. * denotes a significant difference at levels of 0.05.

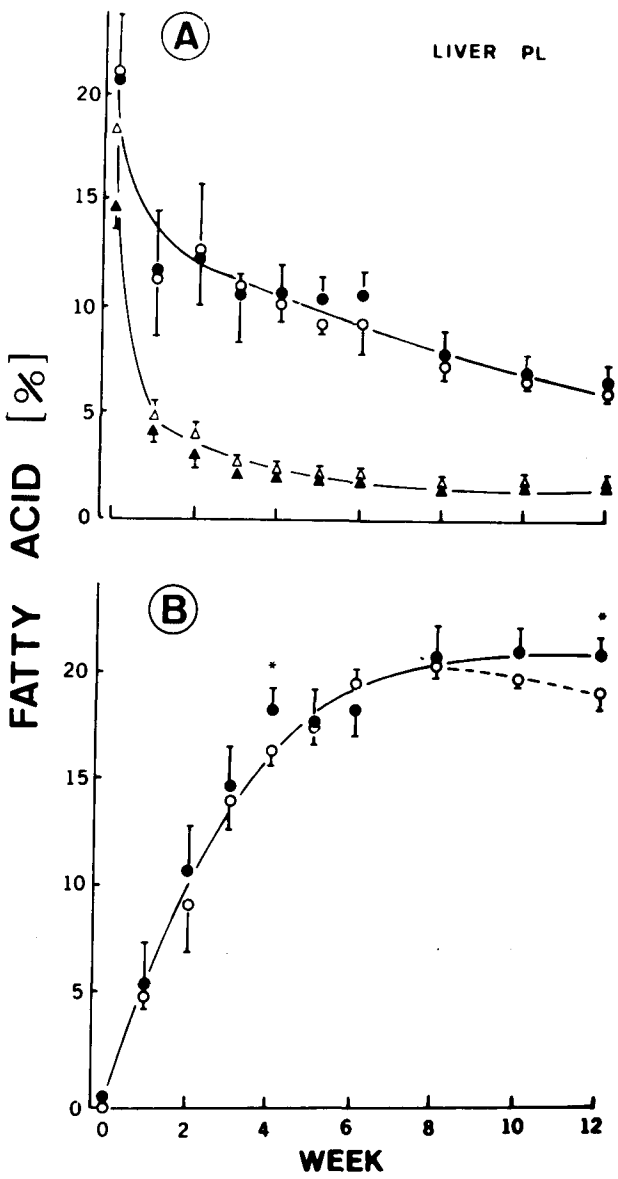

FIG. 3. Changes of 18:2n-6 (△,▲) (A) and of 20:3n-9 (○,●) levels (B) in liver phospholipids from SHR (open symbols) and WKY (solid symbols) after feeding a fat-free diet for a period of 12 weeks. Each data point represents mean ± SD of four animals. * denotes a significant difference at levels of 0.05.

(ranging from 0.94 to 1.45% of total PL fatty acids) were also not significantly different from those in SHR (ranging from 0.69 to 1.66%). However, the levels of 18:2n-6 were reduced to a greater extent in WKY than in SHR after three weeks on the diet. The levels of 20:3n-9 were also increased to a greater extent in WKY than in SHR (Fig. 4B). As a result, the elevation of triene/tetraene (T/T, 20:3n-9/20:4n-6) ratios, which reflects the development of EFA deficiency (18), was greater (but not statistically significant) in WKY than in SHR (3.3 ± 0.5 vs. 2.7 ± 0.5 at the end of the 12-week period).

Kidney phospholipids. Figure 5 shows the changes of fatty acids in kidney PL of the SHR and WKY rats during the 12-week feeding period. As in other tissues, the levels of 18:2n-6 and 20:4n-6 fell gradually as the feeding period lengthened (Fig. 5A). There were no significant differences in the rates of decrease of 18:2n-6 between the SHR and the WKY. However, the levels of 20:4n-6 in SHR were reduced at a slower rate than in WKY. On the other hand, the levels of C22 n-6 fatty acids in SHR (ranging from 0.54 to 0.78%) tended to be lower than those in WKY (ranging from 1.02 to 2.04%). As a result, the ratio of C22n-6/20:4n-6 was lower in SHR (0.03 ± 0.01) than in WKY (0.10 ± 0.04). The levels of 20:3n-9 in the WKY rats were similar to the trend found in heart PL, it increased faster than those in the SHR (Fig. 5B). Consequently, the WKY rats, in comparison with the SHR rats, had significantly ($P < 0.01$) higher T/T ratios (1.37 ± 0.18 vs. 0.96 ± 0.16 at 12 weeks).

Skin total lipids. The major fatty acids in skin total lipids were 16:0, 18:0, 18:1n-9, and 18:2n-6. The levels of 20- and 22-carbon fatty acids were substantially lower.

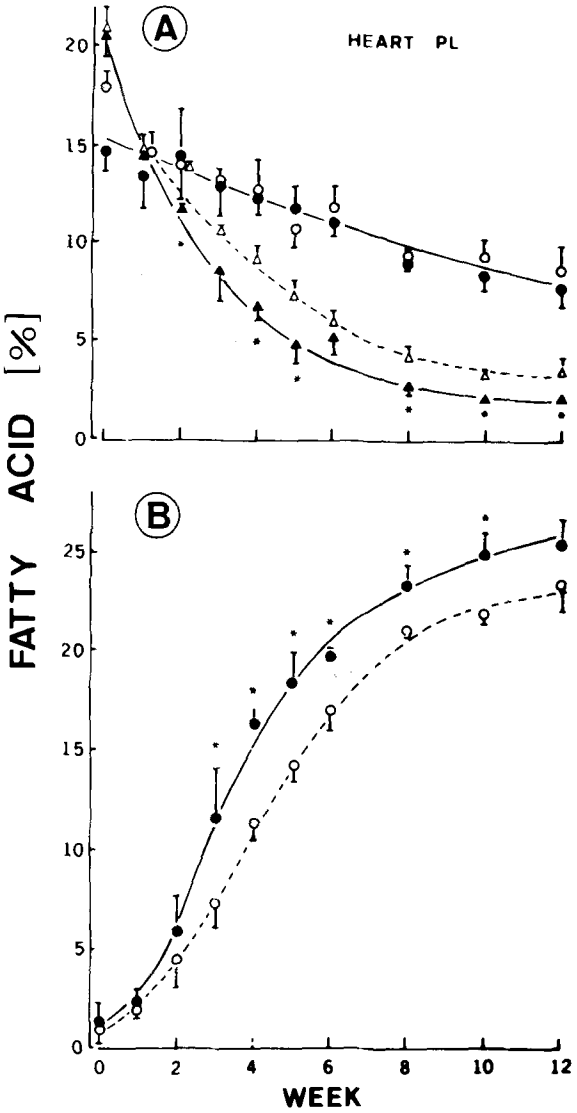

FIG. 4. Changes of 18:2n-6 (△,▲) and 20:4n-6 (○,●) (A) and of 20:3n-9 (○,●) levels (B) in heart phospholipids from SHR (open symbols) and WKY (solid symbols) after feeding a fat-free diet for a period of 12 weeks. Each data point represents mean ± SD of four animals. * denotes a significant difference at levels of 0.05.

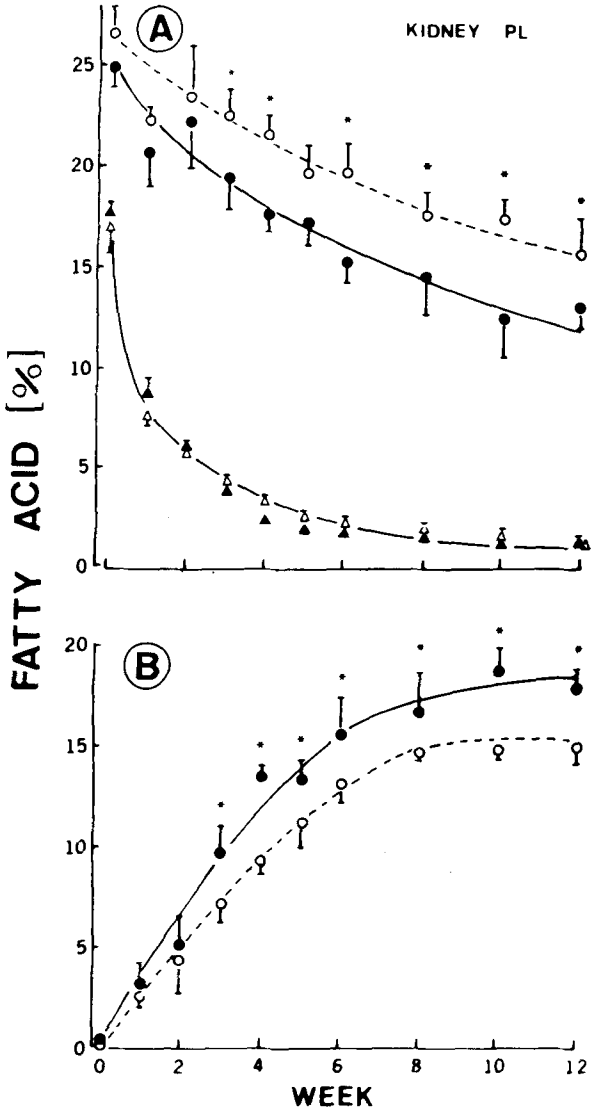

FIG. 5. Changes of 18:2n-6 (△,▲) and 20:4n-6 (○,●) (A) and of 20:3n-9 (○,●) levels (B) in kidney phospholipids from SHR (open symbols) and WKY (solid symbols) after feeding a fat-free diet for a period of 12 weeks. Each data point represents mean ± SD of four animals. * denotes a significant difference at levels of 0.05.

The initial levels of 18:2n-6 were higher ($p < 0.01$) in WKY than in SHR (33.6 ± 2.1 vs. 26.8 ± 0.9%). The opposite was true for the levels of 20:4n-6 (1.9 ± 0.3 vs. 2.4 ± 0.3%, $p < 0.05$). After three weeks on the diet they fell rapidly to minimal levels and remained there. Figure 6 shows the progressive changes of 18:1n-9 and 18:2n-6. It is evident that the reduction of 18:2n-6 was compensated by the elevation of 18:1n-9.

Summary of tissue PL fatty acid profiles. Table 1 summarizes tissue PL fatty acid compositions obtained from animals fed the fat-free diet at onset and then at five weeks. The five-week time point was chosen because the differences in growth rates and EFA deficiency scores between the SHR and the WKY began to be statistically significant at that point (Fig. 1). In general, fat depletion reduced the proportions of saturated and polyunsaturated fatty acids, whereas it reduced those of monounsaturated fatty acids (mainly 18:1n-9). However, there were no significant differences observed of the extent of changes or the P/S ratios (both the initial and the five-week values) between the SHR and the WKY.

DISCUSSION

Church *et al.* (4) have previously shown that EFA deficiencies exacerbated the development of hypertension in SHR. Singer *et al.* (5) have also reported a decreased 18:2n-6 level and a low P/S ratio in SHR as compared to the normotensive Wistar rats in all age groups. To examine whether such differences could account for the earlier onset of EFA deficiency in SHR, we have compared the tissue PL fatty acid compositions in growing SHRs and WKYs. Results in Table 1 show that there were no significant differences of the P/S ratios, the

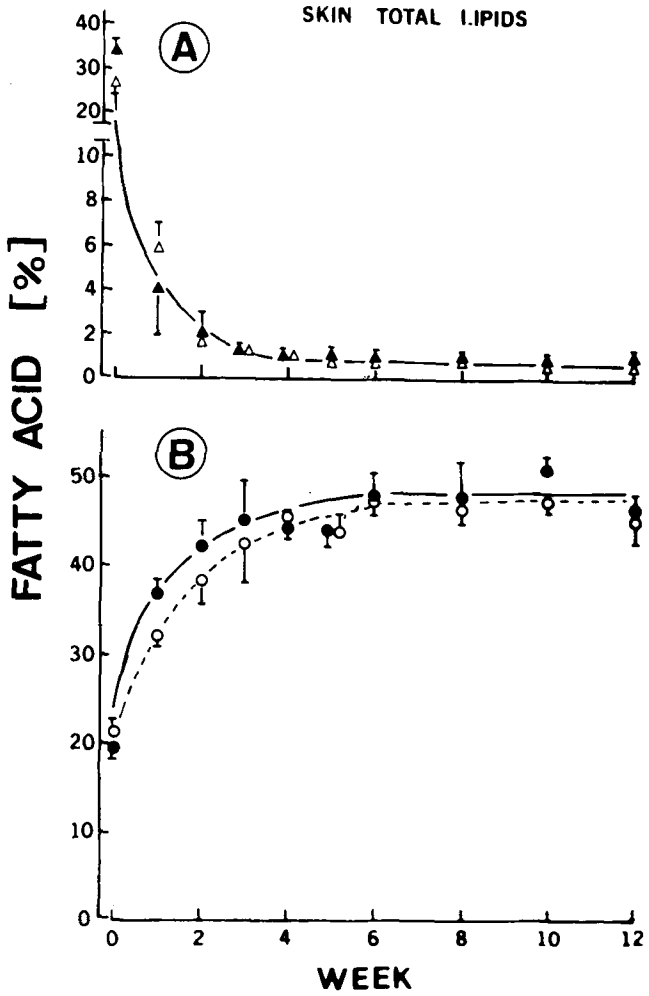

FIG. 6. Changes of 18:2n-6 (△,▲) (A) and of 18:1n-9 (○, ●) levels (B) in skin total lipids from SHR (open symbols) and WKY (solid symbols) after feeding a fat-free diet for a period of 12 weeks. Each data point represents mean ± SD of four animals. * denotes a significant difference at levels of 0.05.

initial values, and the patterns of n-6 fatty acid reduction between these two strains. In comparison with the WKY, the SHR tended to retain more 18:2n-6 in heart PL (Fig. 4) and 20:4n-6 in kidney PL (Fig. 5) during fat-depletion. Therefore, our results did not confirm the report of a lower level of 18:2n-6 and/or 20:4n-6 in tissue membrane PL (Figs. 2-5) in SHR (5) or the theory that a reduced n-6 fatty acid level might be responsible for the earlier onset of EFA deficiency symptoms in SHR.

The T/T ratio is generally used as a biochemical indicator of the state of EFA deficiency (18). In the present study the levels of 20:3n-9 (in plasma, heart, and kidney PL), which increased as fat-depletion progressed, were generally lower in SHR than in WKY. This finding, in conjunction with the non-changing 20:4n-6 levels, resulted in a consistently lower T/T ratio in SHR than in WKY, which is contrary to what the morphological symptoms implied. Hence, our results did not support the view that the elevation of 20:3n-9, or that of the T/T ratio, was responsible for the earlier development of EFA deficiency symptoms in SHR.

The most abundant EFA in skin is 18:2n-6 (Fig. 6). Skin EFAs are principally located in cell membrane PL fraction, and thus play an important role in regulating structural functions such as fluidity and permeability. Since 20:4n-6 is not synthesized per se in the skin due to lack of metabolic enzymes in this tissue (19), 18:2n-6 and 20:4n-6 are both derived from the liver via plasma. Theoretically, a reduced supply of EFA (18:2n-6 and/or 20:4n-6) to the skin membrane might initiate the earlier onset of EFA deficiency symptoms in SHR. While the initial levels (0 week) of skin n-6 fatty acids of 18:2n-6 were lower, the 20:4n-6 levels were higher in the SHR than in the WKY skin lipids. During the course of EFA depletion, however, they both fell rapidly (20). The patterns of the reduction were not significantly different between the two strains (Fig. 6).

A second important role of skin EFA is as a precursor of prostaglandins (21,22). Prottey (23) has shown that topical application of 18:2n-6 or 18:3n-6 (but not of 20:3n-6 or 20:4n-6) improved defective transdermal water loss in EFA deficient animals. This result implies that 18-carbon n-6 EFA are more potent than 20-carbon n-6 EFA in alleviating the skin EFA deficiency symptoms. An alternative explanation is that the topical absorption of 20-carbon n-6 fatty acids by the skin is less effective in comparison with that of 18-carbon n-6 fatty acids. Prottey's observation did not negate the possibility of an abnormal skin PG metabolism which could be responsible for the development of EFA deficiency symptoms. Indeed, Ziboh and Hsia (24) have successfully applied PGE_2 to alleviate EFA deficiency symptoms in the skin. This indicates the possibility of defective PGE_2 synthesis in the EFA-deficient skin.

In this study, we did not find any enhanced depletion of tissue 20:4n-6, or other n-6 fatty acids in SHR as compared to WKY. Since the level of 20:4n-6 required for PG synthesis is generally smaller than that present in the tissue PL pool, the levels of tissue PL fatty acids simply do not reflect the levels of PG synthesis. Nevertheless, it is possible that the levels of precursor fatty acids entering into the process of skin PG synthesis are not equal between the SHR and the WKY.

Earlier reports have shown that rates of PG production in kidney, aorta, and other tissues are significantly enhanced in the SHR (4,25). These observations, together with the fact of a reduced skin PG synthesis during fat-depletion, raised the possibility that an active PG metabolism in other tissues might reduce the availability of 20:4n-6 for PG synthesis in the skin. In other words, an excessive flow of 20:4n-6 for PG synthesis in other tissues might reduce the delivery of 20-carbon n-6 EFA, and cause a functional 20:4n-6 deficiency in SHR skin. This would result in an altered PG synthesis and an earlier onset of EFA deficiency symptoms.

We have observed a significant reduction of both liver TG concentratoins and adipose tissue weights in SHR, indicating that liver and adipose tissue TG were more actively catabolized in SHR than in WKY. This view is supported by results from preliminary data of an *in vivo* incubation study. In this study we found that more radioactive CO_2 is exhaled by EFA deficient SHR than by EFA deficient WKY 24 hours after they were fed labeled 18:2n-6 by gastric intubation (Mills, Ward, and Huang, unpublished data). This difference could be attributed to

TABLE 1

Effect of Fat Depletion (0 vs. 5 wk) on Percent Fatty Acid Compositions in Liver, Heart, and Kidney Phospholipids (WKY vs. SHR) Grouped According to Their Degree of Unsaturation or the Location from the Methyl End of the First Unsaturated Bond

	WKY		SHR	
	0	5	0	5
Plasma				
Saturate	41.9 ± 0.6	36.0 ± 2.5[b]	44.1 ± 0.8[d]	33.8 ± 0.9[a]
Monoene	10.0 ± 1.6	28.7 ± 2.6[b]	6.7 ± 0.7[c]	34.1 ± 2.3[b,c]
Polyene	47.7 ± 1.8	34.1 ± 4.0[b]	49.1 ± 1.1	31.4 ± 2.3[b]
Σ(n-3)	7.8 ± 0.5	1.5 ± 0.2[b]	8.3 ± 0.6	2.6 ± 1.0[b]
Σ(n-6)	39.8 ± 1.3	9.4 ± 2.2[b]	40.8 ± 0.7	10.3 ± 1.6[b]
Σ(n-9)	9.5 ± 1.4	48.8 ± 0.5[b]	6.7 ± 0.7[c]	48.0 ± 3.0[b]
P/S	1.1 ± 0.05	1.0 ± 0.2	1.1 ± 0.04	0.9 ± 0.1[a]
Liver				
Saturate	37.7 ± 1.5	33.2 ± 2.7[a]	30.8 ± 10.9	33.9 ± 0.8
Monoene	6.3 ± 0.7	30.5 ± 4.4[b]	12.6 ± 4.7	31.3 ± 0.7[b]
Polyene	55.1 ± 1.3	35.2 ± 1.9[b]	55.8 ± 10.9	33.7 ± 0.5[a]
Σ(n-3)	17.1 ± 0.4	2.3 ± 0.3[b]	11.6 ± 5.7	2.7 ± 0.3[a]
Σ(n-6)	38.0 ± 1.3	15.4 ± 1.5[b]	44.2 ± 7.9	13.6 ± 0.2[b]
Σ(n-9)	5.4 ± 0.7	40.5 ± 0.9[b]	10.3 ± 3.7	41.9 ± 0.9[a]
P/S	1.5 ± 0.1	1.1 ± 0.1[b]	1.8 ± 1.6	1.0 ± 0.03[a]
Heart				
Saturate	36.0 ± 1.7	30.7 ± 1.3[b]	34.4 ± 1.2	29.2 ± 0.9[b]
Monoene	9.2 ± 1.0	27.0 ± 2.3[b]	8.0 ± 1.1	29.6 ± 1.2[b]
Polyene	54.1 ± 0.9	40.2 ± 0.8[b]	56.9 ± 0.8[d]	39.1 ± 1.2[b]
Σ(n-3)	14.8 ± 1.8	3.8 ± 0.5[b]	15.6 ± 1.0	3.2 ± 0.2[b]
Σ(n-6)	38.0 ± 0.8	17.8 ± 1.8[b]	40.4 ± 0.6[d]	21.7 ± 1.6[b,c]
Σ(n-9)	10.2 ± 1.5	41.0 ± 2.6[b]	8.5 ± 1.8	39.3 ± 1.5[b]
P/S	1.5 ± 0.1	1.3 ± 0.1[a]	1.7 ± 0.1	1.3 ± 0.1[b]
Kidney				
Saturate	35.2 ± 0.6	32.8 ± 0.7[b]	36.6 ± 2.3	34.9 ± 1.2[c]
Monoene	9.0 ± 0.6	24.7 ± 1.3[b]	10.2 ± 0.2[c]	22.7 ± 1.4[b]
Polyene	55.9 ± 5.7	37.0 ± 1.2[b]	52.0 ± 2.8	37.7 ± 0.8[b]
Σ(n-3)	8.0 ± 0.4	1.0 ± 0.1[b]	6.1 ± 0.4[d]	1.4 ± 0.2[b,c]
Σ(n-6)	47.3 ± 5.9	22.6 ± 1.5[b]	45.4 ± 2.5	25.2 ± 2.0[b]
Σ(n-9)	8.9 ± 0.6	32.4 ± 1.3[b]	9.9 ± 0.4[c]	28.9 ± 2.4[b,c]
P/S	1.6 ± 0.1	1.1 ± 0.1[b]	1.4 ± 0.2	1.1 ± 0.04[b]

(n-9) includes 20:3n-9 and 18:1n-9.

[a]Significantly different ($p < 0.05$) from that in 0 week.

[b]Significantly different ($p < 0.01$) from that in 0 week.

[c]Significantly different ($p < 0.05$) from that in WKY.

[d]Significantly different ($p < 0.01$) from that in WKY.

a more active catabolism of labeled fatty acids in SHR than in WKY. Since plasma TG which is released from liver or adipose tissues may also serve as the source of the skin PG precursor fatty acids, it is possible that excessive TG catabolism in SHR reduces the availability of PG precursors in plasma for skin PG synthesis.

ACKNOWLEDGMENTS

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Increased Biliary Calcium in Cholesterol and Pigment Gallstone Disease: The Role of Altered Bile Acid Composition

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The present study was undertaken to define the relationship between calcium metabolism and bile acid composition in animal models of diet induced cholesterol and pigment gallstones. Groups of prairie dogs were fed either a control non-lithogenic chow (N = 12), a 1.2% cholesterol enriched chow (N = 6, XOL) for two weeks, or a high carbohydrate diet deficient in iron (N = 6, CHO-FeD), or a high carbohydrate diet with normal iron levels (N = 6, CHO) for eight weeks. Hepatic (HB) and gallbladder (GB) bile samples were analyzed for total calcium, cholesterol, phospholipids, total bile acids (TBA), and individual bile acid composition.

In each of the four groups, TBA concentrations were essentially similar and taurine conjugates accounted for approximately 90% of TBA in HB bile and about 98% in GB bile. In the control group, cholic acid (CA) was the predominant bile acid and comprised 76% of TBA and chenodeoxycholic (CDCA) accounted for about 13% of the total. Feeding a diet rich in cholesterol caused a significant change in the relative concentrations of individual bile acids of hepatic bile—such that CA decreased significantly ($p < 0.001$) while CDCA increased by 300% ($p < 0.001$). The changes in secondary bile acids were insignificant. An identical shift in individual bile acid composition was noted in animals maintained on high carbohydrate diet, irrespective of iron content. Similar changes were observed in the GB in the experimental groups.

Calcium concentrations of GB bile with or without gallstone formation showed a positive linear relationship with TBA ($y = 4.35 + 0.14X$, $p < 0.001$) and taurochenodoxycholic acid (TCDCA) ($y = 15.04 + 0.46X$, $p < 0.001$), but an inverse relationship with taurocholic acid (TCA) ($Y = 55.16 - 0.41X$, $p < 0.008$). However, such relationships were absent in hepatic bile. These data indicate that diet-induced alterations in bile acid composition may modify calcium solubility or GB function, thereby contributing to the increased GB calcium observed during cholesterol and pigment gallstone formation.
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It has long been recognized that biliary calcium is an important factor in the pathogenesis of pigment gallstones (1,2). Calcium is also present as a salt in the central matrix of some, if not all, cholesterol gallstones (3,4). Recent studies suggest that both pigment (5) and cholesterol gallstone disease (6,7) are characterized by increased biliary levels of calcium. Nonetheless, the factors which are responsible for these changes in calcium metabolism remain obscure. *In vitro* studies by Moore *et al.* (8) indicate that bile acids act as important buffers for free calcium in bile and may be important determinants of calcium solubility. In addition, bile acid composition influences biliary secretion of calcium (9). In an effort to further define the relationship between bile acids and calcium metabolism during gallstone formation, the present study was undertaken to determine the bile acid composition in animal models of diet induced cholesterol and pigment gallstones. The prairie dog develops cholesterol gallstones when fed a synthetic diet containing cholesterol, has biliary lipid secretory kinetics similar to that in human subjects, and provides a model in which the discrete events in the evolution of cholesterol gallstones may be studied (10-12). Additionally, recent studies from our laboratory indicate that prairie dogs maintained on a high carbohydrate, iron deficient diet, have increased biliary concentrations of calcium, phospholipids, and cholesterol, and develop pigment gallstones (13).

MATERIAL AND METHODS

Experimental design. Adult male prairie dogs *(Cynomys ludovicianus),* trapped in the wild state and obtained from Otto Marten Locke, New Braunfels, TX, were caged in thermo-regulated (23°C) rooms with 12 hour light cycles. Four groups of animals were maintained on either a control non-lithogenic chow (N = 12, Purina Laboratory Chow®, Ralston Purina, St. Louis, MO), a 1.2% cholesterol enriched diet (N = 6, Teklad Test Diets®, Harlan Sprague-Dawley, Inc., Madison, W) for two weeks, or a high carbohydrate diet deficient in iron (N = 6, Teklad), or a high carbohydrate diet with normal iron levels (N = 6, Teklad) for eight weeks. The precise composition of the four diets is summarized in Table 1. The 1.2% cholesterol enriched diet has been previously shown to induce cholesterol crystals and gallstones within ten and fourteen days, respectively (14). Prairie dogs maintained on the high carbohydrate, iron deficient diet for eight weeks developed calcium bilirubinate crystals and microscopic stones (13). Iron supplementation has been shown to significantly reduce the incidence of stones in this model (13). All diets contain ample essential fatty acids, minerals, and fiber. All animals tolerated the diets, with maintenance of weight and fur, and had only minimal diarrhea.

After a 16 hour fast with water *ad libitum,* animals were anesthetized with ketamine (100 mg/kg of body weight) and diazepam (0.15 mg/kg of body weight) (15). After a midline laparotomy, the cystic duct was ligated and the gallbladder was carefully removed. The distal common bile duct was ligated, and a 25 cm long silastic catheter (i.d. 0.02 in., o.d. 0.032 in.) was placed into the common bile duct. Hourly hepatic bile (HB) samples were collected on ice in tared tubes wrapped with aluminum foil for

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Abbreviations: CA, cholic acid; CDCA, chenodexycholic acid; CHO, high carbohydrate diet; CHO-FeD, high carbohydrate diet, deficient in iron; CSI, cholesterol saturation index; GB, gallbladder bile; HB, hepatic bile; HPLC, high performance liquid chromatography; i.d., inner diameter; TBA, total bile acids; TCA, taurocholic acid; TCDCA, taurochenodexycholic acid; XOL, cholesterol-enriched diet.

TABLE 1

Composition of Diets

	Control (%)	XOL (%)	CHO (%)	CHO-FeD (%)
Carbohydrate	51	58	63	63
Corn	37	13	0	0
Wheat	13	0	0	0
Sucrose	1	45	35	35
Rice	0	0	28	28
Protein	23	20	18	18
Fat	5	9	8	8
Fiber	5	3	1	1
Mineral mix[a]	—	4	3.5	3.5
Vitamin mix[a]	—	1	0.5	0.5
Water	7	2.8	5	5
Misc. (ASH, etc.)	9	1	1	1
Cholesterol	Trace	1.2	Trace	Trace
Iron (ppm)	198	195	220	56

XOL = 1.2% cholesterol enriched chow; CHO = carbohydrate, normal iron diet; CHO-FeD = carbohydrate, iron deficient.

[a]AIN-76 mineral and vitamin mix (32).

protection against light and saved for further analysis. Gallbladders were opened and their mucosal surfaces carefully inspected (using a dissecting microscope) for the presence of sludge or stones. Gallbladder (GB) and HB bile were aliquoted and then stored at −20°C until analyzed for calcium, cholesterol, phospholipids, bile acids, and individual bile acid composition.

Analytic methods. Frozen bile was warmed to room temperature in a water bath at 37°C and biochemical determinations were performed on the entire volume of the aliquot. Biliary total calcium was measured by spectrophotometric methods as described by Anderegg *et al.* (16), and as modified by Connerty and Briggs (17). Unpublished data from our laboratory has shown that this analysis is not significantly altered by the bilirubin level. Bile cholesterol was assayed enzymatically by the method of Röschlau *et al.* (18). Biliary phospholipids were quantified by measuring lipid phosphorus as described by Dryer and co-workers (19). Total bile acids were determined spectrophotometrically using the steroid dehydrogenase method of Iwata (20). The cholesterol saturation index (CSI) was calculated using Carey's critical tables for cholesterol saturation, based on the total lipid concentration (21). Individual bile acids were analyzed by a modification of the high performance liquid chromatographic (HPLC) method described by Nakayama *et al.* (22). The changes made in methodology were necessary to adapt to our system. All changes were validated with authentic standards. Modifications improved resolution and accuracy of the originally described methodology. The HPLC system consisted of a Perkin-Elmer 400 series solvent delivery pump with a fixed loop Rheodyne Model 7125-S bypass injector, a LC-95 high sensitivity variable wave length UV-VIS detector and a LCI-100 laboratory computing integrator (Perkin-Elmer Corporation, Irvine, CA). The analytical column used was a 10-micron μBondapak® C18, 30 cm × 3.9 mm i.d. (Waters Associates, Milford, MA). To prepare bile samples for chromatographic analysis, an aliquot of 50 μl of the bile was mixed with 50 μl of internal standard (testosterone acetate in ethanol, 2 mg/ml). The mixture was extracted with nine times its volume of ethanol, boiled in a water bath for 10 minutes, cooled to room temperature, and then filtered through a 0.45 μm Alpha Metricel® membrane filter (Gelman Sciences, Inc., Ann Arbor, MI). An aliquot of 5–10 μl of the filtrate was then injected into the column through the sampling valve and chromatographed with a solvent system of methanol acetonitrile and water (40:30:30, v/v/v), acidified to pH 3.4 with phosphoric acid. The flow rate was 1 ml/min and detection was made at 210 nm. Individual bile acids were identified by comparison of retention times with those of bile acid reference standards (Calbiochem, La Jolla, CA), and quantitated using relative response factors determined by known amounts of bile salt standards and the same amount of internal standard added to the sample. All bile salt standards were obtained from Calbiochem. They were in the form of sodium salts of either taurine or glycine conjugates of respective bile acids, and their purity was better than 97%. The internal standard (testosterone acetate) was purchased from Sigma Chemicals and its purity was better than 99%.

Statistical analysis. All data are presented as the mean ± standard deviation. Fisher's exact test was used to compare the incidences of stones. Statistical comparisons between the dietary groups were made using analysis of variance and the Student's t-test for unpaired variables.

RESULTS

Gallstone formation. As expected, no control animals had either crystals or gallstones. All cholesterol-fed animals had cholesterol crystals, and five of the six had typical yellow cholesterol gallstones ($p < 0.002$ vs. controls). Of the six animals maintained on a high carbohydrate, normal iron diet (CHO), only one had calcium bilirubinate crystals. In the high carbohydrate iron deficient group (CHO-FeD), three of six animals had both stones ($p < 0.04$ vs. controls) and crystals, and one animal had crystals alone. The microscopic appearances of the crystals in carbohydrate-fed animals were quite different from cholesterol gallstone animals and were brown-yellowish, comparable to calcium bilirubinate crystals previously reported by other investigators.

Hepatic bile composition. The data summarizing the effects of cholesterol and carbohydrate feeding on HB composition are listed in Table 2. The HB concentrations of total bile acids (TBA) and calcium were similar in all four experimental groups. However, animals fed either the cholesterol or the high carbohydrate diets, demonstrated a significant increase in HB phospholipid concentration ($p < 0.001$ vs. control). In addition, there was a significant increase in cholesterol concentration as compared to control, in both cholesterol fed (XOL) ($p < 0.001$) and iron deficient carbohydrate-fed (CHO-FeD) animals ($p < 0.005$). Prairie dogs maintained on the high carbohydrate (CHO), normal iron diet had similar cholesterol concentrations to controls. The cholesterol-fed animals had cholesterol saturated bile as reflected by a CSI of 1.14 ± 0.35, in contrast to the control (0.52 ± 0.22), CHO (0.38 ± 0.09) and CHO-FeD (0.64 ± 0.20) animals.

The effects of cholesterol and carbohydrate feeding on individual bile acid composition are summarized in

TABLE 2

The Effects of Cholesterol and Carbohydrate Feeding on Hepatic Bile Composition in Prairie Dogs[a]

Dietary group	Total bile acids (μM/ml)	Phospholipids (μM/ml)	Cholesterol (μM/ml)	Total lipids (g/dl)	CSI	Total C_a^{++} (mg/dl)
Control	38.4 ± 12.1	4.9 ± 2.0	0.7 ± 0.2	2.3 ± 0.6	0.52 ± 0.22	9.3 ± 1.6
XOL	31.8 ± 14.5	8.9 ± 3.5[c]	2.2 ± 1.0[c]	2.6 ± 0.9	1.14 ± 0.35[c]	9.4 ± 1.6
CHO	39.3 ± 10.4	8.8 ± 2.3[c]	0.8 ± 0.2[d]	2.4 ± 0.5	0.38 ± 0.09[e]	10.4 ± 1.8
CHO-FeD	35.3 ± 19.6	9.1 ± 2.6[c]	1.5 ± 0.8[b]	2.5 ± 1.2	0.64 ± 0.20	10.1 ± 1.6

[a] A portion of this data has been previously published in Reference 13.

[b] $p < 0.005$, [c] $p < 0.001$, vs. corresponding control.

[d] $p < 0.005$, [e] $p < 0.001$, vs. corresponding XOL.

XOL = 1.2% cholesterol-enriched diet; CHO = high-carbohydrate, normal iron diet; CHO-FeD = high-carbohydrate, iron deficient diet.

TABLE 3

Effect of Diets on Bile Acid Composition of Hepatic Bile

	Biliary bile acids (% TBA)										
	Taurine conjugates						Glycine conjugates				
Diet	UDCA	CA	CDCA	DCA	LCA	Total	CA	CDCA	DCA	LCA	Total
Control	1.2 ± 1.1	75.0 ± 3.5	11.3 ± 2.6	3.5 ± 2.7	1.0 ± 0.5	92.0 ± 2.1	0.8 ± 0.4	1.7 ± 1.0	2.4 ± 0.7	3.1 ± 1.3	8.0 ± 2.1
XOL	1.1 ± 0.5	52.1 ± 1.9[b]	32.8 ± 3.2[b]	6.6 ± 3.4	0.6 ± 0.3	93.2 ± 2.7	0.9 ± 0.5	1.4 ± 0.9	1.1 ± 0.9	3.4 ± 2.8	6.8 ± 2.7
CHO	0.8 ± 0.3	51.7 ± 7.3[b]	34.2 ± 6.7[b]	3.4 ± 2.8	1.1 ± 0.3	91.2 ± 2.3	0.8 ± 0.4	2.3 ± 1.3	1.1 ± 0.9	4.6 ± 0.8	8.8 ± 2.3
CHO-FeD	0.6 ± 0.3	54.6 ± 11.5[b]	34.5 ± 9.9[b]	2.2 ± 1.1	1.2 ± 0.4	93.1 ± 2.9	1.1 ± 0.5	2.2 ± 1.1	1.1 ± 0.3[a]	3.5 ± 1.2	6.9 ± 2.4

[a] $p < 0.005$, [b] $p < 0.001$ vs. corresponding controls.

UDCA = Ursodeoxycholic acid; CA = cholic acid; CDCA = chenodeoxycholic acid; DCA = deoxycholic acid; and LCA = lithocholic acid.

XOL = 1.2% cholesterol-enriched diet; CHO = high-carbohydrate, normal iron diet; CHO-FeD = high-carbohydrate, iron-deficient diet.

TABLE 4

Effect of Diets on Individual Bile Acids of Gallbladder Bile

	Biliary bile acids (% TBA)										
	Taurine conjugates						Glycine conjugates				
Diet	UDCA	CA	CDCA	DCA	LCA	Total	CA	CDCA	DCA	LCA	Total
Control	1.3 ± 0.7	77.4 ± 2.9	15.4 ± 4.4	2.2 ± 1.7	0.5 ± 0.2	96.8 ± 1.5	0.5 ± 0.3	0.5 ± 0.3	0.5 ± 0.4	1.7 ± 0.9	3.2 − 1.5
XOL	0.8 ± 0.5	62.4 ± 8.4[d]	32.7 ± 10.2[d]	1.7 ± 1.1	0.6 ± 0.3	98.0 ± 1.0[a]	0.2 ± 0.1[b]	0.5 ± 0.2	0.2 ± 0.1	0.9 ± 0.5	1.8 ± 0.9[c]
CHO	2.8 ± 1.7	57.7 ± 5.6[d]	35.8 ± 6.8[d]	1.1 ± 0.9	0.2 ± 0.1	97.6 ± 1.5	0.2 ± 0.1[b]	0.3 ± 0.2	0.3 ± 0.2	1.6 ± 1.2	2.4 ± 1.5
CHO-FeD	1.8 ± 1.1	65.1 ± 12.0[c]	30.7 ± 11.7[d]	0.8 ± 0.6	0.2 ± 0.1	98.6 ± 1.6[a]	0.1 ± 0.1[b]	0.2 ± 0.1	0.3 ± 0.3	0.8 ± 0.3	1.4 ± 0.5[b]

[a] $p < 0.05$, [b] $p < 0.01$, [c] $p < 0.005$, [d] $p < 0.001$ vs. corresponding control.

UDCA = Ursodeoxycholic acid; CA = cholic acid; CDCA = chenodeoxycholic acid; DCA = deoxycholic acid; and LCA = lithocholic acid.

XOL = 1.2% cholesterol-enriched diet; CHO = high-carbohydrate, normal iron diet; CHO-FeD = high-carbohydrate, iron-deficient diet.

Table 3. In each of the four groups, taurine conjugates accounted for approximately 90% of the TBA. In the control group, cholic acid (CA) was the predominant bile acid and comprised 76% of the TBA. The other primary bile acid, chenodeoxycholic acid (CDCA), accounted for approximately 13% of the total. The secondary bile acids, deoxycholic and lithocholic acid, accounted for 5% and 4%, respectively. Cholesterol feeding caused a significant alteration in the relative concentration of the individual bile acids such that CA decreased significantly ($p < 0.001$), while CDCA increased by 300% ($p < 0.001$). The changes in the secondary bile acids were insignificant. There was an identical shift in individual bile acid composition noted in animals maintained on the high carbohydrate diet, irrespective of iron content. Once again, CA decreased significantly ($p < 0.001$) while there was a three-fold increase in CDCA.

Gallbladder bile composition. The effects of diet on GB composition are displayed in Figure 1. Cholesterol feeding resulted in a significant increase in TBA concentration ($p < 0.02$), phospholipids, cholesterol, and total calcium ($p < 0.001$) as compared to controls. Animals maintained on the high carbohydrate diets also had significantly increased GB concentrations of phospholipids and calcium. However, biliary cholesterol was significantly elevated only in the CHO-FeD animals. The CSI was significantly higher ($p < 0.001$) in cholesterol-fed animals compared to controls and the carbohydrate animals. The values were similar for controls and CHO-FeD animals, but significantly lower in CHO animals ($P < 0.01$ vs. controls).

Data summarizing the effects of the diet on GB concentrations of individual bile acids are listed in Table 4. Taurine conjugates accounted for 95 to 98% of the total bile acids in each of the four groups. As was noted in HB, CA was the predominant bile acid in control animals, with CDCA comprising 15% of the total. Once again, feeding either cholesterol or high carbohydrate diets caused significant alterations in the relative concentrations of bile acids. All three of the experimental groups had a significant decrease in the percentage of CA, which was associated with a concomitant doubling of CDCA.

Biliary calcium and bile acids. The relationships between the concentrations of biliary calcium and bile acids in both hepatic and gallbladder bile were analyzed irrespective of stone formation. There was no significant correlation noted between HB concentrations of calcium and either total or individual bile acids. Data summarizing the relationships between GB concentrations of calcium and bile acids are displayed in Figure 2. There was a linear correlation ($y = 4.35 + 0.14x$, $p < 0.001$) when the gallbladder concentration of TBA was compared to calcium. The finding can be explained on the basis of the observed correlation ($y = 15.04 + 0.46x$, $p < 0.001$) between GB calcium concentration and the amount of taurochenodeoxycholic acid (TCDCA), expressed as a percentage of the total bile acids. In contrast, there was an inverse linear relationship ($Y = 55.16 - 0.41X$, $p < 0.008$) when taurocholic acid (TCA) was measured instead of TCDCA. Although the presence of linearity between concentrations of bile and calcium is noteworthy, a cause and effect relationship has not yet been established.

DISCUSSION

Data from this study indicates that in the prairie dog, consumption of diets rich in cholesterol or carbohydrates results in a significant alteration in the hepatic metabolism and secretion of individual bile acids. These changes occur despite the apparent absence of any significant change in the TBA concentration. Prairie dogs

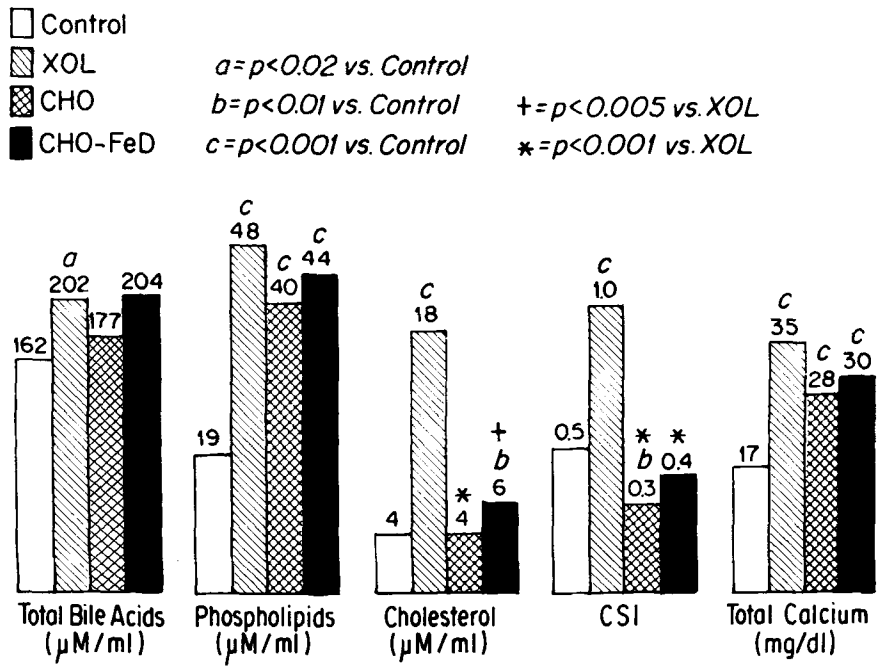

FIG. 1. The effects of diet on gallbladder bile composition. XOL = 1.2% cholesterol enriched diet; CHO = high carbohydrate, normal iron diet; CHO-FeD = high carbohydrate, iron-deficient diet. A portion of this data has been previously published in Reference 13.

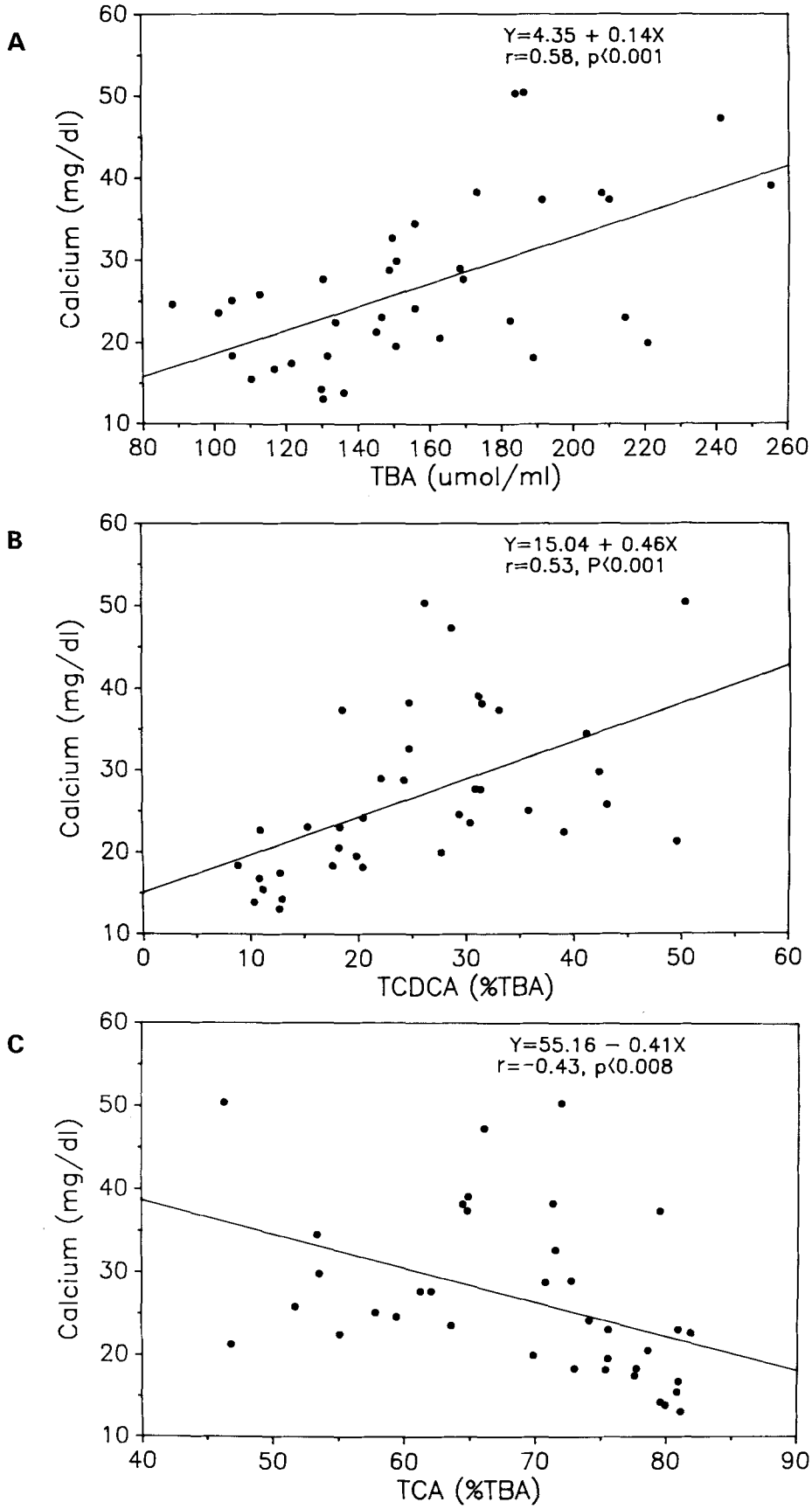

FIG. 2. The linear relationships between gallbladder bile total calcium and biliary bile acid concentrations. (A) Total bile acids (TBA), correlation coefficient (r) is 0.58, $p < 0.001$; (B) taurochenodeoxycholic acid (TCDCA), $r = 0.53$, $p < 0.001$; (C) taurocholic acid (TCA), $r = -0.53$, $p < 0.008$.

maintained on XOL, which is known to induce cholesterol gallstones in this model (10-12), were noted to have a significant shift from TA to TCDCA. Similar changes were noted in animals maintained on the CHO-FeD diet. This latter diet has recently been shown to induce calcium bilirubinate sludge and microscopic pigment gallstones in the prairie dog model (13,23). Therefore, our findings indicate that both cholesterol and pigment gallstone formation are characterized by comparable changes in biliary lipid metabolism and individual bile acid concentrations.

Although the data were scattered, our results demonstrate a significant correlation between the changes in bile acids induced during gallstone formation, and increases in GB concentrations of calcium. The conspicuous absence of these relationships in HB provides further evidence for the hypothesis that the observed increase in GB calcium is a gallbladder, rather than a hepatic phenomenon (7). Furthermore, it is conceivable that the diet induced changes in bile acid composition predisposed to calcium precipitation either by altering the buffering capacity of bile (8) or by modulating gallbladder absorptive function and ion transport (24,25).

A shift from cholic to chenodeoxycholic acid during cholesterol gallstone formation has been previously reported in the prairie dog model (12,26,27). These changes are noted within five days of initiation of cholesterol feeding (14). Similar changes have been observed in humans with cholesterol gallstones (28). It has been previously suggested that the observed changes in individual bile acids may facilitate the formation of cholesterol gallstones (12). Early reports suggested that micelles containing dihydroxy bile acids are larger and less stable than those containing trihydroxy bile acids (29). The implication is that the predominance of the less stable dihydroxy micelles might provide a milieu in which cholesterol precipitation could be facilitated, thereby initiating the cascade of events ultimately resulting in cholesterol gallstones. This suggestion is particularly interesting given the fact that CDCA has been well documented to be effective in cholesterol gallstone dissolution. The apparent dichotomy in terms of potential effects remains unexplained, but may be related to changes in physical structure as opposed to inhibition of cholesterol synthesis. Recent studies have implicated increases in biliary concentrations of cholesterol in the pathogenesis of mixed or pigment gallstone disease as well (13,23,30). Although bile is not saturated with cholesterol in these models, it has been suggested that biliary concentrations of cholesterol may be critical to the formation of non-cholesterol gallstones.

The factors responsible for the shift from CA to CDCA remain unclear. It is conceivable that the relative activities of 12 α-hydroxylase and 26 hydroxylase may act as regulators of these changes. However, the factors responsible for the preferential initiation of 26 hydroxylase and the associated inhibition of 12 α-hydroxylase are not well defined. However, it is interesting to speculate that cholesterol may play a role in this mechanism. It has previously been suggested that the alterations in bile acid composition represent a compensatory response to cholesterol overloading in the hepatocytes (27). Delivery of an increased amount of cholesterol to the liver may induce a subtle alteration in cholesterol metabolism, favoring the synthesis of CDCA. This, in turn, would tend to limit cholesterol production via the negative feedback of CDCA on HMG-CoA reductase, the rate limiting enzyme for cholesterol synthesis (31). The potential interrelationship between cholesterol metabolism and bile acid composition would appear to warrant further investigation. The finding that changes in bile composition were noted despite the presence of normal biliary cholesterol levels (animals fed the CHO diet) suggest that other factors as well may play an important role in the alteration of bile acid compostion.

Increasing evidence suggests that the precipitation of calcium in bile with either carbonate, bilirubinate, phosphate, or palmitate may be a common factor in the pathogenesis of both cholesterol and pigment gallstones. Theoretically, calcium precipitation is thermodynamically possible when the ion-product of calcium and any one of the above mentioned ions exceeds the solubility product of the calcium salt of corresponding ions. In the current study, gallbladder biliary concentrations of total calcium and phospholipids were increased in animals developing either pigment or cholesterol stones. The observation that hepatic biliary calcium levels are unchanged in these animals suggests that a gallbladder mechanism may be ultimately responsible for the increase in gallbladder calcium, as opposed to alterations in hepatic secretion of calcium. One possible explanation is that the changes in the relative concentrations of individual bile acids may alter the phospholipid-bile acid micelles, resulting in a reduction in calcium solubility in bile. Further studies are clearly needed to elucidate the factors in bile which determine calcium solubility.

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Age-related Changes in the Lipid Compositions of Rat and Human Tissues

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The levels of cholesterol, ubiquinone, dolichol, dolichyl-P, and total phospholipids in human lung, heart, spleen, liver, kidney, pancreas, and adrenal from individuals from one-day-old to 81 years of age were investigated and compared with the corresponding organs from 2 to 300 day-old rats. The amount of cholesterol in human tissues did not change significantly during aging, but the level of this lipid in the rat was moderately elevated in the organs of the oldest animals. In human pancreas and adrenal the ubiquinone content was highest at one year of age, whereas in other organs the corresponding peak value was at 20 years of age, and was followed by a continuous decrease upon further aging. A similar pattern was observed in the rats, with the highest concentration of ubiquinone being observed at 30 days of age. Dolichol levels in human tissues increase with aging, but they increase to very different extents. In the lungs this increase is seven-fold, and in the pancreas it is 150-fold. The elevation in the dolichol contents of rat tissues ranges from 20 to 30-fold in our material. In contrast, the levels of the phosphorylated derivative of dolichol increased to a more limited extent, i.e., 2 to 6-fold in human tissues and even less in the rat. These results demonstrate that the levels of a number of lipids in human and rat organs are modified in a characteristic manner during the life-span. This is in contrast to phospholipids, which constitute the bulk of the cellular lipid mass.
Lipids 24, 579–584 (1989).

Dolichol is known to be widely distributed in animal tissues and is present in all cells and membranes (1). Depending on the tissue, smaller or larger portions of these polyisoprenols are esterified with fatty acids (2). While the level of dolichol varies extensively under different conditions, the tissue concentration of dolichyl-P is generally quite stable (3). The best known function of the phosphorylated derivative is its participation in the biosynthesis of *N*-glycosidically-linked oligosaccharide chains (4). Obviously, glycosylation of proteins is a basic cellular function requiring the presence of dolichyl-P.

At least in model membranes, dolichol profoundly influences membrane fluidity, stability, and permeability (5–9). However, it seems unlikely that large variations in membrane dolichol content are compatible with the maintenance of membrane functions. Large amounts of cellular polyprenols are probably not associated with the membranes itself, but are localized in the luminal compartments of organelles.

Several investigators have demonstrated increases in the dolichol contents of human brain (10–13), blood (14), and of a number of organs in mice and rats during aging (15,16). In a recent study on various organs of the rat, Keller and Nellis (17) found significant increases in the dolichol contents in all cases, increases in the dolichyl-P levels of some tissues, but no changes in the levels of cholesterol with increasing age. In this investigation no rats older than 100 days were examined. In the experiments of Kabakoff and Kandutsch on mice liver, the amount of ubiquinone decreased shortly after birth, followed by an increase as the mice grew (18).

In the present study we have investigated changes in the lipid contents of organs of rats between the ages of 2 and 300 days and of human organs (using autopsy material). The lipid compositions of human and rat tissues were found to display characteristic patterns during the life-span.

MATERIALS AND METHODS

Male Sprague-Dawley rats of various ages and with free access to food and water were used. When 2- and 10-day-old rats were studied, different organs from 8–10 rats were pooled. Human tissues were obtained during autopsies within one day after death. Parallel samples were subjected to histological examination in order to exclude tissue with significant pathological changes.

The tissues were homogenized with an Ultra-Turrax blender for two minutes (24,000 rpm) in 0.25 M sucrose to give a 10% homogenate. Part of this homogenate (corresponding to 0.3 g tissue) was removed for the determination of phospholipid, cholesterol, and ubiquinone. Another portion (0.6 g) was adjusted to contain 26 ml chloroform/methanol/water (CMW), 1:1:0.3 containing 0.1 M HCl, and 1 nmol of dolichol-23 and dolichyl-23-P were added as internal standards. The mixture was incubated for 60 min at 20°C, for 45 min at 55°C and, finally, for 10 min at 90°C. Initial mild acid hydrolysis removed monosaccharides from dolichyl phosphates, and acid hydrolysis at higher temperature removed oligosaccharide phosphate from dolichyl pyrophosphates (19,20). The suspension was neutralized and evaporated to dryness. Alkaline hydrolysis of the residue was then performed using 10 ml methanol/water (MW)/60% KOH (1:1:0.5) for 45 min at 90°C. The pH was then adjusted to 6.0, and chloroform and methanol were added to obtain a CMW ratio of 3:2:1. The chloroform phase was subsequently washed three times with 3 ml theoretical upper phase (CMW, 3:48:47). After evaporation, the lipids were dissolved in 200 μl chloroform/methanol (2:1) and supplemented with 15 ml MW (98:2) containing 20 mM H_3PO_4. This mixture was applied to a C18 Sep-Pak column (Waters), which was first washed with 10 ml MW (98:2) containing 20 mM H_3PO_4. The C18 Sep-Pak was washed with MW 10 ml (98:2), and 20 ml CM (2:1) was used to elute both dolichol and dolichyl-P. After the addition of NH_4OH to the eluate (to a final concentration of 1%), the mixture was placed on a silica Sep-Pak column and dolichol eluted with 20 ml CM (2:1), including 1%

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Abbreviations: Abbreviations: MW, methanol-water; CMW, chloroform-methanol-water; CM, chloroform-methanol; HPLC, high performance liquid chromatography.

NH_4OH. For the elution of dolichyl-P 20 ml CMW (1:1:0.3) was used. For high performance liquid chromatography (HPLC), dolichol was dissolved in 200 μl CM (2:1) and dolichyl-P in 200 μl of the solvent system used in pump system A for HPLC.

These lipid samples were analyzed by HPLC using a Hewlett-Packard Hypersil ODS 3 μm reversed-phase column. A convex gradient was used (no. 6, Waters 840 solvent program) from the initial 2-propanol/methanol/water (40:60:5) in pump system A, to hexane/2-propanol/methanol (20:40:60) in pump system B, at a flow rate of 2 ml/min and with a program time of 20 min. In the case of dolichyl-P, solutions A and B also contained 20 mM phosphoric acid. The eluate was monitored at 210 nm.

The portion of the homogenate which was to be used for measuring phospholipid, cholesterol, and ubiquinone was supplied with ubiquinone-6 and [^{14}C]cholesterol as internal standards, and extracted with CMW (1:1:0.3) at 37°C for 1 hr with continuous stirring. This mixture was centrifuged and the protein residue in the pellet was reextracted in the same manner. The extracts were pooled and adjusted to a final CMW ratio of 3:2:1 and the two lower phases were subsequently collected. After a triple wash with theoretical upper phase the solvent was evaporated and the residue dissolved in 200 μl CM, 2:1, and thereafter diluted with 5 ml chloroform. This extract was placed onto a silica Sep-Pak and the neutral lipid fraction was eluted with 25 ml chloroform. Phospholipids were eluted with 25 ml methanol and total phospholipid were determined as previously described (21). The chloroform eluate from the silica Sep-Pak was evaporated to dryness, redissolved in 400 μl CM, 2:1, supplemented with 10 ml methanol and poured onto a C18 Sep-Pak cartridge. This cartridge was then eluted with an additional 25 ml methanol, the eluate was evaporated to dryness, and the lipids redissolved in 200 μl CM (2:1).

HPLC analyses were performed on a Waters 840 system. The sample was injected onto a Hewlett-Packard Hypersil ODS 3 μm reversed phase column. A linear gradient from the initial (A) methanol/water, 9:1, to (B) methanol/2-propanol, 4:1, was run for 20 min. The absorbance of the eluate at 210 nm was monitored for quantitation of cholesterol and ubiquinone. The cholesterol peaks were removed for the determination of radioactivity by scintillation counting. This value was used to correct for recovery.

Data obtained with the autopsy material used in these investigations were compared with those for fresh biopsy material from several tissues removed by operation and extracted immediately. Material for extraction was taken from histologically normal regions of the biopsies. In agreement with previous findings (22), the lipid contents of biopsies were identical with those found in autopsy material, thereby justifying the use of the latter material.

RESULTS AND DISCUSSION

For the quantitation of phospholipids, cholesterol, and ubiquinone after extraction without alkaline hydrolysis, initial separation of these lipids was necessary. Analysis of ubiquinone has previously often been performed after alkaline hydrolysis, which causes partial breakdown of this lipid (23). On the other hand, alkaline hydrolysis is required for the complete recovery of total dolichol and dolichyl-P, and the conditions used here also resulted in hydrolysis of the glycosylated derivatives of dolichyl-P.

The HPLC procedure involving a C18 reversed phase column allowed quantitation not only of the total dolichol and dolichyl-P, but also of the amounts of individual isoprenes (Fig. 1A and B). Cholesterol and ubiquinone were well separated by HPLC and the use of initial purification eliminated the contamination which interfered with further analysis (Fig. 2).

Rat lungs, heart, spleen, liver, and kidney demonstrated only small increases in cholesterol content (on a wet weight basis) between 2 and 90 days of age (Table 1). When, however, the 300-day-old rats were investigated, the corresponding increases were considerable, particularly in the heart and the liver. The behavior of ubiquinone-9 was characteristic—its levels increased during the 30 days following birth, after which a decrease occurred in the various organs. The only exception was the heart, where the ubiquinone content increased continuously throughout the time period investigated.

The dolichol content exhibited a dramatic increase ranging between 10–30 times the 2-day-old value. The level of its phosphorylated counterpart was unchanged in the liver, but in other organs this level was elevated, as much as three times in the spleen and kidney. The total levels of phospholipids (which represent the bulk of the tissue lipid mass) are stable, showing little changes during aging. Thus, characteristic and extensive changes occur in the lipid compositions of various organs during the aging process in rats. The dolichol content of different rat tissues observed here varies to some extent from the earlier data, including some from our own laboratory. This discrepancy probably reflects the fact that there are considerable seasonal variations in the content of this lipid, and this parameter is also affected by the strain of rats used and the animal housing environment.

Histologically normal human autopsy material was collected from the lung, heart, spleen, liver, kidney, pancreas, and adrenals of individuals between the ages of 1 day and 81 years (Table 2). The cholesterol content in all organs exhibited limited variability during the life-span, no major changes were observed even when the oldest age-group was compared to the youngest.

In the case of ubiquinone-10 the pattern for man is somewhat similar to that observed in the rat, i.e., first an increase followed by a decrease. The highest concentrations of this lipid are reached at 20 years of age in the heart, liver, and kidney. In the pancreas and adrenal the highest levels of ubiquinone were found at the age of one year (38 and 58 μg/g, respectively), which was followed by a continuous decrease, so that the amount of this lipid in these same organs was 6–7 times less at 81 years of age.

In agreement with previous investigations, human organs were found to be rich in dolichol (22,24). During the life-span the level of this lipid increased many-fold (i.e., 10–30-fold) in most tissues examined. The increase was more pronounced in the adrenal and pancreas, where it was found to be as much as 100- and 150-fold, respectively.

The increase in dolichyl-P content was considerably less than that of the free alcohol and ranged between 2- and 6-fold. The exception was the heart, where this elevation was found to be more than 20-fold. There were some variations in the total phospholipid content depending on the

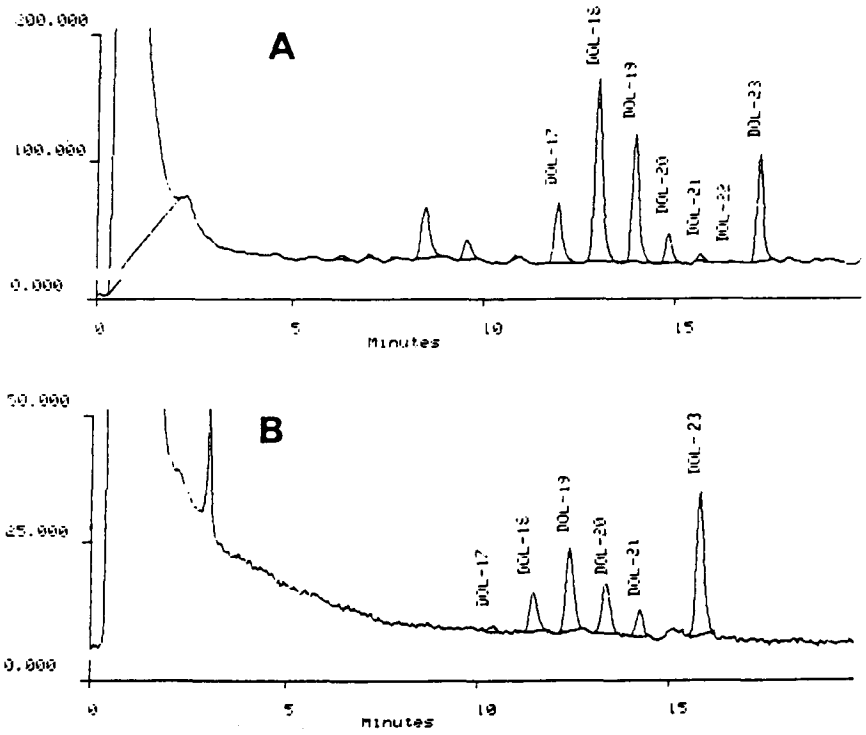

FIG. 1. HPLC patterns for dolichol and dolichyl-P. (A) Dolichol from rat kidney (300-day-old). Dol = dolichol. Dolichol-23 added as internal standard. (B) Dolichyl-P from rat liver (10-day-old). Dol = dolichyl-P. Dolichyl-23-P added as internal standard.

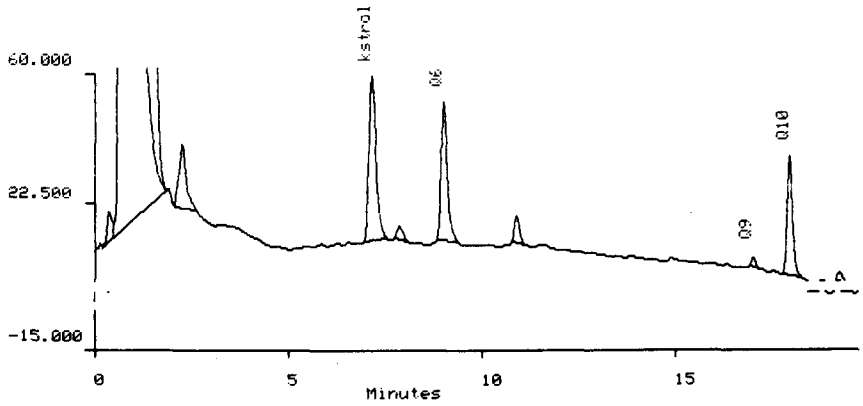

FIG. 2. HPLC pattern for cholesterol and ubiquinone from human heart. Kstrol = Cholesterol. Q6 = ubiquinone-6 added as internal standard. Q9 and Q10 = ubiquinone-9 and 10.

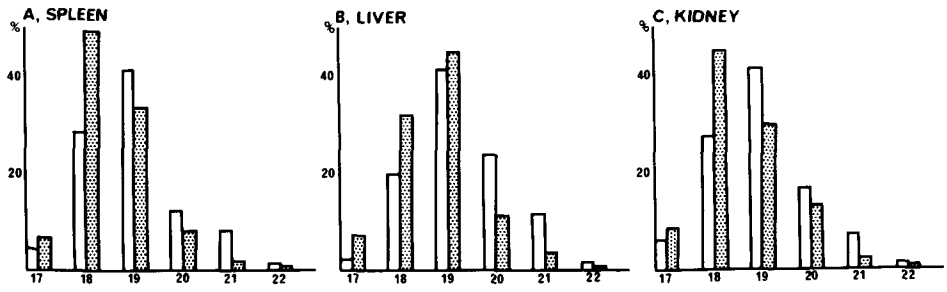

FIG. 3. Distributions of individual species of dolichyl-P in rat organs. (A) Spleen; (B) liver; and (C) kidney. The open bars give values for 10-day-old rats and the patched bars represent 300-day-old animals.

TABLE 1

Distribution of Various Lipids in Organs of the Rat

Organ	Age (days)	Cholesterol (mg/g ww)	Ubiquinone-9 (μg/g ww)	Dolichol (μg/g ww)	Dolichyl-P (μg/g ww)	PLP (mg/g ww)
Lung	2	3.2 ± 0.1	9.6 ± 1.6	1.5 ± 0.1	3.5 ± 0.2	12.5 ± 2.0
	10	3.0 ± 0.4	16.1 ± 2.0	2.3 ± 0.2	3.2 ± 0.3	12.6 ± 1.8
	30	3.1 ± 0.3	17.3 ± 1.0	5.7 ± 0.1	3.3 ± 0.1	12.3 ± 1.0
	90	3.7 ± 0.5	15.1 ± 1.6	16.9 ± 1.3	5.9 ± 0.4	12.5 ± 1.3
	300	4.5 ± 0.8	4.8 ± 0.8	35.6 ± 4.0	5.2 ± 0.3	13.9 ± 1.7
Heart	2	1.5 ± 0.2	33.8 ± 1.7	1.0 ± 0.1	2.6 ± 0.3	9.6 ± 1.3
	10	1.8 ± 0.3	68.0 ± 5.0	1.7 ± 0.2	3.3 ± 0.2	12.7 ± 2.0
	30	1.4 ± 0.1	125.1 ± 6.0	3.8 ± 0.5	3.6 ± 0.4	13.2 ± 1.5
	90	1.9 ± 0.2	129.5 ± 5.3	8.1 ± 1.0	4.6 ± 0.3	13.0 ± 1.8
	300	6.4 ± 0.8	134.5 ± 9.2	32.7 ± 2.6	4.5 ± 0.6	13.7 ± 1.9
Spleen	2	2.2 ± 0.3	8.8 ± 1.1	3.0 ± 0.4	3.7 ± 0.2	8.6 ± 1.7
	10	1.8 ± 0.3	17.4 ± 2.1	2.6 ± 0.3	6.3 ± 0.7	10.1 ± 1.5
	30	2.8 ± 0.4	18.3 ± 2.0	17.0 ± 2.1	6.2 ± 0.5	10.3 ± 1.4
	90	3.6 ± 0.5	11.7 ± 1.5	63.8 ± 4.3	11.5 ± 0.8	12.0 ± 1.4
	300	4.4 ± 0.5	9.4 ± 1.2	83.8 ± 6.4	12.3 ± 0.7	11.6 ± 1.6
Liver	2	2.6 ± 0.1	55.1 ± 7.3	7.2 ± 1.1	7.3 ± 0.9	18.9 ± 2.6
	10	2.2 ± 0.3	143.6 ± 9.3	4.6 ± 0.7	10.1 ± 1.2	23.5 ± 2.4
	30	2.4 ± 0.3	127.6 ± 11.1	16.9 ± 1.3	8.5 ± 0.5	22.0 ± 2.4
	90	2.5 ± 0.2	112.6 ± 10.8	29.1 ± 1.4	8.2 ± 1.0	24.1 ± 2.6
	300	5.7 ± 0.8	34.6 ± 5.0	63.3 ± 4.6	7.8 ± 1.3	23.1 ± 2.1
Kidney	2	3.0 ± 0.4	23.8 ± 3.2	4.5 ± 0.3	6.0 ± 0.4	12.6 ± 1.8
	10	3.2 ± 0.5	63.1 ± 5.0	9.0 ± 1.2	6.5 ± 0.8	13.7 ± 1.5
	30	3.6 ± 0.6	115.6 ± 13.1	12.9 ± 0.8	7.7 ± 0.9	14.6 ± 1.6
	90	4.5 ± 0.5	103.3 ± 9.3	15.6 ± 1.2	15.5 ± 1.2	15.0 ± 2.0
	300	5.7 ± 0.6	50.3 ± 6.2	31.5 ± 2.7	19.5 ± 0.8	15.1 ± 2.1

The values are the means ± SEM of six samples. PLP = phospholipid; ww = tissue wet weight.

TABLE 2

Distribution of Various Lipids in Human Organs

Organ	Age group (years)	Cholesterol (mg/g ww)	Ubiquinone-10 (μg/g ww)	Dolichol (μg/g ww)	Dolichyl-P (μg/g ww)	PLP (mg/g ww)
Lung	1-3 days	3.0 ± 0.4	2.2 ± 0.3	15.2 ± 1.4	1.7 ± 0.2	10.7 ± 0.9
	0.7-2 years	2.3 ± 0.3	6.4 ± 0.4	19.2 ± 1.5	3.6 ± 0.3	9.9 ± 1.0
	19-21 years	1.6 ± 0.2	6.0 ± 0.4	113.6 ± 1.6	4.1 ± 0.5	7.8 ± 0.9
	39-43 years	2.1 ± 0.3	6.5 ± 0.6	130.2 ± 11.2	5.8 ± 0.4	7.9 ± 0.9
	77-81 years	3.2 ± 0.4	3.1 ± 0.4	137.0 ± 11.1	8.9 ± 0.6	6.5 ± 0.7
Heart	1-3 days	1.7 ± 0.2	36.7 ± 1.8	36.7 ± 2.0	1.1 ± 0.1	10.0 ± 1.1
	0.7-2 years	1.2 ± 0.2	78.5 ± 4.6	49.3 ± 3.1	3.8 ± 0.5	9.3 ± 1.2
	19-21 years	1.0 ± 0.1	110.0 ± 9.4	48.3 ± 5.2	4.4 ± 0.4	8.2 ± 1.1
	39-43 years	1.2 ± 0.2	75.0 ± 8.3	133.6 ± 10.2	11.4 ± 0.8	10.4 ± 1.5
	77-81 years	1.4 ± 0.2	47.2 ± 3.9	292.0 ± 15.3	26.0 ± 2.3	10.9 ± 1.4
Spleen	1-3 days	3.2 ± 0.5	20.7 ± 3.2	9.5 ± 1.1	8.8 ± 1.2	8.0 ± 1.1
	0.7-2 years	3.1 ± 0.4	30.2 ± 2.7	54.0 ± 4.9	7.1 ± 0.9	9.0 ± 1.1
	19-21 years	3.5 ± 0.5	32.8 ± 2.6	86.6 ± 3.9	7.0 ± 1.0	7.7 ± 1.0
	39-43 years	3.3 ± 0.4	28.6 ± 2.3	106.3 ± 9.6	8.2 ± 0.7	8.2 ± 1.2
	77-81 years	3.7 ± 0.3	13.1 ± 1.8	97.1 ± 9.3	14.2 ± 1.1	10.0 ± 1.3
Liver	1-3 days	2.5 ± 0.3	12.9 ± 1.5	16.7 ± 2.0	4.1 ± 0.5	12.1 ± 1.5
	0.7-2 years	1.6 ± 0.2	45.1 ± 5.2	66.0 ± 5.3	3.3 ± 0.5	10.9 ± 1.2
	19-21 years	2.0 ± 0.3	61.2 ± 7.3	472.0 ± 30.3	5.2 ± 0.4	12.7 ± 1.5
	39-43 years	1.9 ± 0.2	58.3 ± 6.1	465.0 ± 32.1	5.9 ± 0.7	10.3 ± 1.4
	77-81 years	2.9 ± 0.4	50.8 ± 7.1	500.0 ± 29.2	9.8 ± 1.1	14.1 ± 1.5
Kidney	1-3 days	2.6 ± 0.3	17.4 ± 2.1	17.4 ± 1.3	2.5 ± 3.0	8.0 ± 1.1
	0.7- 2 years	2.4 ± 0.2	53.4 ± 6.6	87.0 ± 9.5	4.8 ± 0.6	8.1 ± 1.0
	19-21 years	2.7 ± 0.3	98.0 ± 10.4	114.2 ± 10.1	4.6 ± 0.5	7.0 ± 0.8
	39-43 years	2.2 ± 0.2	71.1 ± 6.8	120.4 ± 11.3	4.4 ± 0.3	7.6 ± 0.9
	77-81 years	3.2 ± 0.4	64.0 ± 6.6	222.8 ± 18.9	9.5 ± 1.3	7.1 ± 1.0
Pancreas	1-3 days	2.8 ± 0.2	9.2 ± 1.2	20.5 ± 1.8	4.6 ± 0.5	7.0 ± 0.9
	0.7-2 years	1.9 ± 0.1	38.2 ± 4.1	17.8 ± 2.0	7.3 ± 0.6	8.9 ± 0.8
	19-21 years	1.4 ± 0.2	21.0 ± 3.0	1117.3 ± 78.3	8.1 ± 1.0	9.8 ± 1.2
	39-43 years	1.8 ± 0.2	19.3 ± 2.1	2033.1 ± 99.6	21.3 ± 1.8	11.5 ± 1.6
	77-81 years	1.9 ± 0.3	6.5 ± 0.5	3058.8 ± 140.3	23.0 ± 1.7	12.7 ± 1.4
Adrenal	1-3 days	3.4 ± 0.5	17.5 ± 2.1	17.9 ± 2.1	14.3 ± 1.8	11.0 ± 1.2
	0.7-2 years	2.8 ± 0.4	57.9 ± 6.8	164.0 ± 10.2	16.3 ± 1.5	10.2 ± 1.3
	19-21 years	3.7 ± 0.5	16.1 ± 2.3	645.0 ± 50.3	14.4 ± 1.9	9.3 ± 1.4
	39-43 years	4.4 ± 0.6	12.2 ± 1.5	869.0 ± 52.2	17.8 ± 2.3	11.0 ± 1.6
	77-81 years	5.5 ± 0.5	8.5 ± 1.0	1752.0 ± 82.3	57.8 ± 6.1	12.7 ± 1.7

The values are the means ± SEM of 6-8 samples. ww = tissue wet weight.

TABLE 3

Distribution of Individual Species of Dolichols and Dolichyl-P in Organs of the Human and Rat[a]

	Isoprene distribution (%)											
	Dolichol						Dolichyl-P					
Organ	D17	D18	D19	D20	D21	D22	DP17	DP18	DP19	DP20	DP21	DP22
Spleen												
Rat	24.9	46.1	15.3	9.6	2.1	2.0	4.7	28.5	41.2	17.4	7.9	0.3
Human	4.9	21.3	46.7	22.0	3.3	1.8	1.2	11.2	49.4	28.3	6.0	3.9
Liver												
Rat	20.4	41.1	20.2	13.3	3.1	1.9	2.3	19.8	41.5	23.8	11.7	0.9
Human	3.0	13.5	40.5	32.3	9.5	1.2	1.1	5.7	42.9	34.5	15.6	0.2
Kidney												
Rat	6.5	43.5	28.2	14.8	6.8	0.2	6.1	27.6	41.5	16.9	7.6	0.3
Human	2.6	22.2	50.3	20.9	3.9	0.1	3.1	12.6	56.7	24.9	2.6	0.1
Myocardium												
Rat	9.4	35.2	29.4	20.3	4.4	1.3	12.1	42.4	38.0	6.0	1.2	0.3
Human	4.7	33.1	42.8	13.3	4.1	2.0	4.0	10.5	59.1	21.4	4.5	0.5

[a]The human organs were collected from 1-3-day-old individuals and the rat organs were samples from 10-day-old animals. The values are the means of six experiments.

tissue and age. Such variation may be caused by physiological, dietary, or other influences not associated with the aging process.

Dolichol and dolichyl-P are present in both rats and humans as a family of isomers containing between 17 and 22 isoprene residues. In Table 3 the polyisoprenoid compositions of rat and human organs from neonates, an age group not previously investigated in detail, are presented. In rat organs the individual polyisoprenols present in the highest amount in dolichol is that containing 18 residues, which constitutes as much as 35–45% of the total. In contrast, in human tissues the dominating polyisoprenol is dolichol-19. The distribution pattern for dolichyl-P varies in different rat organs. Either dolichyl-18 or 19-P was present in the highest relative amount. The human pattern is more uniform—in all tissues dolichyl-P with 19 residues is dominant, followed by dolichyl-20-P.

In the present study the polyisoprenoid patterns for dolichol were different in rats and humans, but the differences between tissues or in the same tissue with increasing age were small. The distribution pattern for dolichyl-P in human organs was unchanged during the life-span. On the other hand, there was a tendency in rat tissue for this pattern to change, as is illustrated in Figure 3. It is apparent that in the organs shown, i.e., spleen, liver, and kidney, the relative amounts of shorter isoprenols were higher in the 300-day-old than in the 10-day-old animals.

The experiments described in this study demonstrate that considerable age-dependent changes in the lipid compositions occur in both rat and human tissues. Several changes in the lipid compositions are obviously a characteristic part of the aging process. Cholesterol levels in cells and tissues are not an important factor in this process, since these change very moderately in the rat and insignificantly in human tissues during aging.

The behavior of ubiquinone is remarkable, considering the early elevation in its levels, followed by a continuous decrease. This behavior may be explained, at least in part, by the central role of this lipid in mitochondrial respiration, which is rapid during growth and diminishes under steady conditions. The decreases in ubiquinone levels in older people may be harmful. Since ubiquinone is present not only in mitochondria, but in other membranes as well (25), its role as an antioxidant may also be of great importance (26).

Previous investigations have revealed that in human brain and blood the amount of dolichol increases with age (10,12,14). However, the organs studied here have not been previously analyzed. All human tissues examined in the present study display an extensive increase in dolichol content during aging. The dolichol content, which was previously investigated in the organs of young rats, also exhibited a pronounced and continuous elevation in the older ages. Dolichol has a profound influence on the fluidity, stability and permeability of model membranes, which may be also the case in biological membranes. If this is so, the physical properties of membranes may be modified in older ages by the changes in polyisoprenoid content. On the other hand, it is probable that at least part of the dolichol which accumulates during aging is not associated with the membrane itself, but accumulates in the luminal compartment of organelles, and thus does not directly affect membrane properties. This possibility should be investigated in the future.

So far, no pathway for dolichol degradation has been described, and it appears that the only mechanism for the removal of this lipid is excretion (23,27). An auxillary mechanism could be, at least theoretically, the accumulation and storage of dolichol in specific cellular compartments. In light of the fact that the half-lives of dolichol in the endoplasmic reticulum and lysosomes range between 80 and 137 hours (28), the accumulation observed could account only for a very minor portion of the newly synthesized dolichol. Accumulation of dolichol probably has metabolic and pathophysiological explanations, the nature of which must be clarified in future experiments.

The relatively moderate changes in dolichyl-P content upon aging, in contrast to the large changes in the level of the free alcohol, may have several explanations. The enzymes regulating interconversions of the free alcohol and dolichyl-P may change differently during aging, i.e.,

an increase in the phosphatase and/or a decrease in the CTP-kinase activities may occur (29,30). Alternatively, if dolichol and dolichyl-P are synthesized by different pathways (31), then their rates of synthesis may be modified independently during aging. The major known function of dolichyl-P (i.e., participation in glycoprotein synthesis) is required in all tissues at all ages, since glycosylation is a basic part of the living process. For this reason, the levels of dolichyl-P must be sufficiently high even at young ages.

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Molecular Species Analysis of Phosphoglycerides from the Ripe Roes of Cod (*Gadus morhua*)

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Molecular species of the 3,5-dinitrobenzoyl derivatives of phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylinositol (PI) were quantitated by UV detection at 254 nm after reversed-phase HPLC using solvent systems modified from Takamura *et al.* (*Lipids 21,* 356–361, 1986). Three isocratic solvent systems were used and a total of 39 different molecular species detected. Four species, 16:0/20:5, 18:1/20:5, 16:0/22:6 and 18:1/22:6 contributed 67.2% and 61.8% of PC and PE respectively but only 23.0% of PI. In PI the most important species was 18:0/20:4 at 36.7% but this species only constituted 0.7% in each of PC and PE. Small amounts of dipolyunsaturated species were also found in PC and PE.
Lipids 24, 585–588 (1989).

The determination of the fatty acid composition of tissue lipids by gas liquid chromatography of fatty acid methyl esters has been a routine analytical tool of the lipid biochemist for many years. Recently the development of HPLC methods for analyzing the molecular species composition of phosphoglycerides has permitted an investigation of biomembrane phospholipids at a new level of complexity and has given fresh insight into the organization of the biomembrane. A variety of methods has been developed using intact or derivatized phospholipids and several types of detection systems. One of the most successful methods is that of Patton *et al.* (1) which employed an isocratic elution system of intact phosphoglycerides on a reversed-phase (C18) column with UV detection at 205 nm. This system has been widely used for studies involving polyunsaturated fatty acids (PUFA) but cannot give quantitative information on all the molecular species present since detection at 205 nm depends on the presence of double bonds in the unsaturated fatty acids. Post-column fluorescence detection or flame ionization detection does allow quantitative analysis of intact phosphoglycerides (2,3).

However, the most common quantitative methods rely on preparing derivatives of the phosphoglycerides by hydrolysis with phospholipase C followed by acylation of the diacylglycerol to give acetyl, benzoyl or dinitrobenzoyl derivatives (4–7). These derivatives are separated into molecular species on reversed-phase columns and detected with refractive index or UV detectors (3,6). Most molecular species analyses have been of PC but an additional advantage of derivatization is that all the glycerides are converted to similar compounds which can therefore all be separated using the same chromatography conditions. In this paper molecular species analyses of PC, PE and PI from the ripe roes of cod *(Gadus morhua)* were determined as 3,5-dinitrobenzoyl derivatives. A third solvent system was developed to complement the system of Takamura *et al.* (8) and resolve the coeluting species found by these workers.

Abbreviations: PUFA, polyunsaturated fatty acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol. Molecular species are abbreviated as follows: e.g., 16:0/20:5 PC is 1-palmitoyl-2-eicosapentaenoyl-*sn*-glycero-3-phosphocholine.

MATERIALS

Ripe roes were excised from cod caught off Gourdon, south of Aberdeen, Scotland, and stored at −20°C. *Bacillus thuringiensis* type strain IAM 12077 was obtained from the National Culture of Industrial and Marine Bacteria, Torry Research Station, Aberdeen, Scotland.

Phospholipase C from *Bacillus cereus* (Sigma Type XIII), butylated hydroxytoluene (BHT), 3,5-dinitrobenzoyl chloride, dipalmitolein, diolein and the following phosphatidylcholines were obtained from Sigma Chemical Co., Poole, Dorset, UK: egg yolk PC, dilauryl PC, dimyristoyl PC, dipalmitoyl PC, distearoyl PC, 1-stearoyl 2-arachidonyl PC.

Merck TLC and 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, UK. 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 UK Ltd., Progress Road, Sands Industrial Estate, High Wycombe, Bucks, UK).

METHODS

Purification of phosphoglycerides. Total lipid was extracted from freeze-dried homogenates of eggs taken from four fish by the method of Folch *et al.* (9). Approximately 3 g of lipid were obtained from 100 g wet weight of cod roe (10). Solvents routinely contained 0.01% (w/v) BHT and samples were stored at −20°C under N_2 between preparative procedures.

Neutral lipids were separated by preparative TLC using hexane/diethyl ether/acetic acid (70:30:1, v/v/v). The origin material was eluted from the silica with chloroform/methanol/water (5:5:1, v/v/v), and the eluate was dried by rotary evaporation under vacuum at 35°C. The polar lipid fraction contained 46% PC, 20% PE, 3.0% PI, 1.4% PS and smaller amounts of sphingomyelin, lyso-PC and phosphatidic acid determined as phospholipid-bound phosphorus (10).

Polar lipid classes were separated by TLC using methyl acetate/propan-1-ol/chloroform/methanol/0.25% aqueous KCl (25:25:25:10:9, v/v/v/v/v) (11), and visualized under UV light after spraying with 0.1% (w/v) 2,7-dichlorofluorescein in methanol. The PC and PI fractions were further purified by TLC in chloroform/acetone/methanol/acetic acid/water (10:4:2:2:1, v/v/v/v/v) and detected as before. From the lipids 2,7-dichlorofluorescein was removed by extracting with a 2% (w/v) solution of $KHCO_3$. The fatty acid composition of the phospholipids

was determined by GLC of fatty acid methyl esters (FAME) in a Canberra Packard 436 gas chromatograph fitted with a CP Wax 52 CB fused capillary column (50 m × 0.32 mm i.d.) (Chrompack UK Ltd., London) using hydrogen as carrier gas (12).

Preparation of 3,5-dinitrobenzoyl derivatives. One mg portions of PC and PE were hydrolyzed with phospholipase C from *B. cereus* using a two phase system of 1 ml diethyl ether and 1 ml 0.01 M Tris-SO_4 pH 7.4 at room temperature (13). Phosphatidylinositol was hydrolyzed with a PI-specific phospholipase C prepared from *B. thuringiensis* by the method of Ikezawa and Taguchi (14). At the end of the incubation diacylglycerol (DAG) and residual phospholipid were extracted by the addition of 1 ml 0.88% (w/v) KCl and 8 ml chloroform/methanol (2:1, v/v). Lipids were dried under N_2 and stored overnight *in vacuo*. By TLC 1,2-diacylglycerol was purified using hexane/diethyl ether/acetic acid (50:50:1, v/v/v), detected with 2,7-dichlorofluorescein, eluted with hexane/diethyl ether (1:1, v/v) and dried under N_2 and finally *in vacuo* for 2 hr before derivatization. Only trace amounts (estimated to be 1 or 2%) of unhydrolyzed phospholipid remained after phospholipase C digestion. The 1,2-diacylglycerols were acylated in dry pyridine with 3,5-dinitrobenzoyl chloride at 60°C under N_2 for 45 min (8). After extraction and washing (8) the purity of the 1,2-diacyl, 3-dinitrobenzoyl glycerol derivatives was checked by HPTLC in hexane/diethyl ether/acetic acid (70:30:1, v/v/v). All the diacylglycerol was converted to the dinitrobenzoyl derivative.

HPLC of 1,2-diacyl-3-dinitrobenzoyl glycerol derivatives. Molecular species were separated on reversed-phase columns at room temperature (19–21°C) using three isocratic solvent systems with a Pye Unicam 4010 pump, detected at 254 nm with a Pye Unicam 4020 detector and quantitated using a Shimadzu C-R3A integrator. Solvent 1 (methanol/propan-2-ol, 95:5, v/v, 1.0 ml/min) and solvent 2 (acetonitrile/propan-2-ol, 80:20, v/v, 1.0 ml/min) were used with a C18 column as described by Takamura *et al.* (8). A third solvent (methanol/water/acetonitrile, 93:5:2, v/v/v, 1.2 ml/min) was developed for use with a C8 column.

The molecular species containing PUFA in cod roe PC were identified by collecting the peaks after HPLC of the intact PC using the solvent system of Patton *et al.* (1), and preparing FAME to identify the component fatty acids by GLC. A number of synthetic standards were also used to construct plots of log relative retention time (RRT) against effective carbon number at the C1 position of the glyceride (1,8). A convenient reference peak was 16:0/22:6 and RRTs were calculated relative to this species. Sometimes added to samples as additional

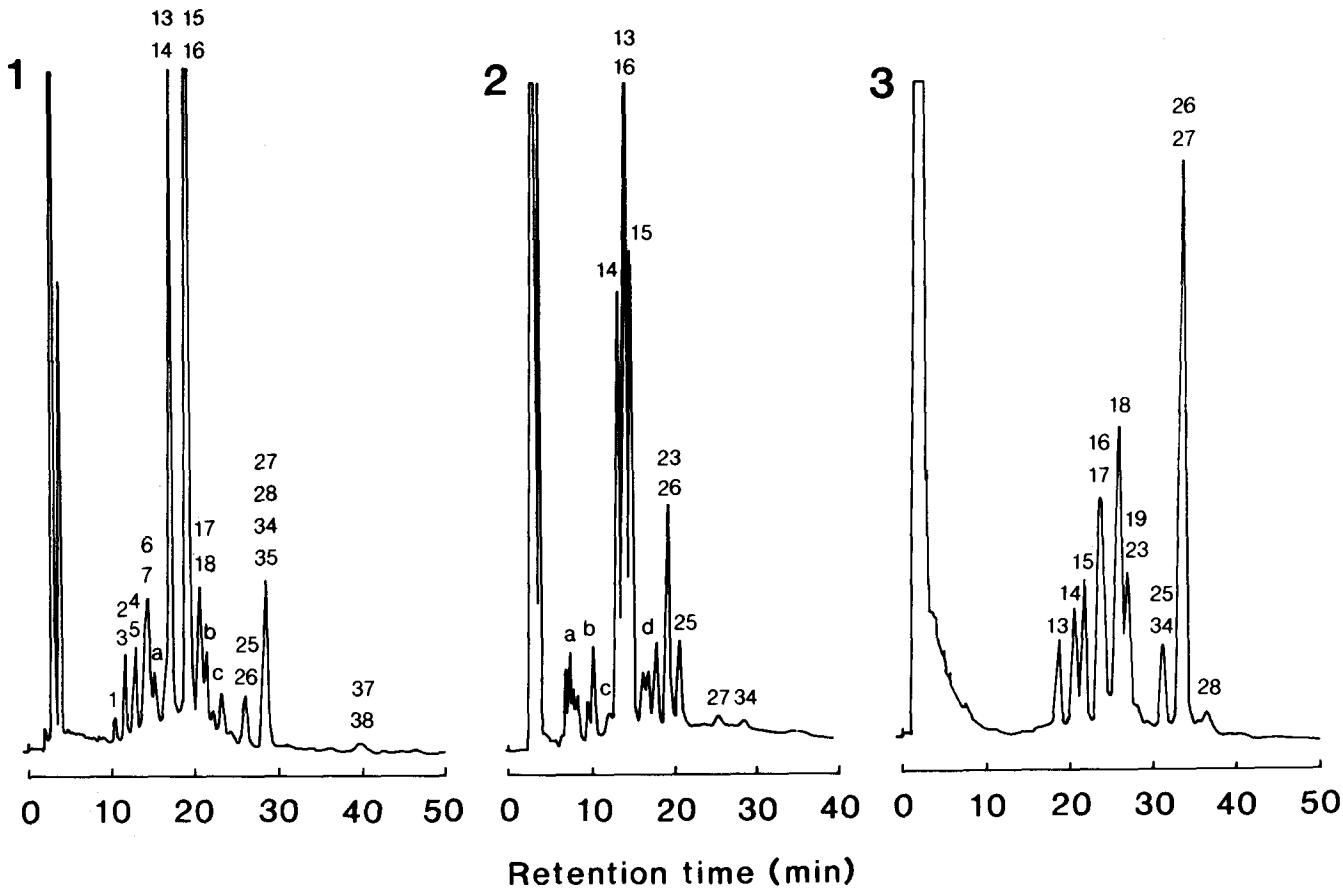

FIG. 1. Molecular species separations of the 3,5-dinitrobenzoyl derivatives of cod roe PC (1) in methanol/propan-2-ol 95:5 (v/v) 1.0 ml/min, cod roe PE (2) in acetonitrile/propan-2-ol 80:20 (v/v) 1.0 ml/min and cod roe P1 (3) in methanol/water/acetonitrile 93:5:2 (v/v/v) 1.2 ml/min. The absorbance range at 254 nm was 0.04 units in 1 and 2, and 0.02 units in 3, with c. 40 μg, c. 40 μg and c. 10 μg of material injected respectively. Peak numbers refer to the molecular species shown in Table 2, but space did not permit the individual numbering of all peaks. Lettered peaks or groups of peaks contain the following species: in profile 1, a = 8, 9, 10, 11; b = 19, 20; c = 21, 22 and 23, 24; in profile 2, a = species 1, 2, 3 and 5; b = 4, 7 and 6; c = 9, 10; d = 20, and 18, 19 and 17, 24.

reference species were 12:0/12:0 and 16:0/16:0. Solvent 1 was used to give the basic information on peak areas and the other two solvents were used to give the peak areas of individual species either directly or by subtraction. Each sample was chromatographed three times in each of the solvent systems and the standard deviations calculated. Where final peak areas were calculated by subtraction, the standard deviations of the contributing peaks were added to give the final error. All final analyses were corrected to 100%.

RESULTS AND DISCUSSION

The protocol described here employing three different isocratic solvent systems to separate 1,2-diacyl, 3-dinitrobenzoyl derivatives of phosphoglycerides offers significant advantages over earlier procedures. The method of Takamura *et al.* (8) which used the same derivatives and solvents 1 and 2 resulted in some coeluting species, including ones of major importance in tissues rich in (n-3)PUFA, e.g., 18:1/22:6 running with 16:0/22:6 in solvent 1 and with 16:0/20:5 in solvent 2. Therefore, a third solvent was developed from the system of Patton *et al.* (1), used for separating intact PC molecules, by removal of the choline-chloride and adjustment of the proportion of methanol, water and acetonitrile to give solvent 3 (methanol/water/acetonitrile, 93:5:2, v/v/v). However, with a C18 column this solvent gave very long run times with consequent peak broadening and diminished resolution, but it was found that a C8 column gave good separations in a much shorter time. The use of these three solvent systems allowed all coeluting species to be separated by at least one of the solvents. As little as 10 μg of material per run was used routinely. Typical separations are shown in Figure 1.

Each solvent system has slightly different characteristics. Solvent 1 separates according to the PUFA on C2 but does not resolve the saturated species from the monoene two carbons longer so that certain pairs such as 14:0/22:6 + 16:1/22:6 and 16:0/22:6 + 18:1/22:6 are unresolved. Solvent 2 separates these pairs with the monoene species running faster but unfortunately gives other coeluting pairs, e.g., 14:0/20:5 + 16:1/22:6 and 16:0/20:5 + 18:1/22:6. Solvent 3 separated all these pairs but there were other coeluting species later in the profile, e.g., 18:0/20:4 + 20:1/22:6.

Comparison of the fatty acid compositions of the phospholipids deduced from the HPLC molecular species analysis with those found by GLC of fatty acid methyl esters shows good agreement and validates the method (Table 1). The molecular species analysis often underestimates the minor fatty acids, e.g., 16:1, 18:2, 20:1 and 22:5, since small peaks containing these species can be unresolved from the large peaks of the major species.

Cod roe PC was found to comprise four main molecular species, with 18:1/20:5, 16:0/20:5, 18:1/22:6 and 16:0/22:6 making up two thirds of the sample, the last species contributing a third of the PC (Table 2). While a further 30 minor species were detected, 16:1/22:6 (4.7%) and 16:0/18:1 (4.9%) were the only other species present at greater than 2.6%. It was assumed in all the separations here that the more unsaturated fatty acid was on position 2 and it appeared that solvent systems 1 and 2 should, in some cases, be able to separate some 1,2 positional isomers,

TABLE 1

Comparison of the Fatty Acid Compositions Found by GLC and HPLC

	Mol % composition					
	PC		PE		PI	
	GLC	HPLC	GLC	HPLC	GLC	HPLC
14:0	1.8	2.1	1.2	0.6	0.3	tr
16:0	31.4	27.2	13.0	14.1	10.4	8.2
16:1(n-7 + n-9)	3.9	3.7	2.1	3.6	1.4	tr
18:0	1.7	2.4	5.4	3.5	21.2	24.5
18:1(n-7 + n-9)	14.7	16.1	21.8	22.1	22.4	16.1
18:2(n-6)	0.7	0.5	1.3	nd	nd	nd
20:1(n-7 + n-9)	1.0	0.8	5.4	5.3	2.5	1.4
20:4(n-6)	2.8	3.4	2.9	3.6	23.2	30.0
20:5(n-3)	14.1	12.9	16.0	16.3	7.7	7.9
22:5(n-3)	1.8	2.2	2.2	2.9	1.2	1.0
22:6(n-3)	26.2	29.1	28.7	27.9	9.6	10.8

The fatty acid composition of PC, PE and PI from cod roe found by GLC of FAME were converted from weight % to mol % using correction factors calculated from the molecular weights of the methyl esters relative to 16:0. The HPLC molecular species analysis gave area % which equates to mol % with a UV detection system. The fatty acid compositions were all corrected to 100%. tr = <0.1%; nd = not detected.

TABLE 2

Molecular Species Composition of PC, PE and PI from Cod Roe

	PC (mol %)	PE (mol %)	PI (mol %)
1. 20:5/20:5	0.5 ± 0.1	0.3 ± 0.1	—
2. 20:5/22:6 22:6/20:5	0.6 ± 0.2	0.8 ± 0.1	—
3. 22:6/22:6	1.3 ± 0.2	1.5 ± 0.3	—
4. 14:0/20:5	0.8 ± 0.2	tr	—
5. 16:1/20:5	1.1 ± 0.2	2.3 ± 0.1	tr
6. 14:0/22:6	2.1 ± 0.2	0.9 ± 0.1	tr
7. 16:1/22:6	4.7 ± 0.2	1.8 ± 0.1	tr
8. 14:0/20:4	0.6 ± 0.1	tr	—
9. 16:1/20:4	1.1 ± 0.2	0.3 ± 0.1	—
10. 14:0/22:5	0.8 ± 0.2	tr	—
11. 16:1/22:5	0.4 ± 0.1	2.0 ± 0.1	—
12. 16:1/18:2	tr	—	—
13. 16:0/20:5	15.2 ± 0.4	8.5 ± 0.6	2.6 ± 0.2
14. 18:1/20:5	6.1 ± 0.5	12.8 ± 0.6	5.1 ± 0.3
15. 16:0/22:6	31.0 ± 0.8	14.8 ± 0.4	5.9 ± 0.2
16. 18:1/22:6	14.9 ± 1.0	25.7 ± 0.7	9.4 ± 0.4
17. 16:0/20:4	2.6 ± 0.1	3.2 ± 0.1	6.2 ± 0.1
18. 18:1/20:4	1.9 ± 0.1	2.2 ± 0.2	16.7 ± 0.3
19. 16:0/22:5	0.5 ± 0.1	1.0 ± 0.2	1.3 ± 0.2
20. 18:1/22:5	1.3 ± 0.1	2.8 ± 0.2	0.4 ± 0.1
21. 16:0/18:2	tr	—	—
22. 18:1/18:2	tr	—	—
23. 18:0/20:5	0.9 ± 0.1	2.5 ± 0.2	7.5 ± 0.7
24. 20:1/20:5	0.3 ± 0.1	4.6 ± 0.2	0.6 ± 0.1
25. 18:0/22:6	1.7 ± 0.2	3.5 ± 0.4	4.8 ± 0.2
26. 20:1/22:6	0.6 ± 0.1	5.2 ± 0.5	1.6 ± 0.2
27. 18:0/20:4	0.7 ± 0.1	0.7 ± 0.3	36.7 ± 2.0
28. 20:1/20:4	—	0.7 ± 0.3	0.5 ± 0.3
29. 18:0/22:5	1.0 ± 0.2	—	tr
30. 20:1/22:5	0.3 ± 0.1	—	—
31. 18:0/18:2	tr	—	—
32. 20:1/18:2	tr	—	—
33. 16:0/16:0	—	—	tr
34. 16:0/18:1	4.9 ± 0.1	0.7 ± 0.1	0.2 ± 0.1
35. 18:1/18:1	1.1 ± 0.1	—	0.2 ± 0.1
36. 18:0/16:1	—	0.3 ± 0.1	—
37. 18:0/18:1	0.4 ± 0.1	—	—
38. 20:1/18:1 18:1/20:1	0.3 ± 0.1	—	—
39. 18:0/18:0	tr	—	—

The errors are given as ±1 standard deviation, rounded to the nearest decimal place (see text). For most of the minor species this considerably over estimates the error. It was assumed that the most saturated fatty acid was on the 1 position of the glyceride. tr = <0.2%; — = not detected.

TABLE 3

The Favored 1- and 2-Position Fatty Acids in Phosphatidylinositol from Cod Roe

	2-Position fatty acid (%)		
1-Position fatty acid	20:4(n-6)	20:5(n-3)	22:6(n-3)
16:0	38.7	16.2	36.9
18:0	74.9	15.3	9.8
18:1	52.2	15.9	29.4
	1-Position fatty acid (%)		
2-Position fatty acid	16:0	18:0	18:1
20:4(n-6)	10.3	61.1	27.8
20:5(n-3)	16.5	47.5	32.3
22:6(n-3)	27.2	22.1	43.3

e.g., 16:0/22:6 from 22:6/16:0, but not 16:0/18:1 from 18:1/16:0. In cod roe PC only 63.4% of species had a saturated fatty acid on C1, 34.1% had a monoene and 2.4% a PUFA. Of the 30 minor species detected, the most interesting were the diPUFA species 20:5/20:5, 20:5/22:6, 22:6/20:5 and 22:6/22:6 totalling 2.4% with the last species being the most important. Molecular species analyses are increasingly finding these highly unsaturated species in a variety of samples including fish muscle PC (4) and phosphoglycerides from bovine retina (15). The intriguing presence of diPUFA molecular species is contrary to earlier ideas on phospholipid structure, and their properties and role in biomembrane function remains to be elucidated.

In PE from cod roe the same four molecular species (16:0/20:5, 18:1/20:5, 16:0/22:6, 18:1/22:6) contributed over 60% of the total but their relative contributions were different, reflecting the greater abundance of 18:1 in PE. The major species contributing a quarter of the total sample was 18:1/22:6 (Table 2). In PE 18:0 and 20:1 species were much more important, totalling 17.2% compared with only 4.2% in PC. In PE only 36.5% of species contained a saturated fatty acid on C1, 60.9% held a monoene and again there was a small amount of diPUFA species totalling 2.6%. No alkenyl species were detected suggesting that PE plasmalogen was not present in this sample. Alkenyl species were readily separated in a sample of bovine brain PE containing about 50% plasmalogen.

It has long been known that mammalian PI is rich in 18:0 and 20:4(n-6) (16), and more recently this was also shown to be the case in fish tissues rich in (n-3)PUFA (10,17). It is therefore not surprising that 18:0/20:4 should be the major molecular species in PI as has been shown in several mammalian tissues (6,16). However, this appears to be the first molecular species analysis of PI from a tissue rich in (n-3)PUFA. The specificity of PI for 20:4(n-6), and especially the predominance of the 18:0/20:4 species is impressive. In PI 36.7% is this species while PC and PE each had only 0.7% (Table 2).

Table 3 shows the favored pairings for the six main fatty acids in PI. Phosphatidylinositol clearly shows a selectivity for an 18 carbon fatty acid on the C1 position and a 20 carbon PUFA on the C2 position. Thus 74.9% of 18:0 is paired with 20:4(n-6) while 61.1% of the 20:4(n-6) is paired with 18:0. Palmitic, stearic and oleic acids all pair with about 15% of 20:5(n-3) on C2, but 22:6(n-3) is selectively paired with 16:0 or 18:1, the major species in PC and PE. Almost half the 20:5(n-3) is paired with 18:0 but only 22% of the 22:6(n-3) is with 18:0. Thus the pairing 18:0/22:6 is not favored and apparently selected against in PI, while in PC and PE there is no such specificity.

The unique molecular species composition of PI, most clearly shown in a tissue rich in (n-3)PUFA, reflects the pivotal metabolic role of PI in signal transduction through the biomembrane (18). In marine species rich in (n-3)PUFA, PI is clearly the main source of arachidonic acid for eicosanoid biosynthesis, while in terrestrial animals this role can also be filled by PC or PE plasmalogen (19,20). The extent to which the 18:0/20:5 species could be involved in such signalling activity is presently unclear both in fish and especially in humans, where dietary supplementation with fish oils to elevate the (n-3)PUFA content of tissue phospholipids is currently the basis for alleviating some circulatory and inflammatory disorders (21,22).

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The Suppression of Eicosanoid Synthesis by Peritoneal Macrophages Is Influenced by the Ratio of Dietary Docosahexaenoic Acid to Linoleic Acid

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The efficacy of docosahexaenoic acid (DHA) in suppressing eicosanoid synthesis by peritoneal macrophages *in vivo* was influenced by the relative amount of dietary DHA when linoleic acid (LA) was held constant. Increasing DHA from 0 to 37% of the fatty acids in diets containing 10 weight % fat (of which LA was 40%) caused an eleven-fold increase in the DHA content of macrophages. Limited retroconversion of DHA to eicosapentaenoic acid was observed. Macrophages in animals consuming DHA synthesized significantly lower amounts of leukotriene E_4, prostaglandin E_2 and 6-keto prostaglandin $F_{1\alpha}$ *in vivo* upon stimulation with zymosan. The maximum inhibition of eicosanoid synthesis was observed when the dietary DHA/LA ratio was 0.16 and no further inhibition occurred when the ratio was increased up to 0.81.
Lipids 24, 589-593 (1989).

Leukotrienes (LT) influence inflammatory and immune functions by regulating extravasation, chemotaxis and degranulation of neutrophils (1-3). Many inflammatory diseases such as arthritis, bowel disease, lupus and psoriasis are exacerbated by a higher production of LT by monocytes-macrophages and polymorphonuclear leukocytes (4,5). The synthesis of LT by these cells is influenced by the fatty acid composition of membrane lipids which can be altered by dietary fats (6,7). LT production is enhanced in cells enriched in n-6 polyunsaturated fatty acid (n-6 PUFA) (6). However, enrichment with n-3 PUFA suppresses LT and prostanoid production (6,7). A number of studies have shown that the diets containing high levels of fish oils containing n-3 PUFA can decrease LT production by neutrophils and monocytes and ameliorate inflammatory diseases, e.g., arthritis, lupus and psoriasis (8-11).

However, no information is available concerning the minimum amount of dietary n-3 PUFA required to suppress LT synthesis in subjects on regular diets though this may be affected by the concurrent intake of other fatty acids, especially linoleic acid.

In a previous study, we demonstrated that the eicosanoid synthesis in mouse macrophages was responsive to dietary fatty acids (6,12) and that dietary DHA was as effective as EPA in suppressing eicosanoid synthesis by macrophages in response to an inflammatory stimulus using an *in vivo* model system (13). Therefore, using this system we examined the effects of the increasing amounts of dietary DHA at a fixed intake of linoleic acid on the synthesis of eicosanoids by mouse peritoneal macrophages *in vivo*.

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Abbreviations: AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoate; HPLC, high performance liquid chromatography; HPTLC, high performance thin-layer chromatography; LA, linoleic acid; LT, leukotrienes; PGF, LTE, PGE, eicosanoids; PUFA, polyunsaturated fatty acid.

MATERIALS AND METHODS

Materials. The ethyl esters of docosahexaenoic acid (>95.0% pure) and linoleic acid (>99.5% pure) were purchased from Nucheck Prep (Elysian, MN). Tristearin and triolein were purchased from ICN Biochemicals, Cleveland, OH. Zymosan A (lot #81F-7721) was purchased from Sigma Chemical Company, St. Louis, MO. The zymosan (10 mg/ml) was boiled in 0.9% NaCl for 60 minutes. The boiled zymosan was suspended in 0.9% NaCl (2 mg/ml) and used as an inflammatory stimulus for the release of eicosanoids from peritoneal macrophages (6,14). Leukotriene standards were obtained from Merck Frosst, Montreal, Canada. Radioimmunoassay kits for quantification of prostaglandins (prostaglandin E_2, 6-keto prostaglandin $F_{1\alpha}$) were purchased from Seragen, Inc., Boston, MA. Analytical grade solvents were used for the extraction of lipids and eicosanoids.

Animals. Male mice (CD-1 type) weighing 18-20 g were purchased from Charles River, Wilmington, MA, and housed in groups of five per cage. The animal room was maintained at 22°C with a 12-hour light-dark cycle.

Diets. The fat-free diet was purchased from ICN Nutritional Biochemicals (Cleveland, OH). The diets were thoroughly mixed with ethyl esters of LA (4 g/100 g diet) and then supplemented with 0, 0.4, 0.8 and 4.0 g of ethyl esters of DHA and designated Diet I-IV, respectively. All diets contained 10% fat by weight. Diet I contained 40% of the total fat as LA ethyl ester and 0% DHA. Diets II, III and IV also contained 40% of the total fat as LA ethyl ester and in addition contained 4, 8 and 37% DHA ethyl esters, respectively. The ratio of DHA ethyl ester to LA ethyl ester in Diets I, II, III and IV were 0, 0.08, 0.16, 0.81, respectively and on a weight basis; this approximately corresponded to 0:1, 1:10, 1:5 and 1:1 for DHA/LA in the diets.

The total fat content of the diet was made up to 10 wt% by adding a mixture of tristearin and triolein (1:1 mix). The diets were adequately balanced with AIN-76 mineral mix and AIN-76-vitamin mix (containing 250 IU of vitamin E/g diet) as described previously (12). The diets were thoroughly mixed after the addition of each component, and small amounts (30 g) were transferred to Whirl-Pak plastic bags, flushed with nitrogen and stored at −70°C in the dark. The mice received a fresh diet every day. There was no negligible oxidation of dietary fats as measured by thiobarbituric acid reactive materials and by the analysis of fatty acids (15). The fatty acid composition of the experimental diets is shown in Table 1.

Mice were fed a fat-free diet for 1 week prior to being placed on the experimental diets which were fed to the mice for 14 days to obtain maximum incorporation of n-3 PUFA into cellular phospholipids (12).

Quantification of eicosanoids in mouse peritoneum. The mouse peritoneum was used as an *in vivo* model for quantifying eicosanoid synthesis by peritoneal macrophages

TABLE 1

Fatty Acid Composition of Dietary Fats Fed to Experimental Mice for 14 Days (Weight %)

Fatty acid	Diet I (wt %)	Diet II (wt %)	Diet III (wt %)	Diet IV (wt %)
14:0	0.88	0.72	0.61	—
16:0	3.60	3.34	3.42	1.02
16:1	2.31	2.09	1.92	0.59
18:0	7.32	7.59	7.46	4.32
18:1	29.40	26.70	24.63	8.75
18:2n-6	56.10	54.42	52.72	45.98
22:5n-6	—	0.32	0.44	1.85
22:6n-3	—	4.31	8.46	37.42
22:6n-3/ 18:2n-6 ratio	0	0.08	0.16	0.81

following stimulation with zymosan as an inflammatory agent (6,14). Mice were lightly anaesthetized with ether, and 0.5 ml zymosan (2 mg) in 0.9% NaCl was injected intraperitoneally. After 30 min, the mice were killed by ether inhalation. The peritoneum was flushed with 2 ml of 0.9% NaCl and the contents were quantitatively removed with a Pasteur pipette. The cells in the peritoneal extracts were then sedimented by centrifugation and the clear supernatant was aspirated and adjusted to pH 3 with 3% formic acid; 2 ml methanol was added and the total volume was made up to 10 ml with deionized distilled water for the extraction of leukotrienes (16). The leukotrienes (total recovery was 60%) in the 20% methanolic extract were isolated by C18 reversed phase columns [Sep Pak, Waters, Milford, MA]. The leukotrienes were then separated by HPLC (Waters, Milford, MA) using a Supelco ODS reversed phase column and a mobile phase of methanol/water (75:25, v/v) in 5 mM ammonium acetate buffer (pH 5.6) containing 1 mM EDTA (16). Leukotriene E_4, which was the only lipoxygenase product detected under the conditions used (6), was quantified by specific absorbance at 280 nm using a photodiode array detector (Hewlett Packard, PA) as described previously (6,16). The prostaglandins were extracted from the peritoneal medium using equal volumes of ethyl acetate (3 times) and quantified by radioimmunoassay as described (17). The PGE_2 antiserum had a cross reactivity of less than 1% with TXB_2, 6-keto $PGF_{1\alpha}$, 5- and 12-HETE and arachidonic acid. The 6-keto $PGF_{1\alpha}$ antiserum showed a cross reactivity of 0.6% with PGE_2 and less than 0.1% with TXB_2, 5- and 12-HETE and arachidonic acid, respectively.

Fatty acid analysis. The peritoneal cells recovered by centrifugation were suspended in 0.8 ml of 0.9% NaCl and sonicated at 4°C for 60 seconds in 3 × 20 second bursts with an ultrasonic sonicator (model W-10 Ultrasonics, Plainview, NY). Lipids were extracted from the homogenates by the method of Bligh and Dyer (18). The total phospholipids were separated from neutral lipids by HPTLC using chloroform/methanol (8°;10, v/v) (19). Lipids were saponified with 0.5 N KOH in methanol and the fatty acids were methylated with diazomethane. Fatty acid methyl esters were separated by gas chromatography (5880A, Hewlett Packard, PA) using a 0.75 mm by 60 meter SP2330 capillary column (Supelco, Inc., Bellefonte, PA) with hydrogen as carrier gas. Fatty acids were identified by comparing retention times with fatty acid methyl ester standards and quantified using pentadecanoic acid as an internal standard (20).

Statistical methods. Results were statistically evaluated by SAS using the General Linear Models procedure in the Tukey's mean separation test (SAS Institute, Chapel Hill, NC).

RESULTS AND DISCUSSION

All the animals consumed a similar amount of food (approx. 5.5 g/day/mouse) and showed similar weight gains (approx. 7.4 g/mouse) during the study. The number of resident peritoneal cells obtained from these mice was also similar (4.8 to 50.0 × 10^6 cells/mouse) between each dietary treatment.

Lipid analysis. The concentration of DHA in the phospholipids of the peritoneal macrophages increased in the mice receiving increased amounts of dietary DHA (Fig. 1). Thus, DHA increased by 8.1-, 9.5- and 11.5-fold as the DHA content in the diet was increased from zero to 4%, 8% and 37% of the dietary fat respectively. This was accompanied by a concurrent decrease of 21%, 49% and 68% in the arachidonic acid (AA, 20:4n-6) levels in phospholipids (Fig. 1). Docosatetraenoic (22:4n-6) acid was also decreased by 8%, 54% and 77% respectively. However, increasing dietary DHA (Diest I-IV) did not affect the concentration of LA nor oleic acid in cellular lipids (Table 2).

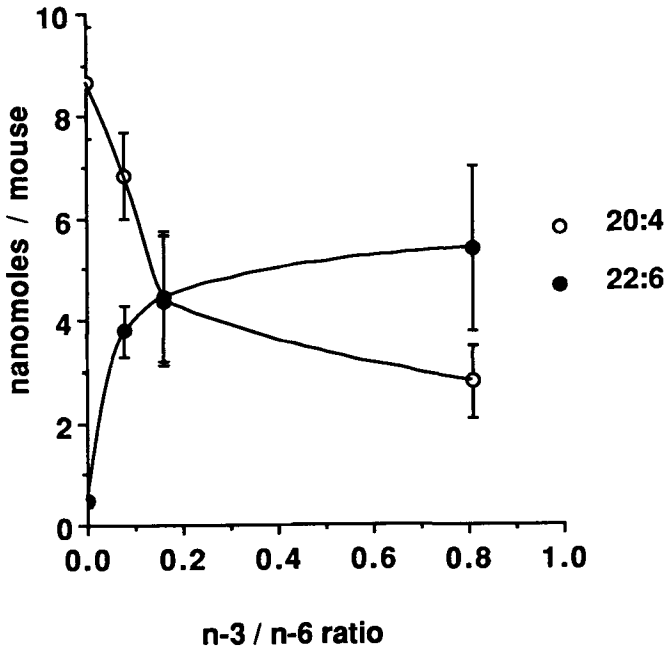

FIG. 1. The relative concentration of arachidonic acid (20:4n-6) and docosahexaenoic acid (22:6n-3) in phospholipids extracted from peritoneal cells of mice consuming diets with increasing concentration of docosahexaenoic acid for 14 days. Mice were maintained on the experimental diets with varying ratios of n-3/n-6 polyunsaturated fatty acids for 2 weeks. The macrophage lipids were isolated and fatty acids were analyzed by gas chromatography. Results are mean ± SEM (n = 4).

TABLE 2

Fatty Acid Composition of Phospholipids of Macrophages Isolated from the Peritoneum of Mice Consuming a Diet with Differing Ratios of Dietary Polyunsaturated Fatty Acids for 14 Days[a]

Fatty acid	Diet I	Diet II	Diet III	Diet IV
		(nanomoles/mouse)		
14:0	0.07 ± 0.39	0.75 ± 0.25	0.28 ± 0.05	0.76 ± 0.27
16:0	6.64 ± 0.57	9.08 ± 0.67	7.83 ± 0.74	8.66 ± 1.89
16:0	0.79 ± 0.08	0.06 ± 0.11	1.18 ± 0.19	1.00 ± 0.16
18:0	5.95 ± 0.91	10.03 ± 0.85	7.56 ± 2.22	6.90 ± 1.54
18:1n-9	3.50 ± 0.54	5.56 ± 0.58	4.58 ± 1.11	4.32 ± 1.09
18:2n-6	2.22 ± 0.43	4.56 ± 0.56	3.82 ± 1.01	3.77 ± 1.07
20:3n-6	0.20 ± 0.09^{a,b}	0.56 ± 0.07^{a}	0.34 ± 0.14^{a,b}	0.08 ± 0.08^{b}
20:4n-6	8.67 ± 1.08^{a,b}	7.84 ± 0.85^{a,b}	4.39 ± 1.29^{a}	2.79 ± 0.71^{b}
20:5n-3	0.00^{a}	0.00^{a}	0.11 ± 0.11^{a}	0.53 ± 0.16^{b}
22:4n-6	2.22 ± 0.17^{a}	2.06 ± 0.46^{a}	1.04 ± 0.29^{a,b}	0.51 ± 0.07^{b}
22:5n-3	0.00^{a}	0.46 ± 0.05^{a,b}	0.64 ± 0.22^{b}	0.59 ± 0.15^{b}
22:6n-3	0.47 ± 0.11^{a}	3.88 ± 0.51^{a,b}	4.47 ± 1.27^{a,b}	5.40 ± 1.62^{b}

[a]Results are mean ± SEM (n = 4). Means with different letters in the same row are significantly different ($p < 0.05$).

At relatively high dietary DHA concentrations (Diets III and IV) there was only a small accumulation of 20:5n-3 and 22:5n-3 compared to a significant accumulation of 22:6n-3 in the phospholipids of peritoneal cells. These data indicate that dietary DHA was transferred directly into macrophages without significant retroconversion and that DHA displaced 20:4n-6 from phospholipids. This is consistent with previous observations of murine macrophages following *in vivo* and *in vitro* modification (12,16) and contrasts with rat platelets which take up little DHA (19) and endothelial cells where some retroconversion of 22:6n-3 may occur (21).

Eicosanoid synthesis. When the peritoneal macrophages were stimulated with zymosan *in vivo,* the eicosanoids (LTE_4, PGE_2 and 6-keto $PGF_{1\alpha}$) were released (6). However, the amount of eicosanoids released by the cells was affected by the dietary n-3/n-6 PUFA ratio (Fig. 2). When the dietary DHA/LA ratio increased from 0 to 0.08 (Diet II), LTE_4 synthesis was decreased by 50% (Fig. 2A) and when the ratio was further increased to 0.16 (Diet III), LTE_4 synthesis was decreased by 67%. Further increases in the dietary n-3/n-6 PUFA ratios, up to 0.81 (Diet IV), did not cause any further decrease in LTE_4 synthesis. This also reflected the ratios of 22:6n-3/20:4n-6 in the macrophage phospholipids which changed from 0.05 to 0.55, 1.02 and 1.92 corresponding to decreases of 50%, 67% and 73% respectively, in LTE_4 synthesis by peritoneal cells.

Prostaglandin synthesis was also depressed by dietary DHA, i.e., PGE_2 decreased by 48%, 51% and 51% (Fig. 2B) and 6-keto $PGF_{1\alpha}$ decreased by 60%, 85% and 87% as the dietary n-3/n-6 PUFA ratio was increased from 0 to 0.08, 0.16 and 0.81 respectively (Fig. 2C).

These data indicate that increasing the 22:6n-3/20:4n-6 ratio in cellular lipids caused a decrease in eicosanoid synthesis by peritoneal macrophages. This is consistent with studies where macrophages enriched in DHA *in vitro* synthesized lower amounts of eicosanoids, and it confirms the observation that dietary DHA is capable of depressing macrophage eicosanoid synthesis *in vivo* (13). Neither the 3-series prostaglandins nor the 5-series leukotrienes were detected in any of these assays. This may reflect the low concentrations of EPA in the macrophages and the fact that negligible amounts of these eicosanoids are found in murine tissues.

The dietary DHA may inhibit the synthesis of eicosanoids either indirectly by reducing the availability of 20:4n-6 substrate from eicosanoid specific phospholipid pools and/or directly by inhibiting the respective cyclooxygenase/lipoxygenase systems. The AA content of phospholipids was progressively diminished with increasing incorporation of DHA into the cellular phospholipids (Fig. 1). Macrophage arachidonic acid decreased when the dietary n-3/n-6 PUFA ratio was increased from 0 to 0.16 and was associated with a similar decrease in eicosanoid synthesis by peritoneal macrophages (Fig. 2). However, when the dietary n-3/n-6 PUFA ratio was increased to 0.81 there was a further decrease in 20:4n-6 levels in the phospholipids (Fig. 1) without a concomitant decrease in eicosanoid synthesis (Fig. 2). This indicated that the 20:4n-6 pool utilized for eicosanoid synthesis was maximally altered at a dietary n-3/n-6 PUFA ratio of approximately 0.16. While further increases in the n-3/n-6 ratio reduced 20:4n-6 in the total phospholipid pool, it did not exert any additional depression of eicosanoid synthesis. This may reflect the existence of separate, more stable pools of AA in certain phospholipids which are differently utilized for eicosanoid synthesis (16,22,23).

To determine if the changes in fatty acid composition resulted in the decreased availability of AA from total phospholipids, the concentration of AA retained in macrophage lipids following zymosan stimulation was quantified by measuring its decrease in phospholipids (Fig. 3). The amount of arachidonic acid utilized did not significantly change as the ratio of dietary n-3/n-6 PUFA was increased from 0 to 0.16 (Fig. 3) though the synthesis of eicosanoids was progressively decreased in this range. This indicated that at low concentrations (Diet II), dietary DHA did not affect the release nor utilization of 20:4n-6 from the phospholipid pool but did cause reduced

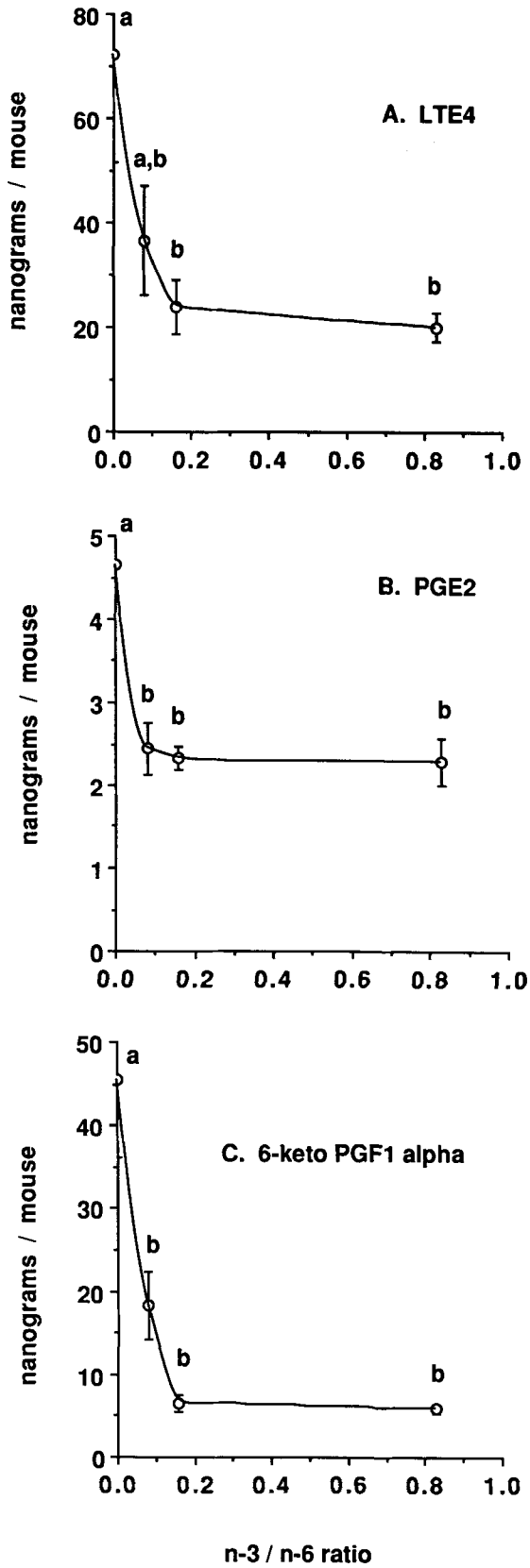

FIG. 2. Eicosanoid production by peritoneal macrophages *in vivo* from mice fed diets with varying ratios of docosahexaenoic/linoleic acid for 14 days. The leukotrienes produced by activated cells were analyzed by HPLC while prostaglandins were analyzed by radioimmunoassay. Results are mean ± SEM (n = 6). Points with different letters above are significantly different ($p < 0.05$).

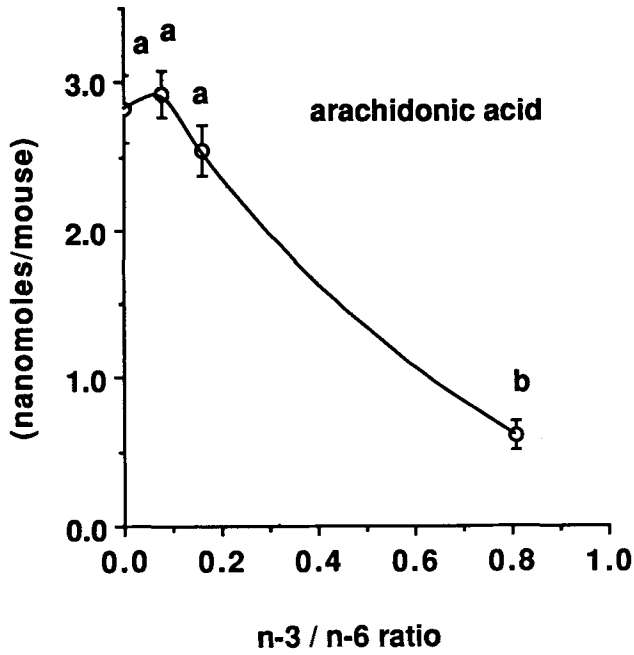

FIG. 3. The relative amount of arachidonic acid in peritoneal macrophages utilized following stimulation with zymosan. Mice were fed the experimental diets with varying ratios of docosahexaenoic/linoleic acid as indicated for 14 days. One group of mice (n = 6) from each dietary treatment was injected with 2 mg zymosan in 0.5 ml saline. Another group on the same treatments (n = 4) was injected with 0.5 ml saline. After 30 minutes the peritoneal cells were isolated, lipids extracted and arachidonic acid remaining in the phospholipid quantified by gas chromatography. The amount of arachidonic acid utilized was calculated from the difference between the concentrations found in zymosan and saline injected animals. Results are mean ± SEM. Points with different letters above are significantly different ($p < 0.05$).

eicosanoid synthesis suggesting that inhibition may have occurred at the cyclooxygenase and lipoxygenase steps. The utilization of AA was more pronouncely decreased at high levels of DHA (Diet IV) though eicosanoid synthesis was not significantly greater than in Diet III.

A reduction in arachidonic acid levels in membrane phospholipids needs not necessarily decrease prostaglandin synthesis, but the presence of n-3 PUFA can significantly decrease eicosanoid production (12,24). Recently Laposata *et al.* (25) demonstrated that eicosanoid production can be decreased in a murine fibrosarcoma cell line (HDSMC) without altering arachidonic acid content. These studies indicate that total arachidonic acid content *per se* may not reflect the capacity of cells to synthesize eicosanoids but rather its concentration in specific pools may determine quantities released to cyclooxygenase and lipoxygenase following specific stimulation.

In summary, dietary DHA was facilely incorporated into peritoneal macrophages and concurrently reduced AA levels in cellular phospholipids. DHA was negligibly retroconverted to 20:5n-3 by peritoneal cells but it significantly decreased eicosanoid synthesis in zymosan-stimulated cells. The extent of inhibition of eicosanoid synthesis was influenced by dietary DHA and maximum diminution occurred at a ratio of dietary DHA/LA of 0.16. The data indicated that DHA may be useful as an antiinflammatory agent for eicosanoid mediated processes and

emphasize the need for more research to define the appropriate intakes of specific dietary fatty acids to ensure balanced eicosanoid synthesis (26,27) and/or amelioration of eicosanoid related pathophysiologies.

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Effects of Omega-3 Fatty Acids on Vascular Smooth Muscle Cells: Reduction in Arachidonic Acid Incorporation into Inositol Phospholipids

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A rapid increase in arachidonic acid incorporation into phosphatidylinositol (PI) occurred following exposure of cultured porcine pulmonary artery smooth muscle cells to calcium ionophore A23187. This response was specific for PI and phosphatidic acid; none of the other phosphoglycerides showed any increase in arachidonic acid incorporation. The incorporation of [^{3}H]inositol also was increased, indicating that complete synthesis of PI rather than only fatty acylation occurred in response to the ionophore. The presence of omega-3 fatty acids, especially eicosapentaenoic acid (EPA), reduced arachidonic acid but not inositol incorporation into PI. Stimulated incorporation of EPA also occurred under these conditions, suggesting that EPA replaces arachidonic acid in the newly synthesized pool of PI. Although much less arachidonic acid was incorporated into the polyphosphoinositides following exposure to the ionophore, arachidonic acid incorporation into these phosphorylated derivatives also decreased when EPA was present. These findings suggest that when omega-3 fatty acids are available, less arachidonic acid is channeled into the inositol phospholipids of activated smooth muscle cells because of replacement by EPA. This may represent a mechanism whereby omega-3 fatty acids, especially EPA, can accumulate in the metabolically active pools of inositol phospholipids and thereby possibly influence the properties or responsiveness of vascular smooth muscle.
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Eicosapentaenoic acid (EPA), an n-3 fatty acid present in fish oils, appears to protect against thrombosis, coronary heart disease and certain inflammatory diseases (1-4). A number of the metabolic effects of EPA probably contributes to this protective action. When increased amounts of EPA are present, less arachidonic acid is converted to thromboxane A_2 in platelets and leukotriene B_4 in leukocytes (5,6). Although some thromboxane A_3 and leukotriene B_5 are formed, these eicosanoids produced from EPA are less potent than the corresponding products derived from arachidonic acid. In addition, administration of EPA lowers the plasma triglyceride concentration (3) and decreases low density lipoprotein formation (7). While these effects may be largely responsible for the protection against coronary heart disease, recent studies suggest that EPA also may have some direct actions on cells contained in the arterial wall.

Endothelial cells have been shown to incorporate EPA and docosahexaenoic acid (DHA, 22:6), and these omega-3 polyunsaturated fatty acids compete with arachidonic acid for entry into endothelial lipids (8-10). When the endothelial cells accumulate EPA or DHA, their capacity to produce prostacyclin (PGI_2) is reduced (8-10). Similar results have been obtained with smooth muscle cells. For example, rabbit aortic smooth muscle cells take up linolenic acid (18:3), EPA and DHA (11), and enrichment of murine aortic smooth muscle cells with EPA reduces their capacity to form PGI_2 (12). No information is presently available as to whether EPA can influence any aspect of endothelial or smooth muscle cell function other than prostaglandin production. This is a potentially important question because endothelial injury and smooth muscle proliferation are two of the central events in atherogenesis (13).

During studies of polyunsaturated fatty acid metabolism in smooth muscle cells, we noted that arachidonic acid incorporation into inositol phospholipids was stimulated immediately after exposure to a calcium ionophore and that this stimulation was reduced by relatively small amounts of EPA. Because of the importance of arachidonic acid in inositol phospholipid metabolism and the role of these phospholipids in modulating cell proliferation (14), we thought it worthwhile to explore this observation. The present report describes this process in porcine pulmonary artery smooth muscle cells, a system that remains well differentiated over ten or more passages in culture as determined by α-actin measurements (15).

METHODS

Cell culture. Porcine pulmonary artery smooth muscle cells were isolated from explants according to the method of Ross (16). The smooth muscle cells were grown in Dulbecco's Minimal Essential Medium (DMEM, Gibco, Grand Island, NY) containing 10% fetal bovine serum (HyClone, Logan, UT) supplemented with MEM nonessential amino acids, MEM vitamins (Gibco) HEPES buffer (Sigma, St. Louis, MO), and gentamycin (50 μg/ml). The morphology and growth pattern of the cells were consistent with a smooth muscle origin. The cells were further characterized as smooth muscle by staining with an anti-α-isoactin; fluorescence microscopy indicated that 99% of cells were stained. Anti-α-isoactin specifically stains muscle actins and does not cross react with the non-muscle actins of endothelium or fibroblasts. In addition a high α/β actin ratio was obtained when proteins extracted from cultures labeled with [^{35}S]methionine were separated by two-dimensional electrophoresis and visualized by autoradiography (17), indicating again that the cells were well differentiated. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

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Abbreviations: EPA, eicosapentaenoic acid; DHA or 22:6, docosahexaenoic acid; PGI_2, prostacyclin (prostaglandin I_2); 18:3, linolenic acid; BSA, bovine serum albumin; PA, phosphatidic acid; TLC, thin layer chromatography; GLC, gas-liquid chromatography; HPLC, high performance liquid chromatography; PC, choline phosphoglycerides; PE, ethanolamine phosphoglycerides; PS, serine phosphoglycerides; PI, inositol phosphoglycerides; PGE_2, prostaglandin E_2; HETE, hydroxyeicosatetraenoic acid; 16:0, palmitic acid; 18:1, oleic acid; PIP, phosphatidylinositol-4-phosphate; PIP_2, phosphatidylinositol-4,5-bisphosphate.

The cells were subcultured weekly by trypsinization, and cells obtained between passages 5 and 12 were seeded into 6-well plates for each experiment.

Incubations. The confluent smooth muscle cultures were washed with serum-free medium and then incubated with serum-free medium containing different radioactive fatty acids, including [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid (95.1 Ci/mmol), [1-^{14}C]arachidonic acid (51.7 mCi/mmol) and [5,6,8,9,11,12,14,15,17,18-^{3}H]EPA (79.0 Ci/mmol), all from New England Nuclear (Boston, MA); and [1-^{14}C]eicosapentaenoic acid (55 mCi/mmol); [1-^{14}C]-palmitic acid (55 mCi/mmol) and [1-^{14}C]linolenic acid (56 mCi/mmol), all from Amersham (Arlington Heights, IL). Non-radioactive fatty acids were obtained from Nu-Chek Prep (Elysian, MN). The fatty acids were complexed with bovine serum albumin (BSA, Fraction V, Miles Scientific, Naperville, IL). Details regarding each experiment are included in the legends to the figures and footnotes to the tables. After incubation, the cells were harvested by removing the medium, washing with cold phosphate-buffered saline, and adding 1 ml cold methanol/acetic acid (99:1, v/v) to each well. For experiments with radioactive inositol, the cells were incubated with Eagle's medium containing 4 μCi/ml myo-[2-^{3}H]inositol (19 Ci/mmol) for 4 hr before exposure to 2 μM ionophore A23187. In additional experiments, the cells were incubated in serum-free medium containing [^{32}P]phosphate (10 μCi/ml) for either 1 or 24 hr prior to addition of the ionophore.

Lipid extraction. The cells were scraped into 3 ml cold methanol/acetic acid (99:1, v/v) with a rubber policeman. Lipids were extracted by addition of 6 ml chloroform and then 2 ml of 0.15 M NaCl containing 4 mM HCl, a modification of the Folch method (18). After vortexing, the phases were separated by centrifugation at 1000 × *g* for 10 min. The lower phase was collected and upper phase was re-extracted with 5 ml of chloroform/methanol/0.15 M NaCl containing 4 mM HCl (85:14:1, v/v/v). This method, which did not cause any breakdown of ethanolamine plasmalogens, was utilized for all lipid extractions except those where only phosphatidic acid (PA) and the polyphosphoinositides were assayed. The combined chloroform extract was dried under N_2 and redissolved in chloroform/methanol (1:1, v/v). Aliquots were mixed with 4.5 ml of Budget Solve scintillation cocktail (Research Products International, Mt. Prospect, IL) for determination of total lipid radioactivity using a Packard Tri-carb 460 liquid scintillation spectrometer. Quenching was monitored with a ^{226}Ra external standard.

Polyphosphoinositides and PA were extracted with chloroform-methanol mixtures containing 2.4 N HCl as described by Schacht *et al.* (19). Since this strongly acidic extraction system caused the breakdown of ethanolamine plasmalogens, it was utilized only in experiments designed specifically to measure these acidic phospholipids. If additional phospholipids were to be assayed, a second aliquot of cells was extracted by the modified Folch procedure described above. Cells were scraped into 2 ml of methanol. After 2 ml of chloroform and 2 ml of 2.4 N HCl were added, the suspension was mixed with a vortex and the phases separated by centrifugation at 100 × *g* for 10 min. After this procedure, the chloroform phase was collected and the upper phase was re-extracted with 2 ml of chloroform. The combined chloroform phases were washed with 4 ml of methanol/1 N HCl (1:1, v/v), dried under N_2 and redissolved in chloroform/methanol (2:1, v/v).

Thin-layer chromatography (TLC). The extracted cell lipids were chromatographed on LK-5D TLC plates (Whatman, Clifton, NJ) using a chloroform/methanol/40% methylamine (60:36:5, v/v/v) solvent mixture. PA was separated on LK-5D plates with a solvent mixture prepared from ethylacetate/2,2,4-trimethylpentane/acetic acid/water (90:50:20:100, v/v/v/v). The lower phase of this mixture was removed, and 1 ml acetic acid added to 100 ml of the upper phase before use (20). Phospholipid standards were added and the chromatogram was visualized under UV light after spraying with 1 mM 8-anilino-1-naphthalene sulfonate. Radioactivity distribution on the TLC plate was determined with a thin-layer chromatography scanner (Radiomatic Instruments Co. Model R), or by subsequently scraping zones of silica gel from the plate into vials containing 5 ml Budget Solve scintillation cocktail and counting in the liquid scintillation spectrometer.

For separation of polyphosphoinositides, TLC was performed on oxalate impregnated LK-5D plates prepared by method of Jolles *et al.* (21). LK-5D plates were impregnated by developing in a 1% potassium oxalate and 2 mM EDTA solution prepared in water/methanol (2:3, v/v). The plates were air-dried for 30 min and activated at 110°C for 30 min before use. After sample application, the plate was developed in chloroform/methanol/4 N NH_4OH (45:35:10, v/v/v). Distribution of radioactivity in the chromatogram was measured as described above.

Gas-liquid chromatography (GLC). Lipids were transesterified by a modification of the method of Morrison and Smith (22) in a mixture containing 1 ml 14% BF_3 in methanol, 1 ml acetonitrile, and 1 ml methanol. After heating at 90°C for 40 min, the fatty acid methyl esters were extracted into heptane and analyzed with a Hewlett-Packard Model 5890A gas chromatograph equipped with a flame ionization detector and a 2 mm × 1.9 m glass column packed with 10% SP 2330 on 100/120 mesh Chromosorb W, Supelco (Bellafonate, PA). N_2 was used as a carrier gas at a flow rate of 25 ml/min and the oven temperature was programmed from 180 to 220°C at the rate of 2°C/min. The fatty acid methyl esters were identified from the retention times of a PUFA-2 standard mixture (Supelco). Peaks were determined with a Hewlett-Packard Model 3380 A integrator-recorder, and the areas are reported as weight percentage.

High performance liquid chromatography (HPLC). Formation of radioactive eicosanoids was measured by HPLC. Following incubation of the cultures for 20 min with either 7.5 μM [1-^{14}C]arachidonic acid or [1-^{14}C]EPA, the medium was collected and acidified to pH 3.5 with 0.1 M citric acid and then extracted with ethyl acetate. This extract was evaporated to dryness under N_2, redissolved in 100 μl of methanol/acetonitrile (1:1) and analyzed with a Beckman 332 HPLC system (Palo Alto, CA) equipped with 4.5 × 150 mm column containing Adsorbosphere C_{18} reverse phase 3 μm spherical packing (Alltech Associates, Deerfield, IL). The elution gradient was 28% to 100% acetonitrile and water adjusted to pH 3.4 with phosphoric acid (23,24). The system was standardized with mixture of known eicosanoids and fatty acids, and the elution of radioactivity was monitored with an on-line Flo-one/β radioactivity detector and software provided by Radiomatic Instruments & Chemical Co. (Tampa, FL).

RESULTS

Comparison of EPA and arachidonic acid utilization. The porcine pulmonary artery cultures take up [1-^{14}C]EPA to about the same extent as [1-^{14}C]arachidonic acid. This is illustrated in Figure 1. The uptake of both fatty acids increased over a 16 hr incubation, with from 2.5- to 3.5-times more fatty acid being incorporated after 16 hr as compared with 4 hr (top panel). Similar amounts of EPA and arachidonic acid were taken up during a 16 hr incubation over the concentration range of 5 to 30 μM (bottom panel).

After a 16 hr incubation with [1-^{14}C]EPA or [1-^{14}C]arachidonic acid, most of the radioactivity was contained in the smooth muscle cell phospholipids. The distribution among the phospholipid classes when the fatty acid concentration in the medium was in the range of 5 to 30 μM is shown in Figure 2. Similar amounts of EPA and arachidonic acid were incorporated into the choline phosphoglycerides (PC), ethanolamine phosphoglycerides (PE), and serine phosphoglycerides (PS). However, from 2- to 3-times less EPA was incorporated into the inositol phosphoglycerides (PI).

Figure 3 illustrates the radioactive products detected in the medium by HPLC when the smooth muscle cells were incubated with either [1-^{14}C]arachidonic acid or [1-^{14}C]EPA. Large amounts of prostaglandin E_2 (PGE_2) were detected when the cultures were incubated with arachidonic acid (upper panel). It is possible that small amounts of several other metabolites also may have been formed, especially in the region of the chromatogram where the 12-hydroxyeicosatetraenoic acid (HETE) standard elutes, but the baseline variability was too large to indicate this with any certainty. By contrast, no major

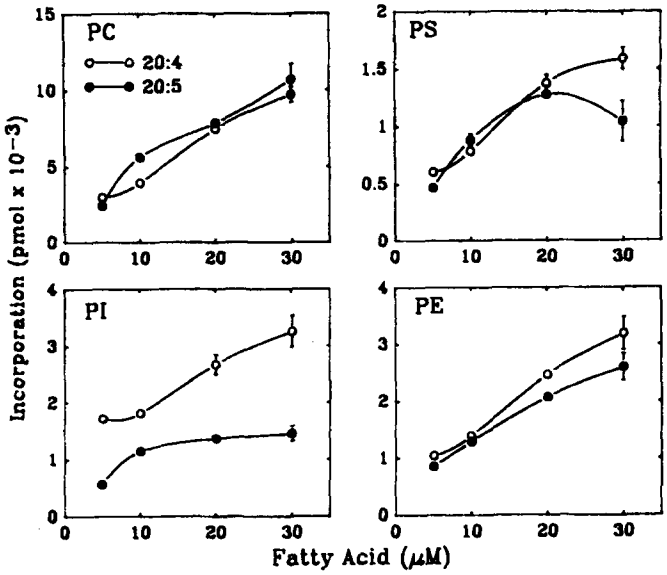

FIG. 2. Comparison of EPA and arachidonic acid incorporation into smooth muscle cell phospholipids. The cultures were incubated for 16 hr in 5 ml of medium containing 1% fetal bovine serum supplemented with varying amounts of fatty acids and 5 μCi [1-^{14}C]-fatty acid. The cell lipids were extracted, and the phospholipids were separated by TLC. Each value is mean ± SE of results obtained from 3 separate cultures.

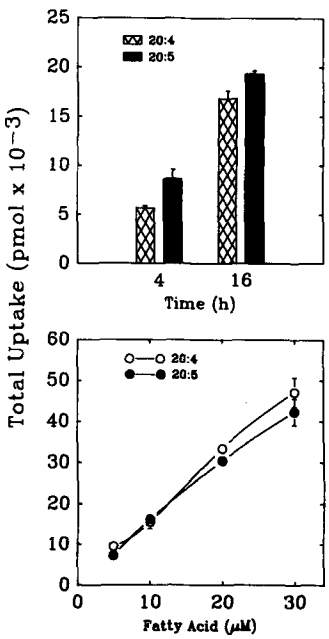

FIG. 1. Comparison of EPA and arachidonic acid uptake by smooth muscle cells. In the upper panel, confluent cultures were incubated with either 10 μM EPA or arachidonic acid in 5 ml medium containing 5 μCi of [1-^{14}C]fatty acid and 1% fetal bovine serum for the indicated periods, and the total radioactivity incorporated into cell lipids was measured. In the lower panel, the cells were incubated with varying amounts of these fatty acids for 16 hr. Each point is the mean ± SE of values obtained from 3 separate cultures. Where the bars are not shown, the SE value is too small to be visible.

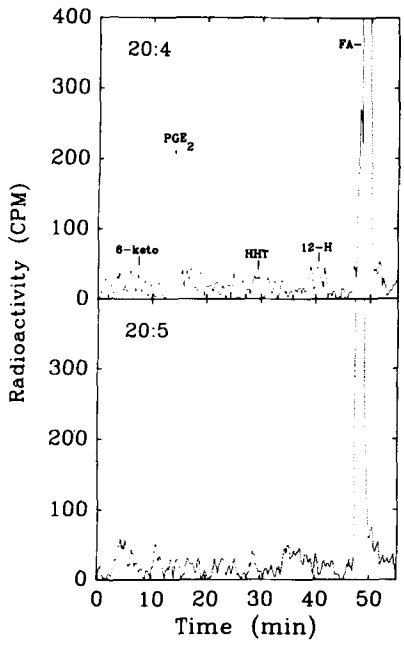

FIG. 3. HPLC analysis of the radioactive eicosanoids formed by smooth muscle cells incubated with labeled arachidonic acid or EPA. The eicosanoids were produced during 20 min incubation of the cultures in 1 ml serum-free medium containing 0.1 μM BSA and 7.5 μM arachidonic acid (top panel) or 7.5 μM EPA (bottom panel) containing 0.4 μCi of the corresponding [1-^{14}C]fatty acid. Retention times of eicosanoid standards are shown on the chromatogram. Abbreviations: 6-keto, 6-keto-prostaglandin $F_{1\alpha}$, the inactivation product of PGI_2; HHT, 12-hydroxy 5,8,10-heptadecatrienoic acid; 12-H, 12-hydroxy 5,8,10,14-eicosatetraenoic acid; PGE_2, prostaglandin E_2.

eicosanoid products were detected in the medium when the cells were incubated with EPA, although it is possible that several of the small peaks may represent values that are slightly above the baseline.

Ionophore-stimulated fatty acid incorporation. Exposure of the smooth muscle cells for short periods to labeled arachidonic acid together with the calcium ionophore A23187 had little effect on the total amount of radioactivity incorporated into the cells. However, the distribution of the radioactivity among the cell lipids was altered substantially. This is seen in Figure 4. In this experiment, the smooth muscle cultures were incubated for 20 min with 1 μM [^{3}H]arachidonic acid. The amount of radioactivity present in the PI fraction was almost 3-times larger when the medium contained the ionophore. By contrast, there was considerably less radioactivity present in PC, PE and neutral lipids when the ionophore was present in the medium.

The increase in labeled arachidonic acid incorporation into PI mediated by the ionophore was observed within 5 min after the start of incubation, the earliest time tested (Fig. 5, top). The magnitude of the increase became greater as the incubation continued. The arachidonic acid concentration in this experiment was 10 μM; this higher concentration was utilized to obtain sufficient incorporation into PI within 5 min to clearly determine whether an increase occurred. This accounts for the larger incorporations into PI in this experiment than those shown in the bottom panel of this figure or in Figure 4, where the concentration was 1 μM.

The magnitude of the increased incorporation into PI after a 20 min incubation with 1 μM arachidonic acid was dependent on the ionophore concentration (Fig. 5, bottom). The maximum increase occurred with 2 μM ionophore, a concentration at which other ionophore-mediated responses such as PGE_2 production occurred maximally. To determine the specificity of the response for ionophore A23187, similar experiments were done with 10 nM bradykinin, 1 U/ml thrombin, 1 μM acetylcholine, 1 μM histamine, 20 pg/ml angiotensin II and 50 pg/ml epinephrine. None of these physiological agonists increased the incorporation of labeled arachidonic acid into PI. Likewise, none of these agonists stimulated PGE_2 production by these smooth muscle cultures (data not shown).

As seen in Figure 6, labeled arachidonic acid incorporation into PA also increased when the smooth muscle cells were exposed to the ionophore. The increase occurred within 5 min and was maintained throughout the remainder of the 20 min incubation.

To examine the specificity of the ionophore-mediated increase in fatty acid incorporation into PI, several radioactive fatty acids were compared. The results are shown in Figure 7. All of the fatty acids tested, including EPA, were incorporated to a greater extent into PI during a 20 min incubation when the ionophore was present. However, only relatively small amounts of 18:3 and palmitic acid (16:0) were incorporated in the basal state, and the magnitude of the increase that occurred in response to the ionophore was small with these fatty acids. By contrast, the magnitude of the stimulated increase with EPA was almost as large as with arachidonic acid.

Figure 8 shows the time dependence of ionophore A23187-mediated enhancement in [^{3}H]EPA incorporation into PI. As in the case of arachidonic acid (Fig. 5), greater EPA incorporation occurred within 5 min, the earliest time tested, when the ionophore was added.

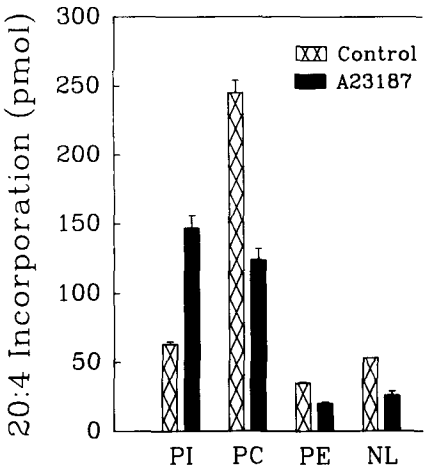

FIG. 4. Effect of ionophore A23187 on arachidonic acid incorporated into smooth muscle cell lipids. Smooth muscle cells were incubated for 20 min in 1 ml serum-free medium containing 1 μM BSA, 10 μM arachidonic acid, 0.2 μCi [^{3}H]arachidonic acid, and 2 μM ionophore A23187. Cell lipids were extracted and analyzed by TLC. Each bar represents the mean ± SE of values obtained from 3 separate cultures; where the error bars are not shown, they are too small to be visible.

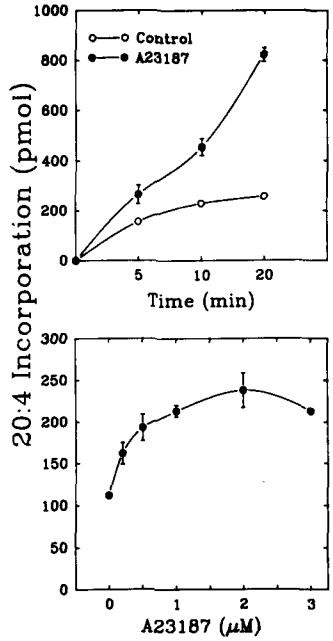

FIG. 5. Time and concentration dependence of ionophore A23187 mediated increase in arachidonic acid incorporation of the smooth muscle cell. In the upper panel, cultures were incubated with 10 μM arachidonic acid in 1 ml serum-free medium containing 0.2 μCi [^{3}H]arachidonic acid and 1 μM BSA, with or without 2 μM ionophore A23187 for the indicated periods. The radioactivity incorporated into PI was measured after TLC separation. In the lower panel, cultures were incubated with 1 μM arachidonic acid, 0.2 μCi [^{3}H]arachidonic acid and 0.1 μM BSA in 1 ml serum-free medium containing varying amounts of ionophore A23187 for 20 min. The radioactivity incorporated into PI was measured. Each point is the mean ± SE of values obtained from 3 separate cultures.

Inositol incorporation. As seen in Figure 9, [^{3}H]inositol incorporation into PI also was increased substantially when ionophore A23187 was added. Supplementation of the medium with either arachidonic acid or EPA did not affect the amount of inositol incorporated in the basal state or when the ionophore was added. In these studies, the cells were incubated with [^{3}H]inositol for 4 hr before exposure to the ionophore. An initial labeling period was necessary in order to get substantial amounts of [^{3}H]inositol into PI during a 20 min incubation with the ionophore. After labeling for 4 hr, the cells contained 116,200 ± 11,700 cpm (n = 3), 98.1% of which was recovered in the aqueous-methanol phase of the chloroform-methanol extract. The cultures were washed and placed in a medium that did not contain any ([^{3}H]inositol before the ionophore was added. Therefore, the increase observed in Figure 9 is due to a direct effect of the ionophore on intracellular inositol incorporation into PI.

Turnover of ^{32}P-labeled phospholipids. To determine the basis for the increased PI formation when the vascular smooth muscle cells were exposed to the ionophore, studies were done using cultures labeled with [^{32}P]phosphate. As seen in Table 1, there was no substantial reduction in the amount of phosphatidylinositol-4′-,5′-bisphos-

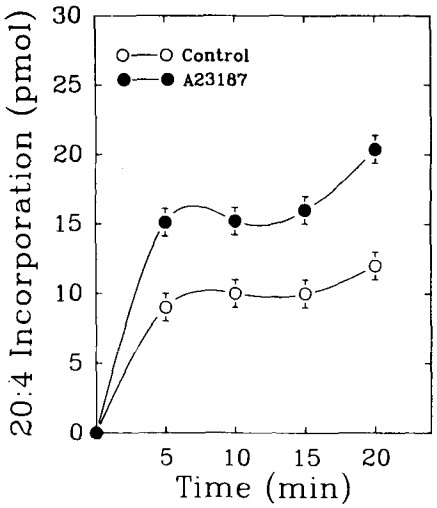

FIG. 6. Ionophore A23187 mediated increase in the incorporation of arachidonic acid into PA. The smooth muscle cultures were incubated in 1 ml of serum-free medium containing 1 μM arachidonic acid and 0.5 μCi [^{3}H]arachidonic acid. Radioactivity incorporated into PA was determined following lipid extraction with the acidic solvent mixture (19) and separation by TLC (20). Each value is the mean ± SE of results obtained from 3 separate cultures.

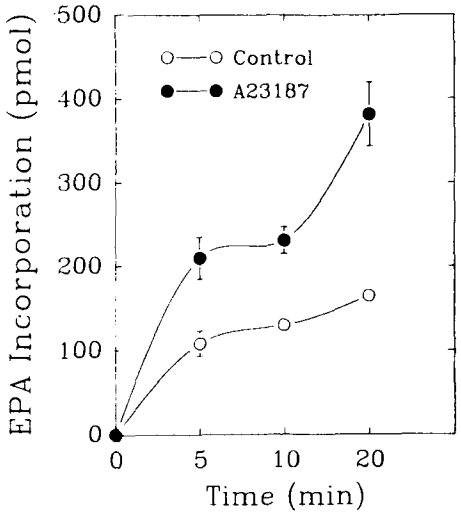

FIG. 8. Time dependence of ionophore A23187-mediated increase in EPA incorporation into PI. Cultures were incubated in 1 ml serum-free medium containing 10 μM EPA, 0.55 μCi [1-^{14}C]EPA, 1 μM BSA and 2 μM ionophore A23187. The radioactivity contained in PI was measured after TLC separation. Each point is mean ± SE of values obtained from 3 separate cultures.

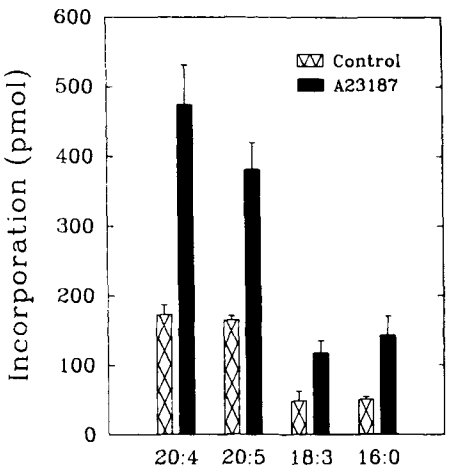

FIG. 7. Ionophore A23187 mediated increase in the incorporation of different fatty acids into smooth muscle cell PI. Cultures were incubated for 20 min in 1 ml serum-free medium containing 0.55 μCi of the corresponding [1-^{14}C]fatty acids, 1 μM BSA, 10 μM fatty acids, and 2 μM ionophore A23187. The radioactivity in PI was determined after TLC separation. Each bar is mean ± SE of values obtained from 3 separate cultures.

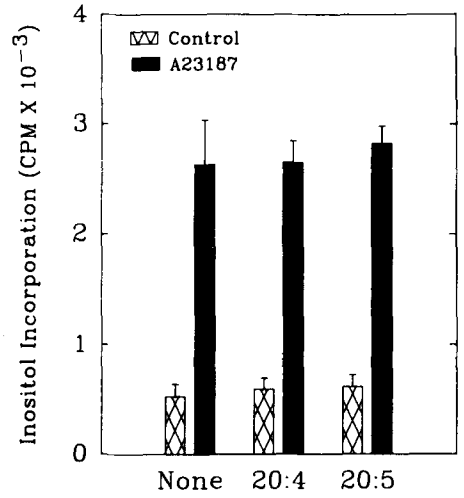

FIG. 9. Effect of ionophore A23187 on inositol incorporation into smooth muscle cell PI. Confluent cultures were incubated with 4 μCi [2-^{3}H]inositol in 1 ml Eagle's medium. After 4 hr, this medium was removed, the cells were washed and then incubated for 20 min in 1 ml serum-free medium containing 2 μM ionophore A23187. Where indicated, 10 μM arachidonic acid or EPA also were present in the medium. Each bar is the mean ± SE of values obtained from 3 separate cultures.

TABLE 1

Changes in ^{32}P Content of Cell Phospholipids Following Exposure to Ionophore A23187

Exp.[a]	Time of incubation with A23187 (min)	Radioactivity (cpm)[b]			
		PIP_2	PIP	PI	PA
1	0	17530 ± 1000	8670 ± 300		
	0.25	15960 ± 1150	9300 ± 510		
	0.50	17670 ± 220	12300 ± 710		
	1	19910 ± 2860	12600 ± 760		
2	0			4290 ± 280	530 ± 40
	20			5430 ± 560	1380 ± 90

[a] In experiment 1, the smooth muscle cultures were labeled for 24 hr in 1 ml serum-free medium containing 30 μC [^{32}P]phosphate before exposure to the ionophore. In experiment 2, the cultures were labeled for 1 hr with 10 μC [^{32}P]phosphate. The ionophore A23187 concentration was 2 μM.

[b] Each value is the mean ± SE of results obtained from three separate cultures.

phate (PIP_2) radioactivity during the first min after exposure of the labeled cells to the ionophore, and the amount of radioactivity contained in phosphatidylinositol-4′-phosphate (PIP) increased during this time. Increases in the radioactivity contained in PI and PA also were observed 20 min following exposure of [^{32}P]phosphate-labeled cells to the ionophore.

Inhibition produced by EPA. The stimulated incorporation of [^{3}H]arachidonic acid into PI that occurred when ionophore A23187 was present decreased when EPA was added. This is illustrated in Figure 10. The magnitude of the reduction was dependent on the concentration of EPA. To determine whether the effect was specific for EPA, several other fatty acids were compared at a concentration of 5 μM. As shown in Figure 11, the decrease produced by DHA in ionophore stimulated [^{3}H]arachidonic acid incorporation was similar to that produced by EPA. Lesser reductions occurred when 18:3, oleic acid (18:1) or 16:0 were added.

In addition to reducing the ionophore-stimulated incorporation of [^{3}H]arachidonic acid into PI, the presence of EPA also decreased incorporation of [^{3}H]arachidonic acid into PIP and PIP_2. This is shown in Table 2. Much more [^{3}H]arachidonic acid was incorporated into PI than either PIP or PIP_2 during the 20 min incubation. However, the percentage decrease in [^{3}H]arachidonic acid incorporation that occurred when EPA was added was roughly similar in each of the three fractions: 20% in PI, 33% in PIP and 26% in PIP_2.

PI fatty acid composition. GLC analysis of the PI fraction was performed to determine whether the arachidonic acid (20:4) content changed appreciably when the smooth muscle cells were activated in the presence of EPA. As shown in Table 3, there was little change in PI fatty acid composition 20 min after the ionophore A23187 was added, and the percentage of 20:4 decreased only slightly. No EPA (20:5) was detected in PI in the basal state or following addition of the ionophore. Similarly, the fatty acid composition did not change appreciably 20 min after addition of the ionophore when 10 μM EPA was present, and there was no significant reduction in the 20:4 percentage as compared with the cells exposed to the ionophore alone. However, the PI fraction accumulated 2.1% 20:5 within 20 min after exposure to the ionophore when the medium contained EPA.

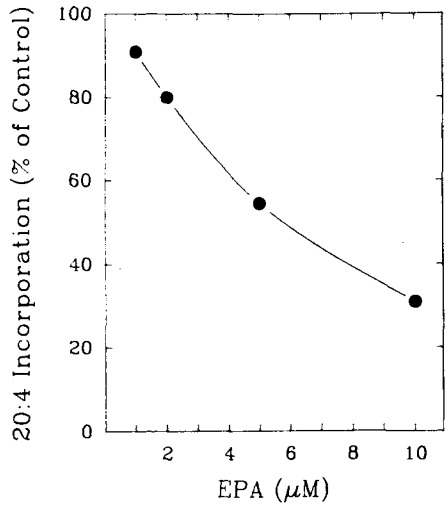

FIG. 10. Reduction in the ionophore A23187 mediated incorporation of [^{3}H]arachidonic acid into smooth muscle cell PI by the presence of increasing concentration of EPA. Smooth muscle cultures were incubated for 20 min in 1 ml serum-free medium containing 1 μM arachidonic acid, 0.2 μCi [^{3}H]arachidonic acid, varying amounts of EPA and 2 μM ionophore A23187. The radioactivity in PI was determined after TLC separation. Each point is mean of values obtained from 3 separate cultures; the SE bars are too small to be visible.

DISCUSSION

These studies indicate that exposure of vascular smooth muscle cells to a calcium ionophore causes an increase in inositol phospholipid synthesis. What role this response plays in smooth muscle function is not presently understood. Since the response occurs rapidly and persists for at least 20 min following exposure to the ionophore, it probably is involved in the recovery phase of the metabolic cycle that follows calcium-mediated activation. Because the incorporation of fatty acids, inositol and phosphate into PI is increased, as well as arachidonic acid and phosphate into PA, complete synthesis of PI must occur rather than fatty acylation of a lysophospholipid precursor. It is questionable as to whether the increase in PI formation is secondary to activation of the PI cycle, for studies with the cells labeled with [^{32}P]phosphate indicate that there is no substantial reduction in either PIP or PIP_2 radioactivity immediately following exposure to the ionophore.

Although there was an increase in the incorporation of other fatty acids into PI, the largest increase occurred with arachidonic acid. This is consistent with the fact that the inositol phospholipids contain relatively large amounts of arachidonic acid (25). When the vascular

TABLE 2

Effect of EPA on [^{3}H]Arachidonic Acid Incorporation into Smooth Muscle Cell Inositol Phospholipids in response to ionophore A23187

Inositol phospholipids	[^{3}H]Arachidonic acid incorporation (dpm)[a]		
	No added fatty acid	EPA added	P
Phosphatidylinositol	159,700 ± 2,270	127,340 ± 5,230	<0.001
Phosphatidylinositol-4-phosphate	6,860 ± 990	4,570 ± 240	<0.05
Phosphatidylinositol-4,5-bisphosphate	3,130 ± 190	2,330 ± 63	<0.01

[a]Cells were incubated for 20 min in 1 ml serum-free medium containing 1 μM arachidonic acid, 1 μCi [^{3}H]arachidonic acid, 0.1 μM BSA, 2 μM ionophore A23187 and where indicated, 5 μM EPA. Each value is the mean ± SE of the radioactivity contained in 3 separate cultures.

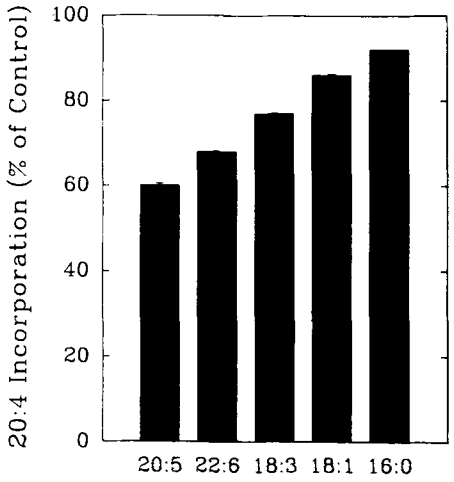

FIG. 11. Comparison of the reductions in ionophore A23187 mediated incorporation of [^{3}H]arachidonic acid into smooth muscle cell PI produced by the presence of various fatty acids. Confluent cultures were incubated for 20 min in 1 ml serum-free medium containing 1 μM arachidonic acid, 0.2 μCi [^{3}H]arachidonic acid, 5 μM additional fatty acids where indicated, and 2 μM ionophore A23187. The radioactivity incorporated into PI was measured after TLC separation. Each bar represents the mean ± SE of values obtained from 3 separate cultures.

TABLE 3

Changes in the Fatty Acid Composition of Smooth Muscle Cell PI After Exposure to Ionophore A23187

Fatty acids	Fatty acid composition (%)[a]		
	Control	A23187	A23187 + EPA
14:0	0.5 ± 0.1	0.8 ± 0.6	1.8 ± 0.5
16:0	7.8 ± 0.3	11.1 ± 0.4[b]	8.9 ± 1.3
16:1 (n-7)	1.7 ± 0.1	2.4 ± 0.1	2.4 ± 0.6
18:0	37.0 ± 0.9	36.7 ± 1.2	32.4 ± 0.6[b]
18:1 (n-9)	11.3 ± 0.3	11.9 ± 0.3	10.9 ± 1.2
18:2 (n-6)	0.9 ± 0.9	0.2 ± 0.2	nd
20:4 (n-6)	18.1 ± 0	16.4 ± 0.3[b]	15.8 ± 0.9[b]
20:5 (n-3)	nd[c]	nd	2.1 ± 0.2[d]
22:4 (n-6)	1.6 ± 0.6	1.8 ± 0.4	1.6 ± 0.1
22:6 (n-3)	4.2 ± 0.8	4.1 ± 0.4	3.3 ± 0.6

[a]Confluent smooth muscle cells were incubated for 20 min in 1 ml serum-free medium containing 2 μM ionophore A23187, or this ionophore and 10 μM EPA. After extracting the total lipids, PI was separated by TLC, transesterified and analyzed by GLC. Only the major fatty acids are listed. Each value is the mean ± SE of results obtained from 4 separate cultures.

[b]P <0.05, as compared with the control.

[c]Not detected, <0.2%.

[d]P < 0.001, as compared with the control.

smooth muscle cells were exposed to omega-3 fatty acids during activation by the calcium ionophore, however, the amount of arachidonic acid incorporated into PI was substantially reduced. By contrast, no decrease in the amount of labeled inositol incorporated into PI occurred. Therefore, the presence of EPA does not reduce the amount of PI that was formed; rather, EPA replaces some of the arachidonic acid that otherwise would be incorporated into the newly synthesized PI. This indicates that EPA effectively competes with arachidonic acid for entry into the inositol phospholipids during the period of accelerated synthesis even though in the basal state, considerably less EPA than arachidonic acid is incorporated into PI (Fig. 2).

The stimulated arachidonic acid incorporation is specific for PA and the inositol phospholipids; all of the other major phosphoglycerides retained less arachidonic acid when the ionophore was added. A possible explanation is that by raising the intracellular calcium concentration, the ionophore caused the activation of a calcium-dependent phospholipase A_2 (26). This would result in increased hydrolysis of fatty acid present in the *sn*-2 position of phosphoglycerides that are substrates for phospholipase A_2, so that much of the arachidonic acid incorporated into these fractions would be almost immediately hydrolyzed. This would account for less of the labeled arachidonic acid accumulating in PC and PE, which ordinarily are substrates for phospholipase A_2. According to this hypothesis, the smooth muscle PI and PA either are poor substrates for the activated phospholipase A_2, or the ionophore-mediated increase in the rate of PI synthesis exceeds any hydrolysis of the newly incorporated arachidonic acid that may occur. Such a mechanism would account for the specificity of the arachidonic acid accumulation in PI.

Even though the amount of arachidonic acid incorporated into PIP and PIP_2 was relatively small after exposure to the ionophore, less incorporation also occurred in these fractions when EPA was present. Moreover, the percentage reductions in arachidonic acid incorporation into PIP and PIP_2 were similar to the percentage decrease observed in PI. This suggests that the PIP and

PIP_2 are formed in response to calcium-mediated activation and are derived from the newly formed PI. Therefore, they also are likely to contain less arachidonic acid if vascular smooth muscle cells are exposed to elevated amounts of omega-3 fatty acids when they are activated.

The function of arachidonic acid in inositol phospholipid metabolism is still uncertain. A number of studies indicate that the inositol phospholipids which undergo rapid turnover following exposure to agonists are highly enriched in arachidonic acid (27-32). Yet, it appears that diacylglycerol does not require arachidonic acid to activate protein kinase C (33,34) or to be incorporated into PI (35,36). It is possible that the high arachidonic acid content is related to eicosanoid synthesis, for prostaglandins can be formed in some systems from the arachidonic acid contained in the diacylglycerol generated by the PI cycle (37-40). Some indirect evidence also suggests that the presence of arachidonic acid may facilitate the hydrolysis of PIP_2 by phospholipase C (41). Therefore, it is possible that replacement of arachidonic acid by EPA in the PI could alter certain functional responses that are specifically dependent on arachidonic acid.

A small decrease in the 20:4 content of the cellular PI was observed by GLC 20 min after exposure to the calcium ionophore. However, no further reduction in 20:4 was noted when the vascular smooth muscle cells were activated in the presence of EPA (Table 2). Therefore, the amount of PI synthesized in response to calcium-mediated activation must represent only a very small fraction of the total PI in the cell. This is consistent with the idea that a small pool of PI highly enriched in arachidonic acid turns over rapidly when cells are activated (26-31). If the arachidonic acid content of this rapidly turning over pool is more extensively modified than indicated by the GLC analysis, which is a measure of the total cellular PI, the potential for an altered functional response may be greater than predicted from the GLC data.

Competition between arachidonic acid and EPA for incorporation into cellular lipids, including PI, has been observed previously. For example, simultaneous incubation of human platelets with 10 μM arachidonic acid and EPA reduced the incorporation of each fatty acid into PI by about 50%, as compared with the incorporation that occurred when either 10 μM arachidonic acid or EPA was incubated with the platelets alone (42). Likewise, the incorporation of 10 μM arachidonic acid into bovine aortic endothelial cell phospholipids, including PI, was reduced progressively when the EPA concentration was raised from 10 to 75 μM, and conversely, EPA incorporation was reduced by the addition of arachidonic acid (9). However, these reductions occurred in the basal state. To our knowledge, the present results with vascular smooth muscle cultures are the first demonstration that competition between arachidonic acid and EPA also occurs during the period after calcium-mediated activation.

In the studies measuring inositol incorporation (Fig. 9), the cells initially were labeled with [^{3}H]inositol and then transferred to another medium before the ionophore was added. Therefore, the greater incorporation into PI cannot be secondary to an effect of the ionophore on inositol transport into the cells; it must be due to increased incorporation of intracellular inositol. Our interpretation is that the larger inositol incorporation signifies an increase in PI synthesis. This is consistent with the increase in [^{32}P]phosphate incorporation into PI following addition of the ionophore (Table 1). However, the percentage increase in [^{3}H]inositol incorporation was considerably greater than the increment in [^{32}P]phosphate incorporation. Because of this, it is possible that some of the [^{3}H]inositol increase mediated by the ionophore could result from a base exchange reaction with existing PI rather than complete synthesis of PI.

Since all of the present results were obtained with ionophore A23187, it is questionable as to whether increased PI formation actually would occur when vascular smooth muscle cells are activated under physiologic conditions. To evaluate this, other agonists were tested, including bradykinin, thrombin, acetylcholine, histamine, angiotensin II and epinephrine. None of these physiologic agonists produced any effect on arachidonic acid incorporation. However, they also did not stimulate PGE_2 production. The failure to obtain a prostaglandin response suggests that as a result of culture, these smooth muscle cells probably have lost the capacity to respond to physiologic agonists. Therefore, the negative results obtained with these agonists do not necessarily mean that the PI effect is an unphysiologic response.

In summary, the present results suggest that inositol phospholipid synthesis may be increased in vascular smooth muscle following calcium-dependent activation. Less 20:4 is incorporated into the inositol phospholipids that are formed if omega-3 fatty acids, especially EPA, are available when the cells are activated. Under these conditions, EPA appears to replace arachidonic acid in the metabolically active pool of inositol phospholipids. Whether this has any effect on the functional properties of the vascular smooth muscle cell is presently being investigated.

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Effect of α-Tocopherol on the Volatile Thermal Decomposition Products of Methyl Linoleate Hydroperoxides

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α-Tocopherol and 1,4-cyclohexadiene were tested for their effect on the thermal decomposition of methyl linoleate hydroperoxide isomers. The volatiles generated by thermolysis in the injector port of a gas chromatograph at 180°C were analyzed by capillary gas chromatography. In the presence of either α-tocopherol or 1,4-cyclohexadiene, which are effective donors of hydrogen by radical abstraction, volatile formation decreased in all tests, and significant shifts were observed in the relative distribution of products in certain hydroperoxide samples. When an isomeric mixture of methyl linoleate hydroperoxides (*cis,trans* and *trans,trans* 9- and 13-hydroperoxides) was decomposed by heat, the presence of α-tocopherol and 1,4-cyclohexadiene caused the relative amounts of pentane and methyl octanoate to decrease and hexanal and methyl 9-oxononanoate to increase. A similar effect of α-tocopherol was observed on the distribution of volatiles formed from a mixture of the *trans,trans* 9- and 13-hydroperoxides. This effect of α-tocopherol was, however, insignificant with pure *cis,trans* 13-hydroperoxide of methyl linoleate. The decrease in total volatiles with the hydrogen donor compounds, α-tocopherol and 1,4-cyclohexadiene, indicates a suppression of homolytic β-scission of the hydroperoxides, resulting in a change in relative distribution of volatiles. The increase in hexanal and methyl 9-oxononanoate at the expense of pentane and methyl octanoate in the presence of hydrogen donor compounds supports the presence of a heat-catalyzed heterolytic cleavage (also known as Hock cleavage), which seems to mainly affect the *trans,trans* isomers of linoleate hydroperoxides.
Lipids 24, 603-608 (1989).

Quite a bit of work has been reported on the volatile oxidation products of unsaturated fats because they cause cellular damage in the body and deterioration of foods (1-4). However, the complicated degradative processes in oils have remained poorly understood. Although fatty ester hydroperoxides are the recognized precursors of volatile secondary products from lipids, the origin of many important degradative products remains obscure.

Two major mechanisms of volatile production from lipid hydroperoxides include a homolytic scission through an alkoxyl radical (also known as β-cleavage), and an acid-catalyzed heterolytic scission (also known as Hock cleavage) (3). A comparative study of the two types of mechanisms has been recently reported (5). Heterolytic cleavage occurs selectively between the carbon bearing the hydroperoxide group and the allylic double bond (4-7). This reaction produces hexanal and 12-oxo-10-dodecenoic acid from the 13-hydroperoxide of linoleic acid, and 2-nonenal and 9-oxononanoic acid from the 9-hydroperoxide. Lewis acids or protic acids in aprotic solvents appear to promote this cleavage (6,7). Protic acids in protic solvents, on the other hand, tend to cause a rearrangement with lower production of aldehydes (8,9). Volatiles formed by thermal decomposition are more diverse, and can usually be explained by homolytic cleavage on either side of the alkoxyl radical derived from the hydroperoxides (3,10). This mechanism predicts the formation of pentane, hexanal, and 13-oxo-9,11-tridecadienoic acid from the 13-hydroperoxide of linoleic acid, and 2,4-decadienal, methyl octanoate, and methyl 9-oxononanoate from the 9-hydroperoxide. Other radical reactions lead to the formation of relatively minor amounts of additional volatiles.

A number of studies have shown an effect of different factors on the decomposition of hydroperoxides. An early report indicated that α-tocopherol and other antioxidants, when tested between 0.01 and 0.98%, accelerate the thermal anaerobic decomposition of hydroperoxides in oxidized lard (11). Various tocopherols were reported to have different effects on the stability of linoleic acid hydroperoxides (12). With α- and γ-tocopherol, the *trans,trans* 13-hydroperoxide isomer decomposes more slowly than the *cis,trans* 13-hydroperoxide isomer; with δ-tocopherol the difference in decomposition rates between the two hydroperoxide isomers is slight. In the copper-catalyzed decomposition of linoleic acid hydroperoxides, α-tocopherol and butylated hydroxyanisole decreased hexanal and increased 2,4-decadienal formation from the 9-hydroperoxide isomer, but these antioxidants have no effect on the corresponding aldehydes from the 13-hydroperoxide isomer (6). Hexanal may form either after rearrangement of the 9-hydroperoxide to the 13-hydroperoxide (13), or after oxidative decomposition of 2,4-decadienal (14). The antioxidants are thought to stabilize unsaturated aldehydes against further oxidation. In another study, the copper-catalyzed oxidation of butterfat in the presence of α-tocopherol promoted the formation of dienals, which cause fishy flavors (15). The mechanism for this effect of α-tocopherol was not explained.

The literature on decomposition of lipid hydroperoxides does not yet provide a clear understanding of the pathways by which the majority of volatile products are formed from different hydroperoxides and how these pathways are influenced by antioxidants. The present study was aimed at clarifying how α-tocopherol affects the decomposition pathways of methyl linoleate hydroperoxide isomers.

MATERIALS AND METHODS

Preparations. Mixtures of methyl *cis,trans/trans,trans* 9- and 13-linoleate hydroperoxides were made by the autoxidation of pure methyl linoleate with oxygen at 40°C and purified by silicic acid chromatography (16). The methyl *trans,trans* 9- and 13-linoleate hydroperoxide isomers were separated from this mixture by semi-preparative high performance liquid chromatography with a reversed phase C-18 column (25.0 × 2.14 mm, 5 microns), using a 70:30 mixture of acetonitrile:water (v/v) at a flow rate

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Abbreviations: EI, electron impact; GC, gas chromatography; UV, ultraviolet.

of 3.0 ml per min and monitored with a UV detector at 235 nm. The *cis,trans* isomers were eluted between 40 and 44 min, and the *trans,trans* isomers between 44 and 52 min. The solutions were concentrated on a rotating evaporator until cloudy and then extracted with hexane; the last portion of water was removed from this hexane extract by azeotropic vacuum distillation with absolute acetone. Pure methyl *cis,trans* 13-linoleate hydroperoxide was prepared by soy lipoxygenase oxidation of linoleic acid, followed by silicic acid column chromatography (17) and esterification of the purified hydroperoxide with diazomethane.

α-Tocopherol was purchased from Eastman Kodak Co. (Rochester, NY), methyl hexanoate from Sigma Chemical Co. (St. Louis, MO), and 1,4-cyclohexadiene from Chemical Samples Co. (Columbus, OH).

Reaction chromatography. To study the decomposition products of methyl linoleate hydroperoxides, a hexane solution of hydroperoxides was subjected to thermolysis in the injector port of a gas chromatograph held at 180°C. The volatile decomposition products generated were trapped in a capillary column, cooled at −65°C, and separated and identified by gas chromatography (GC). Reaction chromatography is commonly used for the study of volatile precursors (3), and provides a convenient and direct analysis of the decomposition products of fatty hydroperoxides. However, this technique varies according to the conditions used for decomposition of hydroperoxides, and more variability in the data can be expected than in standard GC analyses.

Capillary gas chromatography. A Perkin-Elmer gas chromatograph (Model Sigma 300, Norwalk, CT) was used with a capillary column (DB-5, 60 m × 0.315 mm, 1 micron film, J & W Scientific Co., Folsom, CA), cooled to −65°C with liquid nitrogen. After an initial hold of 5 min, the column temperature was programmed to 260°C at 5°C per min with a final hold of 20 min. In a typical run, a one microliter injection was made from a 200 microliter hexane solution containing 13.1 mg methyl linoleate hydroperoxides, 2.4 mg α-tocopherol (15 wt % α-tocopherol), and 1.17 mg methyl hexanoate as internal standard. Total volatiles were calculated as percent of peak areas of volatiles relative to the peak area of the internal standard methyl hexanoate. The relative standard deviations of duplicate GC analyses ranged between 4–5%. Identities of the decomposition products were confirmed by EI mass spectrometry (10,18).

RESULTS

The presence of α-tocopherol affected both the total amount and the relative distribution of individual volatiles formed after thermal decomposition of methyl linoleate *cis,trans* and *trans,trans* 9- and 13-hydroperoxides. When added to linoleate hydroperoxides in different proportions, α-tocopherol decreased the amount of total volatiles as compared to the control (Table 1). The relative distribution of major volatiles formed was also affected by α-tocopherol. The most evident effect was an increase in hexanal and methyl 9-oxononanoate, and a decrease in pentane and methyl octanoate (Fig. 1).

To test the possibility that α-tocopherol was affecting volatile formation by its ability to donate hydrogen by a free radical mechanism, and because α-tocopherol is a weak acid, we designed experiments to test the effect of 1,4-cyclohexadiene, which is well-known for its hydrogen-donor properties (19). As observed with α-tocopherol, we found that 1,4-cyclohexadiene decreased the total volatiles and shifted their relative distribution (Table 1). Pentane and methyl octanoate decreased in relative concentrations, and hexanal and methyl 9-oxononanoate increased correspondingly (Fig. 2).

When α-tocopherol was added to pure *cis,trans* 13-hydroperoxide, the total volatile areas decreased, as seen before with the hydroperoxide isomeric mixture, but the relative distributions of pentane, hexanal, methyl octanoate, and 9-oxononanoate remained constant (Table 2). Linear regression plots of these four volatiles vs the concentration of α-tocopherol gave plots not significantly different from zero (plots not shown). The probabilities of a zero slope were 0.05, 0.01, 0.00, and 0.02 [coefficient of determination (R^2) of 0.49, 0.75, 0.95, and 0.65] for pentane, hexanal, methyl octanoate and 9-oxononanoate, respectively. This unexpected finding that the volatile

TABLE 1

Effect of α-Tocopherol and 1,4-Cyclohexadiene on the Volatile Thermal Decomposition Products of Mixed Isomers of Methyl Linoleate Hydroperoxides, *cis,trans* and *trans,trans* 9- and 13-Hydroperoxides

	Relative percent[a]											
	α-Tocopherol (wt %)								1,4-Cyclohexadiene (wt %)			
Major volatiles	0	11	15	26	30	33	39	58	31	62	95	97
Pentane	16	10	10	9.7	7.8	8.8	6.7	7.1	9.0	9	9.5	12
Hexanal	11	17	16	13	16	22	16	18	17	17	18	16
Me Octanoate	17	13	12	12	11	12	10	10	13	13	13	3.2
2,4-Decadienal	23	23	22	24	23	23	24	20	21	20	20	25
Me 9-oxononanoate	13	18	17	15	20	20	17	23	18	20	20	24
Me 13-oxo-9,11-tridecadienoate	20	19	24	26	22	14	26	22	22	21	20	20
Total peak areas[b]	16	15	7.7	9.2	12	6.9	7.8	6.5	5.5	7.2	5.5	5.0

[a] Individual volatiles are reported as percent relative to the sum of six major volatiles observed. Relative standard deviations from duplicate GC analyses ranged between ±3.9 and ±4.8%.

[b] Relative to that of Me hexanoate used as internal standard.

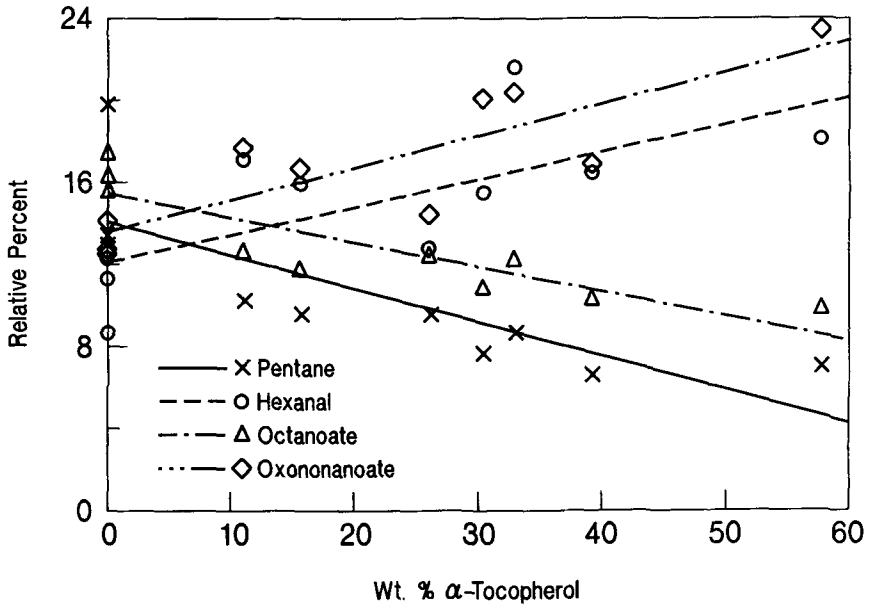


FIG. 1. Effect of α-tocopherol on the formation of methyl 9-oxononanoate, hexanal, methyl octanoate, and pentane from the thermal decomposition of *cis,trans* and *trans,trans* 9- and 13-linoleate hydroperoxides. The probabilities of zero slopes for the linear regression plots of pentane, hexanal, methyl octanoate, and 9-oxononanoate were <0.01, 0.03, <0.01, and <0.01 (R^2: 0.64, 0.47, 0.79, and 0.70), respectively.

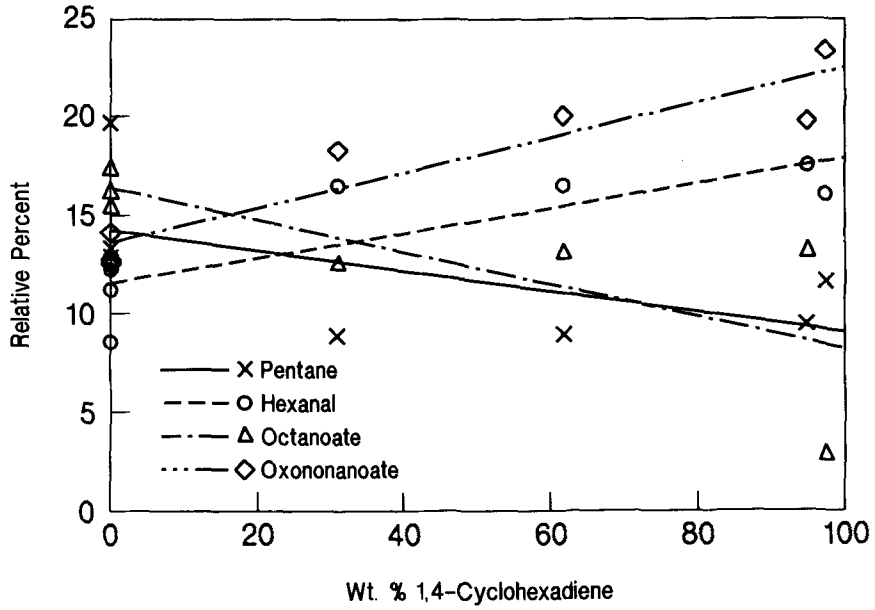


FIG. 2. Effect of 1,4-cyclohexadiene on the formation of methyl 9-oxononanoate, hexanal, methyl octanoate, and pentane from the thermal decomposition of *cis,trans* and *trans,trans* 9- and 13-linoleate hydroperoxides. The probabilities of zero slopes for the linear regression plots of pentane, hexanal, methyl octanoate, and 9-oxononanoate were <0.16, 0.02, 0.05, and <0.01 (R^2: 0.36, 0.68, 0.57, and 0.87), respectively.

distribution formed from pure *cis,trans* 13-hydroperoxide was little affected by α-tocopherol suggests that the *trans,trans* hydroperoxides in the previous isomeric mixture may have been responsible for the observed results. To test this hypothesis, the *trans,trans* hydroperoxide isomers separated from the autoxidation mixture of 9- and 13-linoleate hydroperoxides were also investigated. The effect of α-tocopherol with pure *trans,trans* 9- and 13-hydroperoxides showed the same trend as with the corresponding mixture of *cis,trans* and *trans,trans* 9- and 13-hydroperoxides. With added α-tocopherol, total peak areas decreased and the volatile distribution changed

TABLE 2

Effect of α-Tocopherol on the Volatile Thermal Decomposition Products of Different Isomers of Methyl Linoleate Hydroperoxides, Relative Percent[a]

	α-Tocopherol (wt %)													
	cis,trans-13-hydroperoxide								*trans,trans* 9 + 13-hydroperoxides					
Major volatiles	0	9.4	11	21	35	38	39	48	0	10	11	18	31	52
Pentane	21	23	22	20	22	20	22	24	1.5	2.4	1.9	2.3	1.1	5.6
Hexanal	20	15	17	18	13	14	17	22	13	12	17	16	13	26
Me octanoate	5.0	5.3	3.6	4.1	3.4	6.0	3.1	5.4	13	13	15	9.8	8.3	4.6
2,4-Decadienal	3.5	4.7	2.1	4.4	18	6.9	1.9	5.7	30	32	28	27	29	10
Me 9-oxononanoate	4.3	4.4	3.2	3.9	2.8	5.4	2.9	3.7	26	26	30	30	30	47
Me 13-oxo-9,11-tridecadienoate	46	48	52	50	40	48	53	39	16	15	8.7	15	18	6.3
Total peak areas[b]	2.2	0.77	0.71	0.93	0.54	0.34	0.35	0.33	2.3	1.5	1.3	1.0	0.99	0.77

[a]Individual volatiles are reported as percent relative to the sum of six major volatiles observed. Relative standard deviations from duplicate GC analyses ranged between ±3.9 and ±4.8%.

[b]Relative to that of Me hexanoate used as internal standard.

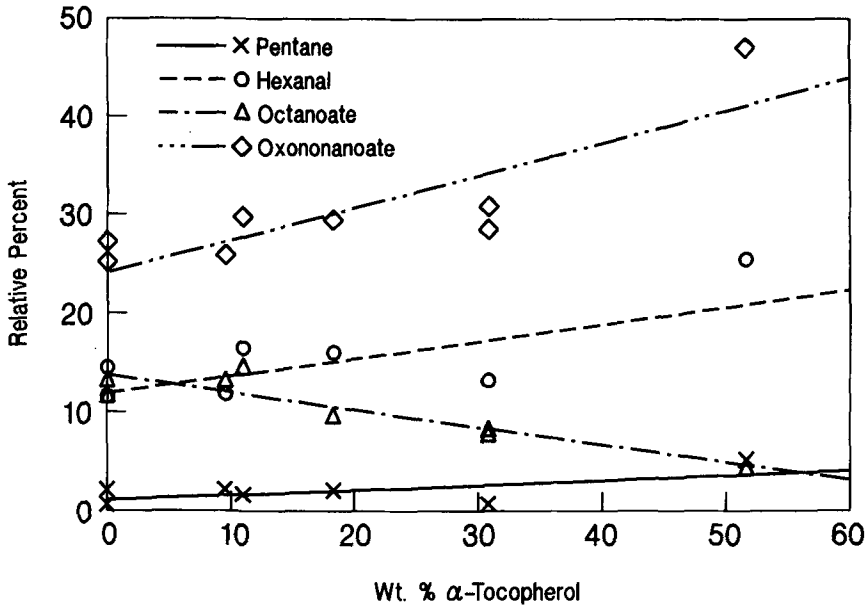


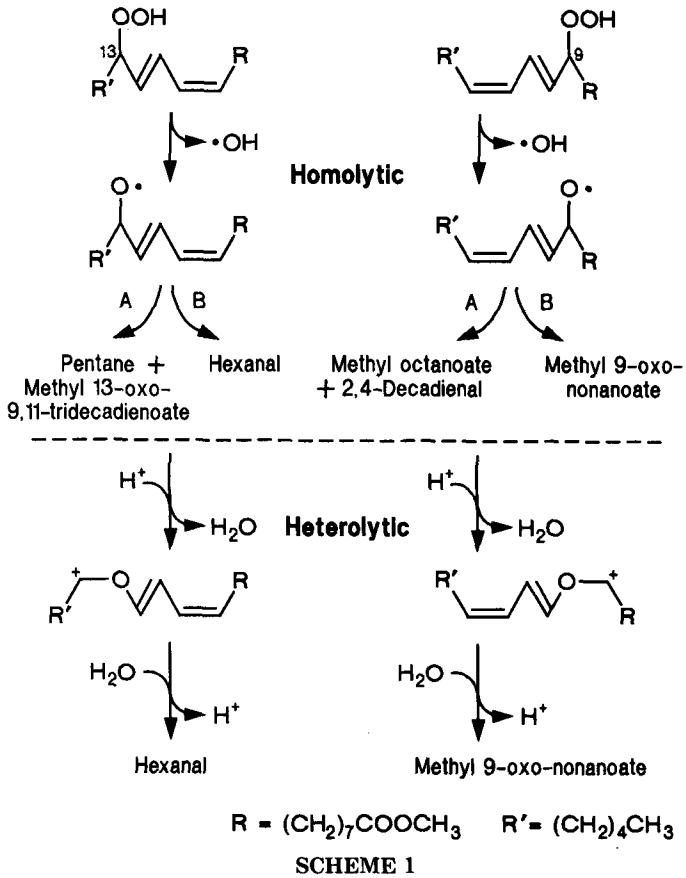
FIG. 3. Effect of α-tocopherol on the formation of methyl 9-oxononanoate, hexanal, methyl octanoate, and pentane from the thermal decomposition of *trans,trans* 9- and 13-linoleate hydroperoxides. The probabilities of zero slopes for the linear regression plots of pentane, hexanal, methyl octanoate, and 9-oxononanoate were 0.13, 0.05, <0.01, and <0.01 (R^2: 0.34, 0.49, 0.82, and 0.72), respectively.

(Table 2). 9-Oxononanoate and hexanal increased in relative concentrations, and pentane and methyl octanoate decreased (Fig. 3). This evidence suggests that the *trans,trans* isomers were indeed responsible for the observed change in volatile composition as a function of added hydrogen-donors.

DISCUSSION

The decrease in total volatiles observed in this study in the presence of α-tocopherol and 1,4-cyclohexadiene support the hypothesis that these compounds inhibit β-scission of an alkoxyl radical to furnish volatiles by their ability to donate a readily abstractable hydrogen. To clarify the mechanism of hydroperoxide decomposition, α-tocopherol and 1,4-cyclohexadiene were used in this study in relatively large concentrations as hydrogen donors to suppress alkoxyl radicals and not as antioxidants, which suppress peroxyl radicals. The total hydrogen donating ability of the medium, a cumulative effect, has been used by Porter *et al.* (20) to suppress peroxyl radicals in their kinetic studies, and hydrogen donation has been the subject of other similar mechanistic studies.

If α-tocopherol and 1,4-cyclohexadiene were suppressing radical formation, their effects may be explained by a partial shift in the mechanism of volatile formation. In



SCHEME 1

Scheme 1 the principal cleavage mechanisms are illustrated with the *cis,trans* 9- and 13-hydroperoxides. The observed increase in hexanal and methyl 9-oxononanoate at the expense of pentane and methyl octanoate (Figs. 1 and 2), and the overall decrease in volatiles (Tables 1 and 2), can be explained if the hydrogen donors block the homolytic pathways A and B without affecting heterolytic pathways. Although pathway B is a possible contributor to the observed shift in volatile distribution, it is energetically less favorable because the heat of formation and the related bond dissociation energy required for the formation of a vinyl radical is larger than the formation of the hydrocarbon radical by pathway A (7). A "mixed" homolytic-heterolytic type mechanism was previously postulated to avoid the formation of unfavorable vinyl radicals by homolytic pathway B (5). Heat used in our experiments may have accelerated the action of the hydroperoxide group as an incipient acid to promote the participation of heterolytic processes. This mechanism is supported by the work of Hamberg and Gotthammar (21), who showed that the 13-hydroperoxide of linoleic acid could be heterolytically transformed into the threo isomer of 12,13-epoxy-11-hydroxy-9-octadecenoic acid by heat (100°C) in ethanol-water solvent and in the absence of added acid catalyst. The same epoxy-hydroxy product, formed heterolytically by acid catalysis in tetrahydrofuran-water solvent, was also accompanied by chain cleavage products (8).

To assess the type of decomposition of methyl linoleate hydroperoxide isomers, the sum of volatiles from the heterolytic and/or homolytic pathway B (hexanal and

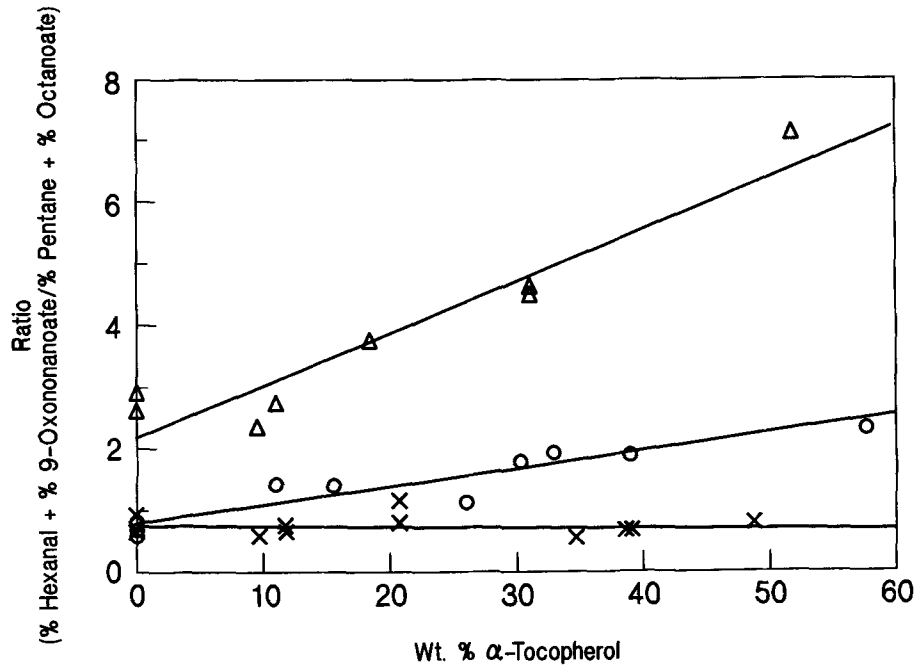


FIG. 4. Effect of α-tocopherol on the ratio of hexanal plus methyl 9-oxononanoate to pentane plus methyl octanoate from the thermal decomposition of: *cis,trans* and *trans,trans* 9- and 13-linoleate hydroperoxides (O—O), *cis,trans* 13-linoleate hydroperoxide (X—X), and *trans,trans* 9- and 13-linoleate hydroperoxides (Δ—Δ).

methyl 9-oxononanoate), was divided by the sum of volatiles from homolytic pathway A (pentane, methyl octanoate). This product ratio increased with increasing α-tocopherol concentration, to a greater extent with the *trans,trans* hydroperoxide isomers than with the mixture of *cis,trans* and *trans,trans* isomers (Fig. 4). No increase was observed with the pure *cis,trans* isomers. These results may indicate a shift in pathways with the participation of a heterolytic cleavage as revealed by suppressing homolytic processes. It is noteworthy that the *trans,trans* isomers of 9- and 13-hydroperoxides of methyl linoleate were previously found to be more susceptible to heterolytic decomposition by acid than the corresponding *cis,trans* isomers (22).

With increasing α-tocopherol concentration, no trends were noted in the relative amounts of methyl 13-oxotridecadienoate and 2,4-decadienal (Tables 1 and 2). These two conjugated dienals, which are expected products of homolytic pathway A in Scheme 1, may be more reactive and less stable than their saturated counterparts under the conditions used in this study.

In summary, this study points to three important factors affecting the types of cleavage occurring in the thermal decomposition of linoleate hydroperoxides—the hydrogen-donating ability of α-tocopherol and 1,4-cyclohexadiene, their effects in decreasing total volatile formation, and their behavior with different geometric isomers of linoleate hydroperoxides. Hydrogen-donor compounds α-tocopherol and 1,4-cyclohexadiene appear to inhibit homolytic β-scission of alkoxyl radicals derived from hydroperoxides, as shown by the decrease in total volatiles as compared to the control without added tocopherol. This decrease in total volatiles was observed for all the hydroperoxide samples tested. A change in mechanism of volatile genesis is indicated by the change in the relative percentages of the various volatiles found for samples containing *trans,trans* hydroperoxide isomers. The increase in hexanal and methyl 9-oxononanoate at the expense of pentane and methyl octanoate suggests the participation of heterolytic cleavage by inhibition of homolytic processes. This work indicates that the addition of varying amounts of tocopherol or other antioxidants may not only decrease the total amount of volatiles formed, but also may control the kinds of volatiles produced.

Relatively high concentrations of α-tocopherol were required in this study to show sufficient changes in the distribution of volatile decomposition products measurable by GC. Therefore, our results are only relevant to clarifying the mechanism of hydroperoxide decomposition and not the antioxidant effect of α-tocopherol. We also did not analyze the products of decomposition of hydroperoxides at lower temperatures than 180°C. Before we can discuss the relevance of these results to biological and food systems, additional studies are needed with methods permitting the analyses of volatile decomposition products at or below physiological conditions.

ACKNOWLEDGMENT

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Autoxidation of Methyl Linoleate Initiated by the Ozonide of Allylbenzene

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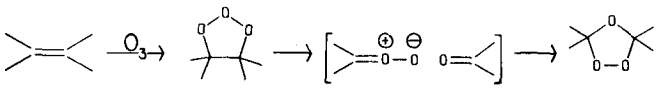
Allylbenzene ozonide (ABO), a model for polyunsaturated fatty acid (PUFA) ozonides, initiates the autoxidation of methyl linoleate (18:2 ME) at 37°C under 760 torr of oxygen. This process is inhibited by d-α-tocopherol (α-T) and 2,6-di-*tert*-butyl-4-methylphenol (BHT). The autoxidation was followed by the appearance of conjugated diene (CD), as well as by oxygen-uptake. The rates of autoxidation are proportional to the square root of ABO concentration, implying that the usual free radical autoxidation rate law is obeyed. Activation parameters for the thermal decomposition of ABO were determined under N_2 in the presence of radical scavengers and found to be E_a = 28.2 ± 0.3 kcal mol^{-1} and log A = 13.6 ± 0.2; k_d (37°C) is calculated to be (5.1 ± 0.3) × 10^{-7} sec^{-1}. Autoxidation data are also reported for ozonides of 18:2 ME and methyl oleate (18:1 ME).
Lipids 24, 609-615 (1989).

Ozone is an important constituent of photochemical smog, reaching levels as high as 0.37 ppm in some urban areas (1). Lung lipids containing polyunsaturated fatty acids (PUFA) are thought to be among the primary targets when air containing ozone is breathed (2,3). We have shown that ozone initiates the autoxidation of PUFA *in vitro* (4); these peroxidative reactions contribute to the disruption of cell membranes, and thus lead to loss of cellular compartmentalization and cell death (3). Our efforts have been aimed at elucidating the mechanism(s) by which ozone, a non-radical, reacts with PUFA to yield radicals that are capable of initiating peroxidative reactions (4-11).

The reaction of ozone with olefins has been the subject of intense study (12). It is generally accepted that ozonation of simple olefins in non-participating (aprotic) solvents proceeds via the 1,2,3-trioxolane, **1**, to yield predominantly the 1,2,4-trioxolane (ozonide), **2** (equation 1) (13).



1 **2**

[1]

Despite the widespread attention given ozonides, these compounds have been largely overlooked as possible sources of free radicals, probably because it is believed that ozonides are stable molecules. However, ozonides are peroxides, and numerous alkyl peroxides are utilized as free radical initiators (14,15).

There are several reports that suggest that ozone-olefin reactions produce free radicals. Firstly, very early studies suggested that ozonation of olefins initiated their polymerization by a free radical mechanism (16-18). More recently, we have used an electron spin resonance (ESR) spin-trapping method to show that ozone reacts with PUFA to yield free radicals (8). And finally, we have shown that ozonated 1,4-pentadiene, a model for PUFA, initiates the autoxidation of 1,4-pentadiene at room temperature (Pryor, W.A., Govindan, C.K., and Church, D.F., unpublished data). This particular study, however, did not allow the identification of the radical-forming species due to difficulties in handling the explosive, low molecular weight ozonation products. In the present report we have utilized allylbenzene, **3**, as a model for PUFA and tested its ozonide, **4**, as an initiator of PUFA autoxidation (19). Preliminary results with ozonides of methyl oleate (18:1 ME) and methyl linoleate (18:2 ME) are also reported (19).

MATERIALS AND METHODS

Materials. Methyl linoleate (18:2 ME, >99%) was obtained from Nu-Chek-Prep., Inc. (Elysian, MN), methyl oleate (18:1 ME, >99%) from Sigma Chemical Co. (St. Louis, MO), and allylbenzene (98%), 2-6-di-*tert*-butyl-4-methylphenol (BHT), chlorobenzene (HPLC grade), 1-butanethiol (99%), and galvinoxyl were obtained from Aldrich Chemical Co. (Milwaukee, WI). Allylbenzene was distilled and passed through neutral alumina immediately before ozonation, and BHT was recrystallized twice from methanol. d-α-Tocopherol (α-T) was obtained from Henkel Corp. (Minneapolis, MN). Methanol suitable for spectrophotometry was obtained from MCB Manufacturing Chemicals, Inc. (Cincinnati, OH). All chemicals were used as received unless otherwise specified.

General ozonolysis procedure. Ozone concentrations were determined by the method of Birdsall *et al.* (20)—ozone was usually delivered in an ozone/oxygen stream at 0.1-0.2 mmol min^{-1}. Ozonolysis was typically carried out to about 90% of the theoretical requirement of olefin. **CAUTION:** *Due to the possibility of detonation, all ozonation mixtures, as well as purified ozonides, should be handled behind a safety shield, and with thick gloves.*

Preparation of allylbenzene ozonide (ABO). A solution of allylbenzene (1 ml) in n-hexane (4 ml) was ozonized at −78°C by the general procedure, flushed with nitrogen, and allowed to warm to room temperature. Solvent was removed *in vacuo*, and 0.2 g of the resulting crude residue was eluted on silica (30 × 1.5 cm o.d.) with n-hexane; pure ABO was obtained as a colorless and transparent liquid in 80% yield: ^{1}H NMR ($CDCl_3$) δ 3.07 (*d*, 2H, *J* = 4.9

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Abbreviations: ABO, allylbenzene ozonide; α-T, d-α-tocopherol; BHT, 2,6-di-*tert*-butyl-4-methylphenol; CD, conjugated diene; DMVN, 2,4-dimethylvaleronitrile; EI, ethyleneimine; ESR, electron spin resonance; HPLC, high performance liquid chromatography; i.d., inner diameter; IR, infrared; KCL, kinetic chain length; MS, mass spectra; o.d., outer diameter; PUFA, polyunsaturated fatty acids; UV, ultraviolet.

Hz), 5.09 (*s*, 1H), 5.18 (*s*, 1H), 5.39 (*t*, 1H, J = 4.9 Hz), and 7.32 ppm (*m*, 5H). ^{13}C NMR ($CDCl_3$) δ 38.3, 94.1, 103.5, 127.0, 128.5, 129.7, and 134.6 ppm. UV (methanol) 256 nm (ε = 125 cm^{-1} M^{-1}). MS (EI) *m/z* (rel. intensity) 166 (M^+, 0.1), 120 (38.4), and 91 (100.0). IR (film) 3065, 3033, 2964, 2892, 1105, 1080, 1056, 753, 738, and 701 cm^{-1}. *Anal*: calcd for $C_9H_{10}O_3$: C, 65.05; H, 6.07. Found: C, 65.01; H, 6.01.

The ^1H NMR chemical shifts of the ozonide ring protons of ABO (5.09, 5.18, and 5.39 ppm) are in good agreement with ^1H NMR chemical shifts reported by Diaper (21) for 1-decene ozonide (5.01, 5.13, and 5.15 ppm).

Preparation of 18:1 ME and 18:2 ME ozonides. This procedure is a modification of a procedure reported by Privett and Nickell (22,23). A solution of alkene (0.5 ml) in dichloromethane (5 ml) was ozonized at 0°C by the general procedure, flushed with N_2, and allowed to warm to room temperature. Solvent was removed *in vacuo*, and pure ozonides (a mixture of *cis* and *trans* isomers eluting at approximately 26 min) were isolated as colorless and transparent oils by semi-preparative HPLC (in >70% yield) (Varian Model 5000 with UV detection at 210 nm; Whatman 50 cm × 9 mm o.d.; 10 μ silica; hexane/diethyl ether [2%] at 4 ml min^{-1}). 18:1 ME ozonide: ^1H NMR ($CDCl_3$) δ 5.17 (*q*, 2H, J = 4.8 Hz), 3.66 (*s*, 3H), and 1.51 ppm (*m*, 31H). ^{13}C NMR ($CDCl_3$) δ 14.1, 22.7, 23.9, 24.0, 25.0, 29.0, 29.2, 29.4, 29.5, 30.8, 31.9, 32.4, 34.1, 51.4, 106.0, and 174.1 ppm. IR (film) 2928, 2857, 1741, and 1109 cm^{-1}. MS(CI/NH_3) *m/z* (rel. intensity) 362 (M + NH_4, 47.5), 220 (35.5), 204 (100.0), and 187 (27.5). 18:2 ME ozonide: ^1H NMR ($CDCl_3$) δ 5.29 (*m*, 4H), 3.67 (*s*, 3H), 2.20 (*m*, 4H), and 1.40 ppm (*m*, 23H). IR (film) 1742, 1105, 1038 cm^{-1}. *Anal.*: calcd for $C_{19}H_{34}O_5$: C, 66.64; H, 10.01. Found: C, 66.48; H, 10.08.

Thermal decomposition of ABO. Rate constants for ABO decomposition, k_d, were determined by measuring loss of ozonide with time in the presence of a radical scavenger (24). In a typical experiment, 1 ml aliquots of stock solutions of ABO and 1-butanethiol (25) in CCl_4 were transferred to a series of ampoules, which were then cooled to −78°C under N_2 and sealed. Ampoules were maintained at a given temperature (98, 70, 50 or 37°C), periodically withdrawn, cooled to 0°C, the contents diluted with n-hexane and analyzed by HPLC (Hewlett Packard 1090 equipped with a UV/VIS diode array detector) using a Zorbax cyanopropyl column (25 cm × 4.6 mm i.d., 5 μ; Dupont, Wilmington, DE) eluted with n-hexane/diethyl ether (1%) at 1 ml min^{-1}. Loss of ozonide was typically followed at 210 nm for 3-4 half-lives except for the 37°C run, which was measured over 2 half-lives. One experiment was performed at 50°C in which galvinoxyl (26) replaced 1-butanethiol as the radical scavenger.

Autoxidation procedures. Oxygen-uptake experiments were performed at 37.00 ± 0.05°C under 760 torr of O_2 in an automatic gas absorption apparatus identical to the one previously described (27). In a typical experiment 2.00 ml 18:2 ME containing a known amount of ABO was rapidly stirred under O_2 in a flask attached to the gas absorption apparatus; a decrease in pressure was measured over time and converted into molar quantities (28). The applied corrections—due to oxygen uptake and release of CO by the primordial radicals from the initiator and oxygen release by termination reaction 7—are small (Table 1) due to long kinetic chain lengths (see Discussion).

Autoxidation experiments in which the appearance of conjugated diene (CD) was monitored were typically performed as follows: 18:2 ME (1 ml) containing a known amount of ozonide was stirred at 37.0 ± 0.1°C in a vial equipped with a silicon septum; oxygen was continuously introduced by means of a capillary bleed tube. Aliquots (5.0 μl) were periodically withdrawn, diluted with methanol, and their absorption at 234 nm measured (Hewlett Packard 8451A diode array spectrophotometer); these absorbencies were converted to CD concentrations using ε = 27,000 M^{-1} cm^{-1} (29). For the slower rates of initiated autoxidation (17 and 68 mM ozonide), the vial

TABLE 1

Kinetic Data for the ABO-Initiated Autoxidation of 18:2 ME at 37°C Under 760 Torr of O_2[a]

[ABO] (× 10^2/M)	[18:2 ME] (M)	R_i (× 10^6/Msec^{-1})	e[b]	$-d[O_2]/dt$ (× 10^6/Msec^{-1}) Measured	Corrected[c]	KCL[d]	Oxidizability (× $10^2/M^{-1/2}sec^{-1/2}$)
1.77	3.01	1.1 ± 0.4	0.046	1.72	1.72	1600 ± 600	1.72
7.03	2.99	3.0 ± 0.6	0.032	2.86	2.88	1000 ± 200	1.75
7.28	2.99	N.D.	—	5.21	—	—	—
28.3	2.90	14 ± 4	0.037	7.35	7.43	500 ± 100	2.14
89.3	2.66	N.D.	—	12.9	—	—	—
93.6	2.62	67 ± 7	0.053	11.9	12.1	200 ± 20	1.75

[a] 18:2 ME, methyl linoleate; ABO, allylbenzene ozonide; KCL, kinetic chain length; oxidizability = $k_p/(2k_t)^{1/2}$; N.D., not determined.

[b] e, Efficiency of chain initiation, $R_i/2k_d$[ABO] where k_d(37°C) = (5.1 ± 0.3) × 10^{-7} sec^{-1}.

[c] Rate corrected for CO given off and O_2 taken up by initiator, and O_2 given off by termination (28); rates of oxygen uptake ($-d[O_2]/dt$) were calculated from the observed changes in pressure (ΔP_{O_2}) using the equation $-d[O_2]/dt = (\Delta P_{O_2})(V_{HS})/RT(V_S)dt$ where V_{HS} is the volume of the headspace above the reaction solution in liters; R is the ideal gas constant (0.0826 atm $mol^{-1}K^{-1}$); T is temperature in K; V_S is the volume of the reaction solution in liters; and dt is the time period over which the pressure change is observed (28).

[d] KCL = $-d[O_2]/dt/R_i$.

containing 1 ml 18:2 mE was maintained under N_2 ca. two hours prior to addition of the ozonide to ensure thermal equilibration at 37°C. This precaution was unnecessary with higher concentrations of ozonide because of the large rates of autoxidation.

RESULTS

Kinetics of ABO decomposition. Rate constants (k_d) for the thermal decomposition of ABO were obtained in homogenous solution in CCl_4 by following the disappearance of ABO with time by HPLC; values for k_d at 50, 70, and 98°C are collected in Table 2. In the absence of a radical scavenger, plots of log (ozonide peak area) vs time are nonlinear (19); these plots are linear in the presence of radical scavengers and all subsequent rate data were obtained in the presence of the radical scavengers 1-butanethiol or galvinoxyl. In the presence of a radical scavenger, decomposition is first order in ABO over at least three half-lives; a typical plot is shown in Figure 1. From the rate data in Table 2, an Arrhenius plot can be constructed (Fig. 2) from which activation parameters $E_a = 28.2 \pm 0.3$ kcal mol^{-1} and log $A = 13.6 \pm 0.2$ for ABO are obtained. A rate constant for ABO decomposition at 37°C was measured and found to be $(6.1 \pm 0.1) \times 10^{-7}$ sec^{-1}(Table 2). Since this value is the result of only one measurement, a more reliable k_d (37°C) can be calculated using the Arrhenius activation parameters given above; this calculation gives k_d (37°C) as $(5.1 \pm 0.3) \times 10^{-7}$ sec^{-1}. The half-life at 37°C is 16 ± 1 days.

Kinetics of autoxidation. When ABO is added to neat 18:2 ME at 37°C under 760 torr oxygen, autoxidation is initiated. This is illustrated in Figure 3, in which plots of conjugated diene (CD) concentration with time are presented. These reactions are inhibited by addition of the radical scavengers d-α-tocopherol (α-T) or 2,6-di-*tert*-butyl-4-methylphenol (BHT). A plot of rate of CD formation (+d[CD]dt) vs $([ABO]^{1/2}[18:2\ ME])$ is linear (Fig. 4).

The ABO-initiated autoxidation of 18:2 ME was also studied by following oxygen-uptake; a typical trace showing both an uninhibited and an α-T-inhibited reaction is presented in Figure 5, and the collected kinetic data are summarized in Table 1. The rate of chain initiation, R_i, was calculated from the length of the induction period caused by known concentrations of α-T or BHT (30). The average efficiency of initiation for ABO in our system is

TABLE 2

Rate Constants for the Thermal Decomposition of Allylbenzene Ozonide (ABO) in CCl_4 Under N_2 in the Presence of Radical Scavengers

ABO (mM)	Scavenger (mM)	Temperature (°C)	k_d ($\times 10^6$)[a]
6.52	9.34[b]	37	0.61 ± 0.01
6.87	8.59[b]	50	3.27 ± 0.03
0.721	0.850[b]	50	3.41 ± 0.05
6.58	14.2[c]	50	3.24 ± 0.07
7.16	8.59[b]	70	50 ± 3
0.709	0.850[b]	70	42.9 ± 0.7
7.16	8.59[b]	98	1010 ± 30
0.710	0.850[b]	98	1010 ± 30

[a] Obtained by least squares analysis of data fit to the equation $\log(ABO) = -k_d t/2.303$, where ABO is the raw peak area from HPLC analysis, t is time, and the slope of the line is equal to $-k_d/2.303$. Units are sec^{-1} (± standard deviation).

[b] 1-Butanethiol.

[c] Galvinoxyl.

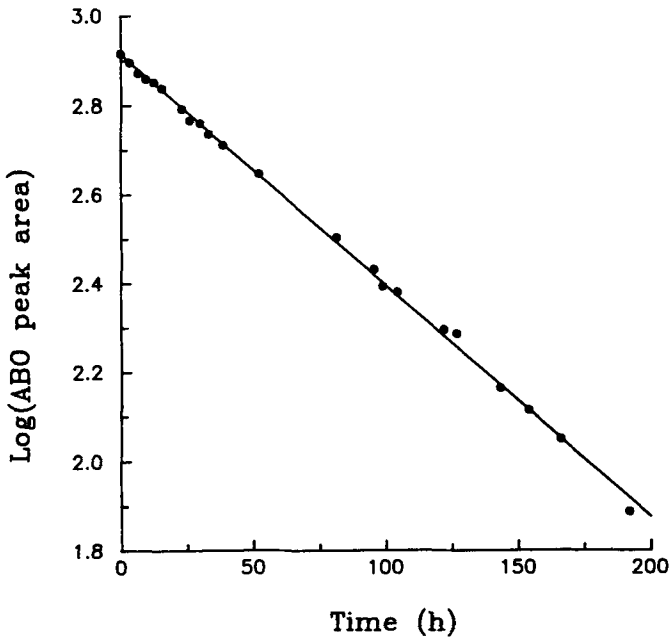


FIG. 1. The thermal decomposition of ABO in CCl_4 (6.87 mM) at 50°C in the presence of 1-butanethiol (8.6 mM). Log (ozonide peak area) vs time is plotted; the slope of the line is equal to $-k_d/2.303$, where areas are from the HPLC analysis.

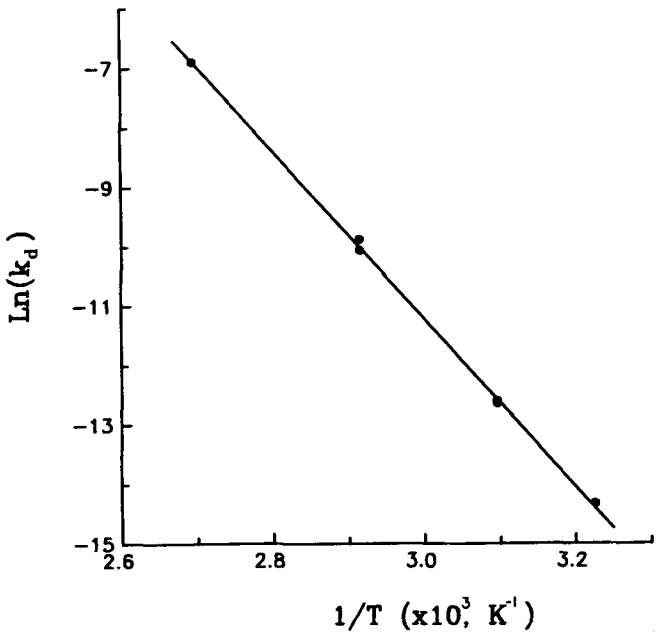


FIG. 2. An Arrhenius plot for the thermal decomposition of 7 mM ABO in the presence of 9 mM 1-butanethiol. The slope of the line equals E_a/R where R = 1.987 cal $mol^{-1}K^{-1}$ and the intercept is ln A.

4.2 ± 0.9%. The final column of Table 1 gives experimentally determined oxidizabilities of 18:2 ME at 37°C, i.e., $k_p/(2k_t)^{1/2}$; an average value of (1.9 ± 0.2) × 10^{-2} $M^{-1/2}sec^{-1/2}$ is obtained.

Preliminary autoxidation studies with 18:1 ME and 18:2 ME ozonides. Experiments with ozonides of 18:2 ME (147 mM) and 18:1 ME (239 mM) show that autoxidation of 18:2 ME at 37°C is initiated with observed rates (+d[CD]dt) of 2.9 × 10^{-6} and 9.3 × 10^{-7} M sec^{-1}, respectively. Table 3 compares these results to the rate obtained using 98 mM ABO as an initiator.

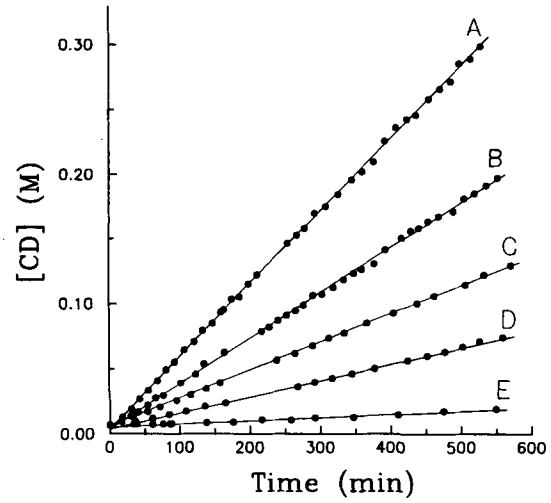


FIG. 3. Plots of conjugated diene (CD) concentration vs time for the ABO-initiated autoxidation of 18:2 ME at 37°C. A, 837 mM ABO, 2.6 M 18:2 ME; B, 272 mM ABO, 2.9 M 18:2 ME; C, 68 mM ABO, 3.0 M 18:2 ME; D, 17 mM ABO, 3.0 M 18:2 ME; E, 3.0 M 18:2 ME.

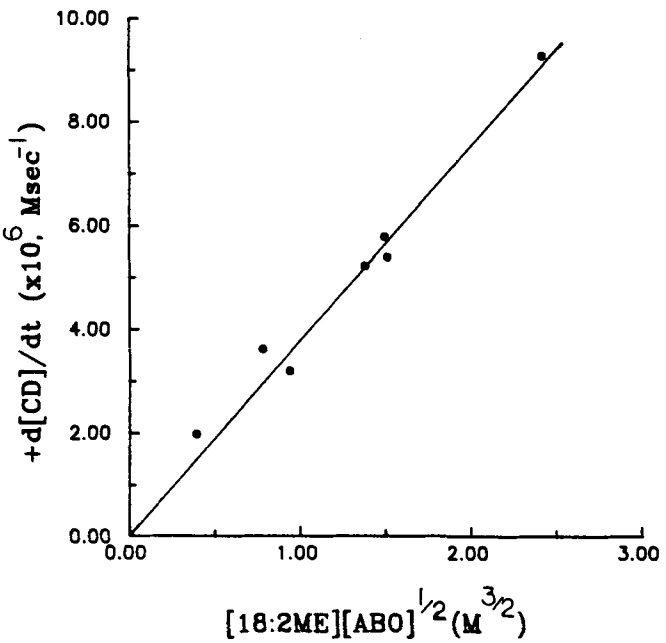
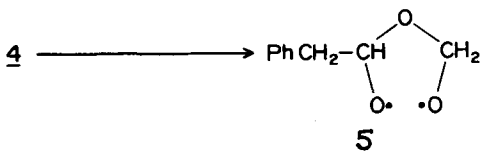


FIG. 4. A plot of d[CD]/dt vs [18:2 ME][ABO]$^{1/2}$ for the ABO-initiated autoxidation of 18:2 ME at 37°C. The line shown was obtained by forced linear regression (confidence level >99.9%).

DISCUSSION

Kinetics of ABO decomposition. Our activation parameters for the thermal decomposition of ABO (E_a = 28.2 ± 0.3 kcal mol^{-1} and log A = 13.6 ± 0.2) are similar to the parameters obtained in the gas phase for the ozonides of ethylene and propene (E_a = 27.5, 25.7 kcal mol^{-1} and log A = 13.6, 11.6, respectively) (31). These parameters are consistent with the initial homolysis of the ozonide peroxide bond to yield the oxy-diradical 5 (32) as shown in equation 2; a more detailed study of the mechanism of ABO decomposition will be reported elsewhere (41).



[2]

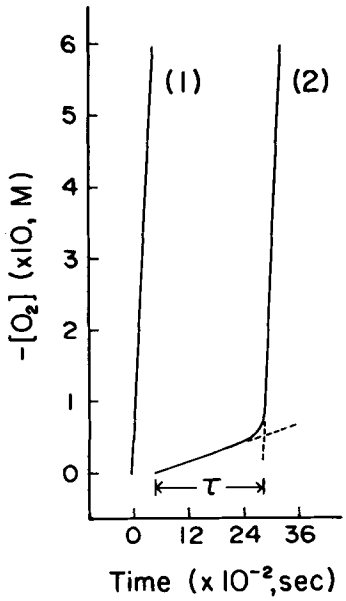


FIG. 5. A plot of oxygen-uptake (−[O_2]) vs time for the autoxidation of 18:2 ME initiated with 280 mM ABO (curve 1). Curve 2 shows inhibition with 2.5 × 10^{-5} M α-T for a period of time, τ.

TABLE 3

A Comparison of 18:1 ME and 18:2 ME Ozonides to ABO as Initiators of Autoxidation of 18:2 ME at 37°C[a]

Ozonide	Ozonide (mM)	Predicted rates[b] (× 10^6/M sec^{-1})	Observed rates[b] (× 10^6/M sec^{-1})
ABO	98	—	3.2
18:1 ME	239	5.0	0.93
18:2 ME	147	3.9	2.9

[a] 18:1 ME, methyl oleate; 18:2 ME, methyl linoleate; ABO, allylbenzene ozonide; CD, conjugated diene.

[b] This is the rate predicted by equation 11 for this concentration of ABO; the rate was measured by following the increase in conjugated diene.

Kinetics of autoxidation. Autoxidation involves a free radical chain; under sufficient oxygen pressure (100 torr) in homogenous solution this chain can be represented by equations 3-7 (Scheme 1) (28,33-36).

Initiation: $\text{Initiator} \rightarrow 2I^{\bullet}$ [3]

$I^{\bullet} + RH \rightarrow IH + R^{\bullet}$ [4]

Propagation: $R^{\bullet} + O_2 \xrightarrow[\text{fast}]{k_o} ROO^{\bullet}$ [5]

$ROO^{\bullet} + RH \xrightarrow{k_p} ROOH + R^{\bullet}$ [6]

Termination: $2\ ROO^{\bullet} \xrightarrow{2k_t}$ non-radical products $+ O_2$ [7]

SCHEME 1

In Scheme 1, $I^{\bullet}$ represents the primordial radicals from the initiator, RH represents the oxidizable substrate, and $R^{\bullet}$ represents the carbon-centered radical derived from RH by H-atom abstraction. Under steady-state conditions, the rate law for autoxidation is expressed by equation 8, where k_p and $2k_t$ are

$$\text{Rate} = \{k_p/(2k_t)^{1/2}\}[RH]R_i^{1/2} \quad [8]$$

rate constants for the rate-controlling chain propagation (equation 6) and chain termination (equation 7) steps, respectively, and R_i is the rate of chain initiation (33,34). The term $k_p/(2k_t)^{1/2}$ is the oxidizability of the organic substrate (36).

Autoxidation studies are usually designed to obtain oxidizability data on substrates susceptible to autoxidation or to investigate effectiveness of potential antioxidants. In these studies, rates of initiation are controlled by using initiators that decompose to radicals at known and constant rates. We have reversed this technique and have studied the autoxidation of a substrate of known oxidizability (18:2 ME) in order to characterize ABO as an initiator. To do this, it is important to establish that the rate of chain initiation, R_i, is constant, and that the system obeys the usual kinetic rate law (equation 8).

Our data show that formation of CD is linear over about nine hours (Fig. 3), implying that R_i is constant over the time range studied. R_i can be measured more directly using the standard induction method (30), in which a known concentration of phenolic antioxidant, ArOH, is added to the system, and the length of time, τ, is measured during which autoxidation is inhibited. Equation 9 relates τ to R_i, where n is the number of

$$R_i = n\,[ArOH]/\tau \quad [9]$$

radicals intercepted by the chain breaking antioxidant (30). We have used this method to measure R_i in oxygen uptake experiments for various concentrations of ABO; these results are listed in the third column of Table 1. Uncertainties in R_i are random within a run, and range from 10 to 36%. Therefore, we believe that R_i is constant within experimental uncertainty. Equation 10 relates R_i to the concentration of the initiator, In, where n is the

$$R_i = nek_d[In] \quad [10]$$

number of radicals produced per initiator molecule (assumed to be 2), e is the efficiency of chain reaction initiation and k_d is the rate constant for thermal decomposition of initiator (36). Substituting this expression into equation 8 gives equation 11.

$$\text{Rate} = \{k_p/(2k_t)^{1/2}\}[RH]\{nek_d[In]\}^{1/2} \quad [11]$$

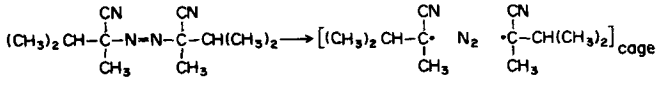
Thus, a plot of Rate vs $[In]^{1/2}[RH]$ should be linear. Figure 4 shows a linear correlation between rates of CD formation and $[ABO]^{1/2}[18{:}2\ ME]$; a similar linear correlation is also found when rates of oxygen-uptake are plotted vs $[ABO]^{1/2}[18{:}2\ ME]$ (19). These results imply that the ABO-initiated autoxidation of 18:2 ME obeys the usual rate law for autoxidation. Furthermore, our mean value of $(1.9 \pm 0.2) \times 10^{-2}\ M^{-1/2}\ sec^{1/2}$ for the oxidizability of 18:2 ME at 37°C (Table 1) is in good agreement with the literature value of $2.03 \times 10^{-2}\ M^{-1/2}\ sec^{-1/2}$ (27).

The kinetic chain length (KCL)—the number of substrate molecules oxidized per initiating radical—can be obtained from equation 12 (35).

$$KCL = (d[O_2]/dt)/R_i \quad [12]$$

Long kinetic chains, ranging from 200 to 1600, are obtained with the longest chains being observed at low ABO concentrations (Table 1).

Efficiency of initiation. Equation 10 can be solved for the efficiency of initiation, and e for ABO is found to be $4.2 \pm 0.9\%$ (Table 1). Low efficiencies of free radical production (and thus, low rates of initiation) are typically associated with a viscous reaction medium that leads to high yields of cage radical combination (37,38). For example autoxidation data for 18:2 ME using the azo compound 2,2′-azobis(2,4-dimethylvaleronitrile) (DMVN) as an initiator show that increasing the concentration of 18:2 ME from 0.20 to 1.9 M results in a decrease in efficiency of initiation from 100 to 76%, respectively (39). We have determined the efficiency of initiation for DMVN in neat 18:2 ME (3.0 M) to be 33% (19). These results with DMVN show that lower efficiencies of initiation are obtained in the viscous medium, 18:2 ME, as expected. However, the effect of solvent viscosity on efficiencies of initiation would not be expected to be equal for ABO and DMVN; ABO decomposes to form an oxygen-centered diradical (5 in Equation 2), whereas DMVN decomposes to give two unattached radicals (Scheme 2). The resulting radical pairs (5 and 6) differ in their ability to diffuse from



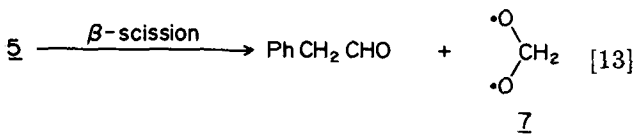
6

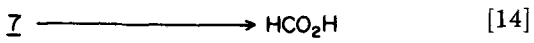
SCHEME 2

each other; radical pair 6 diffusive ability is governed by the usual cage effects. However, the ability of the radical

centers in diradical 5 to diffuse is restricted by their being in the same species. This may help to explain why the efficiency of initiation for ABO (4.2 ± 0.9%) is lower than the value of 33% observed for DMVN.

Another difference between ABO and DMVN also contributes to the observed differences in efficiencies. Equation 2 shows the unimolecular homolysis of ABO to give the oxy-diradical 5; we have proposed the following reactions (Equations 13 and 14) as contributing paths in the mechanism of ABO decomposition (19).





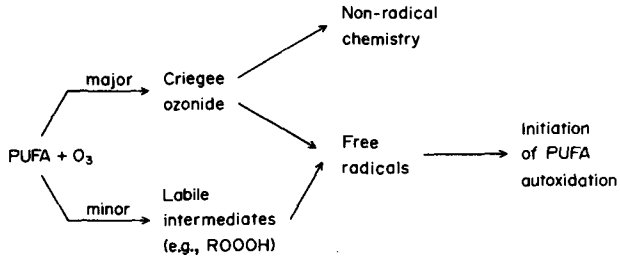
Oxy-diradical 7 is predicted to rapidly isomerize to formic acid (40) (equation 14), and should be too short-lived to initiate radical chains. Therefore, the net result of equations 13 and 14 is a decreased yield of initiating free radicals. In contrast to ABO, DMVN decomposes to yield two unattached radicals that rapidly react with oxygen, resulting in peroxyl radicals that can initiate radical chains. Thus, the yield of initiating radicals is greater for DMVN than ABO, as was previously observed.

Preliminary autoxidation studies with 18:1 ME and 18:2 ME ozonides. The above data were obtained using ABO as a model for the ozonide of a PUFA. However, we have also performed more limited studies using ozonides of 18:2 ME. A comparison of rate data obtained by following appearance of CD with rates of ABO-initiated autoxidation (98 mM) is made in Table 3. If 18:2 ME ozonide initiates as effectively and decomposes with the same rate constant as ABO, then the general rate law predicts an initiated rate of autoxidation of 3.9×10^{-6} M sec^{-1} for 147 mM 18:2 ME ozonide, which is in fair agreement with the observed rate of 2.9×10^{-6} M sec^{-1}. More of a discrepancy is apparent with 18:1 ME ozonide; the observed rate of initiated autoxidation of 9.3×10^{-7} M sec^{-1} is about five-fold lower than the predicted value of 5.0×10^{-6} M sec^{-1}. These results indicate—as might be expected from its structure—that ABO is a better model for the 18:2 ME ozonide than for the 18:1 ME ozonide.

Ozonides as a contributor to initiating activity in ozone-induced autoxidation of 18:2 ME. Our previous work has shown that ozone initiates the autoxidation of 18:2 ME *in vitro* (4). We also have used an ESR spin-trapping method to show that ozone reacts with olefins (e.g., with 18:2 ME at −78°C) to form intermediates that decompose to give radicals starting at about −30°C (7,8). The yield of radicals from these intermediates has not been firmly established, but we have estimated yields between 0.01 and 10% (5-8). It has been suggested that at least a portion of these labile, radical-producing intermediates are hydrotrioxides R-O-O-O-H (5,6).

The present study shows that ABO, a model of a PUFA ozonide, and the ozonide of 18:2 ME initiate the autoxidation of PUFA at 37°C. Criegee ozonides may account for the majority of products obtained when PUFA in lipid bilayers are ozonized.

Thus, at least two types of initiators are formed when ozone reacts with PUFA (Scheme 3). The first type is thermally very labile (such as a hydrotrioxide) and is formed in small yield; the other is the Criegee ozonide, which forms radicals more slowly, but is produced in higher yield.



SCHEME 3

Radicals capable of initiating PUFA autoxidation arise from both of these mechanisms, but it is difficult to calculate the relative contributions of the two routes.

It should be noted that ozonation leads to two types of damage to lipid bilayers. The first is the direct cleavage of the unsaturated fatty acid molecule by production of the ozonide. The second is the decomposition of this ozonide to produce radicals that initiate the chain autoxidation of other, non-ozonated PUFA molecules in the bilayer, thus producing a cascade of damage.

Conclusions. This report shows that ABO, an ozonide structurally similar to those from PUFA, initiates the autoxidation of methyl linoleate, and that the usual kinetic rate law for autoxidation is obeyed. The rate constant for decomposition at 37°C is $(5.1 \pm 0.3) \times 10^{-7}$ sec^{-1}. The ozonide of methyl linoleate initiates about as effectively as ABO does. We suggest that radicals from the thermal decomposition of Criegee ozonides may be a contributing pathway to the overall production of free radicals when PUFA react with ozone. These reactions could be important in the initiation of the autoxidation of pulmonary lipids when smoggy air is breathed (2,3).

ACKNOWLEDGMENTS

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Trans Fatty Acids. 3. Fatty Acid Composition of the Brain and Other Organs in the Newborn Piglet

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The effects of dietary *trans* fatty acids on tissue fatty acid composition were studied in newborn piglets delivered from sows fed partially hydrogenated fish oil (PHFO) (28% *trans*) or partially hydrogenated soybean oil (PHSBO) (36% *trans*) in comparison with lard (0% *trans*) from 3 wk of age and through gestation in Experiment 1, or fed PHFO or "fully" hydrogenated fish oil (HFO) (19% *trans*) in comparison with coconut oil (CF) (0% *trans*) with two levels, 1 and 2.7%, of dietary linoleic acid from conception through gestation in Experiment 2. The piglets were sampled immediately after delivery, without having access to mothers' milk.

Incorporation of *trans* fatty acids into brain PE (phosphatidylethanolamine) were non-detectable or very low (less than 0.1%). The incorporation of 18:1 *trans* into heart-PE, liver mitochondria-PE, total plasma lipids and adipose tissue was low, and 20:1 *trans* was not detected. Dietary *trans* fatty acids had no consistent effects on the overall fatty acid composition of the different tissue lipids. It is concluded that *trans* fatty acids from PHFO, HFO and PHSBO have no significant effects on the fatty acid accretion in the fetal piglet.
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Nervous tissue contains large quantities of long chain polyunsaturated, essential (i.e., n-6 and n-3) fatty acids (LC-PUFA) which are laid down over a relatively brief period of time during late gestation and early post natal life (1-3). Essential fatty acid (EFA) deficiency during brain development may change lipid class and fatty acid composition, and reduce brain weight (4-6). This may lead to changes in the behavioral and electrophysiological activity (6,7). LC-PUFA for brain growth are obtained directly from dietary sources or derived from linoleic acid (18:2n-6 all *cis*) and α-linolenic acid (18:3n-3 all *cis*) through chain elongation and desaturation in the maternal and fetal liver and fetal brain (3,8-10). Since it has been shown that dietary *trans* fatty acids may lower the activity of the Δ-6 and possibly the Δ-5 desaturase, causing reductions in the tissue levels of LC-PUFA (11,12), the effect of dietary *trans* fatty acids on brain development is of considerable interest. This question is of particular interest under conditions of a reduced supply of EFA which are known to exaggerate the biochemical effects of dietary *trans* fatty acids (12-14). Further, the myelinogenesis process demands the accretion of large amounts of oleic acid 18:1 n-9 *cis* (15). The possibility exists that if 18:1 *trans* passes through the blood brain barrier, it may replace the *cis* isomer in the myelin causing changes in the membranes. Data on the effects of dietary *trans* fatty acids on brain development are extremely meager.

Despite the fact that fetal life is a particularly vulnerable period, few studies have dealt with the effects of dietary *trans* fatty acids on fetal development during gestation, and those conducted have given partially contradictory results. Thus, Johnston *et al.* (16) did not find significant percentages of *trans* fatty acids in carcass lipids of newborn rats from mothers fed diets with high contents of *trans* fatty acids. Similarly, Johnston *et al.* (17) did not find significant percentages of *trans* fatty acids in tissue lipids of human fetuses and newborn babies from mothers who themselves had appreciable percentages of *trans* fatty acids in their adipose tissue.

Contrary to these findings several authors investigating the rat (18-20) or the mouse (21) have found a significant transfer of *trans* fatty acids into developing fetuses. These studies showing transport of *trans* fatty acids across the placenta were conducted with rodents. There is therefore a need for studies with species that more physiologically resemble man. Despite a difference in implantation—the pig has a diffuse epitheliochorial placentation compared with discoid and hemochorial in the human being—the domestic pig has been regarded as a suitable model animal for human studies (22). In particular it has been shown that the course of LC-PUFA accretion in the brain is similar in the pig and the human being (1,2,23). The domestic pig may therefore be a suitable model animal in which to study the effects of dietary *trans* fatty acids on the development in humans.

In previous studies two sources of *trans* fatty acids were used: the pure acids (e.g. 18:1 n-9 *trans* or 18:2 n-6 all *trans*) or partially hydrogenated vegetable oil (PHVO). Thus there is a need to examine partially hydrogenated fish oils (PHFO) since these oils, in addition to 18:1 *trans* isomers, also contain *trans* fatty acids with 20 and 22 carbon atoms (24).

The aim of the present study was to examine the nutritional effects of *trans* isomeric fatty acids in PHFO and PHVO, fed with different levels of linoleic acid, on the deposition of *trans* fatty acids and the overall fatty acid profile of the brain and other organ lipids of newborn piglets. Regretfully, our capacity did not permit us to analyze both PC (phosphatidylcholine) and PE (phosphatidylethanolamine). Due to the fatty acid composition, i.e., the high content of long-chain polyunsaturated fatty acids (LC PUFA) in PE, compared with that of PC (25), and the assumed structural importance of these LC PUFA, the fatty acid composition of PE was chosen as an indicator for the metabolic effect of *trans* fatty acids.

Previous publications have reported the results of dietary *trans* fatty acids on brain weight and peroneal histology and nerve conduction velocity (26) and on the fatty acid profile of brain lipids and other organ lipids of the mothers of the piglets used in this study (27).

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Abbreviations: CF, coconut fat; EFA, essential fatty acid; GLC, gas liquid chromatography; HFO, hydrogenated fish oil; HPLC, high performance liquid chromatography; LC-PUFA, long chain polyunsaturated essential fatty acids; PHFO, partially hydrogenated fish oil; PHSBO, partially hydrogenated soybean oil; PHVO, partially hydrogenated vegetable oil; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PUFA, polyunsaturated fatty acids; SFO, sunflowerseed oil.

EXPERIMENTAL

General procedures. Half of the litters (i.e., half of the numbers of each sex) of sows from the Norwegian Landrace breed were electrocuted within four hours of birth and without having suckled their mothers and sampled for fatty acid analysis. In Experiment 1 the piglets were obtained from sows that had been fed 14 wt percent of lard (control, diet 1), PHFO (partially hydrogenated capelin oil, mp 30–32 C, diet 2) or PHSBO (partially hydrogenated soybean oil, mp 40C, diet 3) plus 4 wt percent of SFO (sunflowerseed oil, 65% linoleic acid) as additions to semi-practical diets. The experimental fats were fed from three weeks of age through pregnancy. In Experiment 2, the piglets were from sows that had been fed according to a factorial design 14 wt percent of CF (coconut fat, diets 1.1 and 2.1), PHFO (diets 1.2 and 2.2) or HFO ("fully" hydrogenated fish oil, mp 50–52C, diets 1.3 and 2.3) either without (diets 1.1, 1.2 and 1.3) or with (diets 2.1, 2.2 and 2.3) an extra 3 wt percent addition of SFO to semi-practical diets. In Experiment 2, the diets were fed from gestation at about 6 months of age through pregnancy. Details of the sows' diets and management were presented earlier (26). In both experiments four sows were bred to farrow in each dietary group. The design of the experiments and the fatty acid composition of the sows' diets, determined as previously described (27), are shown in Table 1.

Chemical methods. Total lipids from samples of blood plasma (right ventricle), heart and subcutaneous adipose tissue from the belly were immediately extracted as described by Folch *et al.* (28). This procedure was modified somewhat for the extraction of brain lipids (29). Membrane lipids of the liver mitochondria were immediately isolated, as described by Höy and Hölmer (30). All lipids were stored at −25°C pending further analysis. Phosphatidylethanolamines (PE), including PE-plasmalogenes, of the liver mitochondria, the heart and the brain were prepared by high performance liquid chromatography (HPLC) and the fatty acid compositions were analyzed by capillary gas liquid chromatography (GLC) as described earlier (27). The content of 16:1 *trans* was only analyzed in the dietary fats of Experiment 2 (Table 1). However, in organ lipids the *trans* isomers of 16:1 could not be identified due to interference with other components of the chromatograms.

TABLE 1

Fatty Acid Composition (%) of the Dietary Fats (Total) Fed to the Mother Sows[a]

	Experiment 1			Experiment 2					
	Added SFO, 4%			Added SFO, 0%			Added SFO, 3%		
	Lard Diet 1	PHFO Diet 2	PHSBO Diet 3	CF Diet 1.1	PHFO Diet 1.2	HFO Diet 1.3	CF Diet 2.1	PHFO Diet 2.2	HFO Diet 2.3
8:0				7.1			10.5		
10:0				6.1			5.8		
12:0				40.9	1.0	0.2	34.7	2.1	0.2
14:0				16.7	8.0	8.0	13.0	7.2	6.7
16:0	22.6	16.3	11.2	10.8	15.5	20.5	9.1	14.8	17.6
18:0	14.8	5.6	13.2	3.2	4.2	14.3	3.0	4.7	12.2
20:0	0.3	2.2	0.6		2.6	13.2		2.4	10.6
22:0		2.0			2.1	10.5		2.0	8.5
24:0					0.1	0.6		0.1	0.5
16:1 *cis*	1.5	1.1	0.6	0.2	2.9	0.5	0.2	2.7	0.6
16:1 *trans*[c]					4.8	1.9		3.9	1.9
18:1 *cis*	34.6	14.1	29.1	6.8	10.3	4.2	8.4	9.8	6.6
18:1 *trans*	0.3	6.8	24.3		7.7	3.1		5.8	2.9
20:1 *cis*	0.7	3.9	0.2		5.8	2.0		5.2	2.2
20:1 *trans*		6.0			9.5	5.9		7.9	5.3
22:1 *cis*		5.7			6.0	2.0		5.2	3.2
22:1 *trans*		5.3			6.7	4.4		5.5	3.6
24:1					0.5	0.6		0.4	0.5
18:2n-6	20.8	20.1	18.3	5.2	5.5	5.9	13.2	14.5	13.7
18:3n-3	1.8	2.2	1.9	2.2	1.6	1.4	1.8	1.1	1.3
Others	2.6	8.7	0.6	0.8	5.4	0.8	0.3	4.7	1.9
Sum PUFA	22.6	22.3	20.2	7.4	7.1	7.3	15.0	15.6	15.0
Sum monoenes	37.1	42.9	54.2	7.0	54.2	24.6	8.6	46.4	26.8
Sum saturated	37.7	26.1	25.0	84.8	33.5	67.3	76.1	33.3	56.3
Sum *trans*	0.3	18.1	24.3	0.0	28.7	15.3	0.0	23.1	13.7
P/S[b]	0.60	0.85	0.81	0.09	0.21	0.11	0.20	0.47	0.27

[a]Fed from 3 wk of age through pregnancy in Expt. 1, from gestation through pregnancy in Expt. 2.

[b]Relative amount of sum PUFA (P) to sum saturated fatty acids (S).

[c]16:1 *trans* was not identified in Expt. 1.

PUFA = polyunsaturated fatty acid.

Statistical methods. The basic principle of the statistical methods used was the estimation of the maximum likelihood. The program package chosen was BMDP, program P3V (31).

RESULTS

Brain. The fatty acid composition of the PE in total brain lipids is shown in Table 2. The overall pattern of fatty acids was consistent and similar in both experiments with only minute effects of the dietary regimen of the mothers. No *trans* fatty acids were detected in Experiment 1. In Experiment 2 very low, but significant, levels of 18:1 *trans* were found.

In Experiment 2 offspring from sows fed HFO and, in particular, PHFO had a significantly higher content of 20:3n-9 than those from sows fed CF. A similar, but insignificant trend for hydrogenated fats was also seen in Experiment 1. Further, offspring from sows with a limited supply of linoleic acid (diets 1.1, 1.2 and 1.3) in Experiment 2 had significantly higher contents of 20:3n-9 than those from sows receiving a liberal supply of linoleic acid in their diets (diets 2.1, 2.2 and 2.3).

Offspring from sows fed hydrogenated fats (PHFO and HFO) in Experiment 2 had significantly increased levels of 20:3n-6 when the content of linoleic acid in the diets was low (diets 1.2 and 1.3 vs 1.1), but not when it was high (diets 2.2 and 2.3 vs 2.1). There were no effects of the experimental fats on the content of 20:3 n-6 in Experiment 1 when diets with a liberal supply of linoleic acid were used. Increased dietary levels of linoleic acid in Experiment 2 decreased the level of 20:3n-6.

Feeding hydrogenated fat to the sows gave offspring with significantly reduced contents of 20:4n-6 in Experiment 1. In Experiment 2, hydrogenated fat reduced the content of 20:4n-6 when the diets had a low content of linoleic acid (diets 1.2 and 1.3 vs 1.1), but increased the content of 20:4n-6 when the diets had a high linoleic acid content (diets 2.2 and 2.3 vs 2.1), thus there was a significant interaction between the dietary level of linoleic acid and expermental fats. Further, sows fed diets with a high content of linoleic acid in Experiment 2 had offspring with a significantly higher content of 20:4 n-6 than that found in those from sows fed low levels of linoleic acid.

In Experiment 1 the contents of 22:5n-6 increased significantly when the mothers were fed the hydrogenated fats while the hydrogenated fats apparently did not affect the level of 22:5n-6 in Experiment 2.

Offspring from sows fed restricted levels of linoleic acid in Experiment 2 had significantly higher contents of 22:3 n-6 and 22:6 n-3 than offspring from sows fed high levels of linoleic acid. On the other hand, sows fed liberal

TABLE 2

Fatty Acid Composition (%) in PE of Total Lipids in Brain of Newborn Piglets[a]

	Experiment 1					Experiment 2							
	Added SFO, 4%					Added SFO, 0%			Added SFO, 3%				
	Lard Diet 1	PHFO Diet 2	PHSBO Diet 3	Pooled std. dev.	Significant experimental factors[c]	CF Diet 1.1	PHFO Diet 1.2	HFO Diet 1.3	CF Diet 2.1	PHFO Diet 2.2	HFO Diet 2.3	Pooled std. dev.	Significant experimental factors
14:0						0.1	0.2	0.1	0.1	0.1	0.1	0.2	
16:0	5.4	5.7	5.4	0.4		6.3	7.5	5.2	7.8	6.9	7.2	1.8	
18:0	22.3	21.5	21.2	0.8	C	21.8	21.2	28.1	23.6	21.8	23.7	4.6	C
16:1	0.7	0.9	1.2	0.2	C	0.8	0.9	0.8	0.5	0.7	0.6	0.4	
18:1 *cis*	11.4	12.2	13.1	1.3		14.7	14.1	16.9	15.4	14.2	13.2	2.0	A
18:1 *trans*	n.d.[b]	n.d.	n.d.			0.03	0.06	0.01	n.d.	0.04	0.02	0.04	C
20:1 *cis*	0.5	0.5	0.6	0.1		0.7	0.6	0.8	0.6	0.6	0.5	0.2	B
20:3n-9	0.7	0.9	0.9	0.1		1.0	1.5	1.1	0.6	0.8	0.7	0.3	B,C
18:2n-6	0.6	0.5	0.8	0.3		0.4	0.5	0.5	0.4	0.5	0.6	0.3	
20:3n-6	0.4	0.4	0.5	0.1		0.5	0.6	0.8	0.4	0.5	0.4	0.1	A,B,C
20:4n-6	22.1	20.8	21.1	0.7	C	19.0	17.5	13.1	17.8	19.8	19.0	3.2	A,B,C
22:3n-6	0.6	0.7	0.7	0.2		0.8	1.0	0.9	0.6	0.6	0.6	0.2	B
22:4n-6	11.4	11.3	11.2	0.4		8.6	7.8	7.2	9.9	10.2	10.3	1.0	B
22:5n-6	3.5	5.3	4.4	0.7	C	1.6	1.7	1.9	2.7	2.8	2.8	0.3	B
22:5n-3	0.5	0.4	0.6	0.2		1.2	1.3	1.3	0.5	0.6	0.6	0.4	
22:6n-3	19.7	18.6	17.8	1.3		18.6	20.2	18.8	16.3	18.1	17.5	2.0	B
Sum PUFA n-6	38.6	39.0	37.9			30.9	29.1	24.4	31.8	34.3	33.7		
Sum PUFA n-3	20.2	18.7	18.4			19.8	21.5	20.1	18.7	18.7	18.1	18.1	
Sum PUFA total	59.5	58.6	57.2			51.7	52.1	45.6	49.2	53.8	52.5		
Sum monoenes	12.6	13.6	15.9			16.23	15.66	18.51	16.5	15.54	14.32		
Sum saturated	27.7	27.2	26.6			28.2	28.9	33.4	31.5	28.8	31.0		
20:3 n-9/20:4 n-6	0.03	0.04	0.04			0.05	0.09	0.08	0.03	0.04	0.04		

[a] From mothers fed these diets from 3 wk of age and through pregnancy (Expt. 1) and from gestation through pregnancy (Expt. 2).

[b] n.d. = not detectable.

[c] Significance means ($P < 0.05$): A = Interaction of experimental fats and SFO-level, B = effect of SFO-level, C = effect of experimental fats. Average of eight replicates. Pooled std. dev. = pooled standard deviation, (error mean square)$^{1/2}$.

amounts of linoleic acid gave offspring with higher levels of 22:4 n-6 and 22:5 n-6 than those found in offspring of sows fed low levels of linoleic acid.

Offspring from sows fed hydrogenated fats (PHFO and PHSBO) in Experiment 1 had a significantly reduced content of 18:0 compared with that found in those from sows fed lard. No consistent effects of the hydrogenated fats on the content of 18:0 were seen in Experiment 2.

Sows fed PHFO and PHSBO in Experiment 1 had offspring with significantly more 16:1 than those fed lard. The differences were unrelated to the dietary level of 16:1. Similar, but insignificant, differences between offspring of sows fed unhydrogenated (CF) and hydrogenated fats (PHFO and HFO) were seen in Experiment 2.

In Experiment 2 the content of 18:1 *cis* was significantly increased in offspring from sows fed CF in diets with high levels, but not in diets with low levels of linoleic acid, the interaction between dietary fats and the dietary level of linoleic acid was significant. The contents of 20:1 *cis* increased significantly when the dietary EFA-level was restricted in Experiment 2.

The different dietary regimens had no consistent effects on the relative amounts of saturates, monoenes and PUFAs in the two experiments. Neither were there any effects of the feeding of hydrogenated fats on the content of n-6 and n-3 PUFAs. Increasing the supply of linoleic acid in Experiment 2 caused an increase in the contents of n-6 PUFAs and a decrease in the n-3 PUFAs in the offspring.

Heart. The fatty acid composition of the PE in the total heart lipids is shown in Table 3. In Experiment 1, 18:1 *trans* was found in all dietary groups. Although the contents of 18:1 *trans* was higher in offspring from sows fed hydrogenated fats than in those of sows fed lard, the differences were not significant.

In Experiment 2, the content of 18:1 *trans* was related to the dietary *trans* content and was significantly higher in the offspring of sows fed hydrogenated fats compared with those of sows fed CF. Dietary levels of linoleic acid had no significant effect on the incorporation of *trans* fatty acids in Experiment 2.

Offspring from sows fed partially hydrogenated fat had in both experiments significantly more 18:2 n-6 than those from sows fed the *trans*-free controls.

There were significant interactions between experimental fats and the dietary level of linoleic acid as well as the main effects of the experimental fats on the content of 20:2n-6 in Experiment 2. Thus the feeding of partially hydrogenated fat decreased the level of 20:2n-6 in the groups from sows fed diets with a liberal level

TABLE 3

Fatty Acid Composition (%) in PE of Total Lipids in Heart of Newborn Piglets[a]

	Experiment 1					Experiment 2							
	Added SFO, 4%					Added SFO, 0%			Added SFO, 3%				
	Lard Diet 1	PHFO Diet 2	PHSBO Diet 3	Pooled std. dev.	Significant experimental factors[b]	CF Diet 1.1	PHFO Diet 1.2	HFO Diet 1.3	CF Diet 2.1	PHFO Diet 2.2	HFO Diet 2.3	Pooled std. dev.	Significant experimental factors
14:0						0.3	0.1	trace	0.1	0.1	0.4	0.3	A
16:0	7.7	7.1	8.7	3.2		4.8	5.4	5.9	5.4	5.5	6.9	1.9	
18:0	23.1	16.2	16.2	6.3		18.8	19.5	19.1	18.9	19.7	19.2	1.9	
16:1	1.7	1.2	1.0	0.9		0.8	0.8	1.0	0.7	0.7	0.9	0.3	
18:1 *cis*	16.4	13.5	15.5	3.2		14.6	15.8	15.3	13.7	13.2	14.1	1.7	B
18:1 *trans*	0.5	0.8	1.0	0.5		0.1	0.5	0.1	trace	0.7	0.5	0.4	C
20:1 *cis*	0.1	0.3	0.4	0.1	C	0.1	0.5	0.2	0.1	0.4	0.3	0.2	C
20:3n-9	0.3	0.6	0.6	0.2		0.4	0.5	0.5	0.4	0.3	0.4	0.1	B
18:2n-6	13.9	21.4	20.1	4.3	C	9.5	10.8	10.3	11.3	12.5	11.6	1.3	B,C
20:2n-6	0.4	0.2	0.2	0.3		0.1	0.2	0.2	0.5	0.1	0.2	0.2	A,C
20:3n-6	1.0	1.3	1.1	0.3		1.6	1.6	1.6	1.4	1.3	1.1	0.2	B,C
20:4n-6	26.0	26.5	25.1	6.3		25.6	22.6	24.1	31.9	28.0	27.4	2.2	B,C
22:4n-6	2.0	2.7	2.6	0.8		1.8	1.6	1.8	3.0	2.4	2.6	0.5	B
22:5n-6	1.3	2.2	1.8	0.6		1.7	1.2	1.3	2.0	1.4	1.6	0.5	C
18:3n-3	0.2	0.3	0.3	0.1		0.2	0.3	0.3	0.2	0.1	0.1	0.1	B
20:5n-3	1.5	1.2	1.3	0.5		5.5	5.4	5.6	0.9	1.0	1.5	1.1	B
22:5n-3	1.5	1.9	2.0	0.5		2.7	2.8	3.1	2.0	1.8	2.0	0.4	B
22:6n-3	2.3	2.9	2.3	1.2		4.0	3.4	3.7	3.4	2.6	2.4	0.9	B
Sum PUFA n-6	44.7	54.4	51.0			40.3	38.0	39.3	50.1	45.7	44.5		
Sum PUFA n-3	5.5	6.3	5.9			12.3	11.9	12.7	6.5	5.5	6.0		
Sum PUFA total	50.5	61.3	57.5			53.0	50.4	52.5	57.0	51.5	50.9		
Sum monoenes	18.7	15.8	17.9			15.5	17.5	16.6	14.5	15.2	15.8		
Sum saturated	30.8	23.3	24.9			23.9	25.0	25.0	24.4	25.3	26.5		
20:3n-9/20:4n-6	0.01	0.02	0.02			0.02	0.02	0.02	0.01	0.01	0.01		

[a] From mothers fed these diets from 3 wk of age and through pregnancy (Expt. 1) and from gestation through pregnancy (Expt. 2).

[b] Significance means ($P < 0.05$): A = Interaction of experimental fats and SFO-level, B = effect of SFO-level, C = effect of dietary fats.

Trace, i.e. <0.05%. Average of eight replicates. Pooled std. dev. = pooled standard deviation, $(\text{error mean square})^{1/2}$.

of linoleic acid while increasing the level in those on a low dietary level of linoleic acid. The feeding of hydrogenated fats in Experiment 2 reduced the levels of 20:3n-6, 20:4n-6 and 22:5n-6. No significant effects of hydrogenated fats on these fatty acids were seen in Experiment 1.

Offspring from sows fed hydrogenated fat had a higher content of 20:1 *cis* than those from sows fed either lard or CF as controls in both experiments.

There were no consistent dietary effects on the total PUFA, total monoenes and total saturated fatty acids.

Increasing the dietary level of linoleic acid increased the content of 18:2n-6, 20:4n-6 and 22:4n-6 and reduced the content of 18:1 *cis*, 20:3n-9, 20:3n-6, 18:3n-3, 20:5n-3, 22:5n-3 and 22:6n-3.

Liver. The fatty acid composition of the PE in liver mitochondria is shown in Table 4. In both experiments the 18:1 *trans* was present at significant levels. The level in Experiment 1 was about 3 times higher than that in Experiment 2. However, low to trace levels of 18:1 *trans* were also found in the offspring of control sows fed *trans*-free diets.

Feeding hydrogenated fat to the sows had only marginal and inconsistent effects on the fatty acid composition in the two experiments. In Experiment 2 the contents of 20:3 n-9 and 18:1 *cis* increased significantly when the hydrogenated fats were fed. A somewhat similar trend was seen in Experiment 1. In Experiment 2 the content of 20:3n-9 increased due to both dietary hydrogenated fats and EFA restriction.

In Experiment 2 the content of 20:4n-6 decreased when PHFO and HFO were fed at low dietary levels of linoleic acid, but increased at high levels, the interaction between experimental fats and linoleic acid level was significant. The addition of SFO increased the level of PUFA n-6 and decreased the level of PUFA n-3.

Blood. The fatty acid composition of total lipids in venous blood plasma is shown in Table 5. Offspring from sows fed hydrogenated fat contained in both experiments significant amounts of 18:1 *trans*. As found for the mitochondria-PE, the levels of 18:1 *trans* were about three times higher in Experiment 1 compared with Experiment 2. Trace to low levels of 18:1 *trans* were also found in the offspring of control animals fed *trans*-free fat.

The effects of hydrogenated fat on the overall fatty acid pattern were rather small and inconsistent between the two experiments. Feeding hydrogenated fats increased the level of 20:3n-6 in Experiment 1 and of 20:2n-6 and 22:4n-6 in Experiment 2 and decreased the levels of 20:4n-6, 20:5n-3 and 22:6n-3 in Experiment 2. In both experiments the feeding of hydrogenated fats increased the

TABLE 4

Fatty Acid Composition (%) in PE of Liver Mitochondria of Newborn Piglets[a]

	Experiment 1					Experiment 2							
	Added SFO, 4%					Added SFO, 0%			Added SFO, 3%				
	Lard Diet 1	PHFO Diet 2	PHSBO Diet 3	Pooled std. dev.	Significant experimental factors[b]	CF Diet 1.1	PHFO Diet 1.2	HFO Diet 1.3	CF Diet 2.1	PHFO Diet 2.2	HFO Diet 2.3	Pooled std. dev.	Significant experimental factors
14:0						0.4	0.4	0.3	0.5	0.3	0.4	0.2	A
16:0	12.4	13.4	10.2	5.1		9.4	9.7	8.2	0.9	8.4	8.9	1.8	
18:0	30.4	29.9	29.3	5.3		27.9	25.0	26.2	24.7	26.3	25.9	2.9	
16:1	1.9	2.3	2.0	0.9		2.0	2.2	1.7	1.7	1.4	2.1	0.6	
18:1 *cis*	13.3	16.1	16.0	4.1		13.4	16.5	15.6	12.7	13.0	12.8	1.9	B,C
18:1 *trans*	0.2	2.2	2.6	2.0	C	trace	0.9	0.5	0.3	0.6	0.4	0.4	C
20:1 *cis*	0.3	0.5	0.4	0.3		0.1	0.5	0.4	0.7	0.4	0.2	0.5	
20:3n-9	0.5	0.3	0.6	0.3		0.7	0.8	1.1	0.4	0.7	0.6	0.2	B,C
18:2n-6	5.1	7.6	6.2	2.2		4.2	3.7	3.8	4.5	4.2	5.3	1.3	
20:2n-6	0.4	0.2	0.4	0.4		trace	0.3	0.1	0.2	0.2	0.1	0.2	
20:3n-6	0.6	0.6	0.6	0.3		0.8	0.7	0.9	1.0	0.8	0.8	0.2	
20:4n-6	24.6	18.9	21.5	10.9		24.0	18.3	21.4	25.5	27.0	28.4	4.1	A,B
22:4n-6	0.7	0.6	0.9	0.5		0.9	0.7	1.5	0.7	1.0	1.3	0.7	C
22:5n-6	0.9	1.0	1.6	0.8		0.8	0.6	0.8	1.1	1.1	1.2	0.4	B
18:3n-3	trace	trace	trace			trace	0.1	trace	trace	0.1	trace	0.1	
20:5n-3	0.5	0.5	0.4	0.3		1.9	2.0	2.2	0.3	0.3	0.2	0.5	B
22:5n-3	1.0	0.8	1.2	0.6		1.5	1.3	1.4	1.2	1.1	1.0	0.4	B
22:6n-3	7.5	4.6	5.2	2.8		8.5	6.7	9.6	7.4	6.8	6.6	2.0	B
Sum PUFA n-6	32.3	28.9	31.2			30.7	24.3	28.5	33.0	34.4	37.1		
Sum PUFA n-3	9.0	5.9	6.8			11.9	10.1	13.2	8.9	8.3	7.8		
Sum PUFA total	41.8	35.1	38.6			43.3	35.2	42.8	42.3	43.4	45.5		
Sum monoenes	15.7	21.1	21.0			15.5	20.1	18.2	15.4	15.5	15.5		
Sum saturated	42.8	43.3	39.5			37.7	35.1	34.7	34.2	35.0	35.2		
20:3n-9/20:4n-6	0.02	0.02	0.03			0.03	0.04	0.05	0.02	0.03	0.02		

[a] From mothers fed these diets from 3 wk of age and through pregnancy (Expt. 1) and from gestation through pregnancy (Expt. 2).

[b] Significance means ($P < 0.05$): A = Interaction of experimental fats and SFO-level, B = effect of SFO-level, C = effect of dietary fats.

Trace, i.e. <0.05%. Average of eight replicates. Pooled std. dev. = pooled standard deviation, $(\text{error mean square})^{1/2}$.

level of 18:1 *cis*, while an increase due to hydrogenated fat on 16:1 only was seen in Experiment 1. The feeding of CF in Experiment 2 increased the level of 14:0 with a compensatory decrease in 16:0. The overall effects of feeding hydrogenated fat in Experiment 1 were a reduction in the level of PUFAn-6, PUFAn-3 and saturated fatty acids with an increase in the levels of monoenoic fatty acids. In Experiment 2 the effects of hydrogenated fats on the different classes of fatty acids were negligible.

Increasing the dietary level of linoleic acid in Experiment 2 increased the levels of 18:2n-6, 20:4n-6, 22:4n-6 and 22:5n-6 and decreased the levels of 16:1, 18:1 *cis*, 20:5n-3, 22:5n-3 and of 22:6n-3, the net effects being an increase in PUFAn-6, a reduction in PUFAn-3, an increase in sum PUFA total and a decrease in monoenoic fatty acids.

Adipose tissue. The fatty acid composition of subcutaneous adipose tissue is shown in Table 6. Very low levels of 18:1 *trans* were found in all dietary groups in Experiment 1. In Experiment 2 the concentration of 18:1 *trans* in offspring from sows fed hydrogenated fat was significantly higher than in those of sows fed the *trans*-free fat, although low levels of 18:1 *trans* were found also in the latter groups. The effect on the overall fatty acid composition from the feeding of hydrogenated fat was relatively minor and rather inconsistent between the two experiments. In Experiment 2 the feeding of hydrogenated fats decreased the level of 18:3n-3. Further in Experiment 2, but not in Experiment 1, the feeding of PHFO and HFO led to increased incorporation of the long chain saturated and monoenoic fatty acids, i.e. 20:0, 20:1 *cis* and 22:1 *cis* + *trans*. The content of 12:0 and 14:0 increased significantly when CF was fed in Experiment 2. Adding linoleic acid increased the level of PUFA n-6 and reduced the level of PUFA n-3. It also increased the sum of PUFA and reduced the sum of monoenes, the sum of saturates being unaffected.

DISCUSSION

The present study supports and extends previous understanding (32) of resistance in fetal tissue fatty acid composition to maternal dietary fats. Thus for all tissues examined, variation in fatty acid composition between dietary groups was minor compared with that found for their adult mothers who were fed the experimental fats over a life-span period (27).

This was particularly the case for the incorporation of *trans* fatty acids, as will be seen from Table 7, showing the discrimination factor (i.e., the relation of the percentage *trans* isomers of the total monoene contents of the organ lipids to that of the respective dietary lipids) of

TABLE 5

Fatty Acid Composition (%) of Total Plasma Lipids of Venous Blood from Newborn Piglets[a]

	Experiment 1					Experiment 2							
	Added SFO, 4%					Added SFO, 0%			Added SFO, 3%				
	Lard Diet 1	PHFO Diet 2	PHSBO Diet 3	Pooled std. dev.	Significant experimental factors[b]	CF Diet 1.1	PHFO Diet 1.2	HFO Diet 1.3	CF Diet 2.1	PHFO Diet 2.2	HFO Diet 2.3	Pooled std. dev.	Significant experimental factors
14:0						2.6	1.9	1.6	3.5	1.7	1.7	1.0	C
16:0	24.4	23.3	23.1	1.6		22.1	20.7	20.4	22.6	21.4	21.6	1.5	C
18:0	12.7	11.1	12.5	1.6		10.9	11.2	11.2	10.8	11.2	11.0	0.9	
16:1	3.5	5.1	4.6	0.9	C	5.4	5.0	4.9	4.8	4.7	4.7	0.6	B
18:1 *cis*	23.3	29.3	30.4	3.2	C	26.8	28.8	30.7	24.8	27.5	24.9	2.2	A,B,C
18:1 *trans*	n.d.[c]	2.0	2.1	1.1	C	0.3	0.8	0.5	0.2	0.6	0.5	0.2	C
20:1 *cis*	0.4	0.5	trace	0.5		0.3	1.0	0.3	0.8	0.6	0.3	0.7	
22:1 *cis* + *trans*	trace	trace	trace			0.1	0.6	0.1	0.4	0.1	0.1	0.7	
20:3n-9	trace	trace	trace			0.5	0.6	0.5	0.4	0.6	0.5	0.2	
18:2n-6	19.1	15.3	12.5	3.9		6.6	6.6	6.7	7.4	8.1	7.9	1.3	B
20:2n-6	0.3	trace	0.1	0.2		trace	0.2	0.3	0.1	0.1	0.2	0.2	C
20:3n-6	0.4	0.7	0.6	0.1	C	0.9	0.8	0.8	0.8	0.9	0.9	0.1	
20:4n-6	9.2	8.0	9.4	2.0		8.0	6.4	6.7	10.8	9.8	11.2	1.4	B,C
22:4n-6	0.6	0.2	0.2	0.3		0.3	0.2	0.3	0.4	0.5	0.7	0.2	A,B,C
22:5n-6	trace	trace	trace			0.6	0.3	0.3	0.8	0.6	1.0	0.4	B
18:3n-3	0.7	0.9	1.0	0.3		0.2	0.2	0.2	0.1	0.3	0.2	0.2	
20:5n-3	0.3	0.3	0.4	0.3		1.7	1.4	1.4	0.6	0.5	0.6	0.2	B,C
22:5n-3	0.8	0.4	0.4	0.4		0.9	0.9	0.8	0.5	0.5	0.8	0.2	B
22:6n-3	2.8	1.7	2.0	0.7		4.3	3.9	4.5	3.0	2.8	3.4	0.6	B,C
Sum PUFA n-6	29.6	24.2	22.8			16.4	14.5	15.1	20.3	20.0	21.9		
Sum PUFA n-3	4.6	3.3	3.8			7.1	6.4	6.9	4.2	4.0	5.0		
Sum PUFA total	33.2	27.5	26.6			24.0	21.5	22.5	24.9	24.6	27.4		
Sum monoenes	27.2	36.9	37.1			32.9	36.2	36.5	31.0	32.5	30.5		
Sum saturated	37.1	34.4	35.6			35.6	33.8	33.2	36.9	34.3	34.3		

[a] From mothers fed these diets from 3 wk of age and through pregnancy (Expt. 1) and from gestation through pregnancy (Expt. 2).

[b] Significance means ($P < 0.05$): A = Interaction of experimental fats and SFO-level, B = effect of SFO-level, C = effect of dietary fats.

[c] n.d. = not detectable.

Trace, i.e. <0.05%. Average of eight replicates. Pooled std. dev. = pooled standard deviation, (error mean square)$^{1/2}$.

TABLE 6

Fatty Acid Composition (%) of Subcutaneous Adipose Tissue of Newborn Piglets[a]

	Experiment 1					Experiment 2							
	Added SFO, 4%					Added SFO, 0%			Added SFO, 3%				
	Lard Diet 1	PHFO Diet 2	PHSBO Diet 3	Pooled std. dev.	Significant experimental factors[c]	CF Diet 1.1	PHFO Diet 1.2	HFO Diet 1.3	CF Diet 2.1	PHFO Diet 2.2	HFO Diet 2.3	Pooled std. dev.	Significant experimental factors
12:0						0.7	0.1	0.1	0.8	0.2	0.2	0.3	C
14:0						5.2	3.6	3.3	5.3	3.3	3.7	0.8	C
16:0	33.3	29.5	28.8	3.7		27.6	27.7	28.7	28.0	26.3	26.8	1.5	B
18:0	13.0	12.4	12.2	1.2		10.1	10.5	11.7	10.9	10.9	10.8	1.3	
20:0	n.d.[b]	n.d.	n.d.			0.2	0.4	0.6	0.3	0.4	0.4	0.2	C
16:1	6.3	6.9	6.8	1.2		6.7	6.6	6.4	5.9	5.7	5.6	0.8	B
18:1 *cis*	29.5	29.1	29.6	2.6		28.6	27.9	29.2	25.8	26.7	26.0	1.7	B
18:1 *trans*	n.d.[b]	n.d.	0.1	0.1		0.3	0.9	0.5	0.1	1.0	0.9	0.5	C
20:1 *cis*	0.8	0.6	0.6	0.4		0.4	1.0	0.9	0.3	0.8	1.1	0.6	
22:1 *cis* + *trans*	0.2	0.2	trace	0.2		trace	0.1	—	—	0.1	0.5	0.4	C
18:2n-6	9.0	10.8	9.3	2.5		6.9	6.1	5.3	9.6	10.0	10.6	1.6	B
20:3n-6	0.8	0.8	0.9	0.2		0.8	0.7	0.7	0.9	0.9	0.9	0.2	B
20:4n-6	4.7	6.1	6.7	2.6		4.2	3.5	3.5	5.2	4.5	4.7	1.3	
22:4n-6	1.3	1.5	1.7	0.6		—	0.1	0.9	1.3	0.7	1.5	0.3	B
22:5n-6	0.5	0.5	0.6	0.3		—	—	—	—	—	—		
18:3n-3	0.3	0.4	0.3	0.1		0.8	0.7	0.4	0.4	0.4	0.3	0.2	B,C
22:5n-3	0.6	0.7	0.6	0.4		1.3	1.0	1.0	0.7	0.6	0.6	0.2	B
22:6n-3	1.0	1.0	1.7	0.9		1.7	1.3	1.5	1.1	0.9	1.0	0.4	B
Sum PUFAn-6	16.3	19.7	19.2			12.5	11.0	10.4	17.0	16.7	17.7		
Sum PUFAN-3	1.9	2.1	2.6			3.8	3.0	2.9	2.2	1.9	1.9		
Sum PUFA total	18.2	21.8	21.8			16.3	14.0	13.3	19.2	18.6	19.6		
Sum monoenes	36.8	36.8	37.1			36.0	36.5	37.0	32.1	34.3	34.1		
Sum saturated	46.3	41.9	41.0			43.8	42.3	44.4	45.3	41.1	41.9		

[a]From mothers fed these diets from 3 wk of age and through pregnancy (Expt. 1) and from gestation through pregnancy (Expt. 2).

[b]n.d. = not detectable.

[c]Significance means ($P < 0.05$): A = Interaction of experimental fats and SFO-level, B = effect of SFO-level, C = effect of dietary fats.

Trace, i.e. <0.05%. Average of eight replicates. Pooled std. dev. = pooled standard deviation, $(\text{error mean square})^{1/2}$.

TABLE 7

Ratio of the Percentage *trans* Isomers of the Total Monoene Content of Organ Lipids to That of the Respective Dietary Fats in Newborn Piglets and Their Adult Mothers in Experiment 1[a]

	18:1 *trans*		20:1 *trans*
	PHFO	PHSBO	PHFO
Brain			
Adult	0	0	0
Neonatal	0	0	0
Heart			
Adult	0.45	0.32	0.15
Neonatal	0.17	0.13	0
Liver			
Adult	0.94	0.76	0.41
Neonatal	0.37	0.31	0
Blood			
Adult	0.63	0.51	0
Neonatal	0.20	0.14	0
Adipose			
Adult	0.64	0.58	0.87
Neonatal	0	0.01	0

[a]Fed the diets from 3 wk of age and through gestation.

neonatal piglets compared with that of their mothers in Experiment 1 (27). The incorporation of *trans* fatty acids in the perinatal piglet was 1/3 to 1/4 of that found in their mothers in all tissues except adipose where the incorporation in the perinatal piglet was nil or very low. Table 7 shows that there was no difference in the incorporation of 18:1 *trans* from PHFO and PHSBO and that the 20:1 *trans* from PHFO was not incorporated into the tissue of the piglets. Adipose tissues were mainly triglycerides in both adult females and newborn piglets (results not reported). Differences in lipid class composition, i.e., the relative amounts of triglycerides and phospholipids, can therefore evidently not explain the relatively low levels of *trans* fatty acids in the adipose tissues of newborn piglets compared to adult sows (20). If one assumes that the venous blood of the adult females and the newborn piglets represented the placental blood supply and the fetal blood circulation, respectively, the blood-placenta barrier decreased the level of 18:1 *trans* by 70 percent and excluded the 20:1 *trans*.

Previous studies on placental transport of *trans* fatty acids to the developing fetus are conflicting. Thus, Johnston *et al.* (17) were unable to detect *trans* fatty acids (less than 0.5% *trans* analyzed by I.R. spectrophotometry) in human fetal liver lipids or in total body lipids of

newborn babies. In contrast maternal depot fats contained from 1.5 to 6.8% *trans* fatty acid. The same group (16) did not detect significant levels of *trans* fatty acids in newborn rats from mothers fed hydrogenated margarine stock containing 40.7% *trans* fatty acids.

In contrast to the above findings, McConnell and Sinclair (18) found that newborn rats from mothers fed 48.4% elaidin contained 16% *trans* fatty acids in liver PL and 11% in total carcass lipids. Also Pallansch *et al.* (21) found up to 2.9% *trans* fatty acids (by GLC) in carcass lipids of newborn mice from mothers fed partially hydrogenated corn oil.

Ono and Fredricksen (19) using injection of radiolabeled non-esterified isomers of 18:1 *trans* and 18:2 *trans-trans* into the plasma of pregnant rats found that the *trans* and *trans-trans* isomers crossed the blood-placenta barrier to the same extent as their *cis* homologues.

Similarly, Moore and Dhopeshwarkar (20), also injecting radiolabeled albumine-bond 18:1 *trans* and 18:2 *trans-trans* together with their *cis* counterparts into the jugular vein of rats, found no difference in placenta transfer between the *cis* and *trans* isomers. However, the 18:1 *trans* fatty acid (elaidic acid) was oxidized to a large extent and not incorporated into fetal tissue, a finding that the authors used to explain previous findings of the absence of *trans* fatty acids in fetal lipids. However, Johnston *et al.* (17) did not detect *trans* fatty acids in human placentas.

Our results with pigs support previous experimental data from rodents, that low levels of *trans* fatty acids are incorporated into the fetus, but the levels vary between the different organs.

We have previously reported (27) the absence of *trans* fatty acids in the brain of mother pigs fed the various diets in Experiment 1 from 3 wk to about 2 yr of age. Further, we have previously reported (26) the lack of the effect of *trans* fatty acids on brain size and peroneal nerve histology and conduction velocity in the newborn piglets. The absence or very low levels of *trans* fatty acids found in the brains of the newborn piglets in this study confirms and extends the lack of adverse effects on nervous tissue of dietary *trans* fatty acids.

Our findings of very low to nil levels of *trans* fatty acids in the brain of newborn piglets are in disagreement with the results of Moore and Dhopeshwarkar (20), however, who found almost a similar incorporation of 18:1 *trans* and 18:2 *trans-trans* in the different lipid classes in total body lipids and brain. Obviously, differences in animal species and methodology may explain the difference between our results and those of Moore and Dhopeshwarkar (20).

The subcutaneous adipose tissue of newborn piglets in Experiment 2 contained significantly higher levels of 18:1 *trans* compared to that of Experiment 1. However, in the liver mitochondria-PE and the plasma lipids the contents of 18:1 *trans* were 3–5 times greater in Experiment 1 than in Experiment 2.

It is conceivable that these deviations between Experiment 1 and Experiment 2 may have been due to the differences in the design of the two experiments, i.e., the length of experimental feeding of *trans* fatty acids to the respective mother pigs.

Since it has been postulated that the effect of *trans* fatty acids depends on the dietary level of linoleic acid (12,33,34), Experiment 2 compared diets with 1 and 2.7 percent (2 and 5.5 cal%) linoleic acid. The results revealed no effects of dietary linoleic acid level on the incorporation of *trans* fatty acids in the different tissues, and this is in-line with previous findings (12).

Increasing the dietary levels of linoleic acid in the mothers' diets caused a general increase in the contents of PUFA n-6 and compensatory decreases in the level of PUFA n-3 and 20:3n-9. It has been shown that the appearance of *trans* fatty acids in organ lipids may reduce the biosynthesis of 20:4 n-6, i.e., arachidonic acid (11–13, 35–37), and it has been postulated that *trans* fatty acids may cause EFA deficiency when fed together with a limited supply of linoleic acid (33,34). The triene tetraene ratio (e.g., 20:3 n-9/20:4n-6) has been used as an index of EFA status, values above 0.4 being taken as an evidence of EFA deficiency (38).

In the present study the triene tetraene ratio never exceeded 0.1, thus, far from any signs of EFA deficiency. The lowest level of dietary linoleic used in this study was about 1% (about 2 en%) while the requirement may be as low as 0.2 en% (39). Thus, although the feeding of 10 en% of *trans* fatty acids caused a nominal increase in the level of 20:3n-9, in contrast to previous results (12), the increase was marginal. Therefore the feeding of *trans* fatty acids did not significantly effect the EFA requirement. This is in-line with the previous findings of Zevenbergen *et al.* (12) who performed feeding trials with weanling rats.

Whenever the composition of the n-3 fatty acids of the structural lipids was affected, it was only caused by the dietary EFA-level. The effects of *trans* fatty acids on the PUFA-composition seemed to be on the n-6 fatty acids only.

In Experiment 1, the brain-PE of the piglets contained significantly more 22:5n-6 when the mothers were fed PHFO and PHSBO. Similar effects were found in the brain-PE of the respective mothers (27). This particular effect of dietary *trans* fatty acids was not found in the contents of 22:5n-6 in the other organ lipids. These results are in-line with those of Sanders *et al.* (40) who fed hydrogenated fat to rats, and demonstrate the organ specific influence of dietary *trans* fatty acids.

Some of the experimental fats used in this study contained long-chain monoenoic fatty acids (e.g., *cis/trans* isomers of 20:1 and 22:1 in PHFO), long-chain saturated fatty acids (e.g., 20:0 and 22:0 in HFO) and short-chain saturated fatty acids (e.g., 8:0, 10:0 and 12:0 in CO). It is of interest that only small amounts of these fatty acids were detected in the structural lipids analyzed. Further, the presence of high levels of long-chain saturated fatty acids in HFO did not influence the fatty acid pattern of the organ lipids.

Compared with adult females (27) the levels of 20:1 were low in the various tissues of the newborn piglets. It thus appears that fatty acids which have a low rate of deposition in the adult are further discriminated against in the fetal piglet (Table 7).

Another difference between mother and offspring (Expt. 1) was the higher percentage of linoleic acid in the lipids of blood and adipose tissue in the mothers (27) compared to that of the offspring (almost half the contents registered in the mothers). However, the higher homologues of the n-6 PUFA were present in the fat tissue of

the offspring in higher percentages than in the mothers.

In conclusion this work shows that dietary contents of 3-6% (6-12 cal%) *trans* fatty acids from PHFO or PHSBO equal to 5 to 12 times that found in human diets (33) do not lead to noticeable incorporation of *trans* fatty acids in the brain and to only slight incorporation into other structural lipids in the newborn piglet when fed in diets with liberal or restricted amounts of dietary linoleic acid. Further, the dietary *trans* fatty acid did not cause significant changes in the overall fatty acid pattern. This finding confirms and substantiates previous data (26) showing the lack of teratogenic effects or effects on organ size and neural conduction velocity in newborn pigs.

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Gas Chromatographic Analysis of Intact Steryl Esters in Wild Type *Saccharomyces cerevisiae* and in an Ester Accumulating Mutant

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The steryl ester faction from wild type and mutant strains of the yeast *Saccharomyces cerevisiae* was analyzed without saponification by a non-polar capillary gas chromatographic column. When expressed as μg ester/mg dry wt, the total ester fraction remained constant or declined slightly from log to stationary phase in the wild type. In the mutant the decrease was more dramatic. No individual ergosteryl ester species was dominant throughout the culture cycle in the wild type. A compound tentatively identified as zymosteryl palmitate was the most prevalent ester in wild type log phase cells, ergosta-5,7-dienyl palmitate and ergosta-5,7-dienyl palmitoleate were the major esters in stationary cells. In the mutant strain, ergosteryl esters of palmitate, palmitoleate, oleate, and stearate were the major ester components throughout the culture cycle. Like the wild type, however, the mutant showed an increase in the proportion of ergosta-5,7-dienyl esters in the stationary phase of the culture cycle. The data did not indicate a sterol/fatty acid specificity during the culture cycle.
Lipids 24, 625-629 (1989).

The majority of fungi studied thus far have been shown to contain ergosterol as the dominant sterol, and the yeast *Saccharomyces cerevisiae* is no exception. In non-mammalian systems, little unequivocal data exists regarding the fate of sterol molecules aside from their roles as structural membrane components. Sterols with the unsubstituted 3β-hydroxy moiety, i.e., free sterols, represent the form most likely to be involved in membrane structure owing to their amphipathic nature. In addition, sterols can be esterified to fatty acids, have carbohydrate substituents, or a combination of the two, i.e., steryl esters, steryl glycosides and acylated steryl glycosides, respectively (1,2). Previous efforts in our laboratory did not detect the glycosylated sterols in the yeast investigated. In the present report we will concentrate on the steryl esters of *Saccharomyces cerevisiae.*

Apart from their involvement in vascular sterol transport in mammals, very little conclusive data are found for other possible functions of steryl esters. A similar transport process has been proposed for plant systems (3). There are reports showing that in some plants certain subcellular organelles are more enriched in steryl esters than others, suggesting a possible intracellular transport mechanism (4). Steryl esters are generally regarded as being incapable of membrane interactions, and in yeast are reported to be sequestered into lipid droplets (5). It has been shown in our laboratory that during the culture cycle in yeast, ergosterol precursors and other non-ergosterol sterols were preferentially esterified over ergosterol (6). Furthermore, occurrence of ergosterol in the free or ester fraction, depending on the stage of the culture cycle, has led to the concept of a membrane "proofing" phenomenon (7). This can be envisioned as a mechanism whereby ergosterol and its precursors are esterified as the culture reaches the stationary growth phase, and ergosterol is not needed for membrane biogenesis. Upon re-entry of the cells to active growth ergosteryl esters are selectively hydrolyzed to participate in membrane synthesis.

Typically in yeast sterols are esterified to palmitic, palmitoleic, stearic and oleic acids. If specificity exists for esterification of a given sterol, one can ask whether there is selectivity for the fatty acids that are part of the ester molecule, and if such preferences would change over the culture cycle. Such specificities do not exist in phytosterolemia in man, where plant sterols which are not normally absorbed from food sources are found in the sterol pool, primarily in the ester fraction (8).

Analysis of intact esters may provide insight into mechanisms of ester synthesis, hydrolysis and participation of esters in other cellular phenomena. The difficulty with providing relevant information concerning sterol/fatty acid specificity, and data concerning roles of steryl esters is associated with the technical aspects of assaying intact esters. The classical method of saponification used to identify ester components will not suffice, since once hydrolyzed, only components of the total ester pool can be assessed, but not members of a specific ester. In those experiments designed to purify and identify intact esters, gas chromatography (8-11) and high performance liquid chromatography (8,12) have been most useful in resolving these mixtures. To our knowledge no data exists regarding the intact ester profile in the yeast *Saccharomyces cerevisiae.* In this study we report the results of our investigation of the changes in intact steryl esters in a wild type and a mutant *Saccharomyces cerevisiae* over the culture cycle, and provide a simplistic but useful method of analyzing these lipids.

MATERIALS AND METHODS

Yeast strains and culture conditions. Wild type *Saccharomyces cerevisiae,* strain X2180A (SUC2, mal, gal2, CUP1) and a yeast mutant defective in sterol uptake control designated upc20 (upc 2-1) (13), were grown in rich media containing 2% glucose, 1% yeast extract and 2% peptone. Cells were harvested at various intervals in the culture cycle by centrifugation and the pellet lyophilized. Growth was measured with a Klett-Summerson photoelectric colorimeter equipped with a green filter with Klett units being proportional to cell number (14).

Extraction and separation of lipids. After lyophilization the cell pellet was steamed for one hour in dimethyl sulfoxide (DMSO), followed by the addition of 2M KCl, then hexane. This mixture was vortexed and the phases allowed to separate by low speed centrifugation, resulting

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Abbreviations: DSMO, dimethyl sulfoxide; GC, gas chromatography; HPLC, high performance liquid chromatography; TLC, thin layer chromatography; TMSE, trimethylsilyl ether; UV, ultraviolet.

in the total lipid partitioning into the hexane layer. An aliquot of the total lipid fraction was removed for direct analysis by gas chromatography (GC). This aliquot was dried under a stream of nitrogen and redissolved in hexane containing 1 mg/ml cholesteryl palmitate as a quantitative and reference standard. Quantitation of steryl esters from the GC column was accomplished by comparing the area under the sample with the area of the internal standard. The formula for such calculations is: sample area/standard area × dilutions factor. In addition, periodic, tandem injections (n = 4) of the standard was performed to assure reproducible detection signals. The steryl esters were separated from the remainder of the extract by thin layer chromatography (TLC). The TLC system consisted of 20 cm × 20 cm silica gel F254 analytical or preparatory plates developed in the solvent systems of Skipski *et al.* (15). Bands were visualized with short wavelength UV light, and steryl esters were recovered from the silica with hexane. Individual steryl esters were separated and recovered by high performance liquid chromatography (HPLC).

Identification of fatty acid and sterol moieties in intact esters. Retention times for each individual intact ester recovered from HPLC were determined by GC before saponification. Each was subsequently saponified thus releasing the fatty acid and sterol for further qualitative analysis. Fatty acids were converted to their methyl esters and sterols to their trimethylsilyl ether (TMSE) derivatives for GC purposes (16,17). Cholesterol TMSE was used as an internal GC reference for sterols and fatty acid retention times were compared to authentic standards.

HPLC, GC, conditions. A Beckman model 110 A liquid chromatograph was used to resolve and collect individual esters. This system was interfaced with an IBM 9000 chromatographic work station for data integration and storage. The column employed was a 25 cm × 0.5 mm i.d. semi-preparatory reversed phase (C-18) HPLC column (Supelco Inc., Bellfonte, PA). All solvents were HPLC grade and the system consisted of an isocratic mixture of acetonitrile/isopropanol (6:4, v/v) delivered at 5 mls/min (18). Peaks were detected at 205 nm by a Hitachi Model 100-400 variable wavelength detector.

The gas chromatograph was a Hewlett Packard Model 5890 gas chromatograph equipped for capillary GC analyses. Lipids were analyzed on a 20 meter × 0.32 mm i.d., 0.25 film thickness SPB-1 (bonded methyl silicone, Supelco Inc., Bellfonte, PA) fused silica column. This column was a modification of the original 30 meter column. The column conditions for (a) intact esters were: initial oven temperature 225° (then increased 5°/min to 300°); injector and detector temperature 300°; split ratio 84:1; average linear carrier gas (hydrogen) velocity at 225° oven temperature 72 cm/sec; 9 psi column head pressure; (b) sterol TMSE derivatives: oven temperature 230° isothermal; injector and detector 280°; split ratio 11:1, average linear gas velocity (helium) 66 cm/sec; 25 psi column head pressure; and (c) fatty acid methyl esters: oven temperature 180°; injector and detector 200°; split ratio 30:1; average linear gas velocity (helium) 57 cm/sec; 15 psi column head pressure.

RESULTS AND DISCUSSION

GC and HPLC analysis. During these analyses, some concerns arose that should be addressed. These are related to possible thermal degradation of samples during GC analysis, double bond rearrangement/denaturation during HPLC purification, but most important, the need for extreme care during saponification and recovery of sterols and fatty acids comprising the ester. These stem from the fragility of the homoannular diene in ergosterol and ease of destruction of unsaturated fatty acids. With this in mind various temperature programs were performed and the one chosen provided the best resolution with no apparent degradation. Additionally, any UV absorption during HPLC purification did not appear to have any effect on these analyses. As an example, individual esters such as ergosteryl stearate (Fig. 1k), isolated from the HPLC mixture generally produced a single peak when it was subsequently analyzed by GC. However, if extreme care is not taken during saponification and recovery, multiple components could be indicated due to possible oxidation (or isomerization) products. These appear as smaller components found along with major peaks in individual sterol or fatty acid chromatograms. Alternatively, the possibility exists that minor intact esters co-purify and co-migrate on the GC column along with the major component. Nevertheless, based on chromatographic data from three experiments, peaks identified in this study represent the major sterol and fatty acid in a given fraction. Previous studies have employed polar capillary GC columns (SP-2330) to separate intact esters (8,11). In this study attempts to evoke separation of intact esters on this phase were unsuccessful. It should be mentioned that our attempts were performed with hydrogen as the carrier, since recent data suggests no hydrogenation effects when this phase is employed (8). We did not attempt these analyses on the Sp-2330 using helium as a carrier as previously described (11), but instead chose the non-polar SPB-1 since the problem related to resolution of polyunsaturated fatty acids was not expected. The increased sensitivity associated with this phase for this purpose was beneficial. It is important to note the importance of hydrogen as the carrier gas, since helium does not produce favorable separation under the same conditions. The analytical system used in this study should prove to be

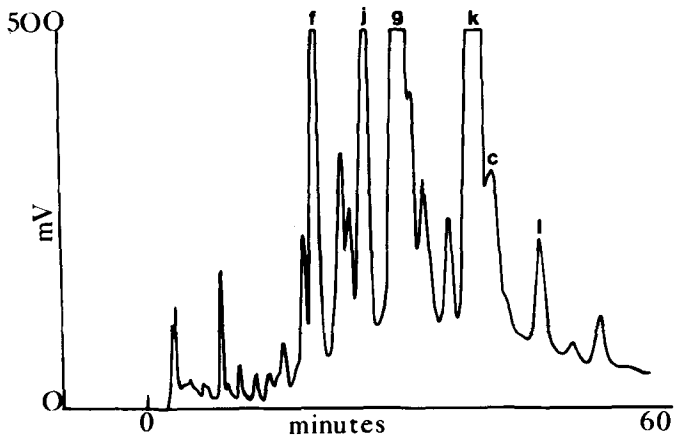

FIG. 1. HPLC separation of the intact steryl ester fraction from an ester accumulating mutant of *Saccharomyces cerevisiae* at stationary phase. HPLC conditions are given in the text. Peaks are identified as in Table 1.

most useful in detailed analyses of steryl esters where it is important to know individual ester species.

Table 1 shows the identity and retention times of the esters relative to cholesteryl palmitate. This method of peak identification was similar to that of Patterson (19) for unesterified sterols. The process entailed the incorporation into the sample of an ester not likely to be a natural yeast constituent, in this case cholesteryl palmitate. Thus the standard would have a relative retention time of 1.00. All retention times of esters found in the sample would be relative to the standard. A greater accuracy is achieved since absolute retention times can vary from analysis to analysis (especially lengthy analyses). The formula for such calculation is retention time of sample/retention time of standard. Surprisingly, the peaks designated zymosteryl palmitate and cholesta-5,7-dienyl palmitate eluted earlier than anticipated based on data for free sterols. However, according to our methods of identification for other peaks, these are tentatively being identified as such. Further efforts should resolve this matter. The data do not show specificity for a given sterol and fatty acid combination as the mixture was relatively heterogeneous with respect to pairings. In some cases quantitative differences existed as will be discussed below. Both HPLC and GC systems are capable of separating steryl esters (Figs. 1-3). However, the HPLC was used primarily as a preparative tool in this study. In addition to its convenience, it also negates the concern of oxidation sometimes associated with argentation TLC methods. There are shortcomings to either system, related to whether samples are composed of mixed esters or esters comprised of one or two sterol species (8). Our data expresses this concern as it is seen that separation of ergosteryl palmitate and ergosteryl palmitoleate is more difficult on the GC than ergosteryl palmitate and ergosta-5, 7-dienyl palmitate (Fig. 3).

It was of interest to determine whether our GC system could simultaneously provide data regarding free sterols as well as esters in a single injection. This has been accomplished in other studies (9,10), however, in that work the mixtures were either relatively simple, or controlled to the extent that known mixtures were analyzed. Our efforts provided limited success. Even though the two

TABLE 1

GC[a] Relative Retention Times[b] of Steryl Esters Identified from *Saccharomyces cerevisiae*

Steryl ester	Relative retention time
a. Cholesta-5,7-dienyl palmitate	0.93
b. 14α-methyl-ergosta-8,24(28)-dienyl myristate	0.94
c. Zymosteryl palmitate	0.98
d. Cholesteryl palmitate	1.00
e. Ergosta-8-enyl myristate	1.03
f. Ergosteryl palmitoleate	1.06
g. Ergosteryl palmitate	1.08
h. Ergosta-8-enyl palmitate	1.14
i. 14α-methyl-ergosta-8,22-dienyl oleate	1.16
j. Ergosteryl oleate	1.27
k. Ergosteryl stearate	1.29
l. Dihydrolanosteryl oleate	1.40
m. Ergosta-5,7-dienyl palmitoleate	1.53
n. Ergosta-5,7-dienyl palmitate	1.56

[a]See text for GC conditions.

[b]Relative to cholesteryl palmitate.

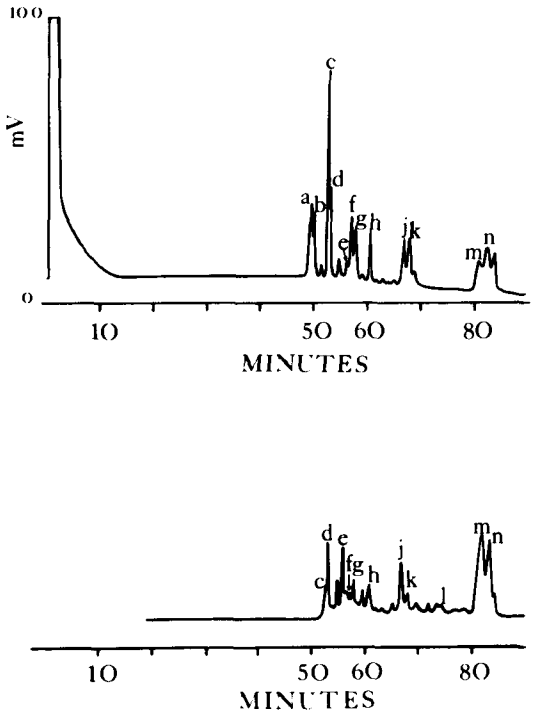

FIG. 2. Comparison of the steryl ester profiles in wild type yeast grown to log phase (A); or late stationary phase (B). Peaks are identified as in Table 1. See the text for chromatographic conditions.

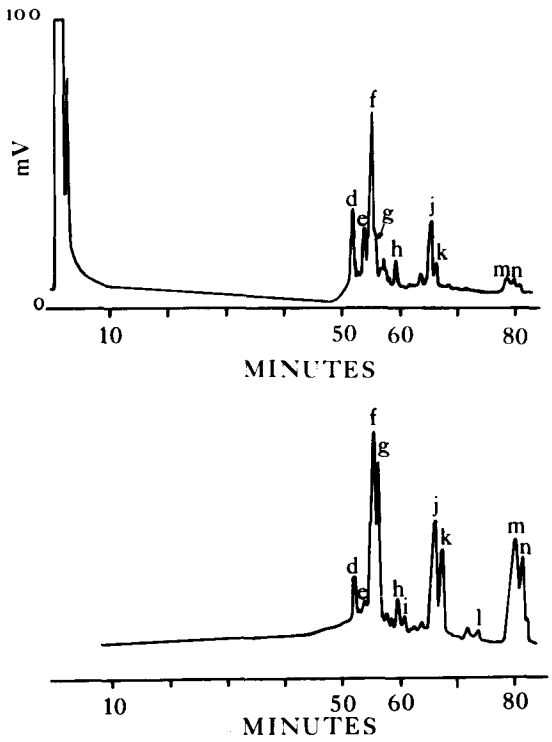

FIG. 3. Comparison of the steryl ester profiles in an ester accumulating mutant grown to log phase (A); or late stationary phase (B). Peaks are identified as in Table 1. See text for chromatographic conditions.

fractions were well separated (data not shown) the retention of free sterols was too short under conditions that allowed optimal separation of the esters. This resulted in peak overlap that was unfavorable for precise data integration. However, this problem may be overcome with other columns and GC conditions. The potential is that once isolated, the mixture may be co-injected after a simple "clean up," thus negating the need for both saponification and separate analysis of free and esterified sterols.

Qualitative and quantitative analyses. Wild type: Qualitatively there is little difference in the steryl ester profile over the culture cycle. As seen in Figure 2, the representative esters are present regardless if the culture was in the log or stationary phase. Other researchers have shown that the addition of inhibitors of sterol synthesis to wild type yeast causes the accumulation of ergosterol precursors in the ester fraction (20,21). No sterol synthetic inhibitors were used in the present study, yet chromatographically, the data show that non-ergosterol moieties are esterified to the same or greater extent as ester species of ergosterol (Fig. 2). It can also be seen that there is no preference for palmitate, palmitoleate, stearate, or oleate. In terms of timing of ester syntheses, there is a slightly higher relative amount of zymosteryl palmitate in the log phase as compared to stationary phase, and at stationary phase there are increased amounts of ergosta-5,7-dienyl palmitate and palmitoleate. This is in agreement with a previous study conducted in our laboratory that showed an increase in ergosta-5,7-dienyl esters as the culture matures to stationary phase (22).

Mutant: The Upc mutant gave a different ester profile. This mutant is characterized by its ability to accumulate sterols aerobically and simultaneously synthesize its own sterols (13). In addition, it was shown to accumulate esters in quantities well in excess of the wild type strain, even in the absence of exogenous sterol. Figure 3 shows the ester profile from this mutant. Most noticeable in this strain is the ability to esterify ergosterol even at early stages in the culture cycle. However, it is similar to the wild type in that the stationary phase shows more palmitate and palmitoleate esters of ergosta-5, 7-dienol. In the original paper which characterized the uptake potential of this strain, Lewis reported the high level of ergosterol in the ester fraction at stationary phase (13).

Most studies involving steryl esters have indicated an increase in the steryl esters as the culture approaches stationary phase. Many of these have relied on radioactive labelling experiments that show an increasing amount of radioactivity going into the ester fraction (7,22). This may be true, however, there has yet to be a report of the quantity of the total ester fraction on a dry weight basis for a non-sterol auxotrophic strain over the culture cycle. In this study the data show that in the wild type yeast the total ester fraction actually remains relatively stable or shows a slight decrease over the cycle, while in the Upc mutant there is a substantial decrease (Table 2). This observation may not be in direct conflict with former studies as it is likely that radioactivity does increase in the ester fraction. However, it cannot be ruled out that as a whole this decrease on a dry weight basis may be due to other cellular events such as storage of other products, increased cell wall bulk, for example. It does seem unlikely, however, that such cellular events could account for the magnitude of decrease seen in the mutant.

TABLE 2

Quantitation[a] of Total Esters from Wild Type and Mutant Strains of *Saccahromyces cerevisiae*

Klett range	μg/mg dry weight
Wild type	
65-160	5.74 ± 0.53 n = 4
206-395	6.80 ± 2.00 n = 3
520-560	3.96 ± 0.10 n = 4
Late stationary[b]	3.90 ± 1.00 n = 4
Upc mutant	
110-135	17.2 ± 1.00 n = 2
282-300	4.89 ± 0.20 n = 2
500-600	5.48 ± 1.00 n = 4
Late stationary	9.96 ± 1.90 n = 4

[a]Values represent the average quantity ± standard deviation for the number of samples analyzed within a given Klett range.

[b]Cultures were allowed to continue an additional day past the previous Klett reading.

Furthermore, previous studies did not show an increased hydrolysis of steryl esters that could account for the decrease (13). In that same study it is worth pointing out that the level of free sterol in the mutant was within the range as that for the wild type.

Knowledge of the identity of intact steryl esters may be crucial in determining the role of esters in cellular processes. One of the obstacles that has to be overcome is a suitable and efficient method to analyze these components. The system described in this report addresses that concern and for the organisms studied, proved to be an adequate system. We were able to show that wild type yeast were normal in their steryl ester profile since they showed ergosterol being only slightly esterified as the culture progressed, and the accumulation of other sterols was apparent. The Upc mutant, on the other hand, was abnormal in the sense that ergosterol was esterified to a larger extent throughout the culture cycle. Whether the analytical system described here holds true for other organisms, especially plants, where the array of sterols are more diverse and pentacyclic triterpenoids are common, remains to be seen. As our analytical capabilities improve so will our ability to address and resolve questions concerning the roles of these compounds.

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Novel Branched-Chain Fatty Acids in Certain Fish Oils[1]

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Methyl-branched fatty acids, which are usually minor components (≤0.1%) in fish oils, were concentrated in the non-urea-complexing fraction along with polyunsaturated fatty acids during the enrichment of omega-3 fatty acids from certain fish oils via the urea complexation process. The methyl-branched fatty acids in the omega-3 polyunsaturated fatty acid concentrates, which were prepared from three fish body oils, were characterized by gas chromatography and gas chromatography/mass spectrometry. Among the major branched-chain fatty acids expected and identified were the known isoprenoid acids—mainly 4,8,12-trimethyltridecanoic, pristanic, and phytanic—and the well-known iso and anteiso structures. Two novel phytol-derived multimethyl-branched fatty acids, 2,2,6,10,14-pentamethylpentadecanoic and 2,3,7,11,15-pentamethylhexadecanoic, were identified in redfish (*Sebastes sp.*) oil. These two fatty acids were absent in oils from menhaden (*Brevoortia tyrannus*) and Pacific salmon (mixed, but mostly from sockeye, *Oncorhynchus nerka*). The major branched-chain fatty acid in the salmon oil, 7-methyl-7-hexadecenoic acid, was also present to a moderate extent in menhaden oil. A novel vicinal dimethyl-branched fatty acid, 7,8-dimethyl-7-hexadecenoic was detected in all of the fish oils examined, but was most important in the salmon oil. Three monomethyl-branched fatty acids, 11-methyltetradecanoic acid, and 11- and 13-methylhexadecanoic, hitherto undescribed in fish lipids, were also detected in salmon, redfish and menhaden oils.
Lipids 24, 630-637 (1989).

Three isoprenoid fatty acids have been known to be associated with marine oils almost since the introduction of gas liquid chromatography (1). Derived from the C_{20} phytol (3,7,11,15-tetramethyl-2-hexadecen-1-ol) of chlorophyll, these are respectively the C_{16} 4,8,12-trimethyltridecanoic acid (4,8,12-TMTD), the C_{19} 2,6,10,14-tetramethylpentadecanoic acid (2,6,10,14-TMPD or pristanic) and the C_{20} 3,7,11,15-tetramethylhexadecanoic (3,7,11,15-TMHD or phytanic). The chlorophyll origin also results in their occurrence in ruminant fats (2).

The human cardiovascular system may benefit from an increased intake of lipids of marine origin (3, 4). Ostensibly eicosapentaenoic acid (20:5n-3 or EPA) is the active principle, but in marine oils and lipids this fatty acid is found in roughly equal proportion with docosahexaenoic acid (22:6n-3 or DHA), the two together comprising 25-45% of many marine lipids and 15-25% of commercial oils. Broadly speaking, saturated fatty acids make about 35% of these lipids and the balance is made up of monoethylenic fatty acids (5-7).

Numerous enriched marine oil preparations have been used experimentally in animal and human clinical studies (7). Moderate enrichment of some oils by "winterizing" to remove a stearine fraction is feasible (7, 8) but the complexity of the fatty acid combinations in the triacylglycerides make it difficult to go beyond a certain point without problems in respect to yield and cost (7). Once the fatty acids are split off the glycerol molecule they are much more amenable to any separation technique. One of the oldest (1, 9) means of enriching the polyunsaturated fatty acids (PUFA) is urea complex formation. The formation of urea clathrates or complexes generally excludes the PUFA such as EPA and DHA and together these give a non-urea-complexing fraction (NUCF) which in our hands (7, 10, 11) includes about 50-60% EPA + DHA in a total of 70-80% omega-3 PUFA. The omega-6 fatty acids, originally totalling no more than about 5% of the fatty acids (6), are not always enriched in proportion and partial removal through formation of urea complexes is usually observed (10, 11).

The isoprenoid fatty acids do not form urea complexes (12-14). It was expected that only the three isoprenoid fatty acids listed above would be concentrated in the NUCF of the fish oils being tested in Halifax for concentration of omega-3 PUFA (10, 11). For example, Sano (15-19) and Sano *et al.* (20) did not report extra compounds of this class in whale oils. Unexpectedly, in addition to the above three isoprenoid fatty acids and other common branched-chain acids (2, 21, 22), we encountered several other branched-chain acids in redfish, menhaden and salmon oils. We report the characterization of these novel branched-chain fatty acids (BCFA) isolated from the NUCF of the above three fish oils.

MATERIALS AND METHODS

Fish oils. Commercial redfish, menhaden, and salmon oils were used in this study. The redfish oil was produced in Canada, at the Canso, N.S. plant of National Sea Products. Menhaden oil was suppled by the Zapata Haynie Corp., VA, USA; and salmon oil was made available by First Alaska Surimi, Inc. Seattle, WA, USA.

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Abbreviations: $AgNO_3$-TLC, silver nitrate-thin layer chromatography; BCFA, branched-chain fatty acid; DHA, docosahexaenoic acid; DMHD, dimethylhexadecanoic acid; ECL, equivalent chain length; EPA, eicosapentaenoic acid; FID, flame ionization detector; GC/MS, gas chromatography/mass-spectrometry; GLC, gas-liquid chromatography; ITD, Ion Trap Detector; MHD, methylhexadecanoic; MTD, methyltetradecanoic; NUCF, non-urea-complexing fraction; PMHD, pentamethylhexadecanoic; PMPD, pentamethylpentadecanoic; PUFA, polyunsaturated fatty acid; TMHD, tetramethylhexadecanoic or phytanic; TMPD, tetramethylpentadecanoic acid or pristanic; TMTD, trimethyltridecanoic acid.

Enrichment of omega-3 PUFA. The omega-3 PUFA concentrate was prepared by urea complexation on a pilot plant scale (7, 10, 11). Initially fish oil was converted to free fatty acids by alkali saponification followed by acidification. The free fatty acids were dissolved in an ethanolic solution of urea at reflux temperature. The solution, in 20:1 lots, was allowed to cool overnight at room temperature and then moved to a 0°C room overnight. The solution was decanted to separate the non-urea-complexing fraction (NUCF) from the complexed solids. Acidification and addition of water yielded NUCF fatty acids enriched with omega-3 PUFA.

Silver nitrate-thin layer chromatography. About 100 mg of the omega-3 PUFA concentrate was converted to methyl esters by heating it with 7% BF_3-MeOH (23). The methyl esters were fractionated according to their degree of unsaturation (24) on silver nitrate-thin layer chromatography ($AgNO_3$-TLC). The development was in hexane:diethyl ether (9:1 V:V). The bands were visualized under UV light after spraying with 2,7-dichlorofluorescein (0.1% in ethanol). The bands were recovered and the silica gel extracted five times with $CHCl_3$. Each $CHCl_3$ extract was evaporated to dryness under N_2, redissolved in hexane (~500 μl) and analysed by gas-liquid chromatography (GLC).

3-Picolinyl ester derivatives. The monounsaturated methyl-branched fatty acid methyl ester fraction of R_f O.54, isolated by $AgNO_3$-TLC from the omega-3 PUFA concentrate prepared from salmon oil, was hydrolysed to the free fatty acid form. The free fatty acid was converted to the 3-picolinyl ester derivative as described by Harvey (25). The positions of the double bonds in 7-methyl-7-hexadecenoic and 7,8-dimethyl-7-hexadecenoic acids were determined by GC/MS of their 3-picolinyl ester derivatives.

Enrichment of branched-chain fatty acids. About 200 mg of the omega-3 PUFA concentrate was completely hydrogenated with hydrogen over PtO_2 (Adams' catalyst). The hydrogenated sample was subjected to urea crystallization on a microscale with 1.5 g urea and 3 ml ethanol (96%). The NUCF was isolated and the fatty acids were recovered. The fatty acids of the NUCF were mainly branched-chain fatty acids. These were analysed by GLC as their methyl esters.

Gas-liquid chromatography (GLC). For GLC analysis all free fatty acids were converted to methyl esters by heating with 7% BF_3-MeOH. The GLC analyses were performed on either Perkin-Elmer model 3920 or 8420 gas chromatographs equipped with flame ionization detectors (FID). The fatty acid methyl ester separation was performed on the following three flexible fused silica capillary columns: bonded Carbowax-20M DB-WAX (30 m × 0.25 mm ID; J and W Scientific Inc., Rancho Cordova, CA); methyl silicone SPB-1 (15 m × 0.25 mm ID; Supelco Inc., Bellefonte PA); SUPELCOWAX-10 (bonded Carbowax-20M; 30 m × 0.32 mm ID; Supelco Inc.) The DB-WAX column was operated isothermally at either 210°C or 170°C. The carrier gas (helium) pressure was 26 psig. The SPB-1 was operated at 165°C with a (helium) pressure of 20 psig. The SUPELCOWAX-10 column was operated at either 190°C or 160°C with a helium pressure of 12 psig.

The Perkin-Elmer Model 3920 was coupled to a Perkin-Elmer LCI-100 computing integrator. The peak area output from Perkin-Elmer 8420 was recorded on a Perkin-Elmer GP-100 graphic printer.

Gas chromatography/Mass spectrometry (GC/MS). Electron impact mass spectra were obtained on a model 700 Finnigan MAT Ion Trap Detector (ITD) system (Finnigan MAT, San Jose, CA) interfaced to a Perkin-Elmer model 990 gas chromatograph. The GLC fused silica capillary column was fed through a heated transfer line directly into the ITD gas inlet. For GC/MS the separations were performed on both polar and non-polar columns. The polar column was the particular DB-WAX column described above. It was operated at 170°C and 10 psig helium. The non-polar chromatography was executed on a methyl silicone DB-1 column (23m × 0.25mm I.D., J and W Scientific Inc.) operated at 160°C and 10 psig helium.

The data system consisted of an IBM PC/XT interfaced to the internal electronics of the ITD. All studies were conducted with version 3.0 of the ITD software supplied by the Finnigan Corp. The ITD was tuned by using the procedure supplied by the manufacturer. The ITD was operated in the full scan mode and scanned with a 1-s cycle time.

RESULTS

The capillary GLC analyses of the hydrogenated branched-chain fatty acid fractions from the three fish oils showed a number of branched-chain fatty acids (Table 1 and Fig. 1). Among these were the known iso and anteiso structures of the C_{14}, C_{15}, C_{16} and C_{17} chain lengths, 7-methylhexadecanoic (21), and the usual isoprenoids of fish oils; 4,8,12-TMTD, pristanic, and phytanic acids. These were easily identified by comparison of their GLC equivalent chain length (ECL) values on both polar and non-polar columns with published literature values (2, 22, 26-29), and verified by their mass-spectral fragmentation patterns (2, 30-33). The structures of the iso and anteiso fatty acids, and those of pristanic and phytanic acids, were further confirmed by comparison of mass spectral patterns with authentic standards.

Altogether seven unusual BCFA were detected. Three were basically isoprenoids, 2,2,6,10,14-pentamethylpentadecanoic (2,2,6,10,14-PMPD), 2,3,7,11,15-pentamethylhexadecanoic (2,3,7,11,15-PMHD), and 5,9,13-trimethyltetradecanoic (5,9,13-TMTD). One with two methyl substituents was shown to be 7,8-dimethylhexadecanoic (7,8-DMHD) acid, and the other three were novel monomethyl-branched fatty acids. The characteristic mass spectral fragments of these fatty acid methyl esters are given in Table 2.

2,2,6,10,14,-PMPD. The mass spectrum of the methyl ester of 2,2,6,10,14-PMPD obtained on the ITDS revealed the structure with unusual clarity (Fig 2). It should be emphasized that a characteristic feature of ITD is that the molecular ion sometimes appears as the protonated ion MH^+ (34). The intensity of MH^+ is a function of the sample introduced into the ITD. The mass spectrum of 2,2,6,10,14-PMPD showed the protonated molecular ion at m/z 327, corresponding to a C_{20} fatty acid methyl ester of mass 326 atomic mass units (amu). However, the ECL values of this acid in

TABLE 1

Proportions (wt% of the Total Fatty Acids[a]) of Branched-Chain Fatty Acids (BCFA) in the Omega-3 PUFA Concentrate and GLC Equivalent Chain Lengths (ECL).

Fatty acid	Abbreviation	Fatty acid composition (wt%)		
		Menhaden	Redfish	Salmon
12-Methyltridecanoic	12-MTD (Iso 14)	TR	TR	TR
4,8,12-Trimethyltridecanoic	4,8,12 TMTD	0.16	0.21	0.25
11-Methyltetradecanoic	11-MTD	0.03	0.10	0.23
13-Methyltetradecanoic	13-MTD (Iso 15:0)	0.10	0.40	0.10
12-Methyltetradecanoic	12-MTD (Anteiso 15:0)	0.10	0.30	0.10
5,9,13-Trimethyltetradecanoic	5,9,13 TMTD	0.02	0.02	0.04
14-Methylpentadecanoic	14-MPD (Iso 16:0)	0.02	0.05	0.12
13-Methylpentadecanoic	13-MPD (Anteiso 16:0)	TR	TR	TR
2,6,10,14-Tetramethylpentadecanoic	2,6,10,14-TMPD	0.20	0.40	0.44
2,2,6,10,14-Pentamethylpentadecanoic	2,2,6,10,14-PMPD	ND	0.48	ND
7-Methylhexadecanoic	7-MHD[b]	0.40	0.90	3.20
11-Methylhexadecanoic	11-MHD	0.01	TR	0.12
13-Methylhexadecanoic	13-MHD	0.01	ND	0.12
15-Methylhexadecanoic	15-MHD (Iso 17:0)	TR	0.05	0.04
14-Methylhexadecanoic	14-MHD (Anteiso 17:0)	TR	TR	TR
7,8-Dimethylhexadecanoic	7,8-DMHD[c]	0.12	0.08	2.25
3,7,11,15-Tetramethylhexadecanoic	3,7,11,15-TMHD	0.87	0.63	0.44
2,3,7,11,15-Pentamethylhexadecanoic	2,3,7,11,15-PMHD	ND	0.43	ND
16-Methylheptadecanoic	16-MHD (Iso 18:0)	0.02	0.10	0.28
15-Methylheptadecanoic	15-MHD (Anteiso 18:0)	TR	TR	TR
Total straight chain saturated fatty acids[e]		5.3	6.5	6.2
Total branched fatty acids[e]		1.82	3.98	7.69
Total omega-3 PUFA[e]		76.7	72.3	83.4

		ECL-values		
		SUPELCO-WAX-10 (165°C)	DB-WAX (170°C)	SPB-1 (165°C)
12-Methyltridecanoic	12-MTD (Iso 14)	13.49	13.55	13.57
4,8,12-Trimethyltridecanoic	4,8,12 TMTD	14.07	14.08	14.51
11-Methyltetradecanoic	11-MTD	14.28	14.29	14.44
13-Methyltetradecanoic	13-MTD (Iso 15:0)	14.51	14.54	14.61
12-Methyltetradecanoic	12-MTD (Anteiso 15:0)	14.67	14.68	14.71
5,9,13-Trimethyltetradecanoic	5,9,13 TMTD	15.01	15.01	15.44
14-Methylpentadecanoic	14-MPD (Iso 16:0)	15.51	15.51	15.57
13-Methylpentadecanoic	13-MPD (Anteiso 16:0)	15.67	15.66	15.70
2,6,10,14-tetramethylpentadecanoic	2,6,10,14-TMPD	15.76(15.78)[d]	15.79(15.74)[d]	16.72
2,2,6,10,14-Pentamethylpentadecanoic	2,2,6,10,14-PMPD	16.09(16.05)[d]	16.08(16.04)[d]	17.27
7-Methylhexadecanoic	7-MHD[b]	16.30	16.28	16.42
11-Methylhexadecanoic	11-MHD	16:35	16.29	16.44
13-Methylhexadecanoic	13-MHD	16:52	16.50	16.58
15-Methylhexadecanoic	15-MHD (Iso 17:0)	16.50	16.50	16.64
14-Methylhexadecanoic	14-MHD (Anteiso 17:0)	16.67	16.67	16.70
7,8,-Dimethylhexadecanoic	7,8-DMHD[c]	16.25(16.46)[d]	16.26(16.46)[d]	16.63(16.82)[d]
3,7,11,15-Tetramethylhexadecanoic	3,7,11,15-TMHD	16.94(16.98)[d]	16.94(16.97)[d]	17.72
2,3,7,11,15-Pentamethylhexadecanoic	2,3,7,11,15-PMHD	17.27(17.30)[d]	17.28(17.32)[d]	18.29(18.82)[d]
16-Methylheptadecanoic	16-MHD (Iso 18:0)	17.51	17.49	17.58
15-Methylheptadecanoic	15-MHD (Anteiso 18:0)	17.67	17.66	17.69

[a]The wt% were determined on the hydrogenated sample.
[b]The parent compound exists in the unsaturated form 7-methyl-7-hexadecenoic acid.
[c]The parent compound exists in the unsaturated form 7,8-dimethyl-7-hexadecenoic acid.
[d]ECL value of diastereoisomer in parentheses.
[e]Wt% in the omega-3 PUFA concentrate before hydrogenation.

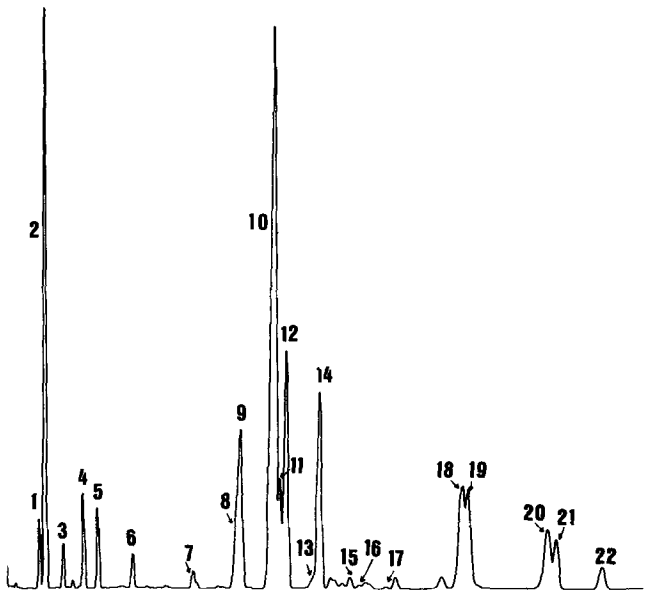

FIG. 1. Partial gas-liquid chromatogram of the hydrogenated branched-chain fatty acid methyl ester enriched fraction isolated from the omega-3 PUFA concentrate prepared from Atlantic redfish (*Sebastes sp.*) oil. Column: SUPELCOWAX-10 fused silica capillary (30 m × 0.32 mm ID) operated isothermally at 165°; He 12 psig. Peak identification: 1=14:0; 2=4,8,12-TMTD; 3=11-MTD; 4=Iso 15:0; 5=Anteiso 15:0; 6=5,9,13-TMTD; 7=Iso 16:0; 8 and 9=2,6,10,14-TMPD; 10=16:0; 11 and 12=2,2,6,10,14-PMPD; 13 and 15=7,8-DMHD; 14=7-MHD; 16=Iso 17:0; 17=Anteiso 17:0; 18 and 19=3,7,11,15-TMHD; 20 and 21=2,3,7,11,15-PMHD; 22=Iso 18:0.

the three liquid phases were lower than 20:0 (Table 1), indicating methyl branching in the chain. On both polar and non-polar columns, isoprenoids as well as other BCFA are eluted in advance of the corresponding straight chain fatty acids (1, 26). The base ion at m/z 102 was very prominent, which demonstrates substituents at C-2, since the base ion is at m/z 74 in the absence of the C-2 substituent (30–33). This branching at the 2-position has to be either a dimethyl or an ethyl group. If it is an ethyl group, both 2,3- and 2,3′-cleavage occur (32). The 2,3′-cleavage will give rise to a significant ion at M-28 due to elimination of a $CH_2{=}CH_2$ molecule. In Fig. 2 an ion at m/z 298 (=326-28) was not detected and therefore the branching at C-2 is 2,2-dimethyl and the base ion at m/z 102 is due to the $[(CH_3)_2C{=}C(OH)OCH_3]^+$ ion. An additional indication of the 2,2-dimethyl group is furnished by the presence of a very intense ion at m/z 115(39.5%); this is due to cleavage of the C_3-C_4 bond resulting in the $[CH_2\text{-}C(CH_3)_2\text{-}COOCH_3]^+$ ion. The side-chain position of fatty acid methyl esters with several methyl chains in many cases can be directly deduced from the cleavage of the bonds adjacent to the tertiary carbon atom (29–32). Cleavage on either side of the methyl-branched carbon gives two prominent ions which differ by 28 amu. These two ions are usually larger than the ion between them, obtained in the absence of methyl branching. Therefore the fragment ions with reduced intensities at m/z 157, 227 and 297 which were flanked by elevated ions at m/z 143 and 171, 213 and 241, and 283 and 311, respectively indicated methyl branching at C-6, C-10 and C-14 positions.

The front shoulder (peak 11, Fig. 1) of the main peak (peak 12) of 2,2,6,10,14-PMPD also had a mass spectral pattern identical to that of 2,2,6,10,14-PMPD. This shows that the shoulder is a diastereoisomer of

TABLE 2

GC-MS Fragmentations of Some Branched-Chain Fatty Acid Methyl Esters Isolated from Menhaden, Redfish and Salmon Oils

Fatty acid	Molecular ion	Base peak	Other useful or diagnostic ions					
4,18,12-TMTD	270(7.2)	43	255(1.0)	227(0.9)	185(0.8)	157(21.1)	87(80.3)	74(30.0)
11-MTD	256(5.4)	74	213(7.7) 87(29.9)	185(9.9)	143(19.9)	129(9.5)	115(5.6)	101(31.2)
5,9,13-TMTD	284(3.4)	74	241(10.2)	199(2.7)	171(6.3)	129(29.1)	115(3.4)	101(30.4)
2,6,10,14-TMPD	312(7.2)	101	297(0.8)	269(2.3)	227(1.3)	199(0.7)	157(10.9)	129(7.8)
2,2,6,10,14-PMPD[a]	327(20.3)	102	311(0.4) 115(39.5)	283(0.4) 74(7.0)	241(0.4)	213(0.6)	171(4.7)	143(2.4)
7-MHD[a]	285(12.5)	74	269(1.1)	157(21.5)	129(12.1)	115(4.0)	101(22.2)	87(30.3)
11-MHD	284(12.0)	57	241(3.2) 101(18.4)	213(8.4) 87(30.2)	185(9.7) 74(88.0)	157(3.5)	143(20.7)	129(30.2)
13-MHD	284(7.5)	74	255(2.9) 143(22.1)	241(3.4) 129(27.5)	213(5.8) 115(4.9)	199(3.5) 101(24.2)	185(6.4) 87(29.2)	171(5.2)
7,8-DMHD[a]	299(31.0)	57	185(5.9) 101(17.7)	171(1.1) 87(19.0)	157(18.3) 74(73.6)	143(1.0)	129(12.8)	115(5.3)
3,7,11,15-TMHD[a]	327(1.0)	101	311(0.7)	283(1.1)	241(0.6)	213(0.7)	171(4.1)	143(2.5)
2,3,7,11,15-PMHD[a]	341(3.1)	115	325(1.0) 88(27.7)	297(2.7) 83(3.3)	255(5.2)	227(1.4)	185(6.9)	157(4.6)

[a]Molecular ion appeared in the protonated form MH^+.

Intensity in parentheses.

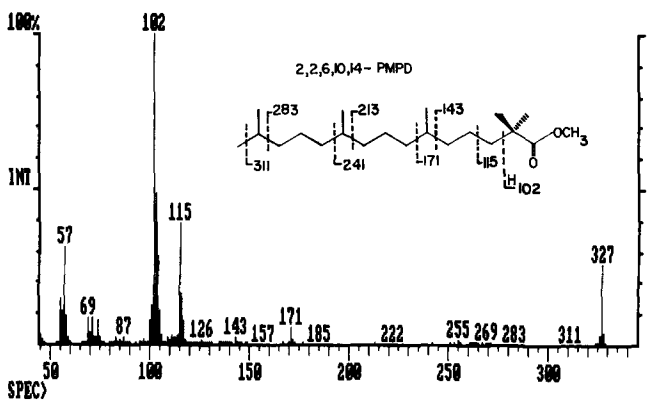

FIG. 2. Electron ionization mass-spectrum of methyl 2,2,6,10,14-pentamethylpentadecanoate.

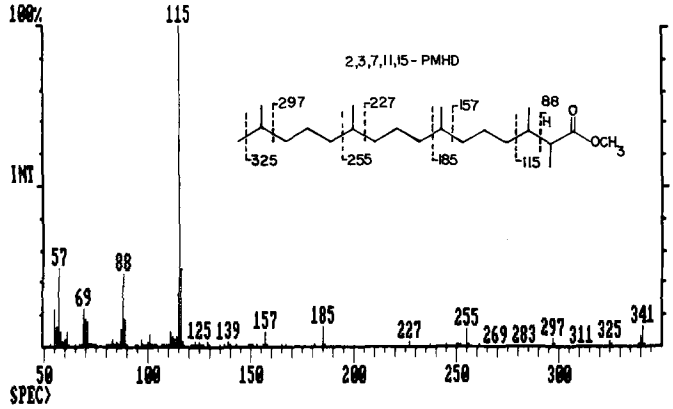

FIG. 3. Electron ionization mass-spectrum of methyl 2,3,7,11,15-pentamethylhexadecanoate.

2,2,6,10,14-PMPD. Very efficient capillary columns are often capable of resolving the diastereoisomers of isoprenoid fatty acids (2, 27, 35), as is clearly demonstrated in Fig. 1 for phytanic acid and to a certain extent with pristanic acid. The resolution could be improved by lowering column temperatures.

2,3,7,11,15-PMHD. The 2,3,7,11,15-PMHD fatty acid methyl ester had a molecular weight of 340, as indicated by the protonated molecular ion at m/z 341 (Fig. 3), which corresponds to a C_{21} fatty acid. However, this C_{21} fatty acid presented extremely low ECL values on all three capillary columns (Table 1), indicating multiple (methyl) branching. The ion of reduced intensity at m/z 74 and an enhanced ion at m/z 88 clearly disclosed a methyl branch at C-2. The ion at m/z 88 is probably due to the fragment ion $[CH_3CH{=}C(OH)OCH_3]^+$ which is characteristic of fatty acids with a methyl branch at the 2-position (2,32). The base ion was at m/z 115 which indicates a methyl branch at C-3. The presence of a significant and characteristic ion at m/z 83 (3.3%, Fig. 3 and Table 2) gives further evidence for the presence of a 3-methyl group. The m/z 83 ion is most likely due to the ketene $[CH(CH_3)C(CH_3){=}C{=}O]^+$ ion formed from the m/z 115 ion $[CH(CH_3)CH(CH_3)CO_2CH_3]^+$ through the loss of a molecule of CH_3OH. The relatively intense ions at m/z 157 (4.6%) and 185 (6.9%), and absence of an ion at m/z 171, indicated a 7-methyl substituent. Similarly elevated ions at m/z 227 (1.4%), 255 (5.2%), 297 (2.7%) and 325 (1%), with the absence of ions at m/z 241 and 311, showed the presence of methyl substituents at C-11 and C-15. The above data confirms the structure of the novel branched chain fatty acid as 2,3,7,11,15-pentamethylhexadecanoic acid. This fatty acid also showed the presence of at least two diastereoisomers as shown by the split peaks in Fig. 1. This indicates retention of the basic phytol skeleton with reduction of the ethylenic bond as the origin of the diastereoisomers (35).

5,9,13-TMTD. The methyl ester of the third unusual isoprenoid fatty acid, 5,9,13-TMTD, had a molecular weight of 284 corresponding to a C_{17} fatty acid, and it had only one GLC peak with ECL values of 15.01, 15.01, and 15.44 on DB-WAX, SUPELCOWAX-10 and SPB-1, respectively. The low ECL values indicated the presence of methyl branching. The elevated ions at m/z 101 (30.4%) and m/z 129 (29.1%), and an ion of reduced intensity at m/z 115 (3.4%), showed the presence of a methyl branch at the C-5 position. A methyl branch at C-9 was shown by the presence of elevated ions at m/z 171 (6.3%) and 199 (2.7%) and absence of an ion at m/z 185. An extremely prominent ion at m/z 241 (10.2%) indicated the presence of a methyl group at C-13 position. However, the corresponding ion at m/z 269 was not observed, probably due to the more favorable cleavage of the C_{12}-C_{13} bond.

7,8-DMHD. The unusual dimethyl fatty acid, 7,8-dimethylhexadecanoic acid (7,8-DMHD) had the protonated molecular ion at m/z 299 (Table 2). The molecular weight of 298 amu implied a C_{18} fatty acid methyl ester, and the ECL values (Table 1) indicated possible branching. The base ion was at m/z 57 and the very prominent ion at m/z 74 (73.6%) indicated the absence of methyl branching at the 2-position. Major ions at m/z 129, 157 and 185 and ions of reduced intensity at m/z 143 and 171 indicated methyl branches at the 7-and 8-positions. The 7,8-DMHD acid had two diastereoisomers (Table 1).

Monomethyl-branched fatty acids. The hydrogenated BCFA showed four unusual monomethyl-branched chain fatty acids (Table 1). The mass spectrum of methyl 7-methylhexadecanoate (7-MHD) displayed a protonated molecular ion at m/z 285. The base ion was at m/z 74 and the second largest ion at m/z 87. Elevated fragment ions at m/z 129 and 157 and an ion of reduced intensity at m/z 143 indicated a C-7 methyl side chain. The spectrum is that expected of a methyl 7-methylhexadecanoate (22).

The other three monomethyl-branched chain fatty acids—11-methyltetradecanoic (11-MTD), 11-methylhexadecanoic (11-MHD), and 13-methylhexadecanoic (13-MHD)—were characterized in the usual way by their mass spectral pattern (Table 2). The methyl ester of 11-MTD had a molecular weight at m/z 256 (5.4%) and the base ion was at m/z 74, indicating no alpha substituent; the relatively intense ions at m/z 185 (9.9%) and 213 (7.7%) and the absence of an ion at m/z 199 indicated a methyl group at the 11-position. The methyl

esters of 13- and 11-MHD had their molecular weights at m/z 284 and the base ions at m/z 74. The elevated ions at m/z 213 (5.8%) and m/z 241 (3.4%) and the absence of an ion at m/z 227 established the 13-methyl substituent in 13-MHD. Elevated ions at m/z 185 (9.7%) and m/z 213 (8.4%) and the absence of an ion at m/z 199 established the 11-methyl substituent in 11-MHD.

7,8-Dimethyl-7-hexadecenoic Acid. The $AgNO_3$-TLC of the omega-3 PUFA methyl esters prepared from salmon oil showed a faint band of R_f 0.54 in between the straight chain saturated fatty acid (R_f 0.64) and the usual monoethylenic fatty acid band (R_f 0.46). On GLC, the esters in this band (R_f 0.54) showed mainly two unusual fatty acids. These were later identified through GC/MS as 7,8-dimethyl-7-hexadecenoic (7,8-DM-7HD) and 7-methyl-7-hexadecenoic (7M-7HD). The methyl esters in this particular methyl-branched-monoethylenic fatty acid $AgNO_3$-TLC band were converted to the picolinyl ester derivative (25) and subjected to GC/MS.

The mass spectrum (Fig. 4) of the picolinyl ester of 7,8-DM-7HD gave prominent ions at m/z 92 (base ion), 108, 151 and 164 which are characteristic of picolinyl esters (25, 36). The protonated molecular ion appeared at m/z 374, which is in agreement with the molecular weight of m/z 373 for the picolinyl derivative of 7,8-DM-7HD. There were significant ions at m/z 358 (M-CH_3), then gaps of 14 amu separating the ions at m/z 344, 330, 316, 302, 288, 274, and 260 representing cleavage at successive methylene groups. This was followed by a gap of 54 amu to m/z 206, after which further ions appeared 14 amu apart. In normal monoethylenic fatty acids with no substituents on the two carbons of the double bond the gap is 26 amu representing cleavage at either side of the double bond (25, 36). Thus the 54 amu gap between m/z 260 and 260 in the picolinyl ester of 7,8-DM-7HD is due to the presence of a $CH_3C{=}CCH_3$ group where the double bond is located at the C_7-C_8 position. Another distinctive feature of the picolinyl ester derivative of monoethylenic fatty acids is the presence of a pair of ions 14 amu apart, which were usually more prominent than the rest of the ions resulting from successive cleavage of the methylene groups of the chain (25, 36). It has been suggested that these ions could be rationalized in terms of initial abstraction of the allylic hydrogens to form conjugated dienes with one or two carbon atoms more than the fragment with the single double bond (36). Thus prominent ions at m/z 274 and 288 (Fig. 4) further support the presence of a double bond in C_7-C_8 position.

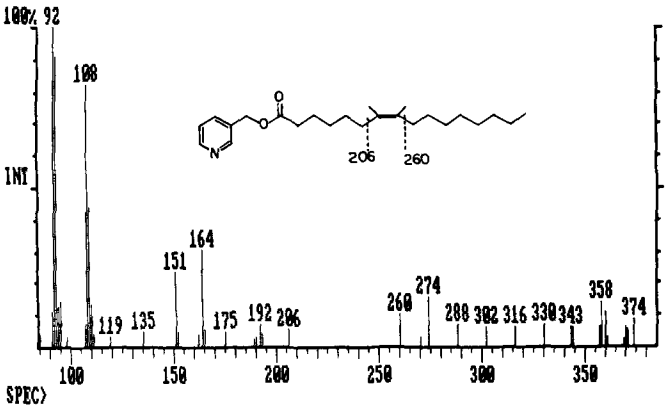

FIG. 4. Electron ionization mass-spectrum of the picolinyl ester derivative of 7,8-dimethyl-7-hexadecenoic acid.

7-Methyl-7-hexadecenoic Acid. The mass spectrum of the picolinyl ester derivative of 7M-7HD gave the prominent characteristic ions of picolinyl ester derivatives of fatty acids at m/z 92 (base ion), 108, 151 and 164. The protonated molecular ion at m/z 360 was very prominent (29%). There were characteristic ions at m/z 344 (M-CH_3), 330, 316, 302, 288, 274, 260 and 246 representing cleavage at successive methylene groups. The ions at m/z 288 and 274 were more prominent than others due to favourable cleavage at the C_9-C_{10} bonds, showing that the double bond is at the C_7-C_8 position. The regular gap pattern of 14 amu was broken at m/z 246, which as followed by a gap of 40 amu to m/z 206, after which further ions appeared with the regular 14 amu gap at m/z 192, 178 and 164. The 40 amu gap between m/z 246 and 206 is due to the $CH_3C{=}CH$ group at the C_7-C_8 position in methyl 7-methyl-7-hexadecenoate.

DISCUSSION

The omega-3 PUFA concentrates prepared from menhaden, redfish and salmon oils on a kg scale (7, 10, 11) are moderately enriched with BCFA. The enrichment was about three- to five-fold with respect to the starting oils, with redfish oil showing the highest enrichment. The concentration of the iso and anteiso fatty acids given in Table 1 may not indicate the actual levels of these fatty acids in the starting omega-3 PUFA concentrate. The iso and anteiso fatty acids are capable to a slight extent of forming urea complexes, more so than other branched-chain fatty acids with centrally-located methyl branches.

The presence of numerous fatty acids in the omega-3 PUFA concentrates (10, 11), and the overlap of some BCFA with PUFA on GLC, make direct characterization of the BCFA by GLC and GC/MS difficult. Therefore for initial detailed skeletal characterization it was necessary to prepare a fraction enriched with BCFA. Hydrogenation (H_2/PtO_2) of the original omega-3 PUFA concentrate and subsequent microscale urea fractionation afforded such a fraction by eliminating longer straight-chain acids. The fatty acids thus isolated from the basic non-urea-complexing fraction were mainly BCFA with minor or moderate amounts of straight saturated fatty acids, especially 14:0 and 16:0 as shown in Fig. 1.

After characterizing the BCFA in the hydrogenated form by GC and GC/MS, the next step was to determine whether any of these BCFA existed naturally in the unsaturated form. For this purpose the omega-3 PUFA concentrates, especially from redfish and salmon oils, were subjected to $AgNO_3$-TLC, and each band was examined by GLC. A small portion of each band was hydrogenated and again examined by GLC for the presence or absence of BCFA. It was found that all the BCFA listed in Table 1 occur naturally in the saturated form, except for 7-methylhexadecanoic (7-MHD) and 7,8-dimethylhexadecanoic (7,8-DMHD). These two occur in the monounsaturated forms.

The two pentamethyl isoprenoid fatty acids, 2,2,6,10,14-PMPD and 2,3,7,11,15-PMHD, have not been previously detected in natural sources. Moreover, these two were detected only in oil from Atlantic redfish (*Sebastes* sp.) caught off eastern Canada and not in oil from menhaden and Pacific salmon. However, additional samples should be examined before attaching much importance to the source. The 2,2,6,10,14-PMPD and 2,3,7,11,15-PMHD are structurally related to pristanic and phytanic acids respectively, and therefore we speculate that there should be a common biosynthetic route for these four isoprenoid acids. It is presumed that the two novel isoprenoid fatty acids arise from insertion of a methylene group across the ethylenic bond initially present in phytol or phytenic acid (3,7,11,15-tetramethyl-2-hexadecenoic). The subsequent opening of the ring could give either the 2,3,7,11,15-PMHD or the 3,3,7,11,15 isomer. The latter would be biochemically shortened to the 2,2,6,10,14-PMPD in the same way as 2,6,10,14-TMPD is related to 3,7,11,15-TMHD (2). This hypothesis suggests the existence of another unusual isoprenoid—3,3,7,11,15-PMHD. However, this isoprenoid was not detected in redfish oil, suggesting a transitory existence during the chain shortening process, or more probably a stereospecific ring opening.

In this work, we report the isolation of yet a third isoprenoid fatty acid not found previously in fish oils, the 5,9,13-TMTD. However, 5,9,13-TMTD had been previously reported in human milk at very low concentrations (37). Synthetically, 5,9,13-TMTD had been prepared by permanganate oxidation of phytol (38). The structural similarity of 5,9,13-TMTD to 4,8,12-TMTD suggests a common oxidative origin from phytol for these two fatty acids. As outlined elsewhere, 4,8,12-TMTD is derived from phytol via oxidation of phytanic acid (1, 39). Bacterial degradation of pristane, an important hydrocarbon in marine lipids (40), also yields 4,8,12-TMTD, and 3,7,11-TMDD (3,7,11-trimethyldodecanoic acid) as well (39). It is possible that the 5,9,13-TMTD isolated from fish oil could be the product of chain elongation of 3,7,11-TMDD.

Another interesting discovery is two novel fatty acids, 7,8-dimethylhexadecanoic (7,8-DMHD) and the corresponding unsaturated fatty acid 7,8-dimethyl-7-hexadecenoic (7,8-DM-7HD). The 7,8-DMHD acid had two distinct diastereoisomers and in constrast to the diastereoisomers of the other BCFA discussed above, those of 7,8-DMHD were well resolved on all three GLC columns (Table 1 and Fig. 1). The two vicinal methyl groups will result in increased steric hindrance and consequently these two distinct diastereoisomers should have important differences in physical properties, accounting for the observed distinct separation of the two isomers by GLC.

The two diastereoisomers of 7,8-DMHD always appeared as a pair in equal proportions in the hydrogenated omega-3 PUFA concentrates prepared from the three fish oils, with the concentrate prepared from the Pacific salmon having about 1.1% of each of the isomers. This observation suggested that the two saturated isomers were artifacts that originated from a common precursor—7,8-DM-7HD—during hydrogenation of the omega-3 PUFA concentrate. This conclusion was verified by $AgNO_3$-TLC studies of the unhydrogenated omega-3 PUFA and by mass spectral examination of the picolinyl derivative of the monounsaturated branched-chain fatty acid band. The GLC analysis of the $AgNO_3$-TLC of the saturated fatty acid methyl ester band from the omega-3 PUFA of salmon oil indicated the absence of 7,8-DMHD. Most branched-chain saturated fatty acids elute slightly ahead of the corresponding straight-chain saturated fatty acids, but in an examination of this concentrate, the $AgNO_3$-TLC area just ahead of the straight-chain saturated band showed no 7,8-DMHD. This $AgNO_3$-TLC confirmed that 7,8-DMHD probably does not exist in this oil. The $AgNO_3$-TLC monounsaturated branched-chain band contained the novel 7,8-DM-7HD acid, as well as the 7M-7HD acid, and exact proportions among these minor components are difficult to assess.

A portion of the $AgNO_3$-TLC monounsaturated branched-chain band of the omega-3 PUFA from salmon oil was hydrogenated and examined by GLC. The peak corresponding to the 7,8-DM-7HD disappeared, but two peaks of equal areas were formed corresponding to the proposed diastereoisomers of 7,8-DMHD. This further proves that the 7,8-DMHD peaks detected in the hydrogenated BCFA fractions were derived from the novel monoethylenic 7,8-dimethyl-7-hexadecenoic acid.

It has been reported that 7-MHD exists naturally in the monounsaturated form, the double bond being in one of the C_6-C_7, C_7-C_8, C_8-C_9 positions (21, 22, 41, 42, 43). In the present work a 7-MHD skeleton was not detected in the saturated fatty acid methyl ester band of the $AgNO_3$-TLC of the omega-3 PUFA concentrate of Pacific salmon oil. However, the parent compound was present in the monounsaturated branched-chain band. The GC-MS of the picolinyl ester derivative of this band afforded the structure of the parent compound as 7-methyl-7-hexadecenoic acid (7M-7HD).

We do not rule out the possibility of the existence of the other possible isomers at very low levels, especially the 7-methyl-6-hexadecenoic isomer (21), since the $AgNO_3$-TLC monounsaturated branched-chain band showed several minor components and these could not be positively identified by GC/MS.

Table 1 shows the major branched-chain fatty acid in salmon oil as 7M-7HD acid followed by the novel 7,8-DM-7HD (quantitated as their hydrogenation products). The higher levels of 7,8-DM-7HD and 7M-7HD acids in salmon oil and their structural similarity suggests a mutual biochemical origin for these two acids. There has been speculation as to the origin of the methyl branch in the branched-chain hexadecenoic acids (21, 22, 43). The most plausible explanation, that of insertion of a methylene group across a pre-existing ethylenic bond, and a rearrangement to give the 7M-7HD structure, could take place in some microsystem such as intestinal bacteria. The coincidence of high levels of both the 7M-7HD and 7,8-DM-7HD in the same species suggests that the system is so active that it acts on the 7M-7HD as soon as it is formed, with the second step necessarily resulting in the 7,8-DM-7HD acid. Curiously, a 2,3-dimethyl-octadecadienoic acid has recently been identified in human skin surface

lipids (44), a source where bacterial involvement is also likely.

Redfish oil is produced from an edible and relatively long-lived deep water fish species. The oil has been used in animal studies on blood lipids (45), and a concentrate such as described in this report was fed to rats with no obvious ill effects (46). Our report is indicative of the variety of minor fatty acids available in the marine milieu. Some have been isolated from lipids of commonly eaten species (47, 48) and some from lipids of rare or inedible species (41). No known effects can be attributed to low level ingestion of these fatty acids in fish or fish products, except for Refsum's syndrome and phytanic acid (49, 50). The potential benefits attributed to the omega-3 fatty acids (51) are an overriding consideration in natural oils and acid or ester concentrates free of artifacts (7) of these particular longer-chain polyunsaturated fatty acids.

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The Effect of Dietary n-6 and n-3 Polyunsaturated Fatty Acids on Blood Pressure and Tissue Fatty Acid Composition in Spontaneously Hypertensive Rats

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Effects of dietary n-6 and n-3 fatty acids (FAs) on blood pressure (BP) and tissue phospholipid (PL) FA composition in spontaneously hypertensive (SHR) and normotensive Wistar-Kyoto (WKY) rats were compared. Male weanling SHR and WKY were fed a fat-free semisynthetic diet supplemented with 10% (w/w) fats containing (a) 78% 18:2n-6 (LA-rich), (b) 20% LA and 55% 18:3n-3 (LN-rich), or (c) 11% LA and 3% LN (CON) for seven weeks. Dietary fats did not affect the BP elevation, but significantly altered the FA composition of brain, adrenal gland, renal medulla and cortex PL in SHR. The LA-rich diet increased n-6 FA while it reduced n-3 FA levels. The levels of 20:4n-6 were not significantly different between animals fed the LA-rich and the CON diets. LN-rich diet increased the levels of n-3 FAs, while it reduced those of n-6 FAs. However, the extent of change was significantly less in SHR than in WKY. In all dietary groups, SHR, as compared to WKY, had a relatively higher level of the 2 series prostaglandin (PG) precursor, 20:4n-6, and a relatively lower level of the 1 and 3 series PG precursors, 20:3n-6 and 20:5n-3. The possibility that the unbalanced eicosanoid FA precursor levels might contribute to the development of hypertension in this animal model is discussed.
Lipids 24, 638-644 (1989).

Dietary supplementation with polyunsaturated fatty acids (PUFAs) has been reported to lower blood pressure (BP) in both hypertensive and normotensive subjects (1-3). An n-6 fatty acid (FA) supplemented diet can also attenuate the development and maintenance of hypertension in salt-loaded hypertensive rats (4-6), Goldblatt hypertensive rats (7), and spontaneously hypertensive rats (SHR) (8-11). Although n-3 FA can reduce BP in humans (12, 13), the antihypertensive effect of n-3 FA in rats is still controversial. Some investigators have reported that n-3 FA, from either vegetable or fish sources, increased BP in Sprague-Dawley rats (14); conversely, others have reported that linseed oil (LSO), rich in 18:3n-3 (linolenic acid, LN), can lower BP in SHR (10, 11). More recently, purified eicosapentaenoic acid (EPA, 20:5n-3) has been reported to reduce BP in SHR (15, 16).

The effects of n-6 FAs and of n-3 FAs on the formation and metabolism of arachidonic acid (AA, 20:4n-6) are totally different. N-6 FAs increase the level of AA and the production of 2 series prostaglandins (PGs), but n-3 FAs competitively inhibit the conversion of n-6 precursor FAs to AA, and also suppress the production of 2 series eicosanoids (17). In addition, n-3 FAs increase the formation of EPA, which leads to the formation of 3 series PGs, particularly the hypotensive prostacyclin (PGI_3) and the relatively inactive thromboxane (TXA_3) (18). Therefore, the reported antihypertensive effects of both FAs cannot be explained only by changes in prostanoid biosynthesis from AA. Indeed, a disassociation between the hypotensive effect of PUFA and prostanoid production has been reported (7, 10, 11). Moreover, there is evidence that olive oil, which is not a prostanoid precursor, can reduce BP in Dahl salt-sensitive rats to the same extent as safflower oil (SFO, rich in linoleic acid, LA or 18:2n-6) (19).

PUFAs are important structural and functional constituents of cell membranes: they modulate membrane properties such as fluidity and permeability, the functions of membrane bound enzymes, and ion transport systems (20). Dietary manipulation of PUFA intake results in substantial alteration of cell membrane FA composition in many tissues. Based on this evidence, there is a possibility that the antihypertensive effect of PUFA may be caused not only by changes in PG synthesis, but also by alteration of membrane FA composition, and by subsequent changes in membrane functions. There is also a possibility that hypertensive animals may have abnormal metabolism or incorporation of PUFAs as part of the prohypertensive mechanism.

Therefore, this experiment was devised to investigate the effects of dietary n-3 and n-6 essential fatty acids (EFAs) on BP and tissue FA composition in SHR. The aim was to compare diets rich in n-6 (mainly LA) and n-3 (mainly LN) FAs, and a diet which provides marginal levels of n-3 and n-6 EFAs.

MATERIALS AND METHODS

Twenty four male weanling (4-week-old) SHR (Okamoto-Aoki strain) and age matched normotensive Wistar-Kyoto rats (WKY) were purchased from Charles River Breeding Laboratories (Montreal). The rats were kept in plastic cages, containing four rats each, with free access to tap water and food. After one week of acclimatization, the rats (5-weeks-old) were randomly allocated into three groups of eight rats each. Then either 10% by weight of SFO (LA-rich), LSO (LN-rich), or a

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Abreviations: AA, arachidonic acid; ANOVA, analysis of variance; BF_3-methanol, boron trifluoride methanol; BP, blood pressure; CON, control oil; DGLA, dihomo-gamma-linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FAs, fatty acids; HCO, hydrogenated coconut oil; LA, linoleic acid; LN, linolenic acid; LSO, linseed oil; MUFAs, monounsaturated fatty acids; PG, prostaglandin; PGI_1, prostacyclin; PGI_3, prostacyclin; PL, phospholipids; PUFAs, polyunsaturated fatty acids; SD, standard deviation; SFAs, saturated fatty acids; SFO, safflower oil; SHR, spontaneously hypertensive rats; TLC, thin-layer chromatography; TX, thromboxane; TXA_3, inactive thromboxane; WKY, normotensive Wistar-Kyoto rats.

mixture of oils (CON, containing 12.5% SFO, 5% LSO, and 82.5% of hydrogenated coconut oil) in a fat-free semisynthetic diet was given to these rats for the following seven weeks. The CON diet contained minimal n-6 and n-3 acids to avoid EFA deficiency. Details of diet composition and of the fatty acid compositions of these oils are shown in Table 1.

The systolic BP of prewarmed, conscious rats were measured weekly in the morning by the tail cuff method (Narco Biosystems, Houston, Texas). After seven-weeks of feeding, rats were killed by withdrawing blood from the heart under light ether anaesthesia. Brain, liver, kidney, and adrenal glands were quickly removed, and immediately frozen in a deep freezer. The extraction of lipids were performed according to the method of Bligh and Dyer (21), and the chloroform layer was collected. Then the extracts were applied to silica gel thin layer chromatography (TLC) plates (Kieselgel 60, F-254, Merck), and developed with the solvent system (Hexane/ether/acetic acid (80:20:1, v/v/v) to separate the major lipid fractions (cholesteryl ester, triglyceride, free fatty acid, and phospholipid). The lipid fraction bands were visualized by spraying with 0.02% 2',7'-dichlorofluorescein in methanol. Then phospholipid (PL) bands were scraped off into individual tubes and were methylated with 14% boron trifluoride in methanol (BF_3-methanol) at 90°C for 30 min. The resulting methyl esters of FAs in PL were analyzed by gas liquid chromatography (Hewlett-Packard model 5880A) equipped with dual-flame ionization detector and level four integrator, using six foot glass columns packed with 10% Silar-10C on Gas Chrom QII for determination of fatty acid composition.

All results were expressed as the means and standard deviations (SD) of eight determinations. Statistical differences within the groups were evaluated using analysis of variance (ANOVA). If ANOVA showed statistical significance at $p<0.05$, then a t-test was used to evaluate the differences between the groups.

RESULTS

Throughout the feeding period, no significant differences in growth rates were found among the different groups (data not shown). The BPs in all SHR groups were significantly increased from seven-weeks of age (eight-weeks for LSO-fed SHR) as compared to those of WKY fed the same diet (Fig. 1). However, dietary fats had no significant effects on BP changes.

Fatty acid composition of renal medullary phospholipids. The fatty acid compositions of renal medullary PL are shown in Table 2. There were significant differences between the SHR and WKY groups on the same diet, as well as those on the different diets. The following points are particularly worthy of emphasis.

(1) SHR, as compared to WKY, showed consistently lower dihomo-gamma-linolenic acid (DGLA, 20:3n-6) levels on all diets.

(2) The ratio of AA/DGLA was consistently higher in the SHR than in the WKY.

(3) In SHR fed LSO diet, the levels of AA were higher, while those of n-3 FAs, especially EPA, were lower than WKY on the same diet.

(4) In all groups the ratio of AA/(DGLA+EPA) was consistently higher in SHR.

Fatty acid composition of renal cortical phospholipids. Table 3 shows the FA compositions of renal cortical PL. The changes in renal cortical PL were generally similar to those in the renal medullary PL, although they were not as striking.

(1) SHR had significantly lower LA and EPA levels than WKY on the HCO diet.

(2) SHR had significantly lower 22:4n-6 and 22:5n-6 levels on the SFO diet as compared to WKY. The

TABLE 1

The Composition of the Diet and Fatty Acid (FA) Composition of Safflower Oil (SFO), Linseed Oil (LSO), and Control Oil (CON, Containing SFO/LSO/Hydrogenated Coconut Oil, 12.5/0.5/82.5 by wt).

Composition of diet		FA composition of the oils (wt%)			
	g/kg	fatty acid	SFO	LSO	CON
Sucrose	602	8:0			5.8
Casein, vitamin-free	200	10:0			4.9
Fat[a]	100	12:0			37.9
Cellulose	50	14:0			15.2
Mineral mix[b], AIN-76	35	16:0	6.9	4.9	8.8
Vitamin mix[c]	10	18:0	2.0	2.6	9.5
DL-methionine	3	18:1n-9	10.5	15.2	3.3
		18:2n-6	78.7	20.2	11.0
		18:3n-3	0.6	55.2	3.0

[a]Either SFO, LSO, or the control oil (CON: SFO/LSO/hydrogenated coconut oil, 12.5/0.5/82.5 by wt).

[b]AIN-76, providing (mg/g mix): $CaHPO_4$, 500; NaCl, 74; K_2SO_4, 52; MgO, 24; KIO_4, 0.01; $CrK(SO_4)\cdot 12H_2O$, 0.55; $MnCO_3$, 3.5; $ZnCO_3$, 1.6; potassium citrate, 220; ferric citrate, 6.0; and sucrose, 118.03.

[c]Providing (mg/g mix): Choline dihydrogen citrate, 349.7; ascorbic acid; 101.7; vitamin E acetate, 24.2; inositol, 11.0; p-aminobenzoic acid, 11.0; niacin, 9.9; calcium pentothenate, 6.6; menadione, 5.0; vitamin A palmitate, 4.0; vitamin B_{12}, 3.0; pyridoxine, 2.2; riboflavin, 2.2; thiamine HCl, 2.2; vitamin D_3, 0.4; folic acid, 0.2; biotin, 0.044; and cornstarch, 466.7.

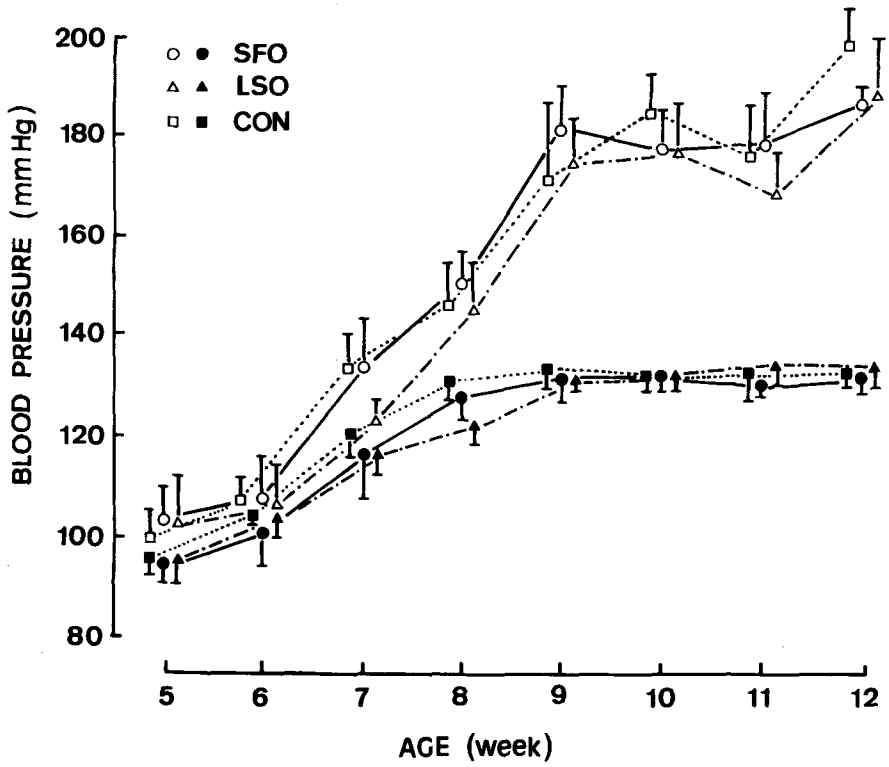

FIG. 1. Systolic blood pressure (mm Hg) of spontaneously hypertensive rats (SHR, open symbols) or Wistar-Kyoto rats (WKY, solid symbols) fed the semisynthetic diets supplemented with 10% by weight of either safflower oil (SFO; ○,●), linseed oil (LSO; Δ,▲), or control oil (CON; containing 12.5% SFO, 5% LSO and 82.5% hydrogenated coconut oil, □,■). The results are expressed as mean ± SD.

TABLE 2

Fatty Acid Composition (% wt) of Renal Medullary Phospholipid in Spontaneously Hypertensive Rats (SHR) or Wistar-Kyoto Rats (WKY) After Seven Weeks Dietary Supplementation with Either 10% by Weight of Safflower Oil (SFO), Linseed Oil (LSO) or Control Oil (CON; SFO/LSO/HCO, 1.25/0.5/8.25) (mean ± SD, n = 8).

	SHR			WKY		
Fatty acid	SFO	LSO	CON	SFO	LSO	CON
SFA	37.0 ± 0.9	36.5 ± 1.3	37.4 ± 1.0	37.6 ± 0.8	37.0 ± 0.9	38.7 ± 0.4
MUFA	11.6 ± 0.6^a	15.1 ± 0.6^b	15.4 ± 0.9^b	10.9 ± 0.5^a	14.4 ± 1.2^b	14.8 ± 0.8^b
18:2n-6	13.2 ± 0.3^a	12.6 ± 0.8^a	7.4 ± 0.3^b	13.1 ± 0.4^a	12.9 ± 0.5^a	7.3 ± 0.3^b
18:3n-6	**0.5** ± 0.3	0.3 ± 0.2	0.3 ± 0.3	0.1 ± 0.2	0.2 ± 0.2	0.1 ± 0.2
20:3n-6	**0.5** ± 0.1^a	**0.5** ± 0.2^a	**0.6** ± 0.1^b	0.6 ± 0.1^a	0.7 ± 0.1^b	0.8 ± 0.0^b
20:4n-6	34.3 ± 1.4^a	**28.6** ± 0.9^b	35.0 ± 0.6^a	33.7 ± 0.6^a	24.3 ± 0.9^b	34.3 ± 1.3^a
22:4n-6	**1.4** ± 0.1^a	0.2 ± 0.1^b	0.8 ± 0.1^c	1.8 ± 0.2^a	0.2 ± 0.0^b	1.0 ± 0.1^c
22:5n-6	**0.9** ± 0.1^a	tr	0.1 ± 0.0^b	1.0 ± 0.1^a	tr	0.1 ± 0.0^b
18:3n-3	tr	1.0 ± 0.2	tr	tr	1.2 ± 0.1	tr
20:5n-3	tr	**2.0** ± 0.8	tr	tr	5.6 ± 0.9	tr
22:5n-3	**0.8** ± 0.5^a	1.8 ± 0.6^b	1.2 ± 0.5^c	0.5 ± 0.2^a	2.2 ± 0.5^b	0.9 ± 0.4^c
22:6n-3	0.4 ± 0.1^a	**1.7** ± 0.1^b	2.0 ± 1.2^b	0.4 ± 0.1^a	1.5 ± 0.1^b	1.5 ± 0.1^b
Σn-6	50.8 ± 1.2^a	**42.2** ± 1.1^b	44.2 ± 0.6^b	50.7 ± 0.5^a	38.3 ± 1.2^b	43.6 ± 1.3^c
Σn-3	1.2 ± 0.6^a	**6.5** ± 1.2^b	3.2 ± 1.5^c	0.9 ± 0.2^a	10.6 ± 1.0^b	2.4 ± 0.4^c
AA/LA	2.6 ± 0.1^a	**2.3** ± 0.2^b	4.7 ± 0.2^c	2.6 ± 0.1^a	1.9 ± 0.1^b	4.7 ± 0.3^c
AA/DGLA	71.2 ± 6.5^a	43.7 ± 16^b	62.2 ± 5.7^c	52.5 ± 5.1^a	33.4 ± 3.4^b	41.8 ± 4.2^c
AA/DGLA &EPA	**71.3** ± 6.5^a	**10.0** ± 1.4^b	**62.2** ± 7.1^c	50.7 ± 5.9^a	3.9 ± 0.6^b	41.8 ± 4.2^c

Abbreviations: HCO = hydrogenated coconut oil; SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; AA = 20:4n-6; LA = 18:2n-6; DGLA = 20:3n-6; EPA = 20:5n-3; tr = trace.

^{a,b,c}Data in the same strain with different superscripts are significantly different from each other at $p<0.05$.

Data in **bold phase** are significantly different from WKY on the same diet ($p<0.05$).

TABLE 3

Fatty Acid Composition (% wt) of Renal Cortical Phospholipid in Spontaneously Hypertensive Rats (SHR) or Wistar-Kyoto Rats (WKY) After Seven Weeks Dietary Supplementation with Either 10% by Weight of Safflower Seed Oil (SFO), Linseed Oil (LSO) or Control Oil (CON; SFO/LSO/HCO, 1.25/0.5/8.25) (mean ± SD, n = 8).

	SHR			WKY		
Fatty acid	SFO	LSO	CON	SFO	LSO	CON
SFA	37.5 ± 2.6	36.6 ± 2.3	37.5 ± 1.4	38.2 ± 2.7	36.6 ± 2.5	36.4 ± 2.9
MUFA	7.2 ± 0.4^a	9.2 ± 0.3^b	**9.2 ± 0.4**^{b}	7.2 ± 0.2^a	9.5 ± 0.4^b	10.5 ± 0.5^c
18:2n-6	12.0 ± 0.7^a	15.1 ± 0.6^b	**8.2** ± 0.5^c	11.8 ± 0.7^a	14.8 ± 0.8^b	9.0 ± 0.7^c
18:3n-6	0.1 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	tr
20:3n-6	0.6 ± 0.1^a	0.7 ± 0.1^b	0.7 ± 0.1^b	0.6 ± 0.1^a	0.7 ± 0.1^b	0.7 ± 0.1^b
20:4n-6	37.9 ± 2.7^a	**28.5** ± 1.8^b	38.9 ± 2.4^a	37.1 ± 2.4^a	22.8 ± 1.9^b	38.6 ± 2.8^a
22:4n-6	**0.9** ± 0.1^a	0.1 ± 0.0^b	0.5 ± 0.1^c	1.1 ± 0.1^a	0.1 ± 0.1^b	0.5 ± 0.1^c
22:5n-6	**1.3** ± 0.1^a	0.0 ± 0.0^b	0.2 ± 0.1^c	1.5 ± 0.1^a	tr	0.2 ± 0.0^b
18:3n-3	tr	**1.3** ± 0.1	tr	0.1 ± 0.1^a	1.7 ± 0.3^b	tr
20:5n-3	0.1 ± 0.0^a	**4.0** ± 0.6^b	**0.1** ± 0.1^a	0.1 ± 0.3^a	8.2 ± 0.8^b	0.2 ± 0.1^a
22:5n-3	0.3 ± 0.1^a	**1.2** ± 0.2^b	0.5 ± 0.2^c	0.3 ± 0.1^a	1.6 ± 0.6^b	0.5 ± 0.1^c
22:6n-3	0.7 ± 0.1^a	2.5 ± 0.1^b	2.4 ± 0.1^b	0.7 ± 0.1^a	2.6 ± 0.2^b	2.6 ± 0.2^b
Σn-6	52.9 ± 3.3^a	**44.5** ± 2.2^b	48.5 ± 2.5^c	52.5 ± 3.0^a	38.5 ± 2.3^b	49.2 ± 3.3^c
Σn-3	1.0 ± 0.1^a	**9.0** ± 0.9^b	3.0 ± 0.3^c	1.1 ± 0.3^a	13.9 ± 1.1^b	3.3 ± 0.3^c
AA/LA	3.2 ± 0.1^a	**1.9** ± 0.1^b	**4.8** ± 0.3^c	3.2 ± 0.1^a	1.6 ± 0.1^b	4.3 ± 0.2^c
AA/DGLA	66.5 ± 8.2^a	**41.8** ± 4.0^b	57.1 ± 8.5^c	67.6 ± 8.5^a	32.2 ± 6.2^b	55.3 ± 8.8^a
AA/DGLA &EPA	64.4 ± 5.9^a	**6.3** ± 0.6^b	**58.1** ± 5.1^c	61.8 ± 11.3^a	2.6 ± 0.3^b	43.6 ± 5.4^c

Abbreviations: HCO = hydrogenated coconut oil; SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; AA = 20:4n-6; LA = 18:2n-6; DGLA = 20:3n-6; EPA = 20:5n-3; tr = trace.

^{a,b,c}Data in the same strain with different superscripts are significantly different from each other at $p<0.05$.

Data in **bold phase** are significantly different from WKY on the same diet ($p<0.05$).

levels of LA were greater in the LSO group than those in the SFO group.

(3) The greater retention of AA and lower of DGLA in the SHR on the LSO diet were observed again. SHR also showed significantly lower LN and 22:5n-3 levels than WKY.

Fatty acid composition of brain phospholipids. The fatty acid compositions of brain PL are shown in Table 4. In brain PL, 22:6n-3 (docosahexaenoic acid, DHA) is the dominant long chain n-3 FA, and the levels of 18 and 20 carbon FAs are very low. As brain FA composition is relatively resistant to dietary influence, the magnitude of dietary effects is generally very small. The main differences between the SHR and WKY were as follows:

(1) SHR showed consistently lower EPA and 22:5n-3 levels, but consistently higher 22:5n-6 level on any diet as compared to WKY.

(2) SHR revealed significantly lower AA, EPA, and 22:5n-3 levels, but higher 22:5n-6 level than WKY on the SFO diet.

(3) When rats were fed the LSO diet, the reductions in 22:4n-6 and 22:5n-6 were significantly smaller in SHR. The increments in EPA and 22:5n-3 in SHR were also significantly smaller than in WKY.

(4) The ratio of AA/(DGLA+EPA) was significantly lower, and the ratio of DHA/EPA was significantly higher, in SHR than in WKY.

(5) The ratios of 22:5n-6/22:4n-6, and of DHA/22:5n-3 in SHR were significantly higher than those in WKY.

Fatty acid composition of adrenal phospholipids. The fatty acid compositions of adrenal PL are shown in Table 5. The patterns of the diet-induced changes in FA composition in adrenal FA were also similar to those in the other tissues.

(1) SHR showed consistently lower LA and DGLA levels as compared to WKY on any diet.

(2) SHR showed a significantly higher level of AA than WKY on the SFO diet.

(3) On the LSO diet, the increments in total n-3 FAs, EPA, 22:5n-3, and the reduction in AA in SHR were significantly smaller than those in WKY.

DISCUSSION

It has been reported that diets supplemented with PUFA rich oils such as SFO, sunflower oil, evening primrose oil, and LSO significantly attenuated the development and maintenance of hypertension in SHR (9–11). However, in this study we observed that BP, which was significantly higher in SHR than in WKY, was not significantly affected by different dietary fats. The discrepancy may be due to differences in the state of animals used and the amount of fat that was supplemented. Other studies examined the effects of PUFA on BP in EFA deficient SHR, or in rats fed low fat diet. EFA deficiency exacerbates hypertension in either SHR (22) or other experimental hypertensive animals (23), and increases BP even in normotensive rats (24). A low fat diet, on the other hand, increases BP in SHR as compared to a high fat diet (25). Thus, our failure to observe significant effects on BP by fat supplementation might be due to animals having high levels of fat intake, and not being EFA deficient.

The diet supplemented with different oils significantly altered tissue FA compositions, but the extents of change differ between SHR and WKY. The levels of AA were generally higher while those of DGLA and EPA were lower in SHR than in WKY. The strain-differences in AA and EPA levels were even greater

TABLE 4

Fatty Acid Composition (% wt) of Brain Phospholipids in Spontaneously Hypertensive Rats (SHR) or Wistar-Kyoto Rats (WKY) After Seven Weeks Dietary Supplementation with Either 10% by Weight of Safflower Oil (SFO), Linseed Oil (LSO) or Control Oil (CON; SFO/LSO/HCO, 1.25/0.5/8.25) (mean ± SD, n = 8).

	SHR			WKY		
Fatty acid	SFO	LSO	CON	SFO	LSO	CON
SFA	42.0 ± 1.2	40.8 ± 3.7	44.0 ± 0.9	41.2 ± 1.0	41.0 ± 1.3	41.1 ± 1.1
MUFA	20.3 ± 1.2^a	22.7 ± 1.4^b	20.8 ± 0.8^a	21.6 ± 1.0^a	23.9 ± 1.9^b	22.5 ± 1.3^a
18:2n-6	1.3 ± 0.1^a	**1.0 ± 0.1^b**	0.7 ± 0.2^c	1.3 ± 0.2^a	1.3 ± 0.2^a	0.7 ± 0.1^b
18:3n-6	tr	tr	tr	tr	tr	tr
20:3n-6	0.4 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.3 ± 0.0
20:4n-6	**10.4 ± 0.2^{ab}**	9.8 ± 0.8^a	10.6 ± 0.6^b	11.2 ± 0.9^a	9.4 ± 0.6^b	11.1 ± 0.9^a
22:4n-6	3.6 ± 0.2^a	**2.8 ± 0.4^b**	3.2 ± 0.3^c	3.7 ± 0.2^a	2.2 ± 0.2^b	3.3 ± 0.3^c
22:5n-6	**3.9 ± 0.2^a**	**0.7 ± 0.1^b**	**1.1 ± 0.1^c**	3.3 ± 0.5^a	0.2 ± 0.1^b	0.7 ± 0.1^c
18:3n-3	0.2 ± 0.1	0.2 ± 0.0	**0.2 ± 0.0**	0.1 ± 0.1	0.2 ± 0.1	0.1 ± 0.1
20:5n-3	**0.1 ± 0.1**	**0.1 ± 0.0**	**0.0 ± 0.0**	0.4 ± 0.2	0.5 ± 0.2	0.4 ± 0.2
22:5n-3	**0.1 ± 0.0^a**	**0.6 ± 0.1^b**	**0.2 ± 0.0^a**	0.9 ± 0.2^a	1.5 ± 0.2^b	0.8 ± 0.2^a
22:6n-3	13.3 ± 0.9^a	16.1 ± 0.6^b	16.8 ± 0.6^b	13.0 ± 0.8^a	17.1 ± 0.9^b	16.3 ± 0.3^b
Σn-6	19.8 ± 0.5^a	**14.8 ± 1.2^b**	15.9 ± 0.9^c	20.0 ± 1.1^a	13.6 ± 0.7^b	16.2 ± 1.2^c
Σn-3	13.9 ± 0.9^a	**17.0 ± 0.6^b**	16.9 ± 0.6^b	14.4 ± 0.6^a	19.3 ± 0.7^b	17.6 ± 0.3^c
AA/LA	8.2 ± 0.7^a	**10.3 ± 1.5^b**	16.1 ± 3.5^c	9.1 ± 1.6^a	7.6 ± 0.1^b	16.6 ± 3.6^c
AA/DGLA	29.8 ± 3.4^a	23.2 ± 2.8^b	**40.4 ± 3.4^c**	33.6 ± 7.9^a	22.8 ± 2.3^b	33.2 ± 5.9^a
AA/C22n-6	**1.4 ± 0.1^a**	**2.9 ± 0.1^b**	**2.5 ± 0.1^c**	1.6 ± 0.1^a	4.0 ± 0.1^b	2.8 ± 0.1^c
22:5/22:4	**1.1 ± 0.1^a**	**0.2 ± 0.1^b**	**0.3 ± 0.1^c**	0.9 ± 0.1^a	0.1 ± 0.1^b	0.2 ± 0.1^c
DHA/DPA	**62.1 ± 12^a**	**25.5 ± 2.5^b**	**95.6 ± 20^c**	15.7 ± 5.9^a	11.5 ± 1.7^b	20.3 ± 4.5^a
DHA/EPA	**120 ± 51^a**	**311 ± 237^b**	407 ± 72^b	38.2 ± 26	36.7 ± 17	46.1 ± 21
AA/DGLA &EPA	**21.9 ± 4.5^a**	**20.2 ± 2.5^a**	**34.9 ± 3.4^b**	13.0 ± 3.7^{ac}	10.5 ± 2.5^a	15.9 ± 5.3^c

Abbreviations: HCO = hydrogenated coconut oil; SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; AA = 20:4n-6; LA = 18:2n-6; DGLA = 20:3n-6; EPA = 20:5n-3; tr = trace.

a,b,cData in the same strain with different superscripts are significantly different from each other at $p<0.05$.

Data in **bold phase** are significantly different from WKY on the same diet ($p<0.05$).

TABLE 5

Fatty Acid Composition (% wt) of Adrenal Phospholipids in Spontaneously Hypertensive Rats (SHR) or Wistar-Kyoto Rats (WKY) After Seven Weeks Dietary Supplementation with Either 10% by Weight of Safflower Oil (SFO), Linseed Oil (LSO) or Control Oil (CON; SFO/LSO/HCO, 1.25/0.5/8.25) (mean ± SD, n = 8).

	SHR			WKY		
Fatty acid	SFO	LSO	CON	SFO	LSO	CON
SFA	40.1 ± 2.2	39.7 ± 1.4	40.9 ± 0.8	39.0 ± 1.6	38.5 ± 1.0	40.0 ± 1.3
MUFA	6.3 ± 0.3^a	10.8 ± 0.5^b	10.7 ± 0.5^b	8.5 ± 1.1^a	13.3 ± 0.8^b	11.6 ± 0.5^b
18:2n-6	**5.6 ± 0.3^a**	**6.2 ± 0.6^b**	**2.7 ± 0.1^c**	6.0 ± 2.3^a	8.8 ± 1.0^b	3.1 ± 0.3^c
18:3n-6	0.2 ± 0.3	tr	tr	0.2 ± 0.3	0.1 ± 0.1	tr
20:3n-6	**0.4 ± 0.2^a**	**0.5 ± 0.1^a**	**0.1 ± 0.1^b**	0.7 ± 0.2^a	1.0 ± 0.2^b	0.3 ± 0.1^c
20:4n-6	**44.8 ± 2.1^a**	**39.2 ± 1.9^b**	43.4 ± 1.0^a	39.8 ± 2.8^a	31.7 ± 1.7^b	42.6 ± 0.9^a
22:4n-6	1.9 ± 0.2^a	0.4 ± 0.1^b	1.8 ± 0.1^a	2.2 ± 0.4^a	0.5 ± 0.2^b	1.7 ± 0.1^a
22:5n-6	0.4 ± 0.4	0.1 ± 0.1	0.1 ± 0.1	0.7 ± 0.2^a	0.1 ± 0.0^b	0.1 ± 0.1^b
18:3n-3	tr	tr	tr	tr	0.4 ± 0.1	tr
20:5n-3	0.1 ± 0.2^a	**1.5 ± 0.2^b**	tr	0.1 ± 0.2^a	2.8 ± 0.6^b	tr
22:5n-3	tr	**1.5 ± 0.2^a**	**0.2 ± 0.1^b**	0.2 ± 0.3^a	1.8 ± 0.2^b	0.3 ± 0.1^a
22:6n-3	tr	0.4 ± 0.1	**0.4 ± 0.1**	0.4 ± 0.3	0.5 ± 0.1	0.5 ± 0.2
Σn-6	**53.2 ± 1.5^a**	**46.4 ± 1.9^b**	48.2 ± 0.9^c	49.8 ± 3.6^a	42.6 ± 1.3^b	48.1 ± 1.2^a
Σn-3	**0.1 ± 0.2^a**	**3.4 ± 0.3^b**	0.5 ± 0.2^c	0.6 ± 0.6^a	5.3 ± 0.8^b	0.7 ± 0.3^a
AA/LA	**8.0 ± 0.8^a**	**6.4 ± 0.8^b**	**16 ± 0.7^c**	5.4 ± 2.3^a	3.7 ± 0.6^b	13.6 ± 1.0^c
AA/DGLA	**116 ± 61^a**	**80 ± 11^a**	**281 ± 44^b**	64 ± 23^a	33 ± 5.4^b	25 ± 4.5^b
AA/C22n-6	**20 ± 4.6^a**	**73 ± 11^b**	24 ± 2.5^a	14 ± 3.1^a	57 ± 15^b	24 ± 2.3^c
AA/DGLA &EPA	**285 ± 177^a**	**20 ± 2.5^b**	**291 ± 38^a**	58 ± 20^a	9 ± 1.4^c	130 ± 14^c

Abbreviations: HCO = hydrogenated coconut oil; SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; AA = 20:4n-6; LA = 18:2n-6; DGLA = 20:3n-6; EPA = 20:5n-3; tr = trace.

a,b,cData in the same strain with different superscripts are significantly different from each other at $p<0.05$.

Data in **bold phase** are significantly different from WKY on the same diet ($p<0.05$).

in animals fed the LSO diet. As a result, the ratios of AA/(DGLA+EPA) in all tissues were consistently higher in SHR than in WKY. In other words, SHR has a higher proportion of eicosanoid precursor (AA), which favors the formation of eicosanoids (including both vasoconstrictors, e.g., thromboxane (TX) A_2, $PGF_{2\alpha}$, and leukotriene C4 (26-28), and vasodilator, such as prostacyclin), and has a lower proportion of DGLA and EPA, which are respectively, the precursors of the vasodilators, PGE_1 and PGI_3, but are not of any known potent vasoconstrictor agents (17, 26-29). Morever, eicosanoid production from AA in SHR tended to favor the synthesis of vasoconstrictor eicosanoids, such as TXA_2 (30-32) and $PGF_{2\alpha}$ (8). Thus, the increased 2 series eicosanoid precursor, AA, and reduced 1 and 3 series eicosanoid precursors, DGLA and EPA in SHR may be related in part to the pathogenesis of hypertension in this model. Abnormalities of eicosanoid production in SHR have been described by many investigators (8, 29, 33-36), but the difficulties associated with measurements of eicosanoids under physiological conditions render the interpretation of many of the results difficult. What one can say, is that the differences between the strains in eicosanoid precursor concentrations are consistent with lower vasodilator and hypotensive effects in SHR.

In this study, we demonstrated consistent differences in tissue FA metabolism between SHR and WKY. These differences are likely to differentially affect membrane function between the strains. PUFAs are important constituents of membrane PL bilayer, and PUFA composition and cholesterol contents of the cell membrane are important factors in influencing membrane properties and functions (20). The membrane differences between the strains may lead to differences in membrane fluidity, in ionic channel behavior, and in the activities of membrane-bound enzymes and receptors. Various membrane abnormalities in SHR have been reported (37-40). The alterations of fatty acid composition of PL in SHR may contribute to the altered membrane properties and functions, and may be related in part to the cause of hypertension in SHR.

In animals fed the LSO diet, the levels of AA were higher and those of EPA were lower in SHR than in WKY suggesting that there were differences between the two strains in their handling of n-3 and n-6 EFAs. LN is known to inhibit the conversion of LA to AA (17, 41). As our results have shown that LSO was significantly more effective at lowering the level of AA in WKY than in SHR, this raises the possibility that the balance between metabolism of n-3 and n-6 EFAs in SHR may be tilted towards the n-6 EFAs as compared to that in WKY. In other words, SHR may either more actively convert LA to AA, or may preferentially incorporate and retain the AA. Only direct assay of enzyme activity will distinguish between these possibilities.

In the brain, SHR has lower total n-3 EFAs and 22:5n-3 levels than WKY. The levels of the 22 carbon n-6 EFAs were, on the other hand, higher in SHR. This might be the result of an enhanced elongation and delta-4-desaturation of 20 carbon n-6 EFAs, or a preferential incorporation or retention of the 22 carbon n-6 EFAs. N-3 EFAs are important in brain development and learning ability in rats (42, 43), and are essential in nervous system function in humans (44, 45). The lower levels of n-3 EFAs in the SHR might contribute to the abnormalities of the central nervous system which have been observed in SHR (46, 47).

The reasons for the observed differences in FA levels are presently uncertain. There may be differences between the strains in the activities of enzymes desaturating and elongating the n-3 and n-6 EFAs. Alternatively, there may be differences in the ways in which the different FAs are incorporated into membranes. Experiments designed to directly investigate the behavior of the various enzymes which could be involved must be done.

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METHODS

HPLC Method for Quantitation of Cholesterol and Four of Its Major Oxidation Products in Muscle and Liver Tissues

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A fast, sensitive, high performance liquid chromatographic method was developed for the quantitation of cholesterol and four of its major oxidation products: 3β-hydroxycholest-5-en-7-one (7-ketocholesterol), cholest-5-ene-3β, 7α-diol (7α-hydroxycholesterol), cholest-5-ene-3β,7β-diol (7β-hydroxycholesterol), and cholest-5-ene-3β,25-diol (25-hydroxycholesterol). In this procedure 2:1 chloroform:methanol (v/v) extracts of tissue homogenate were combined, dried over anhydrous Na_2SO_4, filtered, evaporated to dryness under N_2 and dissolved with a mobile phase of either 97:3 or 93:7 hexane:isopropanol (v/v). After membrane filtration and without further purification, aliquots were directly injected onto a 10-μm pore size, 30 × 0.39 cm μ-Porasil normal phase column. The separation of cholesterol and its oxidation products was monitored by a UV detector at 206 and 233 nm. This method was successfully applied to pork muscle as well as mouse liver tissues and was able to detect cholesterol oxidation products (COP) in the ppm range. The identity of the COP was confirmed by mass spectroscopy.
Lipids 24, 645-651 (1989).

It is known that cholesterol (CH) is unstable under a variety of conditions. Light, heat, radiation, free radicals, and oxygen, among other factors, contribute to its oxidation. Smith (1) has proposed the overall mechanism of CH autoxidation. Numerous products have been reported to result from cholesterol autoxidation (1, 2). Various investigators have suggested that cholesterol oxidation products (COP) may possess undesirable biological characteristics such as interference in membrane functions and morphology, atherogenicity, cytotoxicity, mutagenicity, carcinogenicity, and the inhibition of cholesterol biosynthesis (1-5). The quantitation of cholesterol oxides from biological materials and foods is difficult because their isolation is frequently impeded by large amounts of interfering cholesterol, triglycerides, phospholipids, and some other lipids. The importance of conducting all analytical procedures rapidly, at low temperature, and in the absence of light and oxygen must be emphasized since the mere act of analysis may contribute to artifact formation.

TLC is not a method of choice because it allows exposure of the samples to potential oxidation as a result of its large surface area and also suffers from low loading capacity. Therefore, one is unlikely to recover μg quantities of COP from mg amounts of cogener lipids. The means of detection ultimately limits the usefulness of TLC for quantitation of CH and COP (5, 6, 7).

Column chromatography is limited by loading capacity and specificity (1). Gas chromatography (GC) requires alkaline saponification, which could create artifacts by promoting autoxidation of cholesterol as well as sterol oxide degradation (8, 9, 10, 11, 12). The use of cold saponification may prevent part of the problem (13), but it is very time consuming. Analysis of COP is carried out at relatively high column temperatures making it necessary to use glass columns and tubing (10) and trimethylsilyl derivatives of COP in order to prevent interaction of active hydrogens from the sterol oxides with metal wall surfaces or the stationary phase itself (10, 14, 15). Packed columns have been shown to be inadequate for separation (10, 16, 17) but capillary GC gives good separation of oxidation products (12, 18, 19, 20, 21). However, pretreatment of samples is necessary before chromatography.

The HPLC methods available rely on multi-step preliminary fractionation (9, 22, 23). Some have problems with separation from large amounts of interfering compounds such as lipids from egg yolk (10, 24), butter (23), and tallow (13, 25). A recently published HPLC method used silica gel column chromatography for preliminary purification of samples (26).

With the advent of high resolution HPLC equipment and membrane filtration the need for additional separation steps have been eliminated and the risk of artifact formation during the analytical process reduced. Lipid extracts after membrane filtration can be applied directly onto the HPLC column and quantification is achieved in a single step which saves precedural time.

The presented method is applicable for the quantitation of 7-ketocholesterol, 7α-hydroxycholesterol, 7β-hydroxycholesterol, 25-hydroxycholesterol and cholesterol in various tissues.

The present method is not applicable for the measurement of 5, 6 α-epoxy-5α-cholestan-3β-ol and 5α-cholestane-3β,5,6β-triol because these compounds do not possess adequate U.V. absorption characteristics.

MATERIALS AND METHODS

Reagents. The chemicals used were purchased as follows: hexane and 2-propanol (HPLC grade), chloroform and methanol (ACS grade) from Fisher Scientific Co., Fairlawn, New Jersey; anhydrous sodium sulfate from Mallinckrodt, Inc., Paris, Kentucky; 5,6α-epoxy-5α-

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Abbreviations: AUFS, absorbance units full scale; CH, cholesterol; COP, cholesterol oxidation products; GC, gas chromatography; HPLC, high performance liquid chromatography; TLC, thin-layer chromatography; u.v., ultra-violet; 7-ketocholesterol, 3β-hydroxycholest-5-en-7-one; 7α-hydroxycholesterol, cholest-5-ene-3β, 7α-diol; 7β-hydroxycholesterol, cholest-5-ene-3β, 7β-diol; 25-hydroxycholesterol, cholest-5-ene-3β, 25-diol.

cholestane-3β-ol, 5α-cholestane-3β,5,6β-triol, cholest-5-ene-3β,25-diol and cholest-5-ene-3β,7β-diol from Steraloids, Inc., Wilton, New Hampshire; 3β-hydroxycholest-5-en-7-one, cholest-5-ene-3β,7α-diol, and recrystallized cholest-5-en-3β-ol from Research Plus Laboratories, Inc., Denville, New Jersey.

Tissue Samples. Fresh muscle tissues were obtained from control pigs and those affected by the porcine stress syndrome myopathy. Fresh liver tissues were acquired from mice sacrificed for other experiments.

Ultraviolet Light Irradiation. Porcine (*longissimus dorsi*) muscles were sampled from pigs 3-4 days postmortem, separated from visible fat and bone, and coarsely ground by a Sorvall Omnimixer (Dupont Company, Newton, Connecticut). The sample was divided into 1 g portions and spread over an area of 6 cm^2 on plastic petri dishes, forming a layer approximately 2 mm thick. The uncovered samples were either extracted immediately or irradiated by a Model XX-15 Black-Ray lamp (Ultra-Violet Products, Inc., San Gabriel, California) equipped with two 15-watt black lights (General Electric) at a distance of 10 cm at 40°C.

Extraction Procedure. Extraction of muscle and liver tissue was performed by the method of Folch *et al.* (27), as modified by Csallany and Ayaz (28). A portion (0.25-1.0 g) of tissue was accurately weighed and homogenized in 20 ml of 2:1 chloroform:methanol (v/v) with a Polytron homogenizer at half speed for 10-30 seconds. Before homogenization COP standards previously prepared in 2:1 chloroform:methanol were added to some tissue samples. The homogenate was transferred into a 250 ml separatory funnel and washed twice with 50 ml water. The water washes were combined and re-extracted twice with 25 ml portions of 2:1 chloroform:methanol and the samples were centrifuged at 4000 × g for 15 minutes at 22°C using a GSA rotor in a Sorvall RC-5B Refrigerated Superspeed Centrifuge (DuPont Company). The organic layer was removed, combined, and dried over anhydrous sodium sulfate. After filtration (Whatman #1 filter paper), the solvent was concentrated with a rotary evaporator and dried under N_2. The residue was dissolved and rinsed twice with 3 ml of mobile phase used for HPLC analysis, and filtered by a Swinney filter holder (Gelman Science, Inc., Ann Arbor, Michigan) containing a 0.45 μm Alpha-450 Metrical membrane filter (Gelman Sciences, Inc.). After membrane filtration the sample was reduced to 0.5-2 ml volume under N_2 and mixed. Aliquots of 10 to 250 μl of this filtrate were injected directly into the HPLC column without further purification.

HPLC Equipment and Conditions. An HPLC system equipped with an Altex Model 210 Sample Injection Valve (Beckman Instruments, Inc., Fullerton, California), two Model 110A Pumps controlled by a Model 421 System Controller and a Model SP8440 variable-wavelength detector (Spectra-Physics, Inc., Illinois) was used for quantification of cholesterol and COP. A 10 μm pore size, 30 × 0.39 cm μ-Porasil normal phase column (Waters Associates, Massachusetts) and a Model SP4100 Computing Integrator (Spectra-Physics) were used for analysis.

The HPLC separations for 7-ketocholesterol, 7β-hydroxycholesterol and 7α-hydroxycholesterol were achieved with a mobile phase of 93:7 hexane:isopropanol (v/v) at a flow rate of 0.95 ml/min for 12.5 minutes, followed by a flow rate 0.5 ml/min. The absorbance was monitored for the first 14 minutes at 233 nm and then at 206 nm. Cholesterol and 25-hydroxycholesterol were measured by using a less polar mobile phase of 97:3 hexane:isopropanol (v/v) at a flow rate of 1 ml/min at 206 nm. The detector was operated at the sensitivity level of 0.0025 absorbance units full scale (AUFS) in both separation systems.

Spectrometry. Ultraviolet absorption spectra were measured and recorded for 20 μg/ml solutions of CH and COP standards using a Beckman DU-8 UV/VIS wavelength scanning system (Beckman Instruments Inc.).

Mass spectrometry was used to confirm the identity of COP from freeze dried pork and UV irradiated pork samples. The separately collected peak materials from several HPLC injections (7-ketocholesterol, 7β-hydroxycholesterol, 7α-hydroxycholesterol) individually were combined and were injected into the direct inlet probe on LKB 9000 GC/MS (Bromma, Sweden), interfaced to a PDP 8/e computer (Digital Equipment Corporation, Massachusetts). Ion Source temperature was 290°C and ionizing voltage was 23eV.

Recovery. Standards were prepared by making stock solutions in 2:1 chloroform:methanol of each of the four COP under study. Two levels of all four COP, between 2.5 and 20 ppm, were added to each test tube prior to homogenization. Recovery was measured from peak heights or areas using a standard curve. Results were based on averages of at least three injections of each sample taken from replicate experiments.

RESULTS AND DISCUSSION

Very small amounts of contaminants were observed in the purchased CH standard when large quantities of freshly prepared solutions were injected onto the HPLC column. The reported (29) extinction coefficient for CH is 7,900 in hexane at 190nm versus 8,012 for the CH standard purchased. Repeated recrystallization from hot alcohol did not improve this value; however, the CH fraction collected from the HPLC column after a single chromatography had an extinction coefficient 7909. All the CH and COP standards used in the present study were purified by HPLC to ensure purity. Extinction coefficients for the four COP in the HPLC solvent were not found in the literature; therefore, comparisons with theoretical values were not made. Detection by UV was found to be sensitive and reproducible for CH and the four COP exhibiting UV absorptions. Ultraviolet absorption spectra were measured and recorded (Figure 1) for 20 μg/ml solutions of CH and COP standards in 93:7 hexane:isopropanol (v/v) mobile phase. Maximum absorbance for CH, 25-hydroxycholesterol, 7α-hydroxycholesterol, and 7β-hydroxycholesterol was at 202nm. Absorption maximum for 7-ketocholesterol was at 233nm, but the 5,6 α-epoxy-5α-cholestan-3β-ol and 5α-cholestane-3β,5,6 β-triol showed no UV absorption. Although the HPLC column was able to clearly separate all the above mentioned compounds when the mobile phase was varied between 90:10 (5α-cholestane-3β,5,6β-triol), 93:7 (cholesterol, 25-hydroxycholesterol,

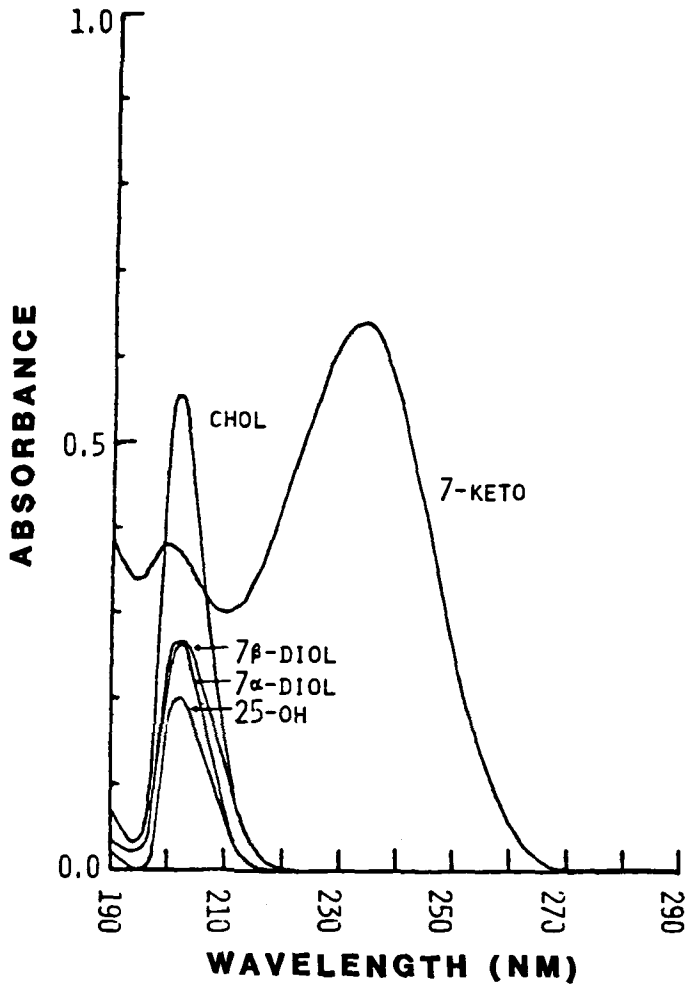

FIG. 1. Ultraviolet absorption spectra of cholesterol and cholesterol oxidation product standards. 20 μg/ml solutions in 93:7 hexane:isopropanol (v/v).

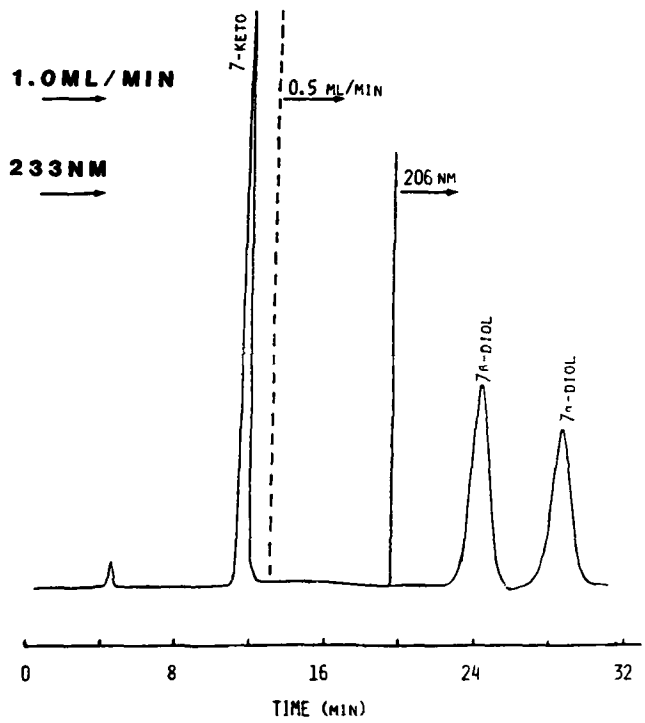

FIG. 2. Chromatogram of C-7 cholesterol oxidation product standards, 93:7, hexane:isopropanol (v/v) mobile phase. Mixture of 1 μg each standard 7-ketocholesterol (7-keto), 7β-hydroxycholesterol (7β-diol), and 7α-hydroxycholesterol (7α-diol) injected.

7-ketocholesterol), 97:3 (7-ketocholesterol, 7α-hydroxycholesterol, 7β-hydroxycholesterol) hexane: isopropanol (v/v), respectively, the detection by a refractive index detector (Model 156 Beckman Instruments) was found to be not sensitive enough for the measurement of minute amounts of COP in the actual tissue samples.

A 10 μm pore size, 30 × 0.39 cm μ-Porasil normal phase column (Waters Associates) was used for the separation of 7-ketocholesterol, 7α-hydroxycholesterol, and 7β-hydroxycholesterol using a 93:7 hexane:isopropanol (v/v) mobile phase system. Separation of the above described oxidation products was achieved within 30 minutes (Figure 2). The separation of cholesterol and 25-hydroxycholesterol was accomplished on the same column by a less polar mobile phase—97:3, hexane:isopropanol (Figure 3). In this less polar system, 7-ketocholesterol can also be measured, however, the 7α- and 7β-hydroxycholesterols have very long retention times and therefore have impaired sensitivity.

Standard curves were made for cholesterol, and each of the COP studied in the range of 0-1 μg. Peak height measurements were found to be more sensitive than peak areas for the detection of COP due to baseline fluctuations and noise. The standard curves for COP were obtained by measuring peak height rather than peak area (Figure 4). However, peak areas were found to be more sensitive than peak heights for the detection of cholesterol because of the relatively high quantity of cholesterol in samples (Figure 5). Correlation coefficients for all least square lines were at least 0.999 in each standard curve. The minimum detectable concentrations of CH and COP by this method were in the 1-2 μg/g range based on a 3:1 signal:noise ratio (Table 1). Because the molecular extinction of 7-ketocholesterol is high, its detection was the most sensitive (Table 1).

The extraction procedure used in the present method was published by Folch *et al.* (27) and modified by Csallany and Ayaz (28). Kaneda *et al.* (30) concluded that 2:1 chloroform:methanol (v/v) was the most suitable solvent for the extraction of total lipids and cholesterol. It was necessary to separate emulsions frequently encountered during the multiple extractions of tissues. To break the emulsions, centrifugation was found to be effective. Furthermore, recovery of COP was found to be higher with than without centrifugation. Recovery for each COP was found to range between 85% and 93% (Table 2). Because of the very small amounts of COP used, higher recoveries could not be achieved. Various tissue samples were tested for COP by the present HPLC method. The objective of the various treatments of tissues was to increase the level of COP in samples for analysis. UV irradiated ground pork tissue samples revealed only 7-ketocholesterol. However, when UV irradiation was increased to 72 hours, 7-ketocholesterol, 7β-hydroxycholesterol,

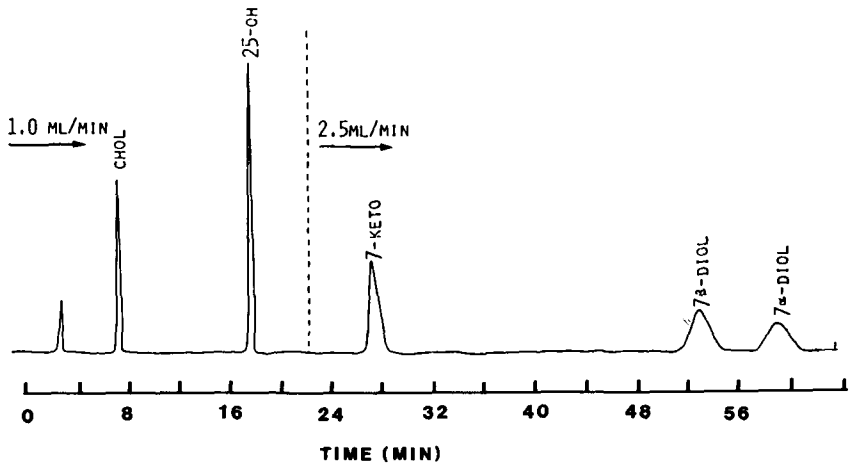

FIG. 3. Chromatogram of cholesterol and cholesterol oxidation product standards. Mixture of 1 μg each of cholesterol and 7-ketocholesterol; 2 μg each 25-hydroxycholesterol (25-OH) and 7β- and 7α-hydroxycholesterol (7β-diol, 7α-diol) injected.

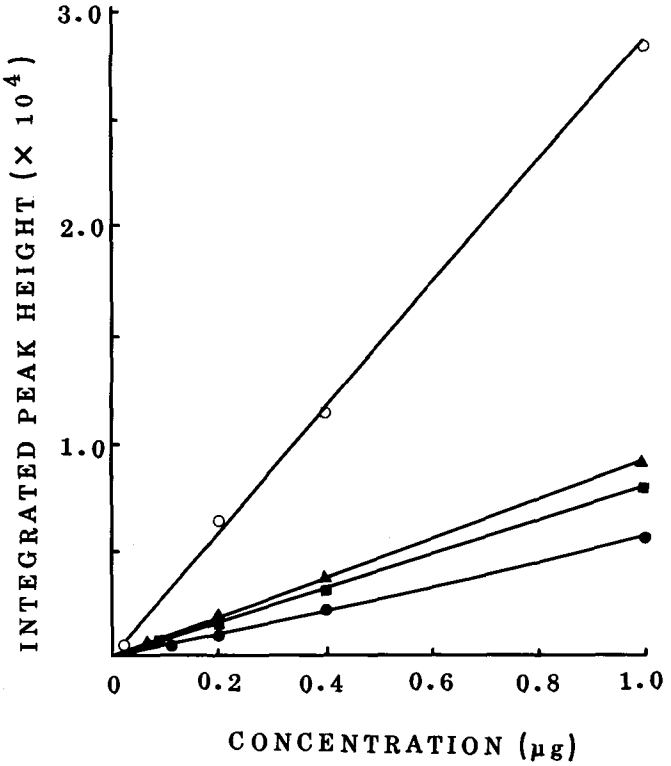

FIG. 4. Standard curves of cholesterol oxidation products. ○: 7-ketocholesterol, ■; 7α-hydroxycholesterol, ▲; 7β-hydroxycholesterol, ●; 25-hydroxycholesterol. Each data point represents mean of three injections at each concentration. Correlation coefficients for all lines were at least 0.999.

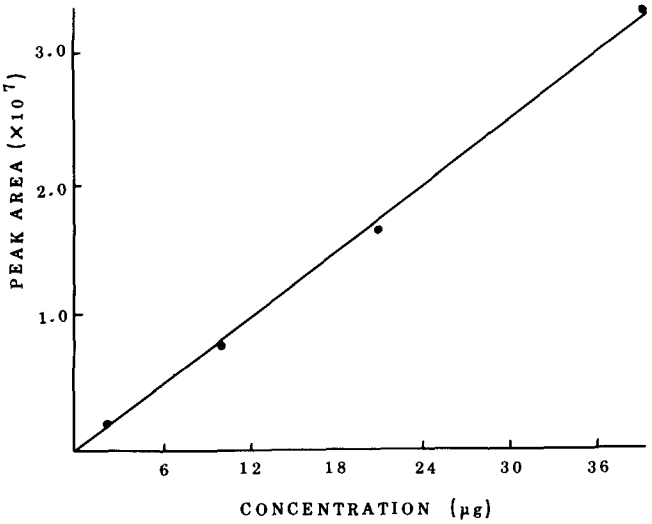

FIG. 5. Standard curves of cholesterol. Each data point represents mean of three injections at each concentration. Correlation coefficients for all lines were at least 0.999.

TABLE 1

Minimum detectable concentrations of cholesterol and cholesterol oxidation products by the present HPLC method.[a]

Sterol	Concentration μg/g
Cholesterol	1.1
25-hydroxycholesterol	2.2
7-ketocholesterol	0.38
7β-hydroxycholesterol	1.5
7α-hydroxycholesterol	1.7

[a]Based on 3:1 signal:noise ratio.

and 7α-hydroxycholesterol were detected (Table 3). No COP were detected in the partially dried samples exposed to UV light for 24 hours, indicating a slower rate of cholesterol oxidation under conditions of reduced moisture content.

No COP were detected in normal fresh or five month frozen pork and fresh porcine stress syndrome pork samples (Table 4). In one ground pork sample, 1.13 ppm of 7-ketocholesterol was found after 10 days of refrigerated storage. No COP were found in fresh pork samples spiked with ten times the amount of cholesterol naturally present in these tissues. This finding demonstrates that the present HPLC procedure does not cause artifact formation.

Freeze-dried pork samples were prepared in our

TABLE 2

Recovery of cholesterol oxidation products from pig muscle.

Cholesterol oxidation products	Spiked amounts μg/g	Recovery amounts μg/g	Recovery (%)
25-hydroxycholesterol	12.5, 20.0	11.9±0.5, 18.0±0.7[a]	93[b]
7-ketocholesterol	2.5, 5.0	2.3±0.1, 4.3±0.2	88
7β-hydroxycholesterol	7.5, 15.0	6.8±0.9, 11.9±0.6	85
7α-hydroxycholesterol	10.0, 20.0	9.2±0.7, 16.2±1.0	87

[a]Mean ± SEM of three injections at each level from replicate experiments.
[b]Average recovery from replicate experiments.

TABLE 3

Measurements of cholesterol and cholesterol oxidation products of UV-treated and control ground pork muscle samples.[a]

Exposure time (hr.)	N	Cholesterol (mg/g)	25-hydroxy-cholesterol (μg/g)	7-keto-cholesterol (μg/d)	7β-hydroxy-cholesterol (μg/g)	7α-hydroxy-cholesterol (μg/g)
0	4	0.30	ND[d]	ND	ND	ND
24[b]	3	0.35	ND	ND	ND	ND
24[c]	1	0.39	ND	1.15	ND	ND
72	3	0.28	ND	1.24	2.29	2.01
72[c]	1	0.28	ND	2.26	—	—

[a]All values are means of triplicate injections for each sample.
[b]Sample was dried to a moisture content of 35% before irradiation.
[c]Sample was stored at 0°C for one day after irradiation.
[d]Not detected.

TABLE 4

Measurements of cholesterol and cholesterol oxidation products of pork muscle and mouse liver tissue samples.[a]

Samples	N	Cholesterol (mg/g)	25-hydroxy-cholesterol (μg/g)	7-keto-cholesterol (μg/g)	7β-hydroxy-cholesterol (μg/g)	7α-hydroxy-cholesterol (μg/g)
Fresh	4	0.30±0.3[b]	ND[h]	ND	ND	ND
Fresh[c]	1	3.30	ND	ND	ND	ND
Refrigerated[d]	1	0.33	ND	1.13	ND	ND
Frozen[e]	1	0.32	ND	ND	ND	ND
Freeze-dried[f]	2	0.38±0.2[b]	ND	126.59±4.96[b]	21.05±1.94[b]	28.98±1.8
PSS muscle fresh[g]	2	0.31±0.2[b]	ND	ND	ND	ND ND
Fresh mouse liver[i]	8	1.38±0.31[b]	ND	1.09±0.41[b]	3.10±0.67[b]	

[a]Mean of triplicate injections for each sample except where indicated.
[b]Mean ± SEM.
[c]Tissue was same as above, but 3.0 mg cholesterol was added to the sample before homogenization.
[d]Stored 10 days at 40°F unwrapped.
[e]Stored five months at -70°C wrapped in butcher paper.
[f]Stored for three years in presence of light and air.
[g]Porcine-stress syndrome.
[h]Not detected.
[i]Normal 17 month-old mice.

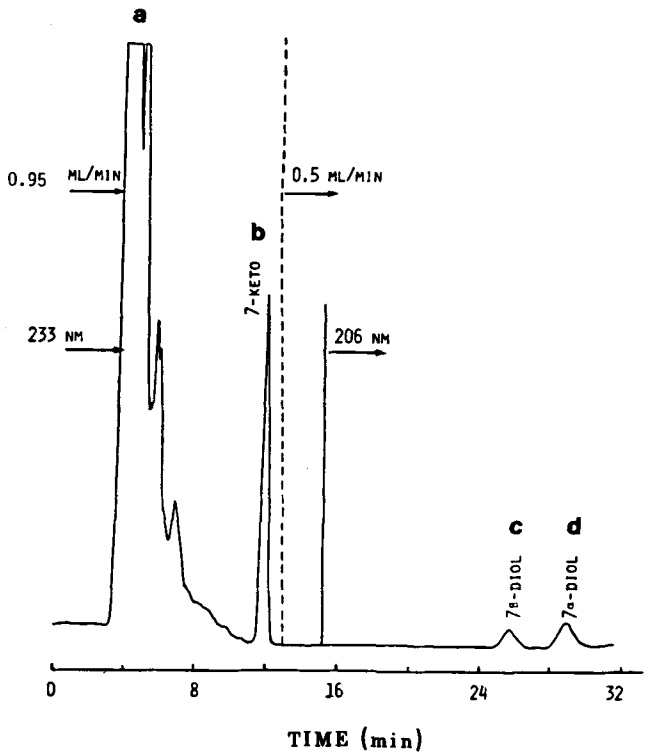

FIG. 6. HPLC chromatogram of freeze-dried pork tissue extract, 93:7 hexane: isopropanol (v/v) mobile phase; a, solvent peak and cholesterol; b, 7-ketocholesterol; c, 7β-hydroxycholesterol; d, 7α-hydroxycholesterol.

laboratory and stored for three years with periods of intermittent exposure to air and light at room temperature. These samples contained high levels of the three COP, but no 25-hydroxycholesterol (Figure 6).

Since 25-hydroxycholesterol was not found in any of the tested tissues, pork muscle homogenates were spiked with 0, 5, and 20 μg/g standard 25-hydroxycholesterol before extraction, and the samples were analyzed to mimic actual situations in the tissue samples (Figure 7).

Fresh mouse liver samples were obtained from our laboratory from mice fed a normal 8% corn oil diet for an extended period as part of another project. Some COP were detected beside cholesterol in mouse livers by the present method.

Mass Spectral Identification. Identification by mass spectral analysis of tissue COP was accomplished in the present experiments since it is believed that retention time and co-chromatography are not specific enough to ensure positive identification. Mass spectral analysis of standard 7-ketocholesterol, 7β-hydroxy-, and 7α-hydroxycholesterol, and their corresponding fractions collected from the freeze-dried pork muscle samples were made and found to be in agreement with those in the literature (21, 31). The mass spectral data confirms the authenticity of the peaks detected by the present method.

In conclusion, we find that the present method is not only sensitive, but fast and simple. It eliminates lengthy prepurifications by using membrane filtration techniques and direct sample injection into the HPLC

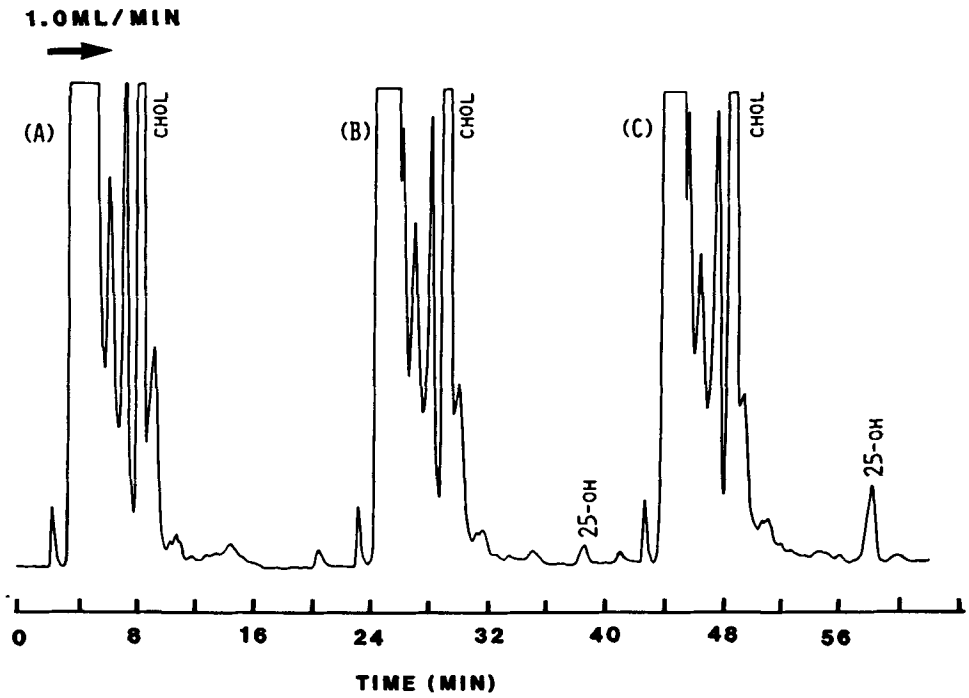

FIG. 7. Chromatogram of pork extracts, 97:3 hexane:isopropanol (v/v) mobile phase. Tissue spiked with (A) 0, (B) 5.0 or (C) 20 ppm 25-hydroxycholesterol before extraction.

column. This fast method can successfully be used for various meat and animal tissues and ensures minimum artifact formation in samples during analysis.

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HPLC Analysis of Desmosterol, 7-Dehydrocholesterol, and Cholesterol

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A simple and sensitive method to analyze mixtures of desmosterol, 7-dehydrocholesterol and cholesterol is described. The method involves the oxidative conversion of the sterols with cholesterol oxidase, followed by high performance liquid chromatographic (HPLC) analysis. A C_{18} reversed phase column (3 μm, 75×4.6 mm) and a mixture of methanol and acetonitrile (1:1, v/v) at a rate of 1 ml/min are used to separate the sterols. The eluted sterols are quantified by measuring UV absorption at 240 nm. As little as 10 pmoles of sterol can be quantified under these conditions.
Lipids 24, 652-655 (1989).

Cholesterol and its immediate precursors, desmosterol and 7-dehydrocholesterol, are structurally and metabolically similar. These similarities are the basis for the experimental use of these precursors as metabolic tracers for cholesterol. In this capacity, desmosterol and 7-dehydrocholesterol have been utilized in estimating the rate of synthesis of cholesterol (1-5) and the movement of cholesterol from one compartment to another (6-7). A simple and sensitive method which can quantitate mixtures containing markedly different amounts of these sterols is needed. The ability of cholesterol and its precursors to absorb ultraviolet (UV) light is poor and high performance liquid chromatographic methods (HPLC) (5-10) which use UV light to quantitate eluted sterols do not have the sensitivity necessary.

Cholesterol oxidase (EC 1.1.3.6) can modify certain sterols by converting them into conjugated enones (11). This markedly increases the ability of the sterols to absorb UV light and hence can be used to quantitate small amounts of 7α-hydroxycholesterol eluted from HPLC (12). Conjugated enone forms of sterols also exhibit mobility patterns in gas liquid chromatography different from the parent sterols and allow the separation of previously inseparable sterol mixtures (13). Similarly, conversion of cholesterol and its precursors into conjugated enone forms also facilitates the separation of closely related sterols by HPLC. A simple and sensitive method for the analysis of cholesterol and its precursors using cholesterol oxidase and HPLC is described here.

MATERIALS AND METHODS

Chromatographic Equipment. The chromatographic system used in this study consists of two Beckman model 110B pumps with a model 420 flow controller, a Beckman 210A sample injection valve equipped with a 50 μl sample loop (Beckman Instruments, Inc., Fullerton, CA), a Vari-chrom UV-Vis detector by Varian Associates, Inc. (Palo Alto, CA), and a dual channel recorder from Cole Palmer (Chicago, IL). The recorder is connected to the auxillary output (1 V per 2 A) of the detector. The input span selector of one of the channels of the recorder is usually set at 100 millivolt (MV) and at 10 MV for the other. The baselines of these channels are set at opposite sides of the chart paper. The attenuators of both channels are left in the calibration position and the chart speed is set at 15 cm/hr. The detector is set at 240 nm with a band pass of 4 nm and a slow time constant. Chromatography is performed at room temperature with a 1:1 (v/v) mixture of methanol and acetronitrile flowing at a rate of 1 ml/min. Several types of columns were investigated. The final selection is an Ultrasphere-XL C_{18} column (Beckman Instruments, Fullerton, CA) with guard cartridge (3 μm, 75 × 4.6 mm). This column is optimal in terms of speed, resolution and reproducibility.

Chemicals. HPLC-grade methanol and acetonitrile were obtained from Fisher (Springfield, NJ). All other biochemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Cholesterol esterase from Pseudomonas Fluorescens (No. C9406) and cholesterol oxidase from *Streptomyces* species (No. C8649) were used in this study. Sterols were used without further purification and standard solutions were prepared in isopropanol.

The total cholesterol assay kit (No. 352-02) was used according to the instructions provided. Briefly, 10 μl aliquots of serum were each incubated with 1 ml of the reconstituted reagent for 20 minutes at 37°C. The optical densities of the incubated mixture were read, and the amounts of total cholesterol in serum were calculated by using cholesterol calibrator (No. C0534) as standard.

Preparation of Samples. A 50 μl aliquot of isopropanol with or without dissolved sterols was transferred to a 13×100 mm screw cap tube. This is the optimal volume for the assay conditions described here; a larger volume of isopropanol causes the inactivation of enzymes added later in the procedure. To the isopropanol was added 250 μl of saline or other aqueous samples such as serum diluted with saline. The mixture was vortexed and a 50 μl aliquot of an enzyme-reaction mixture added. The mixture contained 150 mM sodium phosphate, pH 7.0, 30 mM sodium taurocholate, 1.02 mM carbowax 6000, and 0.1 units of cholesterol oxidase with or without 0.1 units of cholesterol esterase. The latter enzyme was included when total- instead of free-sterol was to be measured. The ability of cholesterol oxidase to convert various sterols to their respective conjugated enones is dependent on the source of the enzyme (11). Cholesterol oxidase from *Streptomyces* was selected for its ability to oxidize all the sterols examined in this study. The sterols and enzyme-reaction mixture were vortexed and the tube was incubated in a 37°C heating block without cap. The tube was removed after 1 hour and allowed to cool.

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Abbreviations: HPLC, high performance liquid chromatography; MV, millivolt. MVPH, millivolt setting of input selector and peak height at that particular input setting; UV, ultraviolet.

A 300 μl aliquot of methanol was added to each tube and the tube was vortexed. Then a 3-ml aliquot of petroleum ether was added, the tube was capped, the sample was vortexed vigorously for one minute, and then centrifuged to separate the phases. Conjugated enone forms of sterols are found in the upper phase and materials deleterious to the HPLC column remained in the lower phase. A 2-ml aliquot of the upper petroleum ether phase was then recovered, transferred to a clean tube and dried under nitrogen. Dried sterols were dissolved in a suitable volume of acetonitrile (usually 250–500 μl), 50 μl of which was injected into the HPLC.

RESULTS AND DISCUSSION

The assay conditions described above represent the optimal conditions for the analysis of cholesterol and its precursors found in a variety of biological samples. Samples such as serum, cell suspension or homogenate can be assayed directly without lipid extraction. The latter procedure is necessary only when the ability of the enzymes to access the sterols is questionable, or when it is used as a means to concentrate trace amounts of sterols found in the biological samples. The amount of esterified sterols can be determined by finding the difference between the amount of total-sterol and free-sterol. This and other previously mentioned features contribute significantly to the flexibility and speed of this HPLC method.

The ability of cholesterol oxidase to convert various free-sterols into the corresponding conjugated enone forms is shown in Figure 1. The Y coordinate is given in MVPH, the product of the millivolt setting of input selector and the peak height at that particular input setting. This expression normalizes peak heights obtained at different millivolt input settings and is easier to use than peak height alone. As is shown in Figure 1, MVPH of each sterol is related to the amount of sterols incubated with cholesterol oxidase. The amount converted is linear with respect to MVPH up to 40 μg. This is used as a limit for the amount of total sterol per sample to be processed under these assay conditions. In this way, quantitative conversion of all sterols in the sample can be assured. The sensitivity (2× MVPH of background noise) for each sterol is about 10 pmole; sensitivity is reduced when excessive noise is present in the HPLC system.

The separation of various conjugated enone forms of free-sterols is shown in Figure 2. Desmosterol, 7-dehydrocholesterol, cholesterol, stigmasterol, and β-sitosterol are all well-separated from one another. Better separated is β-sitosterol from cholesterol than is stigmasterol from cholesterol and was therefore selected for use as the internal standard in the following studies.

The validity of the HPLC method is tested by comparing the amount of human total serum cholesterol obtained with this method and with that obtained in an enzymatic colorimetric method marketed by Sigma. In the latter, the amount of hydrogen peroxide produced by the action of cholesterol oxidase on cholesterol is measured instead of the absorbance of UV light

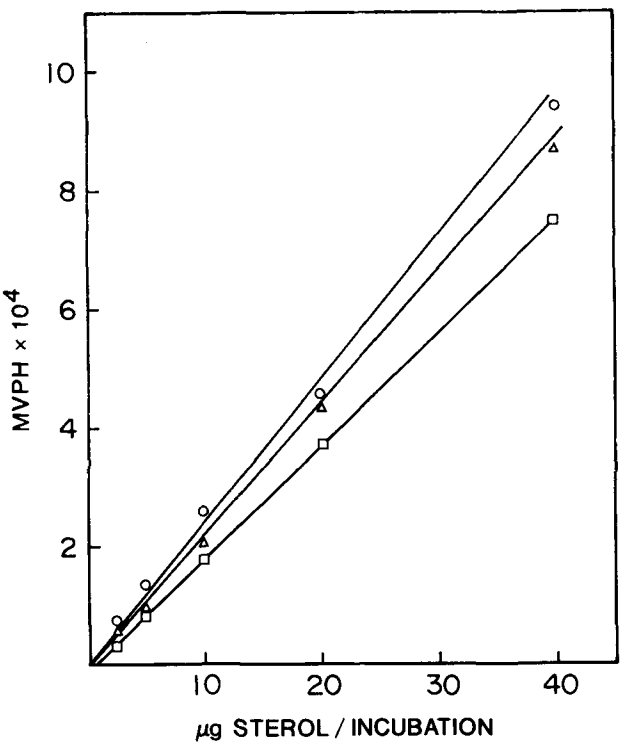

FIG. 1. The conversion of desmosterol (Δ), 7-dehydrocholesterol (□) and cholesterol (○) by cholesterol oxidase. MVPH represents the products of millivolt input setting and peak height in centimeters at that setting.

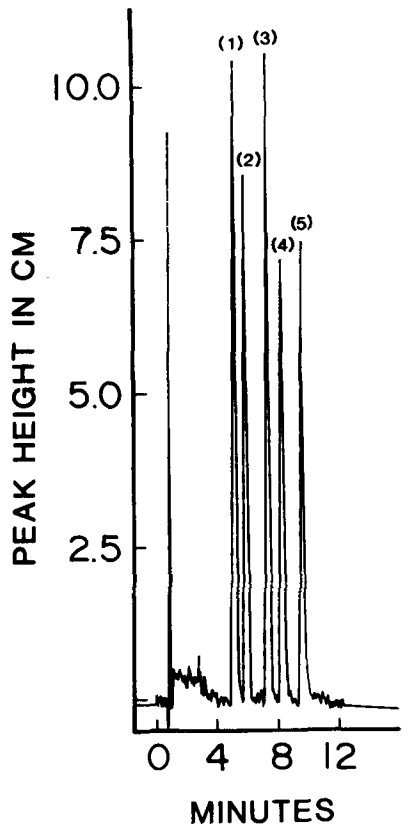

FIG. 2. The separation of a mixture containing 200 pmoles each of desmosterol (1), 7-dehydrocholesterol (2), cholesterol (3), stigmasterol (4), and β-sitosterol (5) after conversion by cholesterol oxidase. The recording is made with an input millivolt setting of 20.

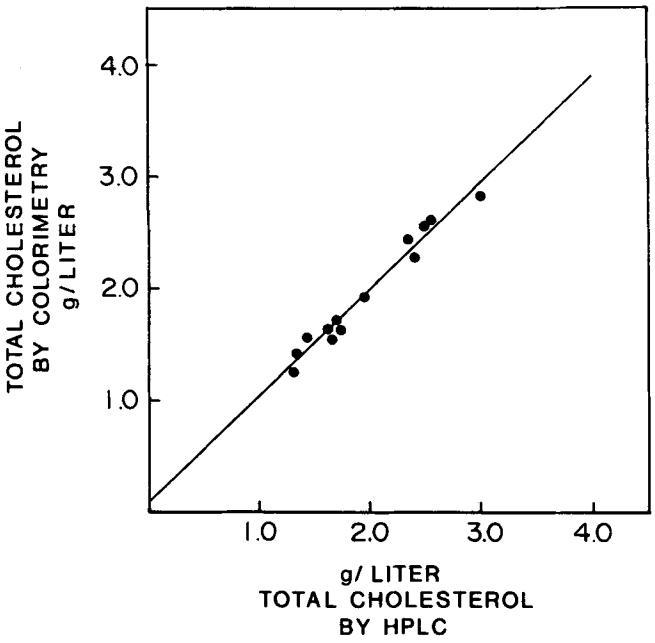

FIG. 3. Human total serum cholesterol concentration as analyzed by the present HPLC method (X axis) correlates with the enzymatic colorimetric method of Sigma (Y axis). The line is represented by $y = 0.11 + 0.94 \times r^2 = 0.99$).

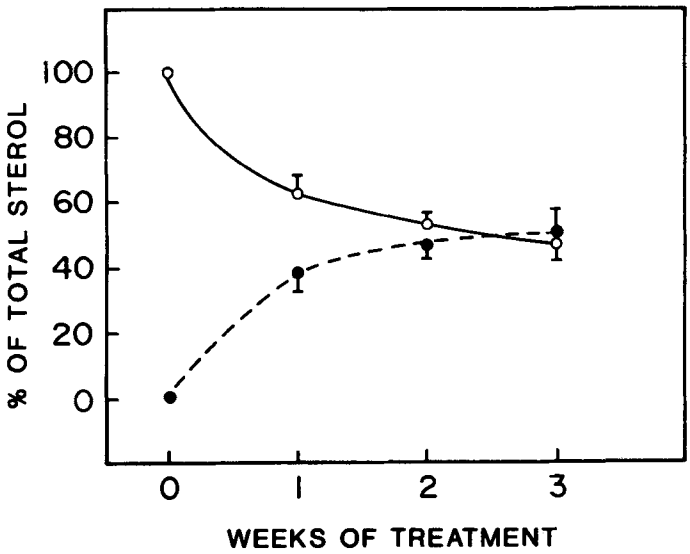

FIG. 4. The replacement of total serum cholesterol (○) by desmosterol (●) in mice after treatment with Triparanol.

by the conjugated enone group in the converted cholesterol. The validity of the HPLC method and its assay conditions is evident by the high degree of correlation between values obtained from these two different methods (Fig. 3).

The ability of the HPLC method to separate and quantitate cholesterol and its precursors is examined by analyzing biological samples containing various amounts of these sterols. For this purpose, a group of mice were treated with Triparanol (50 mg/kg chow) in order to block the conversion of desmosterol to cholesterol (1, 3). Serum samples were collected weekly from the tail vein of each mouse. The mice were then exsanguinated after 3 weeks and a sample of skin was taken for analysis. The skin samples were saponified and the unsaponifiable fractions were recovered and analyzed.

The concentrations of total sterols in the serum remain similar; they are 55 ± 4, 49 ± 14, 58 ± 10, and 55 ± 10 mg/dl, respectively, after 0, 1, 2, and 3 weeks of feeding with Triparanol. The percentage composition of serum sterols is, however, altered (Fig. 4). This suggests a replacement of serum cholesterol by desmosterol. Replacement progresses as treatment continues, and a 50% replacement by desmosterol occurs after 3 weeks of treatment with Triparanol. In contrast to the replacement of cholesterol by desmosterol in the serum, Triparanol elevated the concentration of desmosterol in the skin without changing the concentration of 7-dehydrocholesterol and cholesterol (Table 1). The reason for this difference between the serum and the skin is unclear. It may, in part, be caused by the metabolic differences between blood and skin sterols.

TABLE 1

Concentrations[a] of Various Sterols in Mouse Skin

Sterols	Untreated	Triparanol-Treated
Desmosterol	9 ± 2[b]	600 ± 93[b]
7-Dehydrocholesterol	34 ± 5	31 ± 10
Cholesterol	1055 ± 101	908 ± 67

[a]μg/gm wet weight (n=4).
[b]p>0.05.

It is clear from these data that markedly different amounts of cholesterol and its precursors, desmosterol and 7-dehydrocholesterol, can be analyzed rapidly with the procedure described in this report. The availability of this simple and sensitive method should facilitate investigations into the use and metabolism of cholesterol and its precursors.

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A Modification of the Thiobarbituric Acid Reaction

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A simple modification of the thiobarbituric acid reaction, which removes nonspecific chromogenic reaction products, involves incubation at room temperature for 16–20 hr and a column chromatographic step. The chromogen-concentrating capacity of the latter step is useful for determination of low concentrations of thiobarbituric acid-reactive materials in biological preparations. *Lipids 24,* 656–658 (1989).

Because of its procedural simplicity and nanomolar sensitivity, the thiobarbituric acid (TBA) reaction has been extensively used to estimate products of biological free radical-induced lipid peroxidation, e.g. malondialdehyde, MDA (1–4).

The major problem with use of the TBA reaction in biological samples is a lack of specificity for MDA. When the reaction is run in the usual manner, e.g. acid pH, elevated temperatures, reaction times of 30–60 min or longer, a variety of TBA-reactive materials (TBARM) is generated (3). These include tissue compounds other than MDA (3, 5, 6), acid-hydrolyzable adducts of MDA with amino groups (7, 8), compounds that can be converted to MDA, e.g. lipid hydroperoxides (9, 10), and nonspecific reactants such as iron (10, 11) and ascorbic acid (Bidder, unpublished observations).

The present study was undertaken to ascertain the mildest conditions of temperature and time required for the acid hydrolysis of a labile adduct of MDA, malonaldehyde bis(dimethyl acetal) ($MDAMe_4$), and the subsequent reaction of the liberated aldehyde with TBA. The application of these conditions to the determination of TBARM in biological matrices was studied.

EXPERIMENTAL

Materials. $MDAMe_4$ (Aldrich Chemical Company, Milwaukee, WI); aluminum oxide (Alumina, Acid, 100–200-mesh, AG-4, Bio-Rad Laboratories, Richmond, CA); 2-thiobarbituric acid (Sigma Chemical Company, St. Louis, MO).

Solutions. TBA reagent: 2.5 g thiobarbituric acid, 0.5 g NaOH in 250 ml deionized water (DIW); *Wash solution:* 1 M NaOH (10 g NaOH in 250 ml DIW); *Eluting solution:* 0.1 M Na_3PO_4 (19 g Na_3PO_4 in 500 ml DIW). *Concentrated* and *1 N Hydrochloric Acid Solutions* were commercially available products.

TBA Reaction. Studies employing authentic MDA were carried out by combining equal volumes of solutions, containing approximately 15 nanomolar quantities of $MDAMe_4$ in 0.02 N HCl, and TBA Reagent. Chromogen was developed by allowing this mixture to stand at room temperature (23–24°C) or by heating as specified. With urine, 10.0 ml normal human urine were mixed with 1.0 ml concentrated HCl and 10.0 ml TBA Reagent. Protein-free tissue extracts can be acidified with one-tenth volume of concentrated HCl and combined with an equal volume of TBA Reagent.

Chromogen development entailed standing at room temperature or by heating as described below. TBARM was expressed as the difference (D) between the optical densities at 532 nm and at 600 nm using a DIW blank. Measurements were made with a Turner-Sequoia Spectrophotometer, Model 340D. UV:Visible spectra were determined on a Beckman DU-65 Spectrophotometer.

Isolation of TBARM. Dry aluminum oxide is added to glass or plastic columns (10 mm ID, with reservoir) to a height of 15–20 mm. DIW is added and the suspension slurried to eliminate air bubbles and to achieve uniform packing. Clear, TBARM-containing solutions are carefully added; particulate material is removed by centrifugation or filtration. Column is washed with the minimal volume of Wash solution required to produce a clear effluent; excessive volumes significantly reduce recovery of chromogen from column. One bed-volume of Eluting solution is then added. Since this latter solution can displace TBARM from the solid phase, it should be allowed completely to pass through the column, with pressure extrusion, before additional Eluting solution is added. The red-hued eluate is collected in a five ml, graduated centrifuge tube and made up to the 3.0 ml mark with 1 N or concentrated HCl. The capacity of columns with these dimensions is approximately 35 nanomoles TBARM. Recovery of TBARM prepared from $MDAMe_4$ was in excess of 85%.

RESULTS

Conditions of chromogen development. Comparison of room-and elevated-temperature conditions. The TBA reaction was performed by mixing equal volumes of a solution of $MDAMe_4$ in 0.02 N HCl or human urine (10.0 ml + 1 ml concentrated HCl) and TBA Reagent. Chromogen was developed by heating at 95°C for 30 min, by incubating at 37.5°C for 2.5 hr or by allowing the reaction mixture to stand at 23–24°C for 18 and 28 hr. Chromogenic reaction products were then isolated chromatographically and their optical densities measured. Representative experiments of this type are presented in Table 1.

With $MDAMe_4$, essentially identical D-values were obtained under conditions of elevated temperature and at room temperature. With prolongation of incubation time from 18 to 28 hr, there was a slight reduction in D-values. The true yield of TBARM prepared from $MDAMe_4$ by incubation for 18 hr at 23–24°C was 80% calculated on the basis of Nair's data (λ_{max} 532, ε =

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Abbreviations: TBA, 2-thiobarbituric acid; MDA, malonaldehyde, malondialdehyde, malonyldialdehyde; TBARM, thiobarbituric acid-reactive material; $MDAMe_4$, malonaldehyde bis(dimethyl acetal), 1,1,3,3-tetramethoxy propane, tetramethoxy malonaldehyde; DIW, deionized water; D, difference between optical densities at 532 nm and 600 nm; and OD, optical density.

TABLE 1

D-Values (Differences Between Optical Densities at 532 nm and 600 nm) of Thiobarbituric Acid-Reactive Material Generated from $MDAMe_4$ and from Human Urine under Various Conditions of Temperature and Reaction Times

Experimental conditions	D value	%
$MDAMe_4$		
95°C/30 min	0.350	100
37.5°C/2.5 hr	0.353	100
23-24°C/18 hr	0.352	100
23-24°C/28 hr	0.343	97
Urine:		
95°C/30 min	1.058	207
37.5°C/2.5 hr	0.630	123
23-24°C/18 hr	0.511	100
23-24°C/28 hr	0.515	101

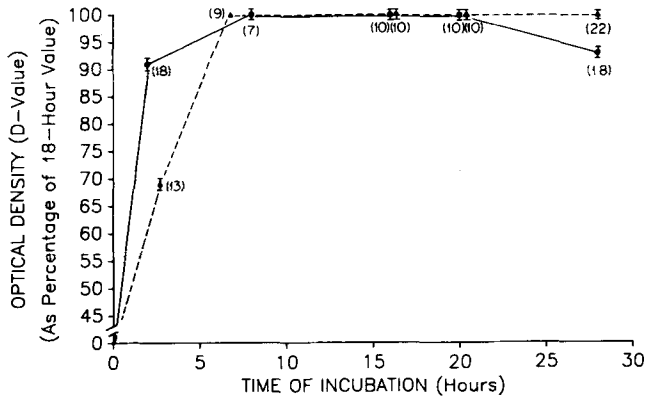

FIG. 1. Time-course of TBA chromogen development at 23-24°C with $MDAMe_4$ (•) and with human urine (▲). Numerals in parentheses are numbers of determinations. Standard deviation for each data point represented by (I). The "100%" values for $MDAMe_4$ represent a true yield of 80% (see text).

159,200) for the 2:1 adduct of TBA and MDA (12).

By contrast, urinary TBARM D-values were elevated to a degree proportional to the temperatures at which the reaction was carried out. Removal of oxygen by helium-purging did not reduce these elevations in urinary TBARM.

Results with antioxidants present in the reaction mixture were as follows. Butylated hydroxytoluene, in concentrations up to 185 mM, did not prevent the higher urinary TBARM values occurring with chromogen development at elevated temperatures (13). $SnCl_2$ interfered with the chromatographic isolation procedure and ascorbic acid reacted in a concentration-dependent fashion with TBA to give a chromogen which significantly absorbed at 532 and 600 nm wavelengths (Bidder, unpublished observations).

Time-course of urinary chromogen development at room temperature. Preliminary experiments, each using a separate urine specimen, and/or employing $MDAMe_4$, established that peak D-values were attained with 23-24°C incubation periods which ranged from 7-20 hr. Because of the variable D-values for the several individual urine specimens used in time-course experiments, an 18-hr incubation sample was run as a reference standard with each set of urine and $MDAMe_4$ experiments and was given the value of 100%. The other samples' values were calculated as percentages of this reference TBARM's value (Fig. 1).

Chromogen development with $MDAMe_4$, in contrast to that with urine, was slightly more rapid and there was a slight decrease in the D-values when reaction time was extended from 20 to 28 hr.

Spectral characteristics of urinary chromogen. The UV-Visible spectrum (200-650 nm) of the urinary chromogen, isolated by the chromatographic technique and in aqueous pH 1 solution, was identical with that of the chromogen prepared from $MDAMe_4$ and subjected to the same isolation procedure. The urinary chromogen showed the same spectral changes as did the $MDAMe_4$ chromogen when the pH was changed from 1 to 9 with NaOH: hue change from red to blue-red and a characteristic bathochromic shift from 532 to 546 nm (Bidder, unpublished observations).

DISCUSSION

Several modifications in the TBA reaction were studied with the objective of reducing the generation of nonspecific TBARM. These included room temperature (23-24°C) incubation, reduction in dissolved oxygen content by purging the reaction mixture with helium, by addition of reducing agents and by chromatographic isolation of the TBARM.

With both $MDAMe_4$ and urine, chromogen development at 23-24°C rapidly reached maximal values at reaction times of 7-20 hr (Fig. 1). When reaction time was extended from 20 to 28 hr, the D-values with $MDAMe_4$, but not with urine, showed a small decrease. Since the TBA Reaction involves a rate-limiting acid-hydrolysis step followed by a combination of the product(s) of this reaction with TBA to form the chromogen, the more rapid development of chromogen with $MDAMe_4$ probably represents the greater susceptibility of the latter compound to acid hydrolysis as compared with that of urinary TBARM.

D-values generated with $MDAMe_4$ were essentially the same irrespective of whether the TBA Reaction was run at room temperature for 18 hr, at 37.5°C for 2.5 hr or at 95°C for 30 min. By contrast, human urinary TBARM D-values were significantly higher when the reaction was run at elevated temperatures in comparison with room temperature/18 hr conditions (Table 1).

These quantitative and time-course characteristics of D-value development were unaffected by reduction in the dissolved oxygen content of reaction mixtures by helium purging. Two reducing agents, $SnCl_2$ (14) or ascorbic acid, were incorporated in reaction mixtures: the former interfered with the subsequent chromatographic step while the ascorbic acid formed a chromogen with TBA which had the spectral characteristics of the MDA-TBA adduct (Bidder, unpublished observations). The antioxidant, butylated hydroxy-toluene,

had no effect on chromogen development under the various conditions employed in this study.

This method of determining TBARM by incubation at room temperature for 16–18 hr (e.g. overnight), and subsequently isolating and concentrating the resulting chromogen by column chromatography provides a simple and convenient procedure which minimizes the formation of TBA-reactive products evoked by running the reaction at elevated temperatures. When this procedure is run on protein-free filtrates of tissues, single-digit nanomolar quantities of TBARM can be readily determined. It has been employed in an *in vitro* assay which measures the capacity of human erythrocytes to inactivate cytotoxic free radicals derived from oxygen (15). This assay has revealed a range of individual radical-inactivating capacities in a human population. Together with a urinary MDARM assay, it is being used further to explore a suggestive correlation of impaired oxygen free-radical-inactivating capacity with the vulnerability of certain humans for developing tissue damage when they are exposed to alcohol or to certain cytotoxic agents.

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COMMUNICATIONS

Antioxidant Activity of β-Carotene-Related Carotenoids in Solution

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The effect of the antioxidant activity of β-carotene and related carotenoids on the free radical-oxidation of methyl linoleate in solution was examined by measuring the production of methyl linoleate hydroperoxides. Canthaxanthin and astaxanthin which possess oxo groups at the 4 and 4′-positions in the β-ionone ring retarded the hydroperoxide formation more efficiently than β-carotene and zeaxanthin which possess no oxo groups. The rates of autocatalytic oxidation of canthaxanthin and astaxanthin were also slower than those of β-carotene and zeaxanthin. These results suggest that canthaxanthin and astaxanthin are more effective antioxidants than β-carotene by stabilizing the trapped radicals.
Lipids 24, 659-661 (1989).

In recent years, epidemiological studies in humans (1-4) have suggested that β-carotene aids in cancer prevention. It was also implied that dietary β-carotene may exert an anticarcinogenic effect by a mechanism independent of its role as a vitamin A precursor (5). On the other hand, β-carotene is an effective singlet oxygen quencher (6), and we have found that β-carotene can prevent singlet oxygen-initiated oxidation of methyl linoleate in cooperation with α-tocopherol (7). Krinsky and Deneke (8,9) demonstrated that carotenoids including β-carotene are capable of inhibiting free radical-induced oxidation in liposomal lipids. Burton and Ingold (10) have shown that β-carotene belongs to a previously unknown class of biological antioxidants especially effective at low oxygen partial pressures such as those found in most tissues under physiological conditions. Therefore, the anticarcinogenic effect of β-carotene may be, at least partly, attributable to its antioxidant effect insofar as oxygen radicals are related to the process leading to human cancer (11).

However, little is known about the antioxidant activity of naturally occurring carotenoids other than β-carotene. We selected β-carotene (structure [1] in Fig. 1) and related carotenoids containing oxo groups and/or hydroxyl groups in the β-ionone rings as a common structural unit (that is, zeaxanthin [2], canthaxanthin [3], and astaxanthin [4] in Fig. 1) and examined their antioxidant effect upon the azo-initiated oxidation of methyl linoleate in solution. The results strongly suggest that the introduction of oxo groups at 4 and 4′ positions enhances the antioxidant activity of carotenoids.

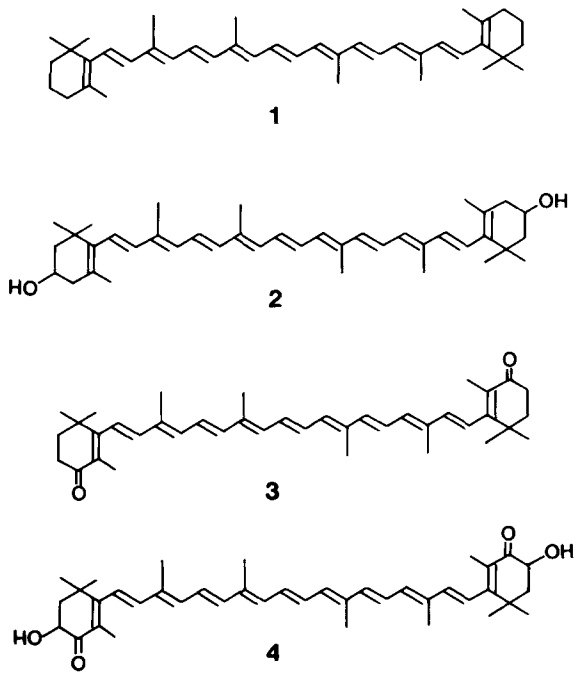

FIG. 1. Structures of carotenoids. (1) β-carotene, (2) zeaxanthin, (3) canthaxanthin, (4) astaxanthin.

MATERIALS AND METHODS

Materials. β-Carotene was obtained from E. Merck, Darmstadt. Canthaxanthin, astaxanthin and zeaxanthin were generously provided by Hoffmann-La Roche. The product of Nacalai Tesque Inc., Kyoto, Japan, was dl-α-tocopherol. Obtained from Wako Pure Chemical Industries, Osaka, Japan, was 2,2′-azo*bis*(2,4-dimethylvaleronitrile) (AMVN). Methyl linoleate (99%), supplied by Nacalai Tesque, was further purified by column chromatography with Florisil (100/200 mesh) (12). Other reagents and solvents were of analytical grade and used without purification.

Procedures. An appropriate amount of carotenoid in tetrahydrofuran (5 μmol/ml) was added to a mixture of hexane/isopropanol (1:1, v/v, 1.0 ml) containing methyl linoleate (100 μmol). Oxidation was initiated by adding a hexane solution of AMVN (10 μmol in 0.1 ml) and the mixture was incubated with continuous shaking under air in the dark at 37°C. At regular intervals, aliquots of the sample (10 μl) were withdrawn and injected into the HPLC column. The HPLC conditions employed and the procedure for the determination of methyl linoleate hydroperoxides have been described in a previous paper (12). Carotenoids and α-tocopherol were also quantified by HPLC using a column of YMC-Pack ODS (6 × 150 mm, 5 μm particle size, Yamamura Kagaku, Japan). The column was eluted with a mixture of acetonitrile/isopropanol (3:1, v/v). The flow rate was maintained at 3.0 ml/min and the effluent was monitored at 470 nm for carotenoids and 290 nm for α-tocopherol.

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Abbreviations: AMVN, 2,2′-azobis(2,4-dimethylvaleronitrile); HPLC, high performance liquid chromatography.

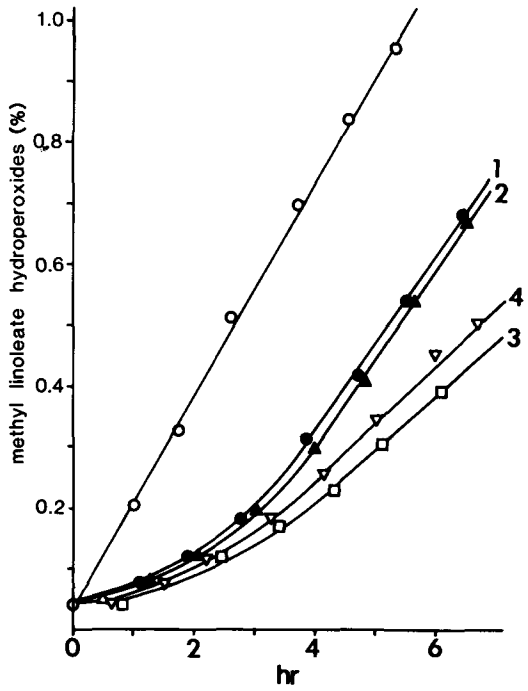

FIG. 2. Effect of carotenoids on the oxidation of methyl linoleate in solution. Reaction system consisted of methyl linoleate (77 mM), carotenoids (0.77 mM) and AMVN (7.7 mM) in a mixture of hexane/isopropanol/tetrahydrofuran (6:5:2, v/v/v, 1.3 ml). ●, β-Carotene; ▲, zeaxanthin; ▽, canthaxanthin; □, astaxanthin; ○, no addition.

RESULTS

Figure 2 shows the effect of the four carotenoids at 0.77 mM (1.0 mol % relative to methyl linoleate) on the rate of formation of methyl linoleate hydroperoxides. In the absence of carotenoids, methyl linoleate hydroperoxides accumulated linearly at the rate of 2.0 $\mu M \cdot min^{-1}$. Each carotenoid suppressed the oxidation of methyl linoleate, although the reaction curve showed no distinct induction period. During the first 2 hr of oxidation, the rate was kept at less than 0.5 $\mu M \cdot min^{-1}$ in the presence of each carotenoid, but thereafter canthaxanthin and astaxanthin retarded the hydroperoxide formation more efficiently than β-carotene and zeaxanthin.

When canthaxanthin or astaxanthin was added to the solution at 0.18 mM (0.2 mol % to methyl linoleate), little effect was observed, compared with α-tocopherol present at the same concentration (Fig. 3A). The concentrations of canthaxanthin and astaxanthin both decreased simultaneously with the formation of methyl linoleate hydroperoxides (Fig. 3B). On the other hand, an obvious induction period (approximately 4 hr) appeared in the presence of α-tocopherol, and the oxidation started only after the α-tocopherol was completely consumed.

In order to compare the reactivity of the carotenoids toward the radical chain reaction, the carotenoids were incubated in the presence of AMVN (8.8 mM) without methyl linoleate (Fig. 4). Evidently canthaxanthin and astaxanthin disappeared more slowly than β-carotene and zeaxanthin. The initial rates of the disappearance of the carotenoids were 2.7 $\mu M \cdot min^{1-}$ (β-carotene), 2.5 $\mu M \cdot min^{-1}$ (zeaxanthin), 1.3 $\mu M \cdot min^{-1}$ (canthaxanthin), and 1.4 $\mu M \cdot min^{-1}$ (astaxanthin), respectively. The rate of radical

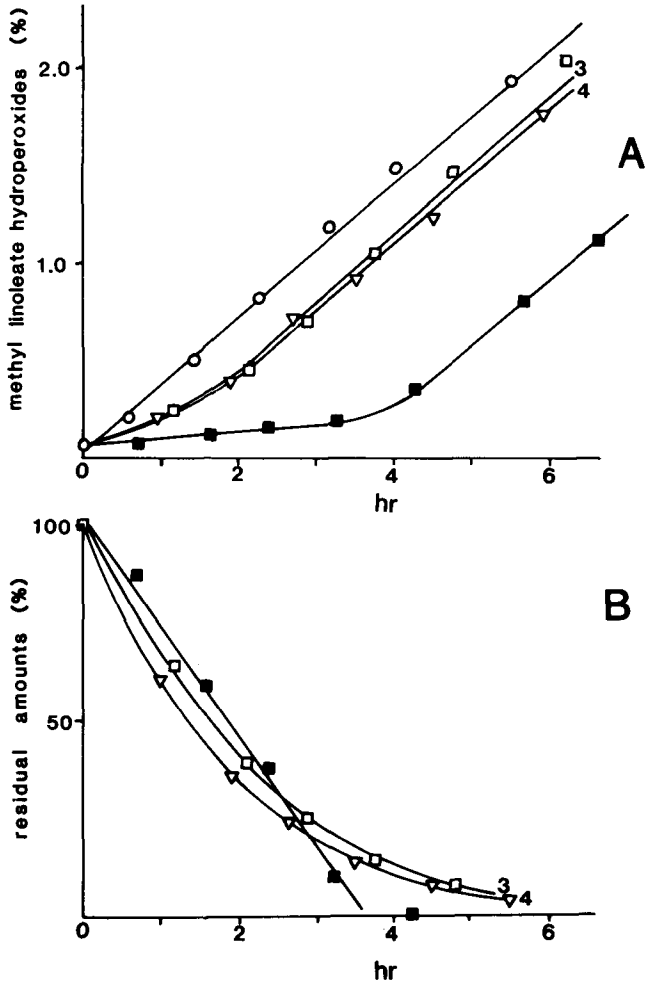

FIG. 3. Formation of methyl linoleate hydroperoxides (A) and loss of canthaxanthin, astaxanthin and α-tocopherol (B) during the oxidation of methyl linoleate. Reaction system consisted of methyl linoleate (88 mM), carotenoids (0.18 mM) or α-tocopherol (0.18 mM) and AMVN (8.8 mM) in a mixture of hexane/isopropanol/tetrahydrofurane (6:5:0.4, v/v/v, 1.14 ml). ▽, Canthaxanthin; □, astaxanthin; ■, α-tocopherol; ○, no addition.

production from AMVN was calculated to be 1.5 $\mu M \cdot min^{-1}$ (2 × 180 μM/240 min) by the induction period in the presence of α-tocopherol shown in Figure 3A (13,14). Thus, the ratio of the rate of carotenoid-disappearance to that of radical production from AMVN (that is, effective chain length of carotenoid oxidation) was determined to be 1.8 (β-carotene), 1.7 (zeaxanthin), 0.9 (canthaxanthin), and 1.0 (astaxanthin), respectively. Accordingly, canthaxanthin and astaxanthin were found to be quite resistant to autocatalytic radical chain reaction.

DISCUSSION

We have used AMVN-induced lipid peroxidation in a solution to measure the antioxidant activity of caroteoids. The unimolecular decomposition of this initiator induces a free radical chain oxidation of methyl linoleate via lipid peroxyl radicals as intermediates resulting in the exclusive formation of methyl linoleate hydroperoxides (15). The inhibition of the hydroperoxide formation by carotenoids has been attributed to their lipid peroxyl radical-trapping ability (10).

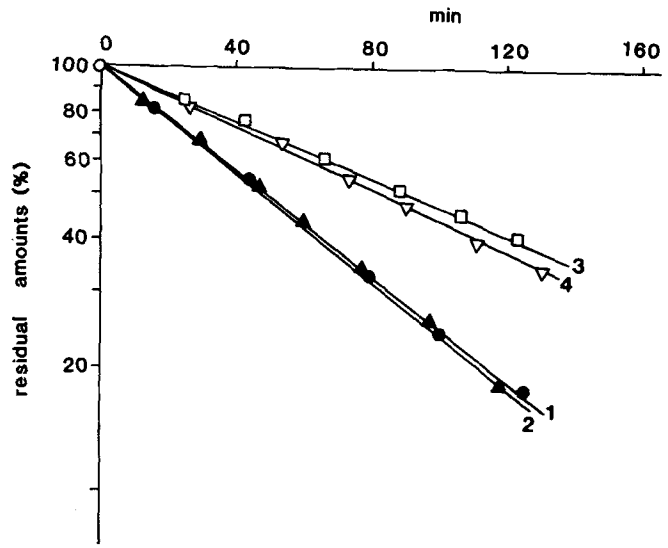

FIG. 4. Loss of carotenoids induced by AMVN. Reaction system consisted of carotenoids (0.18 mM) and AMVN (8.8 mM) in a mixture of hexane/isopropanol/tetrahydrofurane (6:5:0.4, v/v/v, 1.14 ml). ●, β-Carotene; ▲, zeaxanthin; ▽, canthaxanthin; □, astaxanthin.

Conventional chain-breaking antioxidants such as tocopherols trap peroxyl radicals by donating a hydrogen atom. However, β-carotene seems to exert an antioxidant activity by a mechanism in which the chain-propagating peroxyl radical is trapped by addition to the conjugated polyene system of β-carotene rather than the mechanism of hydrogen-donation (10). The resulting carbon-centered radical is resonance-stabilized because of the delocalization of the unpaired electron in the conjugated polyene system, leading to chain termination. This means that the reaction of β-carotene or related carotenoids with the peroxyl radicals competes with the production of methyl linoleate hydroperoxides via a chain reaction. Actually the loss of carotenoids is accompanied by the formation of methyl linoleate hydroperoxides (Fig. 3). The lack of a distinct induction period during the carotenoid-inhibited oxidation, in contrast to α-tocopherol (16), can be explained by the idea that the rate of the antioxidant activity of the carotenoids is similar to the rate of chain propagation of methyl linoleate-hydroperoxidation.

The fact that the antioxidant activity of canthaxanthin and astaxanthin lasted longer than β-carotene and zeaxanthin (Fig. 2) indicates that the substitution of a hydrogen atom by an oxo group at the 4 (4′)-position, but not by the corresponding substitute of a hydroxyl group at the 3 (3′)-position, increases the efficiency of the peroxyl radical-trapping ability of carotenoids containing the β-ionone ring system. It is most likely that the electron-withdrawing character of the oxygen atoms substantially reduces the unpaired electron density on the carbon skeleton resulting in the decrease of the reactivity of the carbon-centered radical toward molecular oxygen. Therefore, the presence of a conjugated carbonyl presumably enhances the stability of the trapped radical by decreasing its tendency for continued chain-propagaton reaction. As shown in Figure 4, carotenoids serve as substrates for autocatalytic oxidation when incubated with a free radical initiator (10). The result that canthaxanthin and astaxanthin are more resistant to such chain reaction than β-carotene is surely indicative of the enhancement of the stability of the chain-propagating radical.

The physiological concentration of carotenoids in human plasma is known to be much higher than that of short-lived primates and nonprimate mammals (17). It was also reported that the major carotenoid species from human plasma (18) has lycopene (0.2–0.5 μg/ml), α-carotene (0.1–0.2 μg/ml) and β-carotene (0.1–0.2 μg/ml). In addition, β-cryptoxanthin, lutein and zeaxanthin are detected in human plasma (19,20), and luten and zeaxanthin are the dominant carotenoids in the whole human retina (21). On the other hand, canthaxanthin has been used as a therapeutic agent in certain photodermatoses, a suntanning agent, and a color-additive in human foodstuffs. Canthaxanthin and other carotenoids are expected to accumulate in the blood plasma beyond the therapeutic level (3–4 μg/ml) when used clinically (22).

In conclusion, canthaxanthin, astaxanthin and probably the other carotenoids containing oxo groups at the 4 (4′)-position in the β-ionone ring system can serve as more effective antioxidants than β-carotene in peroxyl radical-dependent lipid peroxidation. These so-called xanthophylls may be of importance as biological antioxidants, although further studies at different conditions seem to be necessary to fully understand the inhibitory effect of carotenoid pigments on lipid peroxidation.

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Identification of Cholesta-7,24-dien-3β-ol and Desmosterol in Hamster Cauda Epididymal Spermatozoa

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The sterol composition of hamster cauda epididymal spermatozoa was remarkably different from that of several other mammalian spermatozoa. Desmosterol and cholesta-7,24-dien-3β-ol account for as much as 90% of the total sterols. Cholesterol and desmosterol are the major components of mouse cauda epididymal spermatozoa, and rabbit, boar and bull ejaculated spermatozoa. Cholesta-7,24-dien-3β-ol was not detected. Furthermore, cholesterol was the main sterol in hamster caput epididymal spermatozoa, while only a trace amount of desmosterol was detected and cholesta-7,24-dien-3β-ol was hardly detected at all. The sterol content of cauda and caput epididymal spermatozoa was 0.17 ± 0.05 μmol/10^8 spermatozoa. During maturation, the desmosterol and cholesta-7,24-dien-3β-ol levels increase and the cholesterol level decreases. Cholesta-7,24-dien-3β-ol appears as a sterol in mature spermatozoa and seems to be a characteristic sterol of hamster cauda epididymal spermatozoa.

Lipids 24, 662-664 (1989).

Mammalian spermatozoa gradually develop both motility and fertilizing capacity during their transit through the epididymis after spermiation from the testis. This process is called epididymal maturation of spermatozoa, and is accompanied by various physiological, morphological and biochemical changes, including changes in lipids (1-3). Several changes in phospholipids and sterols of the sperm plasma membrane have been reported to occur during epididymal maturation (2,3). There is a decrease in the amount of phospholipids in ram (2). In addition, the sterol composition of spermatozoa also changes during maturation. Sterols are the second major class of lipids in the plasma membrane of mammalian spermatozoa (2,3). Among them, cholesterol is a major component; other sterols, such as desmosterol, are being found only in trace amounts (2,3). The desmosterol/cholesterol ratio in the plasma membrane of ram spermatozoa decreases (2), whereas the ratio increases in boar spermatozoa (3) during maturation. The cholesterol level in the sperm plasma membrane appears to regulate the fertilizing capacity (4). It has been suggested that the time required for capacitation, the process by which mammalian spermatozoa acquire the complete capacity for fertilizing an egg in the female reproductive tract, is closely correlated with the cholesterol/phospholipid ratio of the spermatozoa (4). It was also observed that the removal of cholesterol causes destabilization of the plasma membrane of spermatozoa (5). These observations raise the possibilities that sterols regulate the fluidity of the sperm plasma membrane during maturation or capacitation and the following acrosome reaction. In the present study, the sterols in caput and cauda epididymal spermatozoa of hamster were analyzed and a remarkable change in the sterol composition was found to occur during epididymal maturation.

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Abbreviations: BSA, bovine serum albumin; TMS-, N-(trimethylsilyl)-; GLC, gas-liquid chromatography; GC-MS, gas chromatography-mass spectrometry, cholesterol, cholesta-5-en-3β-ol; desmosterol, cholesta-5,24-dien-3β-ol.

EXPERIMENTAL

Mature 2- to 3-month-old male hamsters were killed by cervical dislocation. Spermatozoa (2 × 10^8 cells) isolated from their cauda epididymides were suspended in 20 ml of Tyrode's solution containing 3 mg/ml BSA and then the suspension was centrifuged at 300 × *g* for 5 min at 20°C. The pellet was washed twice with 20 ml of Tyrode's solution containing 3 mg/ml BSA to remove seminal plasma and contaminating cells (6). The resulting pellet was freeze-dried. Cauda epididymal spermatozoa of mouse, and ejaculated spermatozoa of rabbit, bull and boar were prepared in the same manner. Caput epididymal spermatozoa of hamster were purified by Percoll density gradient centrifugation according to the method of Oshio (7).

Lipids were extracted according to the technique of Bligh-Dyer (8). Freeze-dried sperm (2 × 10^8 cells) were homogenized in 16.5 ml of chloroform/methanol/H_2O (1:2:0.3, v/v/v), followed by centrifugation at 1000 × *g* for 5 min at 4°C. The resulting pellet was extracted with 19 ml of chloroform/methanol/H_2O (1:2:0.8, v/v/v). The chloroform-methanol phase was separated and evaporated to dryness. The extracts were stored at −20°C until use.

An aliquot of total lipids (ca. 50 μg) was saponified at 70°C for 120 min with 3.0 ml of 33% KOH/ethanol (6:94, v/v) (9). Then, 1.0 ml of distilled water was added and the sterols were extracted with 3 ml of hexane. The solvent was evaporated under a stream of argon to a small volume, followed by GLC and GC-MS. TMS derivatives of sterols were obtained by treating sterols with N-(trimethylsilyl)imidazole at room temperature for 30 min (10). Free sterols were analyzed, with a Shimadzu GC-6A gas chromatograph equipped with a flame ionization detector, on a glass column containing 2% OV-1 on Shimalite W (AW-DMCS) (2.6 mm × 2.0 m). Nitrogen was used as the carrier gas, the flow rate being 40 ml/min. The amounts of sterols were determined with 5α-cholestane as an internal standard. The free sterols were identified with a Shimadzu GCMS GP-1000 gas chromatograph-mass spectrometer. The sample was applied to a glass column (2.6 mm × 2.0 m) containing 2% OV-1 on Shimalite W (AW-DMCS). The conditions were as follows: column temperature, 260°C; separator temperature, 270°C; ion-source temperature, 270°C; and bombarding energy, 70 eV. The TMS-derivatives of sterols were analyzed with a JEOL JMS-DX303 gas chromatograph-mass spectrometer on a capillary column of Ultra 1 (0.32 mm × 25.0 m). The conditions were as follows: column temperature, 260°C; injection temperature, 270°C;

separator temperature, 280°C; and inlet temperature, 290°C.

Cholesta-7,24-dien-3β-ol was prepared from cholenic acid as described previously (11).

RESULTS AND DISCUSSION

Sterols of hamster cauda epididymal spermatozoa. A gas chromatogram of free sterols obtained from hamster cauda epididymal spermatozoa is shown in Figure 1. Peak 1 was identified as that of cholesterol and the major component (peak 2) was identified as desmosterol. Figure 2 shows the mass spectrum of peak 3. Molecular ion m/z 384 suggested that the latter sterol was a C_{27}-sterol with two double bonds. The presence of m/z 271 (M-113) suggested that one double bond is located on the side chain, probably at the carbon 24 position (12), and the other on the steroid nucleus. However, its retention time was considerably longer than that of the several C_{27}-sterols reported previously (13). Final proof of its structure was obtained by GC-MS analysis of its TMS ether derivative in comparison with chemically-prepared authentic samples (11) of cholesta-7,24-dien-3β-ol, cholesta-8,24-dien-3β-ol and cholesta-8(14),24-dien-3β-ol. As described recently (11), these three sterol TMS ethers were separated on GC, their relative retention times being 1.25, 1.13 and 1.10, respectively (cholesterol TMS ether, 1.00). The unidentified sterol TMS ether from hamster cauda epididymal spermatozoa comigrated on GC with standard cholesta-7,24-dien-3β-ol TMS ether. Furthermore, its mass spectrum (Fig. 3) was also identical with that of this dienol TMS ether, being distinguishable from the other two isometric dienol TMS ethers. All these data strongly suggest that this sterol is cholesta-7,24-dien-3β-ol.

Sterol content of cauda and caput epididymal spermatozoa. The total sterol content of hamster cauda epididymal spermatozoa was 0.17 ± 0.0.5 μmol/10^8 spermatozoa. Caput epididymal spermatozoa contained nearly the same amount (data not shown). Of the total sterols in cauda epididymal spermatozoa, cholesterol amounted to only 10%. It is interesting to note that cholesta-7,24-dien-3β-ol was not detected in hamster caput epididymal spermatozoa (Fig. 4a). These results suggest that the cholesta-7,24-dien-3β-ol content changed during maturation. Cholesta-7,24-dien-3β-ol was not detected in several other mammalian spermatozoa (Fig. 4c–f). Therefore, it can be said that cholesta-7,24-dien-3β-ol is characteristic of hamster spermatozoa. The occurrence of the cholesta-7,24-dien-3β-ol in mature spermatozoa raises the question of the mode of its formation. It is well known (15) that the final steps of cholesterol biosynthesis in mammals are the isomerization of Δ^7-olefins to Δ^5-olefins, and reduction of the C-24 double bond. Although cholesta-7,24-dien-3β-ol has not yet been definitely identified as an intermediate in cholesterol biosynthesis, it may be that, in hamster cauda epididymal spermatozoa, the final stage of cholesterol biosynthesis is blocked for some reason, resulting in the accumulation of the 7,24-dienic sterol. However, the possibility should also be considered that this sterol is

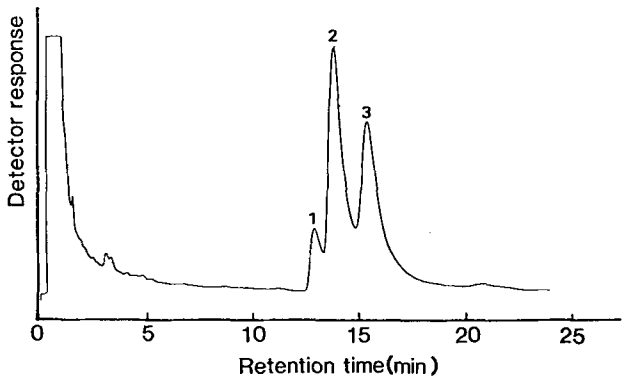

FIG. 1. Gas-chromatogram of free sterols of hamster cauda epididymal spermatozoa. Column temperature, 260°C; detection temperature, 270°C; and injection temperature, 270°C. 1, cholesterol; 2, desmosterol; 3, cholesta-7,24-dien-3β-ol.

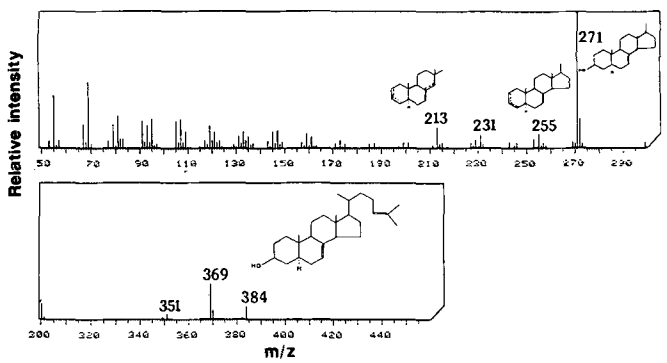

FIG. 2. Mass spectrum of peak 3 in the chromatogram (Fig. 1).

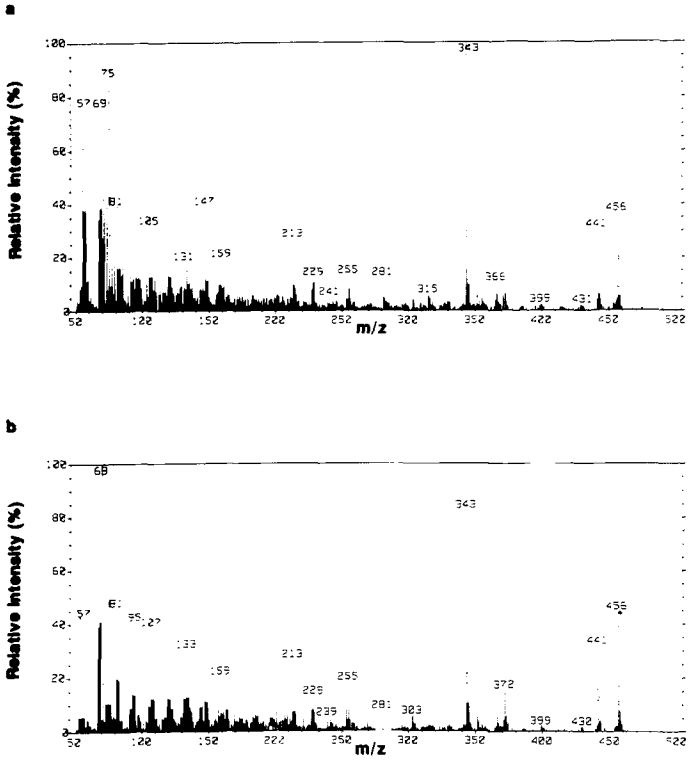

FIG. 3. (a) Mass spectrum of the TMS-derivative of the material in peak 3 in the chromatogram (Fig. 1). (b) Mass spectrum of the TMS-derivative of synthetic cholesta-7,24-dien-3β-ol.

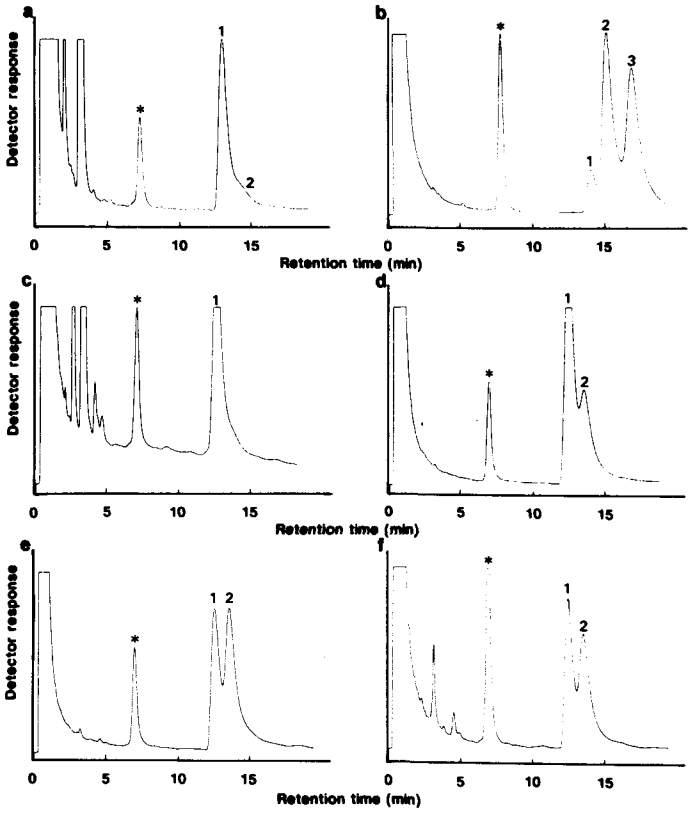

FIG. 4. Gas-liquid chromatographic separation of the free sterols isolated from mammalian spermatozoa. (a) Hamster caput epididymal spermatozoa; (b) hamster cauda epididymal spermatozoa; (c) bull-ejaculated spermatozoa; (d) boar-ejaculated spermatozoa; (e) rabbit-ejaculated spermatozoa; (f) mouse cauda epididymal spermatozoa; (*) 5α-cholestane (internal standard).

produced from cholesterol in a retro-biogenic manner, i.e., the isomerization of the Δ^5-olefins to the Δ^7-olefins and dehydrogenation at the C-24, 25 position.

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On the Isolation of the New Fatty Acid 6,11-Eicosadienoic (20:2) and Related 6,11-Dienoic Acids from the Sponge *Euryspongia rosea*

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The phospholipid fatty acids from the sponge *Euryspongia rosea* were studied revealing the presence of the new 6,11-eicosadienoic (20:2) acid and the rare 6,11-octadecadienoic acid (18:2). The isolation of these 6,11-dienoic acids reveals the presence of new biosynthetic possibilities in sponges and suggests the presence of an active Δ6 desaturase in *E. rosea*. Other acids isolated from *E. rosea* include 12-eicosenoic (20:1) and 17-tetracosenoic acid (24:1). The major phospholipids encountered in the sponge were phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylcholine (PC).
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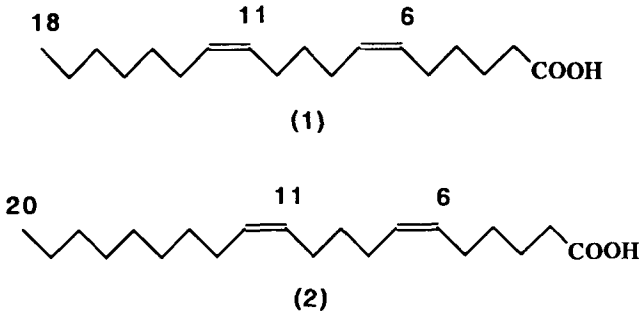
Sponges have attracted the attention of marine natural products chemists in recent years for the bewildering array of new phospholipid fatty acids that they possess (1). Despite the fact that many interesting fatty acids with unusual unsaturation and methyl branching exist in marine sponges, in very few cases are unusual dienoic acids found. Of the very few diunsaturation structures occurring in the phospholipid fatty acids of these marine invertebrates, the Δ5,9 system is certainly the most ubiquitous since it has been recognized to exist in almost every sponge analyzed to date (2). Very few other diunsaturated acids have been reported to occur in sponges; some examples are the 5,8- and 8,12-octadecadienoic acids which were isolated from the sponges, *Microciona prolifera* and *Parasperella psila*, respectively (3). One very interesting and extremely rare case of diunsaturation, not only in sponges but in any animal or plant, is the Δ6,11 arrangement. In fact, the only fully studied case of Δ6,11 diunsaturation has been the case of the rare 6,11-octadecadienoic acid (Scheme 1) which has been found to occur almost exclusively in phosphatidylcholine and the 2-aminoethylphosphonolipids of the ciliated protozoan *Tetrahymena pyriformis* and related species such as *Tetrahymena rostrata* and the mutant *Tetrahymena thermophila* RH179E1 (4-6). The biosynthesis of this intriguing acid, better known as cilienic acid (18:2), has also been elucidated in *Tetrahymena* and has been shown to arise from chain elongation of palmitoleic acid (9-16:1) to *cis*-vaccenic acid (11-18:1) which is further desaturated by a Δ6-desaturase to produce 6,11-18:2 (4). In this paper we wish to report the isolation of yet another hitherto undescribed 6,11 fatty acid, namely 6,11-eicosadienoic acid (Scheme 1), from the phospholipids of the rare dark-red sponge *Euryspongia rosea*. We also report the isolation, from the same sponge, of cilienic acid (6,11-18:2) and the not so common 12-eicosenoic acid. These findings, extremely unusual for these marine invertebrates, suggest the presence of alternative biosynthetic routes in *Euryspongia rosea*, specially the involvement of Δ6 and Δ12 desaturases.

EXPERIMENTAL PROCEDURES

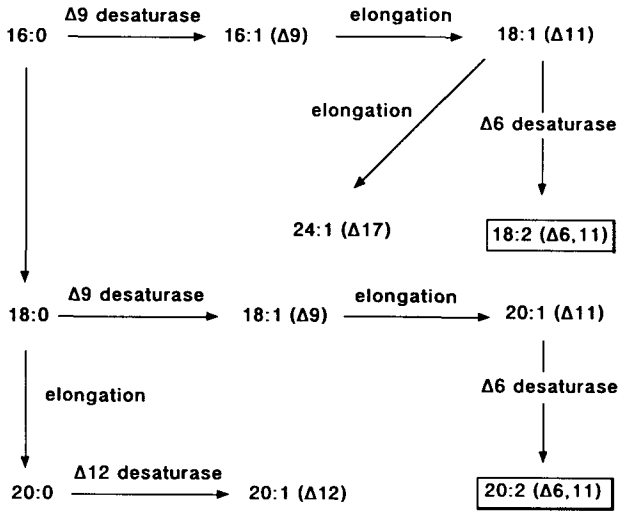
Euryspongia rosea was collected May 3, 1988, near Las Pelotas, Puerto Rico, at a depth of 40 feet. The sponge was washed in seawater, carefully cleaned of all nonsponge debris and cut into small pieces. Immediate extraction of 200 g of wet sponge with 800 mL of chloroform/methanol (1:1, v/v) followed by filtration through Celite (removal of inorganic debris) yielded the total lipids (ca. 400 mg). The neutral lipids (150 mg), glycolipids (50 mg) and phospholipids (150 mg) were separated by column chromatography on silica gel (60-200 mesh) using a similar procedure as that of Privett *et al.* (7). The phospholipid classes were investigated by preparative thin-layer chromatography (TLC) using silica gel and chloroform/methanol/water (65:25:4, v/v/v) as eluent. The fatty acyl components of the phospholipids were obtained as their methyl esters by reaction of the phospholipids with methanolic hydrogen chloride (8) followed by purification on column chromatography eluting with hexane/ether (9:1, v/v). The resulting methyl esters were analyzed by gas chromatography-mass spectrometry (GC-MS) using a Hewlett Packard 5995 A gas chromatograph-mass spectrometer equipped with a 30 m × 0.32 mm 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 (24 h at 100°C) followed by ethereal extraction from the acidified solution and purification by preparative thin-layer chromatography (9). Samples were heated for 24 h since we have found that this procedure affords high yields of pyrrolidide. Hydrogenations were carried out by dis-



SCHEME 1

*To whom correspondence should be addressed.

Abbreviations: GC-MS, gas chromatography; mass spectrometry; PC, phosphatidylcholine; PE, phosphatidylethananolamine; PS, phosphatidylserine; TLC, thin-layer chromatography.



SCHEME 2. Possible biosynthetic pathways for the 6,11-18:2 and 6,11-20:2 acids isolated from *Euryspongia rosea*.

solving 2–3 mg of the fatty acid methyl ester mixture in 5 mL of absolute methanol adding catalytic amounts of platinum oxide (PtO_2) and stirring under a hydrogen atmosphere for 24 h. All the fatty acids in Table 1 were identified by GC-MS. Mass spectral data of key fatty acids for this discussion are presented below.

6,11-Eicosadienoic acid methyl ester. MS *m/z* (rel intensity) 322 (M^+, 4), 292 (0.6), 290(1), 273 (1.4), 248(1.6), 208(4), 207(4), 196(3.3), 195(3.7), 194(3.2), 182(3.1), 181(2.9), 178(3.1), 177(4.1), 165(3.9), 164(15), 154(4), 150(25), 149(11), 140(4), 138(8), 137(13), 136(13), 135(16), 133(6), 124(12), 123(18), 122(16), 121(17), 110(15), 109(32), 108(18), 107(13), 97(25), 96(51), 95(63), 94(35), 91(15), 83(33), 82(58), 81(85), 79(41), 77(10), 74(35), 69(33), 68(25), 67(93), 57(28), 55(100).

6,11-Eicosadienoic acid pyrrolidide. MS *m/z* (rel intensity) 361 (M^+, 1.4), 318(0.1), 290(0.1), 276(0.2), 262(0.5), 248(0.4), 236(0.2), 234(0.4), 222(0.4), 208(0.8), 206(0.3), 195(0.4), 194(0.8), 182(0.3), 180(1), 168(0.3), 167(0.6), 166(1.5), 154(1.1), 152(0.7), 141(0.7), 140(1), 126(11), 114(8), 113(100), 98(8), 93(1), 86(1), 85(9), 81(2), 79(2), 72(6), 70(8), 68(1), 67(5), 55(17).

12-Eicosenoic acid pyrrolidide. MS *m/z* (rel intensity) 363 (M^+, 19), 349(3), 334(2), 320(3), 306(3), 292(4), 278(6), 268(8), 265(3), 264(5), 250(3), 238(3), 236(1), 224(5), 210(5), 196(5), 183(5), 182(6), 168(8), 140(7), 129(8), 127(15), 126(100), 113(33), 98(17), 95(4), 81(4), 72(9), 69(14), 67(8), 55(41).

6,11-Octadecadienoic acid pyrrolidide. MS *m/z* (rel intensity) 335 (M^+, 0.2), 333(1.2), 276(0.2), 262(0.4), 248(0.3), 234(0.3), 222(0.3), 208(0.6), 195(0.3), 194(0.6), 182(0.4), 180(1.2), 168(0.7), 167(0.5), 166(1.2), 154(1), 152(0.7), 140(1), 126(12), 114(8), 113(100), 98(8), 93(1), 86(1), 85(8), 81(2), 80(1), 79(2), 72(5), 71(3), 70(8), 67(3), 55(15).

RESULTS

The complete phospholipid fatty acids from *Euryspongia rosea* are presented in Table 1. *E. rosea* presented a series of short-chain branched acids (18%) and several unsaturated long- and very long-chain acids. The most abundant fatty acid in the mixture was 5,9-hexacosadienoic acid (26:2) which accounted for 17% of the total fatty acids. Other similar acids in the mixture were 5,9-pentacosadienoic (25:2) and 5,9-tetracosadienoic (24:2) which together accounted for another 8%. Characterization of the latter acids was straightforward due to the characteristic base peak at *m/z*=81 of the methyl esters and the allylic cleavage at *m/z*=180 of their corresponding pyrrolidides (1).

Another set of interesting dienoic acids was detected in the mixture. The first of these acids, 2% abundance, presented an ECL value of 17.72, as the methyl ester, and a M^+ peak at *m/z*=294 indicating a possible 18:2 acid. Upon catalytic hydrogenation over PtO_2 this fatty acid was converted to octadecanoic acid (18:0) thus excluding the possibility of any branching. In order to exactly locate the double bonds, pyrrolidide derivatives were prepared and their mass spectra measured. The pyrrolidide derivative of this acid presented a M^+ peak at *m/z*=333 thus confirming an octadecadienoic acid. Critical for the location of the double bonds was the observation that a difference of exactly 12 amu occurred between *m/z*=234 (C11) and *m/z*=222(C10), indicating Δ11 desaturation, and another difference of 12 amu was observed between *m/z*=166(C6) and *m/z*=154(C5) indicating desaturation at Δ6 (Fig. 1). Therefore, from our experimental data we have to conclude that we have identified the rare 6,11-octadecadienoic acid (18:2).

The second dienoic acid of interest presented, as the methyl ester, an ECL value of 19.73 and a molecular ion at *m/z*=322, indicative of a eicosadienoic acid. Upon catalytic hydrogenation this acid was converted to docosanoic acid (20:0) thus excluding the possibility of any branching. The pyrrolidide derivative presented a molecular ion peak at *m/z*=363 and differences of 12

TABLE 1

The Phospholipid Fatty Acids from *Euryspongia rosea*

Fatty acid	ECL	Abundance in weight (%)
Tetradecanoic (14:0)	14.00	5.1
13-Methyltetradecanoic (15:0)	14.61	6.3
Pentadecanoic (15:0)	15.00	1.2
14-Methylpentadecanoic (16:0)	15.55	2.4
Hexadecanoic (16:0)	16.00	7.9
15-Methylhexadecanoic (17:0)	16.60	2.0
14-Methylhexadecanoic (17:0)	16.69	1.2
Heptadecanoic (17:0)	17.00	2.2
6,11-Octadecadienoic (18:2)	17.72	2.0
11-Octadecenoic (18:1)	17.90	6.1
Octadecanoic (18:0)	18.00	5.4
17-Methyloctadecanoic (19:0)	18.62	3.1
16-Methyloctadecanoic (19:0)	18.70	2.3
Nonadecanoic (19:0)	19.00	2.3
6,11-Eicosadienoic (20:2)	19.73	9.0
12-Eicosenoic (20:1)	19.86	1.0
Eicosanoic (20:0)	20.00	0.8
Heneicosanoic (21:0)	21.00	1.5
Docosanoic (22:0)	22.00	4.6
Tricosanoic (23:0)	23.00	1.2
5,9-Tetracosadienoic (24:2)	23.41	1.7
17-Tetracosenoic (24:1)	23.90	4.5
5,9-Pentacosadienoic (25:2)	24.40	6.1
5,9-Hexacosadienoic (26:2)	25.42	16.6
Heptacosanoic (27:0)	27.00	3.5

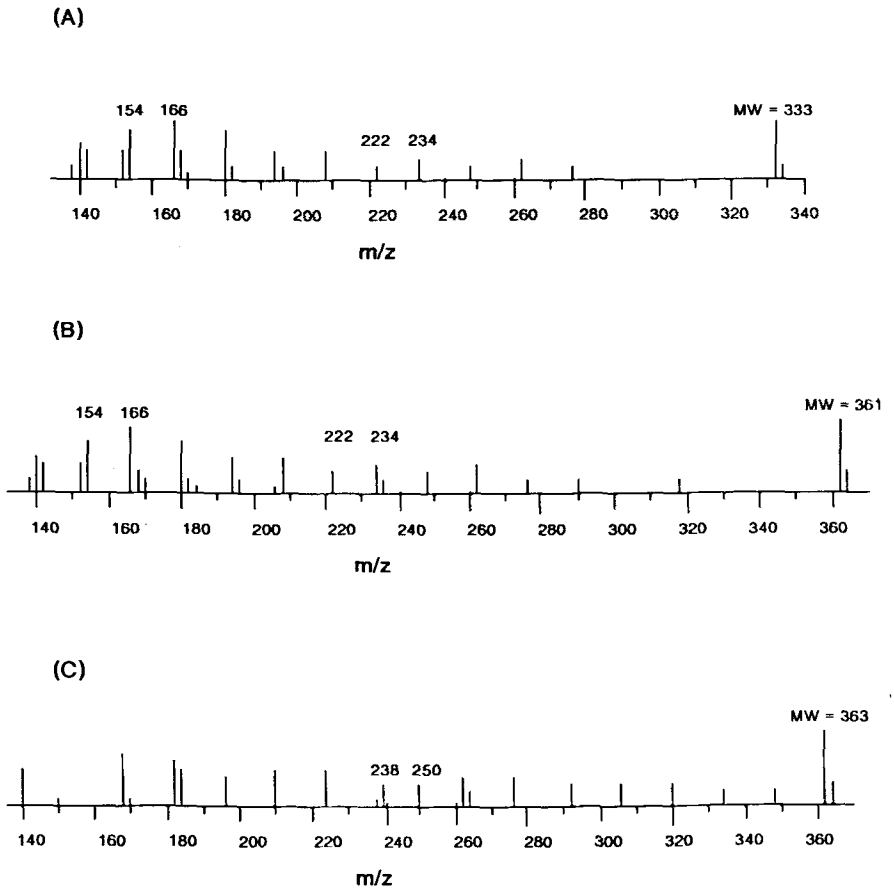


FIG. 1. Partial mass spectra of 6,11-octadecadienoic acid pyrrolidide (A), 6,11-eicosadienoic acid pyrrolidide (B), and 12-eicosenoic acid pyrrolidide (C). Spectra are expanded X9.

amu between $m/z=234$ (C11) and $m/z=222$(C10), indicating again Δ11 desaturation, and another difference of 12 amu between $m/z=166$ (C6) and $m/z=154$ (C5) indicating Δ6 desaturation (Fig. 1). This experimental data suggest that we have identified the unprecedented acid 6,11-eicosadienoic (20:2) which occurred in 9% abundance, certainly one of the principal acids of *E. rosea* (Table 1).

Two very interesting monoenoic acids were also encountered in *E. rosea*. One of these acids presented an ECL value of 19.86 and a molecular ion at $m/z=324$ indicating an eicosenoic acid methyl ester. Upon catalytic hydrogenation this fatty acid methyl ester was also converted to the corresponding eicosanoic acid methyl ester (20:0) excluding the possibility of any branching. The pyrrolidide derivative was the most informative since it presented a 12 amu difference between $m/z=250$ (C12) and $m/z=238$ (C11) indicating Δ12 desaturation. From the experimental data we can conclude that the acid in question is 12-eicosenoic (20:1), which has been isolated from a few sources but not from a sponge (10). The other interesting monoenoic acid, 4% abundance, was readily characterized as 17-tetracosenoic (24:1) due to a 12 amu difference between $m/z=320$ (C17) and $m/z=302$ (C16) in the corresponding pyrrolidide derivative.

The infrared spectrum of the whole fatty acid mixture displayed no prominent absorption at 968-980 cm^{-1}, indicating the absence of *trans* unsaturation in the mixture. In fact, there is no known *trans* double bond in any fatty acid isolated to date from a marine sponge.

The phospholipid composition of *E. rosea* was analyzed with the help of thin-layer chromatography and it was found that the phospholipid fraction consisted of approximately 45% phosphatidylethanolamine (PE), 20% phosphatidylserine (PS), and 35% phosphatidylcholine (PC). The acids reported in this work were evenly distributed in these phospholipids and no phosphonolipids were observed in the mixture. The unusual acids reported in this work were not observed, as sole acids or methyl esters, in the other lipid fractions.

DISCUSSION

The main thrust of this report, namely the isolation of the Δ6,11 acids 6,11-octadecadienoic acid (18:2) and the novel 6,11-eicosadienoic acid (20:2) is very interesting. *E. rosea* is just the second source from which cilienic acid has been isolated; the only previously reported source was the protozoan *Tetrahymena* and related species (4). On the other hand, 6,11-eicosadienoic acid (20:2) is, to the best of our knowledge, unprecedented in nature. These findings open up new alternative biosynthetic pathways in marine sponges. Earlier reports on the ciliated protozoan *Tetrahymena* have revealed that the 6,11-18:2 acid originates from palmitoleic acid

(9-16:1) followed by chain elongation to vaccenic acid (11-18:1) and further desaturation of 6,11-18:2. It is very likely that *E. rosea* utilizes a very similar biosynthetic pathway since vaccenic and palmitoleic acids have been widely recognized to exist in many sponges (3). The new acid 6,11-eicosadienoic (20:2) could probably originate from 9-18:1 (also reported to occur in many sponges) which can elongate to 11-20:1 and further desaturate to 6,11-20:2. Therefore, a very active Δ6 desaturase is operative in *E. rosea*, a fact that has not been observed before in other sponges. It seems that the Δ6 desaturase operating in *E. rosea* is an enzyme with an active site that interacts with an existing double bond at a fixed distance (optimally eleven carbons) from the carboxyl end since no monounsaturated Δ6 acids were detected in this organism. The origin of the 17-24:1 acid in *E. rosea* can be readily explained by a six-carbon elongation of vaccenic acid (11-18:1). Some possible biosynthetic routes to these Δ6,11 acids are presented in Scheme 2.

Another interesting acid detected in *E. rosea* is the rare 12-eicosenoic acid. This acid has been previously reported (10) but has never been encountered before in any sponge. A possible biosynthetic pathway for this acid could be the action of a simple Δ12 desaturase operating on eicosanoic acid (20:0). Work is in progress trying to elucidate the complex biosynthetic routes operating in these invertebrates.

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Estradiol is a Potent Inhibitor of the Hypotriglyceridemic Effect of Levonorgestrel in Female Rats

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The progestin, levonorgestrel, when administered to rats intramuscularly, significantly lowered both total and very low density lipoprotein triglyceride concentrations in the blood plasma by 35–40%. This effect was readily abolished by the simultaneous intramuscular administration of estradiol benzoate. Similarly, estradiol-17β overcame the inhibitory effects of levonorgestrel on the incorporation of [9,10-^{3}H]palmitate into triglycerides of freshly isolated rat hepatocytes studied *in vitro*. However, estradiol alone significantly raised plasma triglycerides by two-fold *in vivo*. Estradiol also significantly enhanced (by 9%) the incorporation of [9,10-^{3}H]palmitate into hepatocyte triglycerides. These results suggest that the effects of estradiol on triglyceride synthesis and concentration dominate over those of levonorgestrel in the rat.
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Progestins and estrogens have been widely administered to women for the purposes of oral contraception (1) or postmenopausal hormonal replacement therapy (2). Progestins such as norethindrone acetate and dl-norgestrel (active isomer levonorgestrel, d-Ng) have been reported to lower plasma triglyceride levels in both humans and rats (3-5), whereas estrogens have been reported to increase plasma triglyceride levels (6, 7). Reduced hepatic triglyceride synthesis and secretion appear to explain at least part of the hypolipidemic effect of progestins (5, 8). Studies in perfused livers of chickens and oophorectomized rats have shown that estrogen increases hepatic triglyceride release (9, 10). However, fed swine receiving ethynyl estradiol *in vivo* failed to show any change in triglyceride secretion (8). Furthermore, increased hepatic catabolism in response to large pharmacological doses of estrogen may independently lower triglycerides (11). Increased efficiency of the hepatic B,E receptor has been found to lower very low density lipoprotein triglycerides (VLDL-TG) without altering apolipoprotein (apo) B production (12). The present study was undertaken to examine the acute effects of d-Ng and/or estradiol-17β (E_2) upon triglyceride synthesis by freshly isolated rat hepatocytes, as well as the chronic effects of intramuscular injections of d-Ng and/or estradiol benzoate (E_2B) on plasma lipids in female rats.

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Abbreviations: AMP, adenosine monophosphate; apo, apolipoprotein; d-Ng, levonorgesterel; E_2, estradiol-17β; E_2B, estradiol benzoate; GMP, guanosine monophosphate; HDL, high density lipoproteins; LDL, low density lipoproteins; mRNA, messenger ribonucleic acid; VLDL, very low density lipoproteins. EDTA, ethylenediamine tetracetic acid; DMSO, dimethyl sulfoxide.

MATERIALS

Female Sprague-Dawley rats (200–250 g) were purchased from Charles River, Canada (Montreal, P.Q.) and maintained on rat chow obtained from Ralston Purina Co. (Minneapolis, MN). [9,10-^{3}H]Palmitic acid (27.5 Ci/mmol), certified over 98% pure, was purchased from New England Nuclear Corp. (Boston, MA). Levonorgestrel, estradiol-17β, estradiol benzoate, collagenase (type IV), and bovine albumin (essentially fatty acid free, fraction V) were purchased from Sigma Chemical Co. (St. Louis, MO). Waymouth's medium (MB 752/1) was obtained from Gibco, Canada (Burlington, Ont). Palmitic acid, dimethyl sulphoxide (DMSO) and Celite 545 were purchased from Fisher Scientific Co. (Fair Lawn, NJ). Trypan blue was purchased from Matheson, Coleman and Bell Manufacturing Chemists (Norwood, OH). Somnotol was obtained from MTC Pharmaceuticals (Hamilton, Ont).

METHODS

Studies in vivo. Rats were housed in individual cages in an animal room with lighting from six a.m. to six p.m. Rat chow and water were freely available throughout the duration of the study. Intramuscular injections of the active hormone (E_2B and/or d-Ng) in sesame seed oil (0.1 ml) were given once a day between four and eight p.m. for 14 days. Control rats received only the oil. Rats treated with E_2B alone received either 0.75 or 3 μg E_2B/day•kg body $wt^{0.75}$ (approximately 0.25 or 1 μg E_2B/day, respectively, for an average rat), while rats treated with d-Ng alone received 0.75 μg d-Ng/day•kg body $wt^{0.75}$ (approximately 0.25 μg d-Ng/day per rat). During therapy with the combined hormones, initially equal amounts of E_2B + d-Ng were injected (0.75 μg/day•kg body $wt^{0.75}$). In subsequent experiments, 3 μg E_2B/day•kg body $wt^{0.75}$ was injected in combination with either 0.75, 7.5, or 75 μg d-Ng/day•kg body $wt^{0.75}$ daily. Kg body $wt^{0.75}$ was used as a unit of body size to facilitate comparisons among species differing widely in size (5, 8, 12, 13).

Blood was withdrawn under ether anesthesia via either aortic or cardiac puncture, and collected into tubes containing 1–2 mg/ml EDTA. Since the rats had eaten throughout the previous night, lipoproteins of $d<1.006$ included both very low density lipoproteins and chylomicrons. Lipoprotein fractions were prepared by the method of Havel *et al.* (14), and lipid extractions were done as described by Folch *et al.* (15).

Studies in vitro using rat hepatocytes. Female Sprague-Dawley rats maintained on rat chow and fed overnight were used for the preparation of hepatocytes by the method of Seglen (5, 16). 4–7 rat livers were used for each experiment to compare the hormone-treated hepatocytes with their untreated controls. Hepatocyte preparations were begun at the same time (8:30–9:30 a.m.) every morning. Viability (mean 80 ± 5%) was determined by the trypan blue dye exclusion test (17).

Experimental and control incubations for metabolic studies were performed with hepatocytes from the same liver. Each pair of control and experimental incubations was carried out with cells freshly isolated from the same rat liver. The hepatocytes (0.6×10^6 viable cells/ml) were initially preincubated at 37°C in a shaking waterbath for 30 min in either the presence or the absence of the steroids. For dispersal in aqueous medium, E_2 and/or d-Ng were dissolved in 0.09% dimethyl sulfoxide (DMSO); a similar amount of DMSO was present in the medium of controls. 25 μCi of [9,10-^{3}H]palmitate (0.14 mM, complexed to bovine albumin, fraction V, molar ratio approximately 0.5:1.0) was added to the medium immediately before the 0 sample, and the incubations continued for 60 more minutes. Samples of suspended cells were taken after 0, 30, and 60 min of incubation, and then centrifuged at 3500 rpm for 15 min at 4°C to sediment the cells. The pellet, re-suspended in 0.5 ml ice-cold saline, was extracted by the method used by Folch *et al.* (15) for the determination of lipids. Phospholipids were removed from the chloroform phase of the lipid extract with silicic acid (5). Labeled free fatty acids were removed by overalkalinization (5, 18) from neutral lipids prior to determining the ^{3}H content of the neutral lipids (essentially triglycerides) of the hepatocytes by the method of Carlson (19). Cholesterol (20) and phospholipids (21) were measured as previously described.

Statistics. Differences between control and treatment groups were evaluated according to the Students t-test for both paired and unpaired samples (22). Variance is expressed as standard error of the mean.

RESULTS

Effects of E_2B and d-Ng, alone and in combination, on the concentration of plasma lipids. Very low doses (0.75 μg/day•kg body wt$^{0.75}$) of d-Ng alone significantly lowered (by 35-40%) the mean plasma concentration of total and VLDL triglycerides as compared to control (Table 1) [Hepatocytes obtained from fed rats (n=10) pretreated for 2 wk with d-Ng (4 μg/d•kg body wt$^{0.75}$, orally, [5, 12, 13] and incubated in the absence of d-Ng showed significantly higher mean rates of incorporation of radiopalmitate into hepatocyte triglycerides at both 30 (31 ± 2 vs 15 ± 2 cpm $\times 10^{-4}/10^6$ cells) and 60 min (34 ± 4 vs 17 ± 2 cpm $\times 10^{-4}/10^6$ cells), as compared to hepatocytes (incubated without d-Ng) from 10 pair-fed untreated controls ($P<0.01$)].

In contrast, a similar intramuscular dose of E_2B (0.75 μg/day•kg body wt$^{0.75}$), either alone or in combination with d-Ng, had no effect on triglyceride levels, indicating complete abolition of the hypotriglyceridemic effect of d-Ng by this low dose of E_2B. However, when the dose of E_2B was increased to 3 μg/day•kg body wt$^{0.75}$, there was a significant elevation in the concentration of total triglycerides which persisted even when the rats received simultaneous injections of d-Ng in doses of 0.75, 7.5 (not shown), or 75 μg/day•kg body wt$^{0.75}$, respectively, over 14 days. The significant elevations in triglycerides during estradiol administration were attributable to significant increases in the concentration of $d<1.006$ lipoprotein triglycerides, which were associated with significant elevations of cholesterol transported in lipoproteins of $d<1.006$. There were no significant changes in plasma total cholesterol.

Effects of estradiol-17β and/or levonorgestrel upon triglyceride synthesis by isolated hepatocytes. The time course of the incorporation of labeled palmitate into hepatocyte triglycerides was essentially linear up to 60 min (Fig. 1). Palmitate incorporation into hepatocyte triglycerides was significantly increased (mean 9%) by E_2 (10^{-4} M) at both 30 and 60 min (Fig. 1A). However, as expected, 10^{-4} M d-Ng alone significantly

TABLE 1

Effects of Estradiol Benzoate (E_2B) and Levonorgestrel (d-Ng) on Plasma Lipid Levels in the Rat[a]

Group	Dose		Triglycerides		Cholesterol	
	E_2	d-Ng	Total	VLDL	Total	VLDL
	(μg/kg body wt$^{0.75}$/d)		(mg/dl)			
C	—	—	95 ± 9	66 ± 8	81 ± 4	3.8 ± 0.5
E_2B	0.75	—	93 ± 7	66 ± 3	77 ± 2	3.5 ± 0.2
C	—	—	88 ± 9	80 ± 9	57 ± 3	3.3 ± 0.5
d-Ng$_2$	—	0.75	57 ± 6[b]	48 ± 7[b]	54 ± 3	2.6 ± 0.3
C	—	—	95 ± 9	66 ± 8	81 ± 4	3.8 ± 0.5
E_2B + d-Ng	0.75	0.75	96 ± 11	77 ± 5	76 ± 3	3.9 ± 0.3
C	—	—	88 ± 9	80 ± 9	57 ± 3	3.3 ± 0.5
E_2B	3	—	148 ± 18[b]	140 ± 22[b]	54 ± 3	7.1 ± 1.1[b]
C	—	—	88 ± 9	80 ± 9	57 ± 3	3.3 ± 0.5
E_2B + d-Ng	3	0.75	155 ± 20[b]	144 ± 10[b]	54 ± 2	8.3 ± 0.5[b]
C	—	—	64 ± 7	51 ± 5	65 ± 2	5.9 ± 0.8
E_2B + d-Ng	3	75	133 ± 13[b]	119 ± 12[b]	63 ± 2	11.4 ± 0.9[b]

[a]Plasma obtained from rats receiving intramuscular placebo injections (Control, C) vs injection of E_2B and/or d-Ng daily for 14 days was separated by ultracentrifugation to isolate the triglyceride-rich VLDL fraction and lipid concentrations levels were determined as described in Materials and Methods. Each value represents the mean ± SE for 15 rats in each of the C vs d-Ng groups, and 8 rats in each of the other groups.

[b]Designates a statistically significant difference from control value at $P<0.05$.

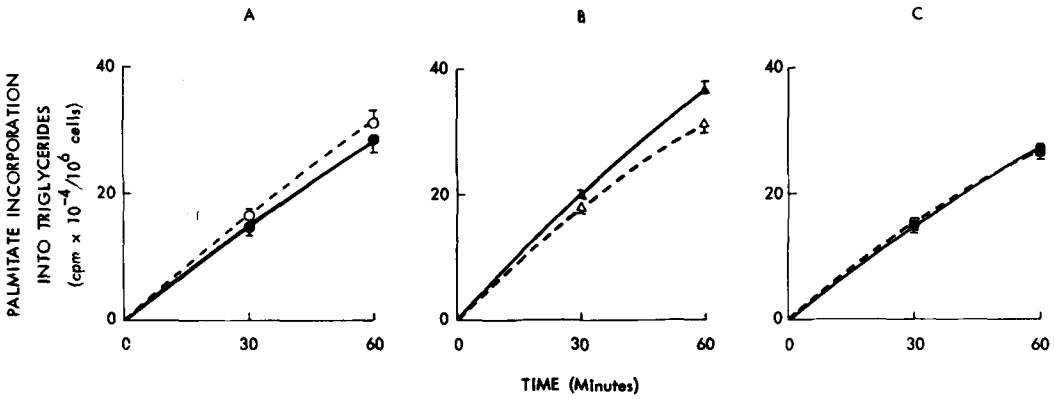

FIG. 1. Time course of incorporation of [9,10^{-3}H]palmitic acid into hepatocyte triglycerides. Isolated rat hepatocyte suspensions were incubated with 25 μl of [9,10^{-3}H]palmitic acid in the presence (O- - -O) or absence of (●—●) of 10^{-4} M estradiol-17β (A, n = 7), the presence (△ · · △) or absence (▲ — ▲) of 10^{-4} M levonorgestrel (B, n = 6), the presence (□- - -□) or absence (■ —■) of 10^{-4} M estradiol-17β and 10^{-4} M levonorgestrel combined (C, n = 7). The samples of hepatocytes removed at 0, 30, and 60 min were extracted and triglyceride counts were determined as described in Materials and Methods. Experimental values (mean ± SEM) were significantly higher for estradiol-17β than control at 30 and 60 min ($P<0.05$) and were significantly lower for levonorgestrel than control at 60 min ($P<0.05$).

decreased the rate of incorporation of palmitate into hepatocyte triglycerides by 12% at 60 min (Fig. 1B). Incubation of the hepatocytes in the presence of both d-Ng and E_2 gave values similar to control (Fig. 1C), indicating that E_2 and d-Ng neutralized their effects on hepatocyte triglyceride synthesis.

DISCUSSION

The present studies demonstrate that a very low dose of E_2B given intramuscularly abolishes the lowering of plasma total and VLDL triglycerides, which occurs with d-Ng administration to fed female rats (Table 1, [5]). These findings differ from a previous report that prior administration of progesterone with estradiol decreases the rate of secretion of triglyceride from perfused rat livers from that observed with prior estradiol alone (23). It is uncertain whether the different findings relate to the use of different progestins (d-Ng vs progesterone) or different doses of the respective progestins (up to 75 μg vs 16,666 μg/kg body wt$^{0.75}$); however, the highest dose of E_2 used in the present study was almost as high as the lowest dose used in the previous study.

The apparently dominant effect of E_2B in the present study could not be overcome, even by a 100-fold increase in the dose of d-Ng above that needed to easily demonstrate a significant reduction in plasma triglycerides in the rat (Table 1). The present studies in isolated rat hepatocytes indicate that at least part of the effect of E_2 is mediated by an increase in the synthesis of hepatocyte triglycerides which counters the corresponding d-Ng-induced inhibition of triglyceride synthesis (Fig. 1).

The present study (Fig. 1B) also demonstrates the novel finding that E_2 (10^{-4} M) alone significantly stimulates triglyceride synthesis in freshly isolated rat hepatocytes which are known to secrete lipoproteins mainly in $d<1.01$ (5, 24). Previous studies have shown that estrogen can enhance triglyceride synthesis and secretion in hepatocyte cultures (25); however, after 17 hours of culture, the ability of hepatocyte cultures to secrete VLDL declines precipitously, while secretion of LDL and HDL are maintained (26). The present findings indicate that E_2 and d-Ng act directly upon hepatic triglyceride production, but in different directions.

Because the effects occurred so rapidly, hepatocyte triglyceride synthesis may have been altered by a mechanism other than by steroid-induced transcription (27). Dibutyryl cyclic AMP has been shown to reduce the amount of triglyceride released into the incubation medium by isolated rat hepatocytes (24) and to reduce triglyceride output from perfused rat livers (28, 29), reflecting increased fatty acid oxidation. However, the reported increase in uterine cyclic GMP by estrogen and its reduction by progesterone (30) may not be accompanied by parallel changes in the liver. Alternatively, the inhibitory effect of cyclic AMP on hepatic triglyceride output, reported to be an order of magnitude more potent than cyclic GMP (31), may predominate. Other possible explanations to account for the rapid effects of E_2 and/or d-Ng on hepatocyte triglyceride synthesis could include a) the selective translation of mRNA, independent of events in the nucleus, and b) a phosphorylation-dephosphorylation mechanism as proposed by Haagsman *et al.* for the inhibition of hepatic triglyceride synthesis by glucagon (32).

It is hazardous to compare *in vitro* studies of hepatocytes incubated in the presence of d-Ng vs controls from the same animal incubated in the absence of d-Ng (Fig. 1B) with *in vitro* studies of hepatocytes obtained from rats treated chronically *in vivo* with d-Ng for 14 days (d-Ng absent from the incubation medium) vs hepatocytes from their untreated pair-fed controls. (See Results.) The results of the latter experiment, which initially appeared to be paradoxical, may reflect a rebound in hepatocyte triglyceride synthesis after withdrawal of d-Ng following earlier *in vivo* exposure to d-Ng. The results of the *in vitro* studies of the acute effects of d-Ng on hepatocytes (Fig. 1) are entirely consistent with chronic plasma triglyceride-lowering

effects of d-Ng observed *in vivo* (Table 1); however, it is apparent that hormone-dependent effects could be missed with an inappropriate *in vitro* protocol.

Administration of the progestin d-Ng over an extended period in the feed (5, 12) or by intramuscular injection (Table 1) similarly lowers the plasma concentration of all components of lipoproteins of $d<1.006$, in a manner similar to norethindrone acetate (33), while estradiol induces proportionate increases (34). This would suggest that changes in the concentrations of the lipid and protein components of lipoproteins of $d<1.006$ with d-Ng and/or E_2 are at least partly attributable to changes in secretion in the number, rather than in the composition of the particles. However, d-Ng-induced inhibition of the glycerol-3-phosphate acyltransferase [E.C.2.3.1.15] step in hepatic triglyceride synthesis (35) could result in VLDL particles which contain relatively less triglyceride per particle. Enhanced clearance of VLDL apo B and decreased clearance of LDL apo B have also been observed in the rat with d-Ng (12). Hepatic triglyceride lipase has been shown to be elevated by d-Ng (36) and depressed by estrogen treatment (37). However, this seems more closely related to plasma HDL_2 than to triglyceride levels (38).

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Interaction of Potentially Toxic Bile Acids with Human Plasma Proteins: Binding of Lithocholic (3α-Hydroxy-5β-Cholan-24-Oic) Acid to Lipoproteins and Albumin[1]

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The binding of lithocholic acid to different plasma fractions was studied. When whole plasma was incubated for 8 hr, approximately 25% of the incubated [^{14}C]lithocholic acid was bound to the lipoprotein and lipoprotein-free, albumin-rich fractions. An average of 87.6% of the bound-lithocholic acid was present in the lipoprotein-free, albumin-rich fraction, 7.2% in high density lipoproteins, 2.2% in low density lipoproteins, 1.0% in intermediate density lipoproteins and 2.0% in very low density lipoproteins. Expressed as binding per μg protein, considerably less [^{14}C]lithocholic acid was bound to the lipoprotein-free, albumin-rich fraction, than to the lipoproteins. The binding of [^{14}C]lithocholic acid after the incubation of the isolated plasma fractions was similar to that found after the incubation of whole plasma. The highest transfer of [^{14}C]lithocholic acid occurred from the lipoprotein-free, albumin-rich fraction to the lipoprotein fractions. The studies indicate, that, although the largest amount of lithocholic acid is bound to the lipoprotein-free, albumin-rich fraction, per μg protein, the binding of lithocholic acid to lipoproteins is more pronounced and stable than that bound to the lipoprotein-free, albumin-rich fraction. Since lipoproteins, in contrast to albumin, are internalized by most tissues, they may be important carriers into cells of lithocholic acid and other potentially toxic or tumorigenic bile acids.
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The transport of bile acids from the intestine to the liver via the portal blood stream represents an integral phase of the enterohepatic circulation (1-3). This transport is facilitated by the binding of bile acids to constituents of blood, in particular to albumin and lipoproteins (4-9). Studies by other authors indicate the comparative binding of different bile acids to plasma proteins to be a function of their hydrophobicity, as evaluated by the octanol-water partition coefficient (5). The most hydrophobic bile acids showed the strongest binding to lipoproteins and albumin. However, the bile acids studied did not include potentially toxic or tumorigenic secondary bile acids, such as lithocholic acid (LC). Because lipoproteins, in contrast to albumin, are internalized by most tissues, they may be important carriers of bile acids into cells. The aim of the present study was to assess the binding of LC to plasma lipoproteins and albumin. LC is a common secondary bile acid and well recognized for both its hepatotoxic and tumorigenic potential (10, 11). After being formed from chenodeoxycholic acid by bacteria in the lower intestine, unconjugated lithocholic acid is absorbed and transported via portal vein to the liver. While, normally, most of lithocholic acid is taken up by the liver and is conjugated, various proportions of the unconjugated compound spill over into the systemic circulation. The latter process is particularly pronounced in disease conditions which are characterized by an impairment of hepatic uptake function, significant porto-systemic shunting or both.

MATERIALS AND METHODS

Materials. [24-^{14}C]LC (specific activity, 55mCi/mmol) was purchased from Amersham and was more than 99% pure by TLC. All other reagents used were of analytical grade available from commercial sources.

Methods. Initially, experiments were carried out to determine the equilibration of [^{14}C]LC between the incubation medium and the interior of incubated dialysis bags. Equilibration was shown to occur after 1 1/2 hr. Three types of bile acid-binding experiments were conducted, using either whole plasma or the isolated lipoprotein and albumin fractions of the plasma from fasting, healthy human subjects. Blood was collected in sterile glass tubes containing 0.15% EDTA. Plasma was separated by centrifugation at 300 *g* for 15 min at 4°C.

In the first type of experiment, the binding of LC to the different plasma fractions was assessed. Aliquots of 3.5 ml of plasma were incubated in triplicate, in a shaking incubator, for 8 hr at a temperature of 37°C and a pH of 7.4 in 200 ml of an 0.9% NaCl solution of [24-^{14}C]LC Na+, at a concentration of 0.12 μM. At the end of the incubation, the lipoprotein and lipoprotein-free, albumin-rich (LFAR) fractions were isolated by density gradient ultracentrifugation, as previously described (12). In brief, samples of plasma were adjusted to d=1.21 *g*/ml with solid potassium bromide, and 4.0 ml aliquots were pipetted into 13.2 ml polyallomer centrifuge tubes (Beckman Instruments, Palo Alto, CA). A discontinuous gradient was prepared as described by Redgrave, Roberts, and West (12). All salt solutions contained EDTA, 0.1 mg/ml, and were prepared from potassium bromide and sodium chloride according to Havel, Eder and Bragdon (13). Ultracentrifugation was carried out at 10°C for 24 hr at 286,000 *g* and 41,000 RPM using the SW41 Beckman rotor. Layers corresponding to the different lipoprotein and LFAR fractions were removed from the tube by needle aspiration corresponding to the following densities (*g*/ml): Very Low Density Lipoproteins (VLDL), <1.006;

[1]Results of this study were presented at the Annual Meeting of the American Society for Clinical Investigation in Washington, D.C. in May 1988.

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Abbreviations: LC, lithocholic acid; LFAR, lipid-free, albumin-rich; VLDL, very low density lipoproteins; IDL, intermediate density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Intermediate Density Lipoproteins (IDL), 1.006-1.019; Low Density Lipoproteins (LDL), 1.019-1.063; High Density Lipoproteins (HDL), 1.063-1.210; LFAR fraction, >1.210 (14). For this experiment and the subsequent two studies, the purity of the fractions was assessed by SDS-PAGE. The lipoprotein and LFAR fractions were also analyzed for protein, cholesterol, phospholipid and triglyceride content. The total protein concentration was measured, using the method of Bradford (15). Enzymatic assays were used to measure cholesterol and triglyceride (Bio-Dynamics, Boehringer Mannheim, Indianapolis, IN) as well as phospholipid (Nippon Shoji Kaisha, Ltd., Higashi-Ko, Osaka, Japan) concentrations. The concentrations of these components in the different fractions were virtually identical to those described in the literature (13). The ^{14}C-radioactivity was measured in all fractions by liquid scintillation counting.

The second type of experiment was conducted to corroborate the results of the first study by excluding the possibility that significant redistribution of [^{14}C]LC had taken place during centrifugation. The different plasma fractions—VLDL, IDL, LDL, HDL and LFAR—were isolated as described above and dialyzed for 12 hr against 0.9% NaCl and 0.01% EDTA at a pH of 7.4 and a temperature of 4°C. Each fraction was diluted to the same volume with 0.9% NaCl at a pH of 7.4. Aliquots of 1.8 ml of each fraction were pipetted into dialysis bags (MW cutoff, 8000 Da) and were incubated together, in triplicate, in a shaking incubator for 3 hr at 37°C in a 0.9% NaCl solution, pH 7.4, containing 0.12 μM [24-^{14}C]LC Na+. The ^{14}C-radioactivity in each of the fractions was measured after the 3 hr incubation. In this and in the following study, the protein-associated radioactivity was assessed by non-denaturing gel electrophoresis (16). Following electrophoretic separation, the protein bands were cut from the gel, dissolved in 1 ml 30% H_2O_2 and assayed for ^{14} C-radioactivity by scintillation counting.

The third type of experiment was carried out to assess the exchange of bound LC among the different plasma fractions. The plasma lipoprotein and LFAR fractions were adjusted to an identical protein concentration of 50 μg/ml. Following dialysis, five bags out of ten for each fraction, respectively, containing 1.8 ml apiece, were incubated with [24-^{14}C]LC Na+, 0.12 μM, for 3 hr. The ^{14}C-radioactivity of the different fractions was measured in 100 μl aliquots which were removed from each bag. Each of the labeled lipoprotein and LFAR fractions was individually incubated with each cold lipoprotein and LFAR fraction in 0.9% NaCl at a pH of 7.4 and a temperature of 37°C for 12 hr. Each was comprised of one bag containing a radioactive fraction and another bag containing a nonradioactive fraction which were placed into 0.9% NaCl. Aliquots of 100 μl were removed at 3 and 12 hr from the bags for the measurement of ^{14}C-radioactivity transferred from the prelabelled to the unlabelled fractions.

Statistical analyses. The statistical significance of the difference in [^{14}C]LC binding between the various lipoprotein fractions and the LFAR was calculated using the Student's t-test.

RESULTS

The results of the first study, in which whole plasma was incubated for 8 hr with [24-^{14}C]LC Na+ at a concentration of 0.12 μM are shown in Figure 1A. Approximately 25% of the incubated [^{14}C]LC was bound to the LFAR and lipoprotein fractions. An average of 87.6% of the bound-LC was present in the LFAR fraction, 7.2% in HDL, 2.2% in LDL, 1.0% in IDL and 2.0% in VLDL. When the distribution of [^{14}C]LC was expressed as binding per μg protein, 5.4% was bound to the LFAR fraction, 26.2% to HDL, 15.8% to LDL, 26.3% to IDL and 26.3% to VLDL. In either calculation, the differences in LC-binding between the LFAR fraction, on the one hand, and the different lipoprotein fractions, on the other, were statistically significant ($p<0.01$).

The results of the second type of experiment, which comprises 3 hr incubations of the different isolated plasma fractions with 0.12 μM [24-^{14}C]LC Na+, are depicted in Figure 1B. Although the distribution of [^{14}C]LC among the different fractions was, in principle, similar to that found after the incubation of whole plasma, differences in the binding pattern of [^{14}C]LC were observed in the two experiments. The differences were mainly represented by the binding of [^{14}C]LC to the LFAR fraction, which was lower after incubation of the isolated fraction than after that of whole plasma. The average binding of [^{14}C]LC to the LFAR fraction was 43.1%. The corresponding binding to HDL, LDL, IDL and VLDL was 32.4%, 13.4%, 5.4% and 5.4%. When the distribution was expressed as % binding per μg of protein, the values were as follows: 0.2% to the LFAR fraction, 5.4% to HDL, 18.8% to LDL, 37.2% to IDL and 38.4% to VLDL.

When, in the third type of study, isolated plasma fractions were adjusted for protein content, and incubated for 3 hr with [^{14}C]LC, the binding of this bile acid was significantly higher in the lipoprotein fractions than in the LFAR fraction (Fig. 1C). Measurement of the transfer of [^{14}C]LC from the [^{14}C]LC Na+ preincubated fractions to cold fractions showed that approximately 2 to 8% of the originally bound compound appeared in each incubated cold fraction after 3 hr (Fig. 2). The highest transfer, which ranged from 7 to 8%, occurred from the LFAR fraction to the lipoprotein fractions. The ^{14}C-transfer to the lipoprotein fractions was about twice as high after 12 hr than after 3 hr (Fig. 2).

DISCUSSION

The present study is, to our knowledge, the first which concerns the comparative binding of LC to the different lipoprotein classes and the LFAR fraction. The principal binding behavior of LC, as far as its distribution among lipoproteins and the LFAR fraction is concerned, was similar to that of deoxycholic, chenodeoxycholic, hyodeoxycholic, ursodeoxycholic, cholic and ursocholic acids reported by other investigators (1-7). Although the majority of LC in plasma was bound to the LFAR fraction, when expressed as binding per μg protein, more LC was found to be bound to lipoproteins than to the LFAR fraction. The difference in LC bind-

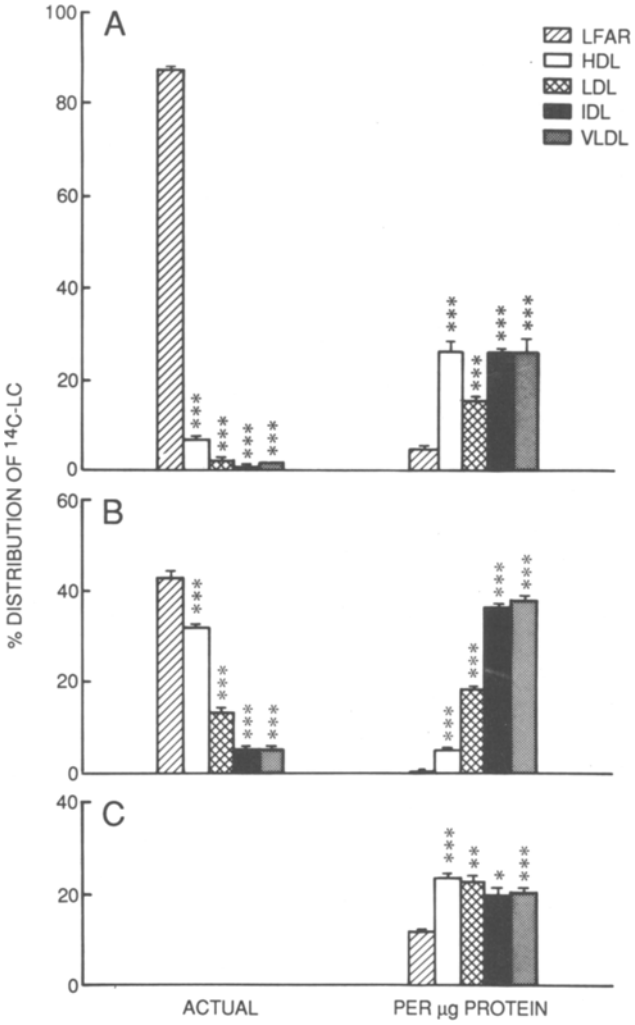

FIG. 1. Distribution of [^{14}C]lithocholic acid: A. in lipoprotein and lipoprotein-free, albumin-rich fractions after 8 hr incubation of whole plasma expressed as actual percent distribution in DPM per volume and relative distribution in DPM/µg protein; B. after 3-hr incubation of fractions isolated from human plasma expressed as actual percent distribution in DPM per volume and relative distribution in DPM/µg protein; C. after 3-hr incubation of fractions adjusted for protein concentration (50 µg/ml). The fractions were isolated from human plasma.*Denotes a statistically significant difference between LFAR and a respective lipoprotein fraction (*p<0.05; **p<0.02; ***p<0.01).

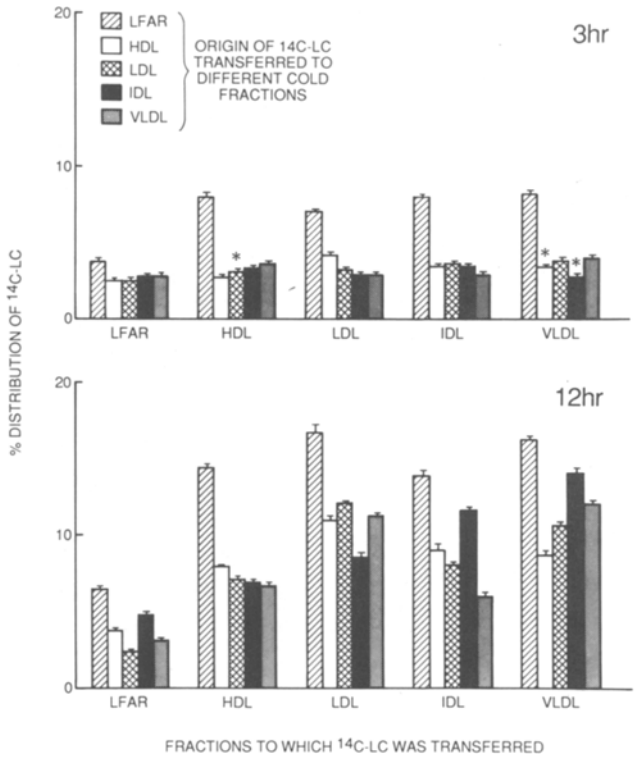

FIG. 2. Transfer of [^{14}C]lithocholic acid from preincubated fractions to unlabelled fractions after 3 and 12 hr, respectively. [^{14}C]LC-prelabelled lipoprotein and LFAR fractions (see Fig. 1C) were incubated with unlabelled lipoprotein and LFAR fractions. Transfer of radioactivity to the unlabelled fractions was assessed at 3 and 12 hr and is expressed as percent radioactivity transferred to the unlabelled fractions from the respective prelabelled fractions.

ing quality between lipoproteins and the LFAR fraction was also evidenced by two findings. First, the transfer of LC from the LFAR fraction was significantly more pronounced than the transfer from lipoproteins, and, second, per µg protein, more LC transferred to lipoproteins than to albumin. The apparently strong affinity of LC to lipoproteins is probably an expression of the marked hydrophobicity of this bile acid (5).

The different possibilities which exist as far as the chemical or physical-chemical nature of the LC binding to proteins is concerned have been discussed by other authors (8, 17). They include peptide linkage, van der Waals forces as well as electrostatic and hydrophobic interactions.

The binding of LC and other potentially toxic or tumorigenic bile acids to and transport by plasma lipoproteins may be of considerable pathophysiological importance. The receptor-mediated uptake of LDL provides for a selective delivery mechanism through which these compounds are internalized in the cells of the various organs and tissues (18, 19). It is possible, if not likely, that the intracellular traffic, processing and effects of LC are significantly influenced by the mode of uptake by the cell. Therefore, the exposure of the different cell organelles to LC may vary considerably depending on whether the lipoproteins or albumin are the plasma carriers. The deleterious effects of LC may, therefore, not only be affected by its plasma concentrations, but also by the concentrations of albumin as well as the level and composition of lipoproteins in plasma.

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Impact of Lemongrass Oil, an Essential Oil, on Serum Cholesterol

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To test the hypothesis that non-sterol mevalonate pathway end products lower serum cholesterol levels, we asked 22 hypercholesterolemic subjects (315 ± 9 mg cholesterol/dl) to take a daily capsule containing 140 mg of lemongrass oil, an essential oil rich in geraniol and citral. The paired difference in serum cholesterol levels of subjects completing the 90-day study approached significance ($P < 0.06$, 2-tailed t-test). The subjects segregated into two groups, one consisting of 14 subjects resistant to the protocol and the other consisting of 8 subjects who responded. Paired differences in cholesterol level at 30, 60 and 90 d for resistant subjects were +2 ± 6, +2 ± 7 and −1 ± 6 mg/dl; paired differences for the responding subjects were −25 ± 10 ($p < 0.05$), −33 ± 8 ($p < 0.01$) and −38 ± 10 ($p < 0.025$), respectively. The paired difference (+8 ± 4) in the cholesterol levels of six responders 90 days after the discontinuation of lemongrass oil was not significant.
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The cardio-protective action of the vegetarian diet in lowering serum cholesterol may be due in part to a variety of non-sterol mevalonate pathway end products (1) which act in the regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) at the non-sterol site of control (2). We found that dietary supplements (50 ppm) of geraniol, the first end product of the branched pathways of plant mevalonate metabolism, and citral, a mixture of the acyclic *cis* and *trans* prenylaldehydes geranial and neral, suppressed avian hepatic mevalonate synthesis (3). These monoterpenes are the major (>80%) constituents of lemongrass oil, a GRAS substance (Generally Regarded as Safe, Flavoring and Extracts Manufacturer's Association 2624) approved for use as a food additive (4,5,6). In this report we present evidence that the constituents of lemongrass oil effectively lowered the cholesterol levels of a subset of hypercholesterolemic human subjects.

METHODS

The putative cholesterol-lowering action of non-sterol end products of the mevalonate pathway was tested by administering capsules containing 140 mg lemongrass oil to hypercholesterolemic subjects recruited from the clientele of the Cardiac Rehabilitation Clinic, University of Wisconsin Hospitals and Clinics. Males who had undergone coronary artery bypass surgery within the past seven years and who maintained an elevated serum cholesterol level while adhering to a diet with strict limitations on fat, energy and cholesterol intakes were invited to participate in the study. Of the 26 patients who volunteered for the study, 22 qualified with a cholesterol level in excess of 250 mg/dl. The volunteers were interviewed by a clinical dietitian (GU) who recorded each subject's medication (β-blockers, calcium channel blockers, aspirin, thiazide diuretics) and dietary history. The dietitian also gave instructions for recording food intake during the three days preceding clinic visits. These visits were scheduled in January and at four week intervals for three months. Four-week supplies of gelatin capsules filled with 140 mg lemongrass oil containing isoprenoid end products (monoterpenoid aldehydes and alcohols), the equivalent of 350 mg cholesterol on a molar basis, were distributed at the initial and two succeeding visits. Fasting blood and dietary records were collected and the dietitian graded the protocol adherence of the volunteers using a scale of 1 (low) to 3 (high). Dietary intakes of energy, cholesterol, total fat, and classes of fatty acids were analyzed using Nutritionist III software (N-Squared Computing, Silverton, OR). The dietitian also reviewed the subject's overall pattern of his response to the cholesterol-lowering action of lemongrass oil. The blood was processed for the analysis of serum lipids. Cholesterol and triglyceride levels were determined using Sigma Diagnostic Kits (No. 351, Cholesterol, total and HDL and No. 338, Triglycerides, Sigma Chemical Company, St. Louis, MO). Low density and very low density lipoproteins were precipitated from the serum (100 μl) with 10 μl each of 9.7 mM phosphotungstic acid + 0.4 M $MgCl_2$. After standing for 5 min at room temperature, the mixtures were centrifuged at 2000 × *g* for 10 min, the supernatants removed and the levels of cholesterol in HDL determined. Sera from normocholesterolemic laboratory workers were interspersed with sera from the experimental subjects for analysis. The mean ± SE for the cholesterol level of the reference volunteers was 201 ± 3 mg/dl. Across the study the SD for the analysis of the 200 mg cholesterol standard was 2.3% of the mean. Triglyceride concentrations were determined in sera samples that had been held at −80°C for 9 months. Statistical evaluation employed the paired Student t-test with the subject's baseline value serving as the base for comparison. Means ± SEM are presented in the text. Lemongrass oil was donated by Aroma Resources Division of Biddle Sawyer Corporation, Keyport, NJ (refractive index @ 25°C, 1.483-1.489), and Bell Flavors and Fragrances, Inc., Northbrook, IL (refractive index @ 25°C, 1.481-1.491). The major constituents of lemongrass oil, quantitated by gas liquid chromatography, included geranial (42%), neral (32%), geraniol (4%), 3% each of myrcene and citronellal and 1-2% each of limonene, linalool and dipentene. The study was approved by the U.S. Center for Health Sciences Human Subjects Committee.

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Abbreviations: GRAS, Generally Regarded As Safe; HDL, high density lipoprotein; HMGR, 3-hydroxy-3-methylglutaryl reductase.

TABLE 1

Summary of Responses to Lemongrass Oil

	n	Paired Difference	t	P
a. Summary of paired t-tests				
30 days				
Combined	21	−7 ± 6[a]	1.313	NS
Resisters	14	+2 ± 6	0.254	NS
Responders	7[b]	−25 ± 10	2.667	0.05
60 days				
Combined	22	−8 ± 6	1.443	NS
Resisters	14	+2 ± 7	0.189	NS
Responders	8	−33 ± 8	3.890	0.01
90 days				
Combined	16[c]	−15 ± 7	2.094[e]	0.06[e]
Resisters	10	−1 ± 6	0.124	NS
Responders	6[d]	−38 ± 10	3.814	0.025

b. Responding subjects who returned for post-test evaluation

	n	Cholesterol (mg/dl)	Paired differences mg	P
Baseline	6	320 ± 17[a]		
30-day	6	290 ± 20	−30 ± 4	<0.025
60-day	6	296 ± 21	−24 ± 3	<0.025
90-day	5[d]	281 ± 15	−34 ± 4	<0.025
Post test	6	328 ± 15	+8 ± 4	NS

[a] SEM.

[b] A subject (responder) was vacationing.

[c] Five subjects (one responder) withdrew following 60-day visit.

[d] One subject (responder) was on vacation.

[e] t = 2.131 for $p < 0.05$, two-tailed.

TABLE 2

Baseline Lipids, Physical Characteristics and Dietary Intakes of Subjects Grouped as Responders (n = 8) and Resisters (n = 14)

	Responders	Resisters
Physical characteristics		
Age, yr	58.6 ± 2.7[a]	56.3 ± 0.4
Weight, kg initial	91.7 ± 5.1	82.9 ± 2.9
Weight, kg final	91.8 ± 4.5	83.2 ± 3.0
BMI, wt/ht	29.9 ± 1.8[b]	26.1 ± 0.9[b]
Baseline lipids		
Triglycerides, mg/dl	445 ± 94[b]	266 ± 75[b]
Cholesterol, mg/dl	318 ± 15	313 ± 12
HDL cholesterol, mg/dl	30.7 ± 3.9[c]	33.0 ± 1.7[d]
Dietary intake		
Energy, kcal	1807 ± 123	2080 ± 217
Cholesterol, mg/1000 kcal	112 ± 14	127 ± 13
SFA, energy %	14.9 ± 1.1	14.2 ± 1.2
MUFA, energy %	12.5 ± 1.0	14.1 ± 1.1
PUFA, energy %	8.5 ± 0.6	7.6 ± 0.8
P/S ratio	0.60 ± 0.12	0.65 ± 0.20

[a] SEM.

[b] Different values, $p < 0.05$.

[c] One subject's HDL cholesterol, 55.6 mg/dl, fell in the normal range.

[d] Two subjects' HDL cholesterol, 43.5 and 43.6 mg/dl, fell in the normal range.

RESULTS

The mean cholesterol level of the 16 hypercholesterolemic subjects who completed this 90-day test of the cholesterol-lowering efficacy of lemongrass oil decreased from 310 ± 11 to 294 ± 11 mg/dl. The paired t-test suggested a modest treatment effect ($p < 0.06$). Baseline and 90-day HDL cholesterol levels for the subjects were 32.2 ± 2.3 and 32.1 ± 1.7 mg/dl. On reviewing the data, we noted a subject-to-subject variability in response to the treatment, a variability in response which has been documented for normocholesterolemic subjects in other studies of dietary inputs which influence cholesterol levels (7). Overall, we identified eight subjects who consistently responded to the treatment; the remaining 14 were resistant. A summary of the paired t-tests of the responses of these subsets of subjects and of the combined subsets is given on Table 1a. The serum cholesterol levels of responders at 30, 60 and 90 days were 91.8 ($n = 7$, $p < 0.05$), 89.6 ($n = 8$, $p < 0.01$) and 87.8 ($n = 6$, $p < 0.025$) percent, respectively, of their baseline values.

To provide some confirmation that the fall in cholesterol was a response to the treatment, we obtained 90-day post-test blood samples from six of the eight responders. The longitudinal responses of these subjects are presented in Table 1b. At each experimental period, the paired difference was significant ($p < 0.025$). Cholesterol levels of these subjects 90 days following the termination of the lemongrass oil protocol were not different from the baseline values.

Medical charts provide some basis for the segregation of the subjects into the two groups. While the body weights of the two groups of subjects did not differ, the body mass index of the responders was greater ($p < 0.05$, Table 2). The charts also suggested that responders tended to have higher serum triglyceride levels. This was confirmed upon analysis of triglycerides in blood sera which had been frozen following collection. Triglyceride levels of responders were higher at the baseline ($p < 0.05$, Table 2) and continued to be 30–40% higher than those of resisters throughout the study. One of eight responders and two of 14 resisters had HDL cholesterol levels falling in the normal range.

The subjects were continued on their drug therapy during the study. The only subject recorded as taking a lipid-lowering agent, a responder, had discontinued gemfibrozil two weeks prior to the study. The single non-insulin dependent diabetic subject, a resister, took glyburide through the study. Eight of the subjects took beta-blockers and eleven took aspirin on a daily basis. Four subjects were taking thiazide diuretics. No relationship between medications and response was indicated.

The subjects recruited for this study had all undergone coronary bypass surgery within the previous seven years. All had serum cholesterol levels in excess of 250 mg/dl three months following the surgery. They had received instruction in the AHA diet (30% fat energy, 10% energy as saturated fat, 300 mg cholesterol and total calories to achieve optimal body weight). The charts suggest a

moderate to good adherence to this diet. Nevertheless, serum lipids were not responding to the diet modification at the time the subjects were recruited (Table 2). This moderate to good adherence to the dietary protocol continued throughout the study (Table 2). The intake data shown in Table 2 are calculated from food intakes recorded during the second experimental period. According to our evaluation, the subjects as a group were successful in limiting their cholesterol intake to less than 300 mg/dl. While the subjects failed to achieve the AHA goals for fat intake, the evaluation does indicate that some restriction was practiced both in terms of percent total dietary energy (36%) and in percent energy supplied by saturated fatty acids (14.5%). Diet intakes of responders did not differ from those of resisters. The subjects maintained their body weights during the trial (Table 2).

DISCUSSION

This study tested the hypothesis that lemongrass oil, an essential oil rich in non-sterol end products of plant mevalonate metabolism, would, by suppressing mevalonate synthesis, lower the serum cholesterol levels of hypercholesterolemic men. The test subjects were recruited from a group of patients who followed a diet limited in cholesterol content under medical supervision. Within the limits of this test we recorded a fall in cholesterol which approached significance at 90 days. The subjects did not respond uniformly to the protocol; 14 subjects maintained their baseline (pretest) cholesterol levels during the treatment phase. Eight subjects experienced a lowering of cholesterol during the test phase, a lowering which was reversed on discontinuation of the protocol.

Responding subjects generally differed from the resistant subjects in being more overweight (BMI, $p < 0.05$) and having higher serum triglycerides ($p < 0.05$). Responders required 20% fewer calories (per kg) to maintain body weight. Whether or not one of these factors or a specific lipoprotein disorder was responsible for the difference in response has not been determined. We note that on testing a large group of normal subjects, McNamara *et al.* (7) found two populations, one of which compensated for changes in dietary intake of cholesterol. If the non-sterol end products of plant mevalonate metabolism suppress HMGR activity and presumably cholesterol synthesis in humans as they do in animals (4,8,9) it is not unlikely that a subject heterogeneity in response to non-sterol end products similar to that reported for cholesterol (7) exists.

In this study, lemongrass oil was given at a level approximately 40% that deemed to be the maximum acceptable dose of citral, the major constituent of lemongrass oil (10). Animals have been challenged with much greater intakes of citral. Citral administered by gavage (2.4 g/kg body wt) induced hepatic peroxisomal proliferation and cyanide-insensitive palmitoyl-CoA oxidative activity in rats (11). This clofibrate-like response to a large dose of citral failed to lower serum triglycerides. Also at high levels of intake (0.5–1.0% of diet), cyclic monoterpenes apparently alter lipoprotein metabolism as HDL cholesterol is reported to be increased (12). Cyclic monoterpenes administered by gavage (13) or fed (14) suppress hepatic HMGR activity. The reduced activity traces to a decrease in enzyme mass (13). It is our hypothesis that the fall in cholesterol level recorded for 36% of our subjects reflects an end product-suppressed HMGR activity in this hypercholesterolemic sub-population.

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Isolation and Characterization of Ganglioside 9-*O*-acetyl-GD_3 from Bovine Buttermilk

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Bovine buttermilk contains a unique ganglioside, 9-*O*-acetyl-GD_3. In order to isolate large quantities of this ganglioside, a simplified isolation scheme which consists of several ion-exchange and silica gel column chromatographic procedures was devised. The isolated 9-*O*-acetyl-GD_3 was characterized on the basis of its thin-layer chromatographic behavior, its immunoreactivity with a specific monoclonal antibody, JONES, and by conversion to authentic GD_3 by mild base treatment.
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The last few years have witnessed a growing interest in the occurrence, regulation and function of *O*-acetylated sialic acids (2) on oligo- or poly-saccharides, in glycoproteins and gangliosides. While the observed effects of *O*-acetylation of sialic acids include such major phenomena as protection from neuraminidase (3), no specific function *in vivo* has been demonstrated yet for any *O*-acetylated species. Biological evidence, however, has now repeatedly shown very specific patterns of expression, during normal development (4) as well as in malignancy (5), of one such molecule, namely 9-*O*-acetyl-GD_3[1]. This ganglioside is consistently found on the surface of human melanoma cells and cell lines (6) as well as on glioma (our unpublished observations). In fact, antibodies specific for this antigen have been proposed as diagnostic tools in melanoma (3,6) and the availability of pure antigen could provide an invaluable aid in the development of a specific and effective therapy.

Our interest in the 9-*O*-acetylated derivative of GD_3 arises from recent studies (4,7) which have shown a very strictly regulated expression of 9-*O*-acetyl-GD_3 and related gangliosides during embryonic, fetal and postnatal development of the rat nervous system. The pattern and timing of this 9-*O*-acetylated ganglioside's expression suggest that its appearance on the surface of neurons and glia may serve to modulate and provide selectivity for the critical events of cell migration and process outgrowth (4).

Because of the growing interest in obtaining large amounts of 9-*O*-acetyl-GD_3 for studying its function and metabolism, we have initiated a study in isolating this ganglioside from a commercially available source, the bovine buttermilk.

The ganglioside content and composition of bovine buttermilk has been investigated previously by several groups (8–11). While the relative abundance of the disialoganglioside GD_3 in buttermilk, as well as its purification from this source (12) have been reported, the presence of 9-*O*-acetylated gangliosides, and, in particular, of 9-*O*-acetyl-GD_3 has been overlooked (13).

In this study, 9-*O*-acetyl-GD_3 was identified as one of the ganglioside species present in bovine buttermilk based on the following three criteria. We first identified this ganglioside based on its HPTLC mobility between GM_1 and GM_2 in our solvent system (see Materials and Methods). Second, this band showed strong immunoreactivity with the monoclonal antibody JONES (14), which was previously shown to recognize an epitope present on 9-*O*-acetyl-GD_3, but not on GD_3, in gangliosides extracted from developing rat central nervous system (CNS) (15). Finally, after base treatment this ganglioside was converted to a new species shown to comigrate with GD_3. A preliminary report of this work has been presented (16).

MATERIALS AND METHODS

Materials. Spray sweet cream buttermilk powder was kindly provided by Land O'Lakes, Inc. (Minneapolis, MN). DEAE-Sephadex (A-25) and Sephadex LH-20 were purchased from Sigma Chemical Co. (St. Louis, MO). Iatrobeads was purchased from Iatron Chemical Co. (Tokyo, Japan). High-performance thin-layer chromatographic plates (silica gel 60, 0.2 μm thick, 10 × 20 cm) were obtained from E. Merck (Darmstadt, FRG). All other chemicals and reagents were of analytical grade and solvents were redistilled before use.

JONES, the mouse monoclonal antibody, was prepared in one of our laboratories (MC-P), which was previously shown to exhibit a high degree of specificity toward 9-*O*-acetyl-GD_3 (14). The monoclonal antibody R24 was a gift from Dr. Kenneth Lloyd, Memorial Sloan-Kettering Cancer Institutes (New York, NY), and had a high degree of specificity toward GD_3 (17,18).

Ganglioside preparation. The starting material, spray sweet cream buttermilk powder (230 g), was extracted with 10 volumes of chloroform/methanol (1:1, v/v) and, after the solvents were evaporated, the extract was resuspended in water and dialyzed against distilled water to remove the small amounts of salts and free oligosaccharides. After lyophilization, the extract was resuspended in chloroform/methanol/water (30:60:8, v/v/v) and passed through a 50 ml DEAE-Sephadex A-25 chromatographic column. Neutral lipids were eluted first with 175 ml of chloroform/methanol/water (30:60:8, v/v/v) (19), followed by 75 ml of methanol; then 5 ml fractions of acidic lipids (among them gangliosides) were eluted using a step gradient system of 150 ml each of 0.05 M, 0.15 M and 0.4 M ammonium acetate in methanol.

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Abbreviations: The ganglioside nomenclature used is based on that of Svennerholm (1). Cer, ceramide (N-acylsphingoid); NeuAc, N-acetylneuraminate; GM_3, NeuAcα2-3Galβ1-4Glcβ1-1Cer; GM_2, GalNAcβ1-4Gal(3-2αNeuAc)β1-4-Glcβ1-1Cer; GM_1, Galβ1-3GalNAcβ-1-4Gal(3-2αNeuAc)β1-4Glcβ1-1Cer; GD_3, NeuAcα2-8NeuAcα2-3Galβ1-4Glcβ1-1Cer; GD_{1a}, NeuAcα2-3Galβ1-3GalNAcβ1-4Gal-(3-2αNeuAc)β1-4Glcβ1-1Cer; GD_{1b}, Galβ1-3GalNacβ1-4Gal(3-2α-NeuAc8-2αNeuAc)β1-1Cer; GT_{1b}, NeuAcα2-3Galβ1-3Gal(3-2NeuAc8-2αNeuAc)β1-4Glcβ1-1Cer; GQ_{1b}, NeuAcα2-8NeuAcβ2-3Galβ1-3GalNAcβ1-4Gal(3-2αNeuAc8-2αNeuAc)β1-4Glcβ1-4Glcβ1-1Cer, HPTLC, high-performance thin-layer chromatography.

Purification of 9-O-acetyl-GD_3 and of GD_3. Ganglioside mapping revealed that 9-*O*-acetyl-GD_3 and GD_3 were contained in fractions 46–77, which contained predominantly disialogangliosides, from the DEAE column. These fractions were combined and dialyzed against distilled water to remove salts. After lyophilization, the resulting 330 mg of material were resuspended in chloroform/methanol/water in the ratio of 65:32:3 (v/v/v) and rechromatographed through an Iatrobeads column (1.5 × 94 cm; 160 ml bed volume). Fifteen ml fractions were collected from a gradient elution with the following continuous gradient solvent system: chloroform/methanol/water (65:32:3, 60:37:3 and 40:57:3, v/v/v) using a three-chamber system as previously described (19). Pooled fractions from this column were either stored (62–71), or rechromatographed (47–61 and 72–161).

Fractions 47–61 (<10 mg) were rechromatographed on an Iatrobeads column (1.0 × 127 cm, 100 ml bed volume) and eluted with the following continuous gradient systems: (A) chloroform/methanol/0.33% $CaCl_2$ in water 70:27:3, 65:32:3 and 40:57:3, v/v/v; (B) chloroform/methanol/0.33% $CaCl_2$ in water 40:57:3, 32:65:3 and 15:82:3, v/v/v. Ten ml fractions were collected and fractions 85–140 contained 9-*O*-acetyl-GD_3.

Fractions 71–161 from the first Iatrobeads column were also combined and rechromatographed on an Iatrobeads column (1.5 × 94 cm, 160 ml bed volume) which we eluted into 15 ml fractions with the following continuous gradient system: chloroform/methanol/water (70:27:3, 28:69:3 and 20:77:3, v/v/v). Individual fractions were desalted on a Sephadex LH-20 column in methanol. The Sephadex LH-20 gel was first equilibrated in methanol and packed in a glass column (bed vol. 20–40 ml). After application of the sample in methanol, we determined the fractions containing 9-*O*-acetyl-GD_3 or GD_3 by HPTLC and immuno-overlay (see below).

Final isolation from preparative HPTLC. The last step in this purification scheme was preparative high-performance thin-layer chromatography. Fractions 37–49 off the last Iatrobeads column were desalted, combined, and spotted as a continuous band on a 10 × 20 cm HPTLC plate, which was then developed in chloroform/methanol/0.2% $CaCl_2$ in water (50:45:10, v/v/v). The running dimension was 10 cm. The plate was then sprayed lightly with water and the 9-*O*-acetyl-GD_3 band was scraped off the glass backing with a razor blade. The ganglioside containing silica powder was collected in chloroform/methanol (1:1, v/v).

Thin-layer chromatography and immunostaining. Fractions were all analyzed by high-performance thin-layer chromatography (HPTLC) on plates and developed in the following solvent system: chloroform/methanol/aq. 0.02% (w/v) $CaCl_2 \cdot 2H_2O$ (50:45:10, v/v/v). Gangliosides were visualized with the resorcinol-HCl reagent (20); total lipids were visualized by charring according to the method of Macala *et al.* (21). 9-*O*-Acetyl-GD_3 and of GD_3 were identified immunologically using the antibodies JONES and R24, respectively. A modified method was used for HPTLC overlay assays (22,23). JONES IgM from ascites fluid was used at a dilution of 1:100; R24 at a dilution of 1:500; HRP-conjugated goat anti-mouse IgM secondary antibodies from Jackson were diluted 1:100. Antibody incubations were carried out in 0.1 M phosphate buffered saline (PBS, pH 7.4) containing 1% bovine serum albumin (BSA); JONES antibody was incubated overnight at 4°C; R24 and secondary antibodies for 1.5 hr at room temperature. The peroxidase reaction was done at room temperature in a phosphate/citrate buffer, pH 5.0, with 0.5 mg/ml 4-chloro-1-naphthol and 0.01% H_2O_2 (Baker) as substrates.

Base treatment. Small amounts of gangliosides were base treated in an aqueous solution of 2.5 N NH_4OH in a shaking water bath at 37°C for 1.5 hr. After treatment, the solution was dried under N_2 and the resulting gangliosides resuspended in chloroform/methanol (1:1, v/v) and chromatographed by HPTLC.

Sialic acid determination. Sialic acid content of purified gangliosides was determined by the method of Svennerholm (20), using a Shimadzu spectrophotometer.

RESULTS

Purification of 9-O-acetyl-GD_3 and GD_3. Our purification scheme consisted simply of a chloroform/methanol extraction, followed by dialysis and three successive column chromatographic steps. Figure 1 represents the ganglioside map obtained after ion-exchange chromatography. We were able to distinguish 6 discrete bands, which we numbered 1 through 6 starting from the bottom of the plate. #1 ran near GQ_{1b} and #2 between GT_{1b} and GD_{1b}; both these species were present in extremely low amounts and were not investigated further. #3, the most abundant, comigrated as a doublet with GD_3; #4 ran just above GM_1 and below GM_2; #5 migrated slightly above GM_2, and #6 migrated near GM_3.

Since preliminary results by base treatment and immunostaining of fraction 58 showed ganglioside #4 to be 9-*O*-acetyl-GD_3, we focused on fractions 46–77 to isolate #3 and #4.

Figure 2 (lane b) shows the dialyzed crude buttermilk extract after HPTLC and resorcinol stain. Several ganglioside species, the predominant of which was GD_3, as well as numerous phospholipids and neutral lipids were present in this crude mixture.

With the first Iatrobeads column, we were able to remove phospholipids, to separate out #1 and #2, and to significantly enrich #3 and #4. The next two Iatrobeads columns achieved separation of #4 from #5 and #6, and of #3 from #4.

Identification of 9-O-acetyl-GD_3. Previous studies (5) have shown that human melanoma derived 9-*O*-acetyl-GD_3 migrates between GM_1 and GM_2 on TLC. In Figure 1 a ganglioside species (#4) which migrates between GM_1 and GM_2, can be seen starting at about fraction 46. After purification, this same species is shown to migrate as a single band by staining with the resorcinol-HCl reagent (Fig. 2, lane c). Land d in Figure 2 illustrates the position of this single band after base treatment. As can be seen, base treatment converted ganglioside #4 to a new species which comigrates with GD_3 (Fig. 2, lane e). Lanes a and f of Figure 2 show the relative migration of standard bovine brain gangliosides for reference.

Figure 3 shows the results of immunostaining with the specific monoclonal antibodies JONES and R24. Lanes c and d show that JONES recognizes the purified 9-*O*-acetyl-GD_3 band (c), but not GD_3 (d); lanes e and f show R24 immunostaining of base-treated 9-*O*-acetyl-GD_3 and GD_3, respectively.

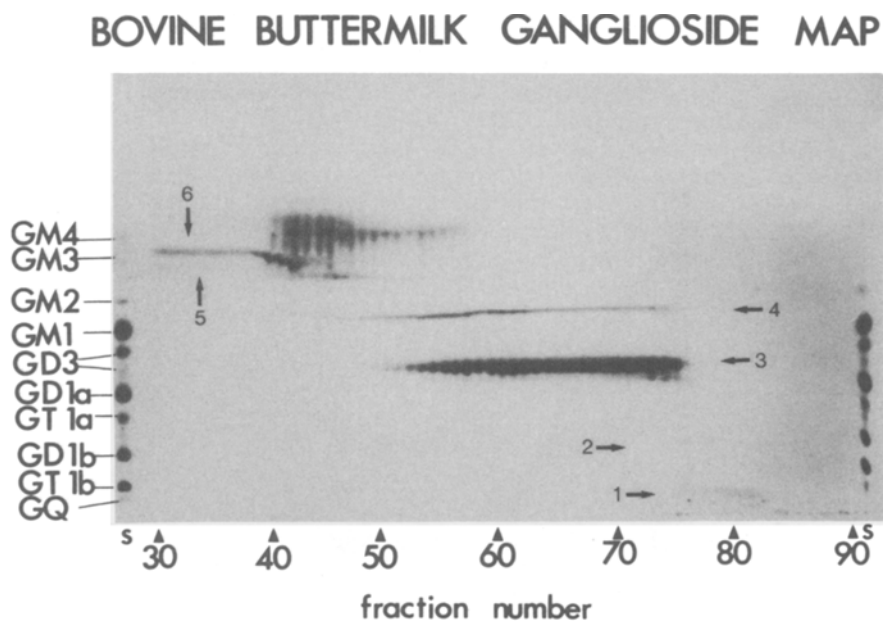

FIG. 1. Ganglioside map of bovine buttermilk after chloroform/methanol extraction, dialysis and ion-exchange chromatography. Human grey matter gangliosides are run on the outside lanes as standards. Resorcinol-HCl stain.

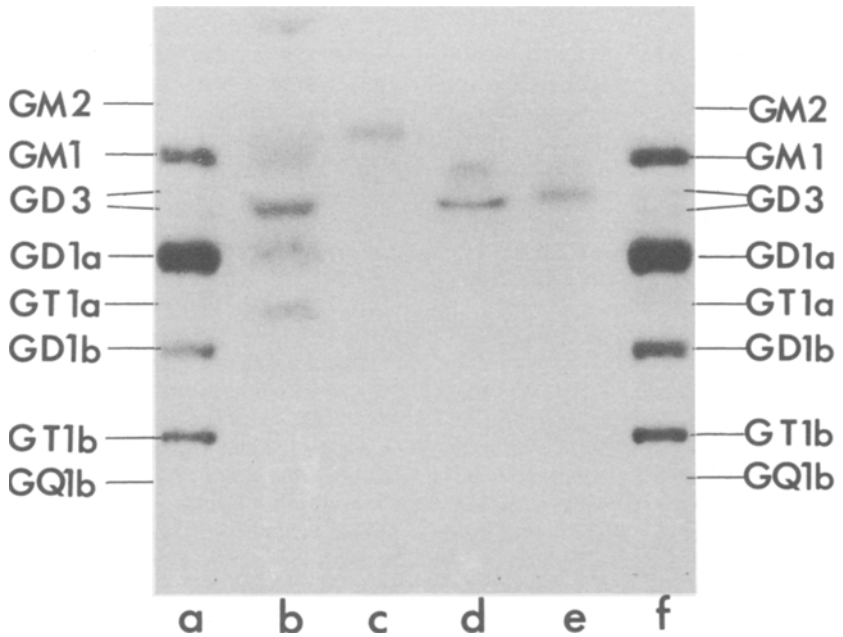

FIG. 2. Lanes a and f: Bovine brain ganglioside standards, about 6 μg. Lane b: Crude buttermilk extract, before ion-exchange chromatography, about 3 μg gangliosides. Lane c: Purified 9-*O*-acetyl-GD_3, about 1.7 μ. Land d: Purified 9-*O*-acetyl-GD_3 after mild base treatment, about 2 μg. Lane e: Purified GD_3, about 1 μg. Resorcinol-HCl stain.

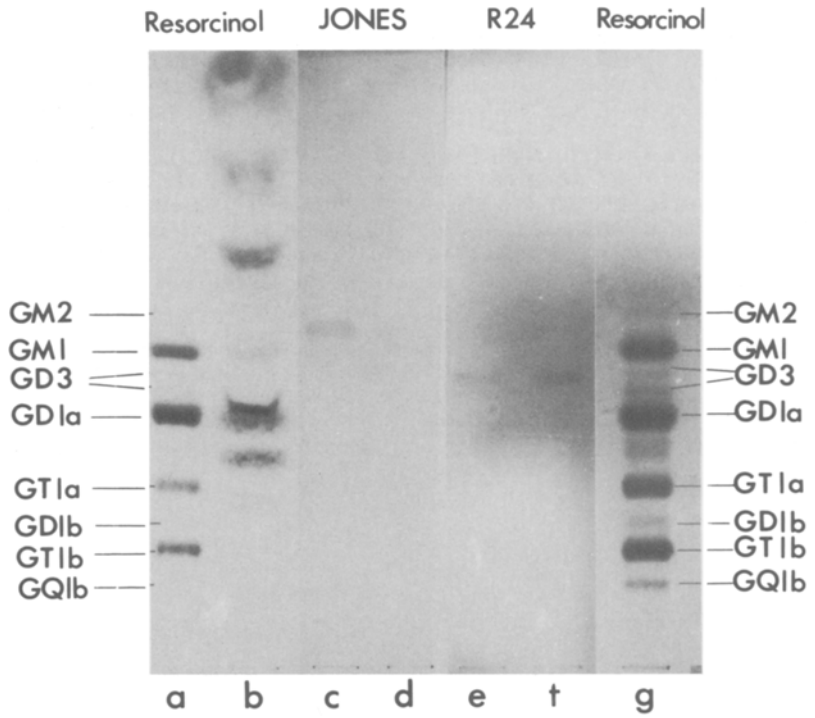

FIG. 3. Lanes a and g: Bovine brain ganglioside standards, about 6 μg, resorcinol-HCl stain. Lane b: Crude buttermilk extract, about 3 μg gangliosides, resorcinol-HCl stain. Lanes c and d: JONES immuno-overlay of purified 9-*O*-acetyl-GD_3, 1.7 μg, and purified GD_3, 1 μg, respectively. Lanes e and f: R24 immuno-overlay of pure, base treated 9-*O*-acetyl-GD_3, 2 μg, and of GD_3, 1 μg, respectively.

We calculated the total amount of purified 9-*O*-acetyl-GD_3 obtained to be about 200 μg, by the method of Svennerholm (20). This represents about 5 μg/g of buttermilk powder, or 0.2% of total gangliosides.

DISCUSSION

This communication is the first report for the presence of ganglioside 9-*O*-acetyl-GD_3 in bovine buttermilk. Since buttermilk is commercially available, it represents a convenient source for the isolation of this ganglioside. We have illustrated a method for the purification of this rare ganglioside in relatively large quantities.

Previous reports on the ganglioside composition of bovine buttermilk exist in the literature (8–12). One of these reports (12) shows each purified species and its relative abundance, as well as identification of some of the ganglioside species. However, 9-*O*-acetyl-GD_3 was never identified by these authors. In the present investigation, we took advantage of some of their purification schemes and introduced some modifications, which simplified the isolation task. First, we reduced the initial extraction by simply extracting the buttermilk powder with chloroform/methanol (1:1, v/v), and passing this crude extract directly through an ion-exchange column (DEAE-Sephadex A25) eluted with an ammonium acetate gradient. This immediately produced somewhat purified fractions, without requiring the laborious Folch partitioning procedure and preliminary ion-exchange and Iatrobeads chromatography. Comparison of the ganglioside map obtained by our simplified procedure with that obtained by Takamizawa *et al.* (12) indicated no significant reduction in the separation obtained. The considerable phospholipid contamination was effectively removed by the ensuing Iatrobeads columns.

Our finding that 9-*O*-acetyl-GD_3 is present as one of the six known gangliosides in bovine buttermilk is of considerable interest, because until now no other reasonable source for this ganglioside was available. Although this species was originally described in developing rat nervous system and in human melanoma, it represents only a minute proportion of these tissues' total ganglioside content, and its purification from such sources would involve prohibitive numbers of rat fetuses (where its concentration is highest) or quantities of melanoma tissue, followed by a very long series of column chromatographies. Furthermore, because of its low abundance in such tissues, its presence in crude extracts could only be monitored by very sensitive, time consuming and expensive immunoassays. On the other hand, when buttermilk is used as a source simple resorcinol-HCl reagent is sufficient to monitor the presence of this now readily available species.

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Lipid Characteristics of RSV-Transformed Balb/c 3T3 Cell Lines with Different Spontaneous Metastatic Potentials

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To determine whether a metastatic phenotype may be correlated with a characteristic lipid pattern, we compared the lipid composition of low metastasizing Balb/c 3T3 cells transformed by the B77 strain of Rous sarcoma virus (B77-3T3 cells) with that of a subclone isolated by growth in 0.6% agar, the B77-AA6 cells, which exhibit a high capacity for spontaneous metastasis. B77-3T3 cells revealed characteristics in their lipid composition common to other systems of transformed cells, i.e., an accumulation of ether-linked lipids, a reduction of the more complex gangliosides, an increase of oleic acid (18:1) and a decrease of arachidonic (20:4) and C22 polyunsaturated fatty acids in phospholipids. High metastatic B77-AA6 cells showed: a) an even more marked decrease of complex gangliosides; b) a more pronounced increase of 18:1 and decrease of 20:4 and 22 polyunsaturated fatty acids in certain phospholipid classes; and c) a higher percentage of alkyl-acyl subfractions in both phosphatidylcholine and phosphatidylethanolamine than B77-3T3 cells.

Comparing the data for other systems of metastatic cells with those of lipid studies of spontaneously metastasizing B77-AA6 cell system leads us to conclude that the metastatic phenotype is characterized by a change in ether-linked lipids, rather than in fatty acids.
Lipids 24, 685-690 (1989).

The notion that metastatic diffusion is a multistep process which implies interactions between the cell surface and the homeostatic mechanisms of the host (1) has prompted interest in the characteristics of cell-surface constitutents of metastatic cells. The recent availability of various model systems of metastatic cell lines (2,3) has been instrumental in the study of the correlation between metastatic potential and alterations of specific cell-surface constituents in malignant cells. Particular attention has been devoted to glycolipids (4-8) which may be implicated in the interactions between metastasizing cells and the host's homeostatic mechanisms (1,9-12) due to the role of these cell-surface components in intercellular recognition (13), cell adhesion (14,15) and receptor activities (13,16,17). The levels of total gangliosides (4) and of individual glycolipid components (5-8) have been found to change in various systems of high metastatic cells compared with their low metastatic counterpart.

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Abbreviations: EGTA, ethylene-bis(oxyethylenenitrilo)tetraacetic acid; DMEM, Dulbecco's modification of Eagle's Minimal Essential Medium; FCS, fetal calf serum; PBS, phosphate-buffered saline; TLC, thin-layer chromatography; GLC, gas-liquid chromatography; HPTLC, high performance TLC; Chol/PL, cholesterol/phospholipid ratio; TG, triacylglycerols; FFA, free fatty acids; ADG, alkyldiacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; DPG, diphosphatidylglycerol; RSV, Rous sarcoma virus.

More recently, interest has been focused on other cell-surface lipid components of metastatic cells, such as cholesterol (18-21), phospholipid classes (19-22) and phospholipid fatty acids (19-22), since they may well affect metastatic properties through their influence on adhesiveness (23,24), permeability (25), antigenicity (26) and enzymatic activities (27). Participation of lipids in the metastatic process is also suggested by the correlation between metastatic behavior and membrane fluidity (22,28-30), which is influenced by acyl chain composition (25,31), cholesterol (32), cholesterol/phospholipid (Chol/PL) molar ratio (26), sphingomyelin (26), and ether-linked lipids (33,34). Indeed, a lower Chol/PL molar ratio and a higher phosphatidylcholine/phosphatidylethanolamine (PC/PE) ratio were found in the high lung-colonizing F10 variant of B16 melanoma compared with the low lung-colonizing F1 variant (19). Polyunsaturated fatty acids were lower in the high lung-colonizing F10 melanoma cell line (19) and in high metastatic human melanoma lines (21) than in the respective low metastatic counterparts, but they were higher in metastases than in local tumors derived from LM cells (22). Moreover, a higher level of alkyl-linked lipids has been found in the F10 variant of B16 melanoma (20), as well as in a series of high metastasizing rat mammary carcinomas (35), compared with their respective low metastatic counterparts. In addition, neutral ether-linked lipids have been correlated with tumorigenicity in a system of mouse fibroblast cell lines (36). However, it cannot be established from these studies whether the metastatic phenotype is associated with a characteristic lipid pattern, due to the limited number and heterogeneity of model systems examined and to discrepancies in the lipid components of different metastatic cells.

This study deals with the relationship between lipid pattern and metastatic potential in a system of virally-transformed murine fibroblasts whose capacity for spontaneous metastasis differs; this system is composed of B77-3T3 cells (a clone of Balb/c 3T3 cells transformed by the B77 strain of Rous sarcoma virus [37]) and of a high metastatic subclone isolated from B77-3T3 cells by growth in 0.6% agar (B77-AA6 cells) (38).

MATERIALS AND METHODS

Cells and culture conditions. B77-3T3 cells were kindly provided by Dr. J. M. Bishop, University of California, San Francisco, while the B77-AA6 cells were isolated by Drs. M. F. Di Renzo and S. Bretti as described in a previous paper (38). Cells were grown at 37°C in a humidified atmosphere containing 5% CO_2 in air, using Dulbecco's modification of Eagle's Minimal Essential Medium (DMEM) (Grand Island Biological Company, Grand Island, NY) supplemented with 10% fetal calf serum (FCS) (Flow Laboratories, Irvine, U.K.). Cells, seeded at 0.8×10^6 cells per dish (10 cm Falcon tissue

culture dishes), were changed to fresh medium the day after plating and then every other day. Cells were harvested by mild trypsinization (0.25% trypsin solution in phosphate-buffered saline [PBS]) on the fourth day, at which time B77-3T3 and B77-AA6 cell cultures reached saturation densities of 0.38×10^6 and 0.48×10^6 cells per cm^2, respectively.

Before examining their metastatic potential and lipid structure, B77-3T3 and B77-AA6 cells were treated with the 5-bromouracil-fluorochrome procedure (39), in order to eradicate *M. orale*, which had been found to contaminate these cells. During the whole experiment, the decontaminated cells were proved to be mycoplasma-free as established by the fluorochrome staining procedure (40) and by a direct culture test (41).

Determination of spontaneous metastatic potential. Subconfluent cultures of B77-3T3 and B77-AA6 cells were harvested by mild trypsinization. Cells were washed twice by resuspension in PBS and centrifugation at $250 \times g$ for 10 min. 5×10^5 cells, resuspended in 0.2 ml of serum-free growth media, were injected subcutaneously into the left abdominal flank of 8-week-old female Balb/c mice (Charles River Italia, Calco, Italy). By the third week, all the animals injected with either B77-3T3 or B77-AA6 cells developed a palpable tumor. Upon the natural death of these animals, there were twelve lung metastases per animal (range 0–71) injected with B77-AA6 cells (90% incidence), whereas one lung metastasis was found in only one of the six animals injected with B77-3T3 cells. These values are similar to those originally reported by Di Renzo and Bretti (38) for these cell types.

Lipid analysis. Lipid analyses were carried out on the same cell cultures which had been examined for spontaneous metastatic potential. Cells were harvested on the fourth day after seeding by incubation with 0.5 mM EGTA, and washed twice by centrifugation and resuspension in PBS. Cell suspensions were sonicated and extracted with 19 vol of a chloroform/methanol mixture (2:1, v/v) following the procedure of Folch *et al.* (42). Total lipid extracts were fractionated into neutral lipids and phospholipids by silicic acid column chromatography.

Neutral lipids and phospholipids were fractionated into individual classes by thin layer chromatography (TLC) under the conditions described in a previous paper (20). Triacylglycerols (TG) and free fatty acids (FFA) were evaluated by quantitative gas-liquid chromatography (GLC) of their fatty acids with an arachidic acid internal standard. Cholesterol was measured in the total lipid extract (total cholesterol) and in the free and esterified cholesterol fractions by quantitative GLC, after saponification with 0.3 N methanolic NaOH. Alkyldiacylglycerols (ADG) were analyzed by quantitative GLC after hydrogenolysis according to Su and Schmid's procedure (43). The various phospholipid classes were assayed for phosphorus content (44) after digestion with sulfuric acid/perchloric acid (3:2, v/v). PC and PE were submitted to ether-lipid analysis following Su and Schmid's procedure (43) in which alkenyl-linked lipids are converted into cyclic acetals, and alkyl-linked lipids into alkylglycerols. Cyclic acetals and isopropylidene derivatives (45) of alkylglycerols were analyzed by quantitative GLC with dimethoxytetradecane and pentadecylglycerol, respectively, as internal standards.

Gangliosides were determined radiometrically by measuring their radioactivity after exposing the cells for 48 hr to growth media containing 1.0 μCi/ml of [^{3}H]-galactose (31.5 Ci/mM, NEN Research Products, Dreieich, FGR). Gangliosides were separated from the other lipid components by Siakotos and Rouser's chromatographic procedure (46). The isolated gangliosides were fractionated on HPTLC plates (Merck) using chloroform/methanol/0.25% $CaCl_2$ (55:40:9, v/v). Under these conditions, gangliosides were separated into four fractions which were identified as GM_3, GM_2, GM_1 and GD_{1a} by comparing their migration rates with those of authentic standards. The labeled ganglioside fractions were made visible by fluorography and quantitatively transferred into scintillation vials for counting.

Gas chromatographic analyses. Methyl ester derivatives of fatty acids of TG, FFA, cholesteryl esters and phospholipid classes were obtained by heating the sample at 80°C for 12 hr in a solution of 5% H_2SO_4 in methanol/benzene (25:1, v/v). Fatty acid methyl esters were extracted into hexane and then analyzed isothermally at 185°C on 6-foot glass columns packed with 10% EGSS-X on Gas-Chrom P 100–120 mesh (Supelco, Bellefonte, PA) and mounted in a Perkin Elmer Gas Chromatograph model 3920 equipped with hydrogen flame detectors. Fatty acid methyl esters were identified by their retention times relative to those of standard mixtures (Supelco, Bellefonte, PA).

The cyclic acetal derivatives of alkenyl-linked lipids and the isopropylidene derivatives (45) of alkylglycerols were analyzed at 195°C on 6-foot glass columns packed with 10% EGSS-X on Gas Chrom P 100–120 mesh.

Cholesterol was analyzed with a coprostanol internal standard at 265°C on a 6-foot glass column packed with 3% OV 17 on Supelcoport 80–100 mesh (Supelco).

Peak areas were measured with a Perkin Elmer Sigma 10 integrator.

Lipid standards. We synthesized the standard of pentadecyl-glycerol (47). Dimethoxytetradecane, coprostanol and arachidic acid were supplied by Supelco.

Statistical tests. Student's *t*-test was used to assess the statistical significance of the differences between B77-3T3 and B77-AA6 cells.

RESULTS AND DISCUSSION

The values of total phospholipids, free and esterified cholesterol, TG, ADG and FFA of B77-3T3 cells (Table 1) were similar to those found in other systems of transformed cell lines grown in tissue culture (49–53). The values of total phospholipids and cholesterol in B77-3T3 cells were lower than those reported for the parental untransformed Balb/c 3T3 cells (53). Compared to B77-3T3 cells, the high metastatic B77-AA6 cells had a higher level of esterified cholesterol, a change also found in a high metastatic rat mammary adenocarcinoma cell line compared with its low metastatic counterpart (18). However, the Chol/PL molar ratio of B77-AA6 cells did not differ significantly from that of B77-3T3 cells (Table 1), while it decreased in the high lung-colonizing F10 melanoma line compared with the low colonizing F1 line (19). The B77-3T3 and B77-AA6 cells did not differ significantly in their TG, ADG and FFA content.

The phospholipid composition of B77-3T3 and B77-AA6 cells showed characteristics comparable to those

TABLE 1

Lipid Composition of B77-3T3 and B77-AA6 Cell Lines[a]

Lipid class	μg/mg protein[b]	
	B77-3T3	B77-AA6
Total phospholipids[c]	102.3 ± 4.0	103.0 ± 5.1
Total cholesterol	16.9 ± 1.7	19.1 ± 2.7
Chol/PL molar ratio	0.31 ± 0.036	0.34 ± 0.028
Esterified cholesterol	1.2 ± 0.07	2.0 ± 0.4[d]
Triacylglycerols	1.2 ± 0.3	1.2 ± 0.6
Alkyldiacylglycerols	1.8 ± 0.1	1.3 ± 0.5
Free fatty acids	2.2 ± 0.3	1.1 ± 0.7

[a] Values are means ± SD of 3-5 separate experiments.

[b] As determined by the method of Lowry *et al.* (48) with a bovine serum albumin standard.

[c] As calculated by multiplying by 25 lipid-phosphorus as assayed on total lipids (44) after digestion with sulfuric acid/perchloric acid (3:2, v/v).

[d] $P < 0.02$.

TABLE 2

Phospholipid Composition of B77-3T3 and B77-AA6 Cell Lines[a]

Phospholipid class	B77-3T3	B77-AA6
Diphosphatidylglycerol	8.3 ± 2.5	5.2 ± 0.6[b]
Phosphatidylehtanolamine	14.8 ± 0.6	16.4 ± 2.6
Phosphatidylcholine	52.0 ± 2.5	55.8 ± 3.2
Phosphatidylinositol + phosphatidylserine	13.1 ± 1.5	13.8 ± 0.7
Sphingomyelin	7.1 ± 1.3	7.0 ± 1.5
Phosphatidic acid	3.9 ± 1.1	1.6 ± 0.4[c]

[a] Values, expressed as percentages of total phospholipid phosphorus (44), are means ± SD of 3-5 separate experiments.

[b] $P < 0.05$.

[c] $P < 0.01$.

found in other cell lines grown in tissue culture (20,49, 51,53) (Table 2). Compared to B77-3T3 cells, B77-AA6 cells had lower proportions of diphosphatidylglycerol (DPG) and phosphatidic acid, but an unchanged PC/PE ratio. This ratio was higher in other systems of metastatic cells (19,22).

In both B77-3T3 and B77-AA6 cells, approximately 50% of PE was composed of an alkenyl-acyl subfraction, which was less abundant in PC; appreciable proportions of alkyl-acyl subfractions were present in both PE and PC (Table 3). The B77-AA6 cells had higher proportions of alkyl-acyl PE and PC than the B77-3T3 cells. An increase of alkyl-acyl phospholipids was also found in the high lung-colonizing F10 melanoma line compared to the low colonizing F1 line (20). Moreover, a correlation between the metastatic potential and the level of alkyl-linked lipids has been established in a series of rat mammary carcinomas (35). The increase of alkyl-linked lipids in metastatic cells may reflect a higher activity of the acyl-hydroxyacetone phosphate pathway (54) which regulates the levels of ether-linked lipids in normal and transformed cells (34,55).

The ganglioside composition of B77-3T3 cells is characterized by a high level of GM_3 and GM_2, and by

TABLE 3

Diacyl, Alkenyl-acyl and Alkyl-acyl Subfractions of PE and PC from B77-3T3 and B77-AA6 Cell Lines

Subfraction	Percentage of total phospholipid class[a]	
	B77-3T3	B77-AA6
Diacyl PE	41.3 ± 3.8	24.1 ± 3.8[c]
Alkenyl-acyl PE	46.7 ± 2.7	54.6 ± 8.6
Alkyl-acyl PE	12.0 ± 0.7	21.3 ± 1.3[d]
Diacyl PC	83.5 ± 0.2	75.4 ± 4.9[b]
Alkenyl-acyl PC	8.0 ± 0.7	4.3 ± 3.0
Alkyl-acyl PC	8.4 ± 0.5	20.3 ± 1.9[d]

[a] Percentages of the various subfractions were calculated as previously described (20). Values are means ± SD of three separate experiments.

[b] $P < 0.05$.

[c] $P < 0.01$.

[d] $P < 0.001$.

TABLE 4

Ganglioside Composition of B77-3T3 and B77-AA6 Cell Lines

Gangliosides[b]	Percentage of the total gangliosides[a]	
	B77-3T3	B77-AA6
GM_3	42.4 ± 2.8	8.3 ± 1.5[d]
GM_2	37.7 ± 7.2	86.1 ± 0.5
GM_1	10.7 ± 4.7	4.3 ± 2.1[c]
GD_{1a}	8.8 ± 1.4	1.0 ± 0.1[c]

[a] Values are percentages of the total ganglioside-associated radioactivity and are means ± SD of three separate experiments. "Total" refers to the sum of the radioactivities of individual gangliosides fractionated by TLC.

[b] Gangliosides are indicated according to the nomenclature of Svennerholm (56). GM_3: (sialyl)galactosyl-glucosyl-ceramide; GM_2: N-acetylgalactosaminyl-(sialyl)galactosyl-glucosyl-ceramide; GM_1: galactosyl-N-acetylgalactosaminyl-(sialyl)galactosyl-glucosyl-ceramide; GD_{1a}: (sialyl) galactosyl-N-acetylgalactosylaminyl-(sialyl)galactosyl-glucosyl-ceramide.

[c] $P < 0.01$.

[d] $P < 0.001$.

a low proportion of the more complex homologs, GM_1 and GD_{1a} (Table 4). These latter, instead, were the major gangliosides in the untransformed parental Balb/c 3T3 cells (57). In SV40-transformed Balb/c 3T3 cells, the reduction of the more complex gangliosides was found to be counterbalanced by the increase of only GM_3 (57). The accumulation of different precursors in the two transformants of Balb/c 3T3 cells may be related to the different levels in the ganglioside biosynthetic pathway that are blocked by DNA or RNA viruses (58,59). In B77-AA6 cells, the decrease of GM_1 and GD_{1a} and the accumulation of GM_2 were even more evident than in B77-3T3 cells. Therefore, the block of ganglioside biosynthesis typical of transformed cells (58,59) is even tighter in the more metastatic variant of the model system examined in this study. In other systems of metastatic cells, complex gangliosides were found to be increased (6,8), or qualitatively different (7) in the more metastasizing

TABLE 5

Fatty Acid Composition of Cholesteryl Esters, Triacylglycerols and Free Fatty Acids from B77-3T3 and B77-AA6 Cell Lines[a]

Fatty acid	Cholesteryl esters		Triacylglycerols		Free fatty acids	
	B77-3T3	B77-AA6	B77-3T3	B77-AA6	B77-3T3	B77-AA6
14:0	1.9 ± 0.5	5.1 ± 1.8[c]	6.8 ± 0.2	5.1 ± 0.7[d]	7.3 ± 2.6	5.0 ± 1.7
16:0	24.5 ± 1.3	22.5 ± 2.9	40.5 ± 1.6	37.6 ± 4.4	50.8 ± 5.4	47.4 ± 4.1
16:1	5.1 ± 4.5	10.8 ± 1.5[c]	3.4 ± 1.0	5.4 ± 1.1	2.2 ± 0.3	2.1 ± 0.6
18:0	19.7 ± 5.1	12.6 ± 2.0[c]	20.6 ± 1.9	16.9 ± 3.8	24.6 ± 1.8	26.9 ± 2.5
18:1	30.6 ± 3.3	43.4 ± 5.8[d]	25.9 ± 2.7	33.0 ± 5.1	10.3 ± 5.8	13.2 ± 3.2
18:2	16.6 ± 5.1	3.4 ± 2.5[e]	1.6 ± 1.2	0.8 ± 0.8	0.5 ± 0	0.6 ± 0.3
20:4	1.2 ± 0.7	1.9 ± 0.6	1.3 ± 0.1	0.5 ± 0.3[d]	3.2 ± 0.05	3.8 ± 1.2
20:5	N.D.[b]	N.D.	N.D.	N.D.	0.5 ± 0	0.7 ± 0.3
22:5	N.D.	N.D.	0.1 ± 0.09	0.2 ± 0.4	N.D.	N.D.
22:6	N.D.	N.D.	1.2 ± 0.2	0.2 ± 0.3[f]	N.D.	N.D.

[a]Values, expressed as % of total fatty acids, are means ± SD of 3-5 separate experiments.
[b]N.D., not detectable under the analytical conditions used.
[c]$P < 0.05$. [d]$P < 0.02$. [e]$P < 0.01$. [f]$P < 0.001$.

TABLE 6

Fatty Acid Composition of Glycerophospholipids from B77-3T3 and B77-AA6 Cell Lines[a]

Fatty acid	Diphosphatidylglycerol		Phosphatidylcholine		Phosphatidylethanolamine		Phosphatidylinositol + phosphatidylserine	
	B77-3T3	B77-AA6	B77-3T3	B77-AA6	B77-3T3	B77-AA6	B77-3T3	B77-AA6
14:0	1.8 ± 0.3	2.1 ± 0.6	2.4 ± 0.5	3.3 ± 0.5[c]	0.9 ± 0.7	0.8 ± 0.2	0.2 ± 0.1	0.3 ± 0
16:0	34.8 ± 4.6	29.0 ± 6.4	30.6 ± 0.7	34.1 ± 1.4[d]	15.8 ± 1.8	19.7 ± 5.9	9.5 ± 0.5	8.3 ± 1.0
16:1	6.4 ± 1.0	12.3 ± 2.1[d]	6.6 ± 0.7	7.9 ± 1.0	1.9 ± 0.7	3.5 ± 0.5[d]	1.9 ± 0.2	2.0 ± 0.4
18:0	6.6 ± 0.9	4.7 ± 3.2	11.2 ± 0.8	10.9 ± 0.8	33.7 ± 3.0	28.4 ± 3.7	41.9 ± 2.9	51.2 ± 2.9[d]
18:1	45.5 ± 4.1	45.0 ± 6.3	44.9 ± 0.6	44.0 ± 3.0	25.3 ± 2.8	30.7 ± 3.2[c]	37.5 ± 2.0	30.0 ± 2.4[d]
18:2	4.6 ± 0.2	5.5 ± 0.8	1.0 ± 0.05	0.9 ± 0.05	0.7 ± 0.1	1.0 ± 0.2	0.5 ± 0.1	0.5 ± 0.1
20:3ω9	N.D.[b]	N.D.	0.2 ± 0.05	0.1 ± 0[d]	2.4 ± 1.0	2.0 ± 0.5	1.7 ± 0.1	2.8 ± 0.7[c]
20:3ω6	0.4 ± 0.1	N.D.	0.5 ± 0.05	0.3 ± 0.02[e]	0.5 ± 0	0.6 ± 0.08	0.8 ± 0.1	0.5 ± 0.1[d]
20:4	0.5 ± 0	N.D.	0.9 ± 0.2	0.2 ± 0.05[e]	8.2 ± 0.8	7.3 ± 2.3	2.7 ± 0.3	3.3 ± 0.6
20:5	N.D.	N.D.	N.D.	N.D.	1.0 ± 0.1	0.4 ± 0.2[d]	N.D.	N.D.
22:5	N.D.	N.D.	0.4 ± 0.1	0.1 ± 0.05[d]	3.7 ± 0.3	3.0 ± 1.0	1.5 ± 0.5	1.0 ± 0.5
22:6	n.D.	N.D.	0.3 ± 0.05	0.1 ± 0.7[d]	4.6 ± 0.5	3.3 ± 1.4	1.1 ± 0.2	0.9 ± 0.3

[a]Values, expressed as % of total fatty acids, are means ± SD of 3-5 separate experiments.
[b]N.D., not detectable under the analytical conditions used.
[c]$P < 0.05$. [d]$P < 0.01$. [e]$P < 0.001$.

TABLE 7

Alkenyl and Alkyl Group Composition of Ether-Linked Subfractions of PE and PC from B77-3T3 and B77-AA6 Cell Lines[a]

Alkenyl or alkyl chain	Alkenyl-acyl PE		Alkenyl-acyl PC		Alkyl-acyl PE		Alkyl-acyl PC	
	B77-3T3	B77-AA6	B77-3T3	B77-AA6	B77-3T3	B77-AA6	B77-3T3	B77-AA6
16:0	41.1 ± 0.8	45.4 ± 4.6	40.0 ± 1.7	48.4 ± 5.7	43.4 ± 0.6	46.1 ± 1.1[b]	48.7 ± 0.8	49.0 ± 2.4
16:1	5.9 ± 0.2	2.1 ± 0.4[d]	17.0 ± 1.4	14.6 ± 6.6	2.2 ± 0.4	2.1 ± 0.9	1.7 ± 0.1	1.3 ± 0.7
18:0	27.8 ± 1.5	32.8 ± 8.8	25.6 ± 1.8	14.6 ± 4.7[c]	38.6 ± 0.7	28.9 ± 0.5[d]	18.2 ± 0.4	17.2 ± 3.7
18:1	25.2 ± 1.4	19.7 ± 3.7	17.4 ± 2.4	22.4 ± 5.1	15.8 ± 1.1	22.9 ± 0.8[d]	31.4 ± 0.9	32.5 ± 6.5

[a]Values, expressed as mol % of total alkenyl and alkyl chains, are means ± SD of three separate experiments.
[b]$P < 0.05$. [c]$P < 0.02$. [d]$P < 0.001$.

variants. These discrepancies may be related to lineage-dependent peculiarities in biosynthetic pathways (13) and differences in transformation-associated blocks of these pathways (58,59) that may occur in different systems of metastatic cells.

The fatty acid composition of individual lipid classes from B77-3T3 and B77-AA6 cells are reported in Tables 5 and 6. In cholesteryl esters of B77-3T3 cells, 18:1 prevailed over the other fatty acids, while in TG and FFA, 16:0 was the major fatty acid (Table 5). In cholesteryl esters, B77-AA6 cells contained higher proportions of 16:1 and 18:1 and a lower level of 18:2 than B77-3T3 cells.

In both B77-3T3 and B77-AA6 cells, the overall fatty acid patterns of DPG, PC, PE, PI+PS (Table 6) were comparable to those found in other cell types grown in tissue cultures (20,52,53,60,61). Moreover, the various glycerophospholipid classes of B77-3T3 and B77-AA6 cells were rich in 18:1 and poor in 20:4 and 22 polyunsaturated fatty acids, as also reported for other types of transformed cells (20,52,53,60,61). Compared to B77-3T3 cells, B77-AA6 cells had: a) an increase of 16:1 in DPG; b) a decrease of 20 and 22 polyunsaturated fatty acids in PC associated with an increase of 16:0; c) higher percentages of 16:1 and 18:1 and a lower level of 20:5 in PE; and d) an increase of 18:0 associated with a decrease of 18:1 in PI+PS. A decreased level of 20 and 22 polyunsaturated fatty acids was also found in murine (19) and human (21) melanoma metastasizing cell lines, while metastases of LM cells showed lower proportions of polyunsaturated fatty acids than local tumors derived from these cells (22). No significant difference was found in the fatty acid composition of sphingomyelin of B77-3T3 and B77-AA6 cells (data not shown).

The alkenyl and alkyl group composition of ether-linked PE and PC from B77-3T3 cells was characterized by the predominance of 16:0 followed by 18:0 and 18:1 chains (Table 7). Compared to B77-3T3 cells, B77-AA6 cells had a lower proportion of the 16:1 chain in alkenyl-PE, and of the 18:0 chain in alkenyl-linked PC and alkyl-linked PE. Alkenyl and alkyl group compositions of ether-linked phospholipids did not differ significantly in the high and low lung-colonizing variants of B16 melanoma (20).

The following three conclusions can be drawn from our lipid studies that compare the spontaneously metastatic B77-AA6 cells and non-metastatic B77-3T3 cells. First, both B77-3T3 and B77-AA6 cells showed one of the more typical lipid characteristics of malignant cells, namely a high proportion of 18:1 associated with low percentages of 20:4 and 22 polyunsaturated fatty acids. However, there has been only a slight difference in these characteristics between the two types of cells. Moreover, a consistent fatty acid pattern has not been found on studying different systems of metastatic cells (19-22); thus, it seems that the metastatic phenotype cannot be related to a specific fatty acid profile. Second, the higher level of alkyl-acyl PE and PC in B77-AA6 cells than in B77-3T3 cells, which is analogous to the higher content of alkyl-linked lipids found in other systems of metastatic cells (20,35), may indicate that an alteration of ether-linked lipids is a molecular characteristic of the metastatic phenotype independent of the origin of the malignant cells and of the procedure used for the selection of the metastatic variant. Third, the transformation-associated reduction of more complex gangliosides (58,59) was more pronounced in the spontaneously metastatic B77-AA6 cells than in their non-metastatic counterpart. Although a low level of complex gangliosides was not common to other systems of metastatic cells (5-8), it may be relevant to the metastatic behavior of B77-AA6 cells. Indeed, metastatic cells of different origin may have specific complements of gangliosides, each capable of interacting successfully with the host's homeostatic mechanisms.

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An Evaluation of NBD-Phospholipids as Substrates for the Measurement of Phospholipase and Lipase Activities

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Because most of the existing assays of phospholipase activity are quite laborious, the use of 1-acyl-2-[6-(7-nitro-1,3-benzoxadiazol-4-yl)amino]caproyl labeled phospholipids (NBD phospholipids) was investigated to determine whether they could be used as substrates in the routine assay of various phospholipases and lipases. NBD-labeled analogues of phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, and phosphatidic acid were evaluated. There was about a 50-fold increase in fluorescence upon hydrolysis of the NBD hexanoic acid from the NBD phospholipid, confirming an earlier report. This change in fluorescence was constant over the normal physiological pH range (pH 5–9). Detergents and bovine serum albumin interfered with the assay in a concentration dependent manner. An increase in fluorescence and a concomitant increase in NBD hexanoic acid was detected with the two phospholipase A_2 enzymes. Although a change in fluorescence was detected with a phospholipase C, careful evaluation revealed that the rate of increase in fluorescence was not proportional to the rate of production of diacylglycerol product. Neither of the two phospholipase D enzymes which were tested were able to cause an increase in fluorescence when incubated with NBD phospholipids. A small increase in fluorescence was detected with each of the four lipases. Of the five NBD lipids tested, the highest rates of hydrolysis were consistently obtained with NBD-phosphatidylglycerol followed by NBD-phosphatidylcholine.
Lipids 24, 691–699 (1989).

In a series of elegant papers, Pagano *et al.* (1–3) employed various NBD-labeled lipids and fluorescence microscopy to study the intracellular transport and metabolism of phospholipids in cultured animal cells. In 1984, Wittenauer *et al.* (4) reported that NBD-phospholipids could also be employed as fluorometric substrates in a convenient assay of lipolytic enzyme activity. Our laboratory has previously reported on the adaptation of this assay technique for several lipolytic enzymes from plant and fungal sources (5–8). In one of these studies (6) we reported that crude extracts from eight plant tissues were able to hydrolyze C_6-NBD-PC and that the rates of hydrolysis ranged from 0.021 to 4090 nmol/min/gram fresh weight of tissue. Although several other laboratories have reported on the use of either phospholipid or sphingolipid analogues which are labeled with fluorescent groups (9–13), the NBD technique which was described by Wittenauer *et al.* (4) appears to be the most convenient because this assay is continuous, and therefore does not require either lipid extractions or chromatographic separations. The present study was undertaken to evaluate the usefulness of the NBD technique to detect the activity of a diverse group of commercially available purified phospholipases and lipases.

Abbreviations: BSA, bovine serum albumin; C_6-NBD or NBD hexanoic acid, 6-(7-nitro-1,3-benzoxadiazol-4-yl)amino]caproic acid; 4MUL, 4-methylumbelliferyl laurate; 4MUP, 4-methylumbelliferyl phosphate; NBD, 6-(7-nitro-1,3-benzoxadiazol-4-yl)amino; C_6-NBD-PC, 1-acyl-2-[6-(7-nitro-1, 3-benzoxadiazol-4-yl)amino]caproyl phosphocholine; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PNP-PC, *p*-nitrophenyl phosphocholine; TAG, triacylglycerol.

MATERIALS AND METHODS

Materials. The lipase from *Rhizopus delemar* was obtained from Seikaguka Kogyo Co. C_6-NBD (the fluorescent cyclic free fatty acid), 4-methylumbelliferyl phosphate, *p*-nitrophenyl phosphocholine, and the other commercially prepared enzymes used in this study were obtained from Sigma Chemical Co. The NBD phospholipids were obtained from Avanti Polar Lipids (Birmingham, AL). These lipids are prepared from egg lecithin and contain a long chain fatty acid in the *sn*-1 position and an NBD-hexanoate or NBD-dodecanoate in the *sn*-2 position. The 4-methylumbelliferyl laurate (4MUL) was obtained from United States Biochemical Co. All other reagents were of the highest purity commercially available.

Enzyme assays. Phospholipase activity was measured using NBD-phospholipids as substrates by the procedure of Wittenauer *et al.* (4) with minor modifications. The reaction mixture (2 ml) contained 5 μM C_6-NBD-PC or other NBD lipid, 50 mM HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid) buffer (pH 7.0) and 1 to 50 μl of enzyme sample. A standard curve of NBD-hexanoic acid concentration (0–7.5 micromolar) vs fluorescence was prepared by diluting 1 mM NBD-hexanoic acid (dissolved in ethanol) into the above 2 ml reaction mixture containing the concentrations of buffer and C_6-NBD-PC as stated above. This standard curve was used to convert changes in fluorescence to nmol of NBD-hexanoic acid produced. Relative fluorescence was measured continuously with a Sequoia-Turner Model 450 Fluorometer equipped with an excitation fiter (460 ± 5 nm) and an emission filter (>535 nm). Esterase activity was measured using 4-methylumbelliferyl laurate (4MUL) as a substrate as previously described (14,15). The reaction mixture (2 ml) contained 50 mM potassium phosphate buffer (pH 8.0), 40 μl of 20 mM 4MUL in ethylene glycol monemethyl ether, and 1–50 μl of enzyme sample. The fluorometer was equipped with an excitation filter (360 ± 5 nm) and an emission filter (>415 nm). Phospholipase C activity was measured with PNP-phosphocholine (PNP-PC), essentially as described (16). The reaction mixture contained 1 mM PNP-PC, and 50 mM HEPES (pH 7.8) and 10–100 μl of enzyme sample. The increase in absorbance at 405 nm was measured with a recording spectrophotometer. Alkaline phosphatase activity was measured with 4-methylumbelliferyl phosphate (4MUP) using the same conditions as listed above for 4MUL except 4MUL was replaced with 4MUP.

Extraction, separation, and quantification of fluorescent products. A 2 ml reaction mixture containing C_6-NBD-PC was prepared as described above. After addition of enzyme, the mixture was incubated at 30°C. At various

time intervals the reactions were stopped by adding 200 μl 1 M HCl. The lipids were immediately extracted by adding 6 ml 2:1 ethyl acetate:acetone. The lipids were removed in the upper organic phase and transferred to a clean test tube where the solvents were evaporated under a stream of N_2. The lipids were redissolved in a small volume of 1:1 chloroform:methanol and spotted on a TLC (250 micron silica gel G) plate and developed in chloroform/methanol/30% ammonium hydroxide, 65/35/5. After development, the plates were thoroughly air dried and scanned for fluorescence with a Shimadzu Model CS-930 dual wavelength TLC scanner equipped with filter #3. The Rf values 0.27 and 0.18 for free C_6-NBD and C_6-NBD-PC, respectively, were determined by spotting samples of these compounds in separate lanes. The Rf values of 0.64 and 0.11 for NBD-diacylglycerol and lyso-NBD-PC, respectively, were deduced from the presence of unique products with phospholipase C and phospholipase A_2, which corresponded well with previously published Rf values (1).

RESULTS AND DISCUSSION

Fluorescence of NBD-phospholipids and NBD-hexanoic acid under various conditions. In the first experiment the relative fluorescence of C_6-NBD-PC and its corresponding free acid, NBD-hexanoic acid or C_6-NBD (the product of hydrolysis by A_2-type phospholipases), were measured in two organic solvents, methanol and chloroform (Fig. 1) and in an aqueous solution buffered at pH 7.0 (Fig. 2). In chloroform and methanol the fluorescence of each compound was much higher than in the aqueous solution. In methanol the fluorescence of C_6-NBD was about 70% higher than that of C_6-NBD-PC. In chloroform, the fluorescence of C_6-NBD was about 140% higher than that of C_6-NBD-PC. In each organic solvent, the fluorescence was proportional to the concentration of either compound. In the aqueous solvent the fluorescence of C_6-NBD-PC was very low, but was detectable and was concentration-dependent. Identical curves were obtained when the aqueous solutions were buffered with 50 mM citrate-KOH, pH 4.2 or 50 mM glycine-HCl, pH 9.0. The fluorescence of C_6-NBD was much higher than that of C_6-NBD-PC, but was about one-tenth of the value observed in methanol or chloroform (note scale change of abscissa). This experiment confirms the previous report of Wittenauer *et al.* (4) who observed that there was a 50-fold increase in fluorescence upon enzymatic removal of C_6-NBD from C_6-NBD-PC. An important finding of this experiment is that the fluorescence

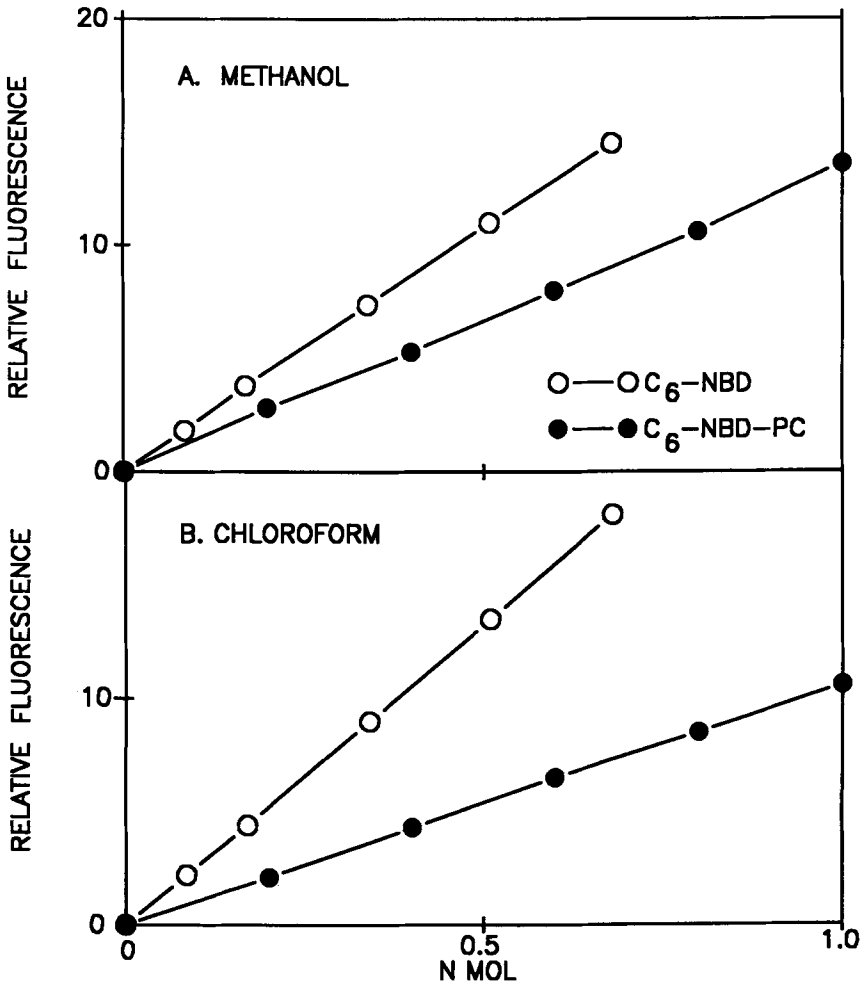

FIG. 1. The relative fluorescence of various concentrations of C_6-NBD and C_6-NBD-PC in methanol and chloroform.

of these two compounds is constant over the range of pH values of 5–9. This insensitivity to pH demonstrates that enzyme assays which employ NBD phospholipids as substrates can be buffered at any point in the physiological pH range. In contrast, several other spectrophotometric and fluorometric esterase substrates (i.e., *p*-nitrophenyl esters and 4-methylumbelliferyl esters) require alkaline pH values for maximum absorbance or fluorescence. There was no evidence of non-enzymatic hydrolysis of NBD lipids (increase in fluorescence) under any of the conditions which were investigated in this study.

In our initial studies, detergents and bovine serum albumin (BSA) appeared to interfere with the fluorescence of NBD lipids. Upon further examination, detergents or BSA induced a dramatic concentration-dependent effect on the fluorescence of C_6-NBD-PC (Fig. 3). However, these compounds had no effect on the fluorescence of C_6-NBD (data not shown). None of the five detergents which were studied had an effect on the fluorescence of C_6-NBD-PC at detergent concentrations below 0.02 mg/ml. However, when the detergent concentration was increased, a unique point was reached for each detergent at which higher concentrations caused a significant increase in the fluorescence of C_6-NBD-PC. These results suggest that the detergent concentration which starts to cause an increase in fluorescence of C_6-NBD-PC may be correlated with the critical micelle concentration (CMC) of the detergent. A similar concentration-dependent effect was observed with BSA, with an increase in fluorescence occurring at concentrations of 0.05–0.1 mg/ml. This experiment indicates that most detergents and BSA should only be used with caution, and even then probably only within the lower concentration ranges described in Figure 3. If detergents had increased the fluorescence of the product, C_6-NBD, then perhaps increased sensitivity could have been attained by including detergents in the assay mixture, but because detergents only increased the fluorescence of the substrate, C_6-NBD-PC, it is unlikely that detergents could provide any real advantage.

Hydrolysis of C_6-NBD-PC by various phospholipases and lipases. In the original paper describing the use of C_6-NBD-PC as a fluorometric substrate (4), it was used at a concentration of 5 μM in an aqueous solution buffered at pH 7.0. We have utilized this convenient assay technique to measure phospholipase activities in crude extracts from a variety of plant tissues (5–8). In the next experiment, commercially prepared samples of five phospholipases and four lipases (Table 1) were tested for their ability to hydrolyze 5 μM C_6-NBD-PC. For each assay 1–100 μg of protein was added to the cuvette and the enzyme activity was calculated from the mean of at least three different levels of protein that yielded product concentrations within the values shown in Figure 2 for a 5 min assay period. Increases in fluorescence were detected with the A_2 and C type phospholipases, and with each of the four lipases, but no increase in fluorescence was detected with the D type phospholipases. Among the enzymes which exhibited activity, the rates of hydrolysis ranged from 0.33 nmol min^{-1} mg $protein^{-1}$ for *R. delemar* lipase, to 1645.4 nmol min^{-1} mg^{-1} for *N. naja* phospholipase A_2 assayed with Ca^{2+}. The phospholipase A_2 activities were dramatically stimulated by Ca^{2+}, which is characteristic of this group of enzymes. The activity of each of the other phospholipases (Table 1) was unaffected by Ca^{2+}. In order to compare this new assay technique with others commonly used for lipolytic enzymes, three other substrates were tested. With 4-methylumbelliferyl laurate, a common fluorometric esterase substrate, only the lipases exhibited activity. With *p*-nitrophenyl phosphocholine, a spectrophotometric substrate for phospholipase C activity, significant levels of activity were detected with the phospholipase C and a low

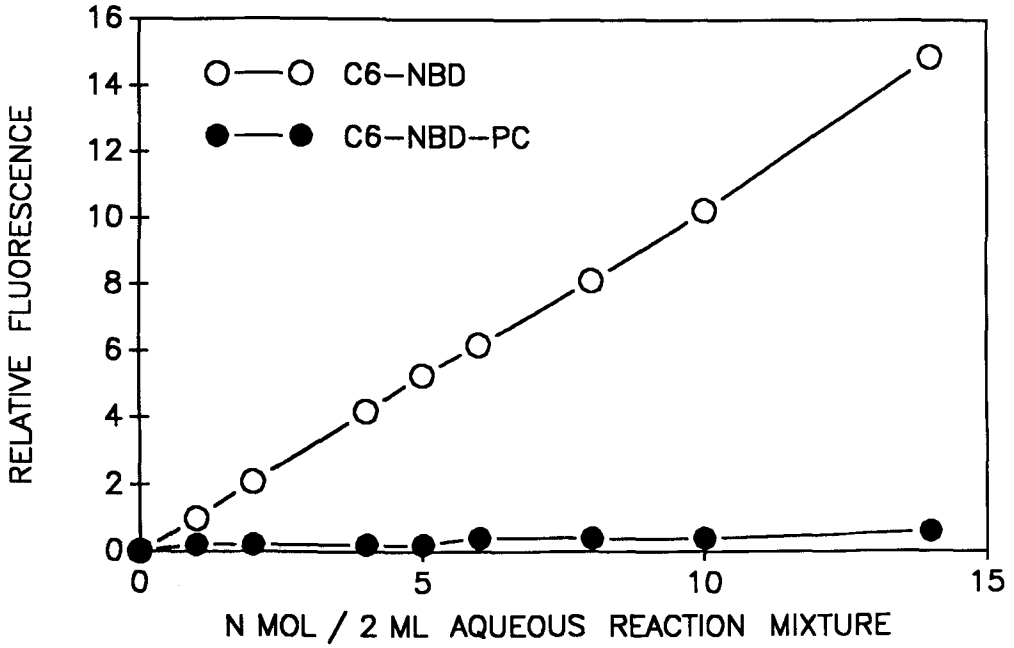

FIG. 2. The relative fluorescence of C_6-NBD and C_6-NBD-PC in 2 ml of an aqueous solution buffered with 50 μM HEPES-NaOH, pH 7.0.

level of activity was detected with the phospholipase D from cabbage. With 4-methylumbelliferyl phosphate, a common fluorometric phosphatase substrate, activity was only detected with the phospholipase D from cabbage. It is not known whether this phosphatase activity is actually catalyzed by phospholipase D or is due to the presence of contaminating enzyme(s), because acid phosphatases are very common in plant tissues.

In order to compare the rates of hydrolysis of artificial substrates by these enzymes, the rates of hydrolysis of two natural substrates (PC for the phospholipases and TAG for the lipases) are also reported in Table 1. These values were not determined in this study but were the specific activities that were reported by the suppliers of each of these commercially-prepared enzymes. It can be seen that for the two phospholipase A_2 enzymes, the rates of hydrolysis of the natural substrates was about 400 to 600-fold higher than the rates of hydrolysis of C_6-NBD-PC. With phospholipase C, PC appeared to be hydrolyzed at a rate that was 60,000-fold higher than that for C_6-NBD-PC, but the last section of this paper will present data which indicated that this value may not be reliable. For each of the lipases, TAG was the preferred substrate, followed by 4MUL, and finally by

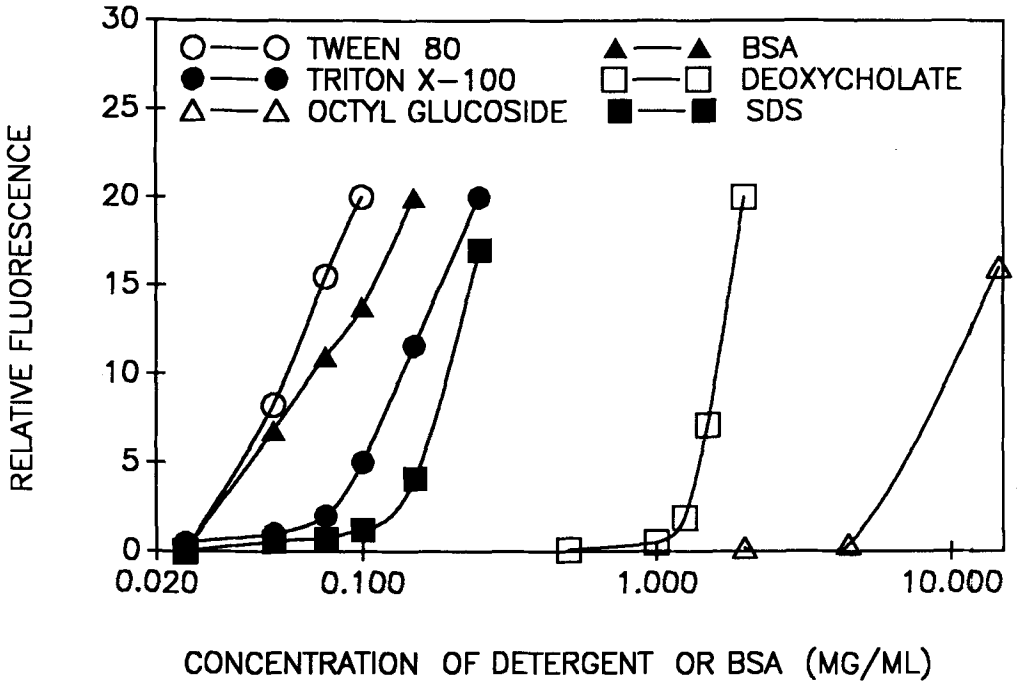

FIG. 3. Effect of various concentrations of detergents and BSA on the fluorescence of C_6-NBD-PC in an aqueous solution buffered with 50 μM HEPES-NaOH, pH 7.0.

TABLE 1

A Comparison of the Rates of Hydrolysis of C_6-NBD-PC and Other Common Substrates with Commercial Lipolytic Enzymes

Enzyme	Ca^{2+} (3 mM)	Specific activity (nmol min^{-1} mg $protein^{-1}$)					
		C_6-NBD-PC	4MUL	PNP-PC	4MUP	PC[a]	TAG[a]
Phospholipase A_2	−	216	0	0	0	—	—
(Naja naja)	+	1645.4	0	0	0	980,000	—
Phospholipase A_2	−	3.84	0	0	0	—	—
(pancreatic)	+	156	0	0	0	600,000	—
Phospholipase C	−	19.2	0	80	0	1,160,000	—
(Bacillus cereus)	+	18.3	0	74	0	—	—
Phospholipase D	−	0	0	0	0	10,000	—
(peanut)	+	0	0	0	0	—	—
Phospholipase D	−	0	0	1.24	60	9,000	—
(cabbage)	+	0	0	0.87	56	—	—
Lipase *(R. delemar)*	−	0.33	1,471	0	0	—	600,000
Lipase *(R. arrhizus)*	−	198	34,470	0	0	—	6,000,000
Lipase *(C. cylindracea)*	−	1.06	4,360	0	0	—	7,000
Lipase (pancreatic)	−	2.33	194	0	0	—	2,000

[a]Rates of hydrolysis of phosphatidylcholine (PC) and triacylglycerol (TAG) as reported by suppliers of the commercially-prepared enzymes.

C_6-NBD-PC. This experiment demonstrates that NBD lipids may be useful as substrates for some lipolytic enzymes, especially for phospholipase A_2 activities, since none of the other three assays in Table 2 were capable of detecting A_2-type enzyme activity.

A comparison of the rates of hydrolysis of five NBD-phospholipids. In addition to C_6-NBD-PC, four other NBD lipids are commercially available. In the next experiment the activities of the three most active enzymes from the previous experiment were compared using the five NBD lipids (Table 2), each measured at a substrate concentration of 5 μM. With the *N. naja* phospholipase A_2 assayed in the presence of Ca^{2+}, the highest activity was obtained with C_6-NBD-phosphatidylglycerol (C_6-NBD-PG), followed by C_{12}-NBD-PC, C_6-NBD-PC, C_6-NBD-phosphatidylethanolamine (C_6-NBD-PE), and finally C_6-NBD-phosphatidic acid (C_6-NBD-PA). With pancreatic phospholipase A_2 in the presence of Ca^{2+} the activity with C_6-NBD-PG was 14 to 25-fold higher than with the other four substrates. Finally, with the lipase from *R. arrhizus* measured in the absence of Ca^{2+}, nearly equal levels of activity were obtained with C_6-NBD-PG, C_6-NBD-PE, and C_6-NBD-PC, and lower levels of activity were obtained with C_6-NBD-PA and C_{12}-NBD-PC. With the lipase, the activity with each of the substrates was inhibited by Ca^{2+}.

Effect of concentration of NBD-lipids on rates of hydrolysis. In their initial description of the use of C_6-NBD-PC to assay enzymes, Wittenauer *et al.* (4) suggested that a concentration of 5 μM was saturating and would probably be optimum for most enzymes. In the next experiment the three enzymes which were studied in Table 2 were assayed with concentrations of C_6-NBD-PC and C_6-NBD-PG ranging from 1-20 μM (Fig. 4). With each of the three enzymes C_6-NBD-PG was hydrolyzed at a higher rate than C_6-NBD-PC. The reason that each enzyme hydrolyzed C_6-NBD-PG at a higher rate than C_6-NBD-PC is not known, but may have to do with a different aggregation state of the two lipids. Only with the lipase did the activity seem to become saturated with substrate and this occurred at about 10 μM. A similar trend was observed with C_6-NBD-PC except that the activities were consistently 20-30% lower than those observed with C_6-NBD-PG. This experiment indicates that for preliminary studies of the properties of an enzyme activity a 5 μM concentration of NBD lipid may be sufficient, but for more detailed kinetic analyses higher concentrations of substrate may be necessary to ensure that saturating concentrations are achieved. Since Wittenauer *et al.* (4) reported the critical micelle concentration of C_6-NBD-PC to be 2×10^{-7} M, at a concentration of 5 μM greater than 90% of the C_6-NBD-PC would be present as aggregates or micelles. We have previously reported that two of the phospholipase activities in plants had Km values of 1.5 and 2.3 μM for C_6-NBD-PC (5). Each of the NBD-phospholipids was quite soluble in buffer solution except C_{12}-NBD-PC, which required shaking for at least 30 min to dissolve it. It should be noted that in this study the rates of hydrolysis of C_{12}-NBD-PC were estimated using the standard curve of C_6-NBD since C_{12}-NBD is not commercially available. We assume that this estimation should be fairly accurate since the fluorescence of C_{12}-NBD should be very similar to that of its C_6 analogue. We did purchase a methylated derivative of C_{12}-NBD, NBD-*N*-methylamino dodecanoic acid, but found that it exhibited very little fluorescence using the fixed excitation and emission wavelengths of this study. During these studies we observed that the fluorescence of each of the substrates appeared to decline gradually during several months even when stored in sealed glass ampoules at −10°C. The manufacturer reports a usable shelf life of 3-6 months in the freezer.

Separation of the products of hydrolysis of C_6-NBD-PC by TLC. In the final experiment (Fig. 5) the validity of measuring direct changes in fluorescence of the aqueous reaction mixture as a measure of activity was assessed by measuring the actual production of fluorescent products. After incubation with the enzymes, the lipids were extracted and separated by TLC and the levels of fluorescent product formed were quantified (4). For this experiment we chose to use C_6-NBD-PC as the substrate in conjunction with the two phospholipase A_2 enzymes and the phospholipase C enzyme. With the two phospholipase A_2 enzymes, there was a very good correlation between the rate of change of fluorescence of the entire solution and the rate of appearance of C_6-NBD. These results indicate that the continuous assay which we are describing is an accurate measurement of the enzyme activity of these two enzymes. In a previous study, Wittenauer *et al.* (4) reported that the levels of lyso NBD-PC were actually higher than the levels of C_6-NBD in similar time-course studies. In our hands there was a low level of fluorescent material which cochromatographed with lyso NBD-PC, but it only occurred in the 5 and 10 min

TABLE 2

A Comparison of the Rates of Hydrolysis of the Various C_6-NBD Phospholipids with Three Common Lipolytic Enzymes

Enzyme	Ca^{2+} (3 mM)	Specific activity (nmol min^{-1} mg $protein^{-1}$)				
		C_6-NBD-PC	C_6-NBD-PE	C_6-NBD-PA	C_6-NBD-PG	C_{12}-NBD-PC
PLA_2	−	216	621	516	172	466
(N. naja)	+	1645	563	360	5310	3040
PLA_2	−	3.84	17.2	144	274	3.9
(pancreatic)	+	156	138	145	2260	91.1
Lipase *(R. arrhizus)*	−	198	215	41.2	235	43.2
	+	145	97.4	25.9	89.4	22.3

[a]Each substrate was tested at a concentration of 5 μM. The values reported are the mean of 3-5 determinations.

readings and was absent in the scans of extractions taken at later times in the time course (data not shown). This transient appearance of lyso NBD-PC was probably due to the presence of some *sn*--1-NBD-PC isomer. In this case the initial hydrolysis of non-NBD fatty acid from the *sn*-2 position (resulting in the appearance of lyso NBD-PC) is probably followed by hydrolysis of the C_6-NBD from the *sn*-2-NBD isomer. A similar phenomenon was observed by Wittenauer *et al.* (4) except that their levels of lyso NBD-PC remained high throughout the experiment.

When the phospholipase C was examined the fluorescence of the aqueous solution increased at a nearly linear rate during the time-course (Fig. 5). However, upon examination of the reaction products by TLC, two important observations were made. The first was that there was no evidence of C_6-NBD at any of the sample times,

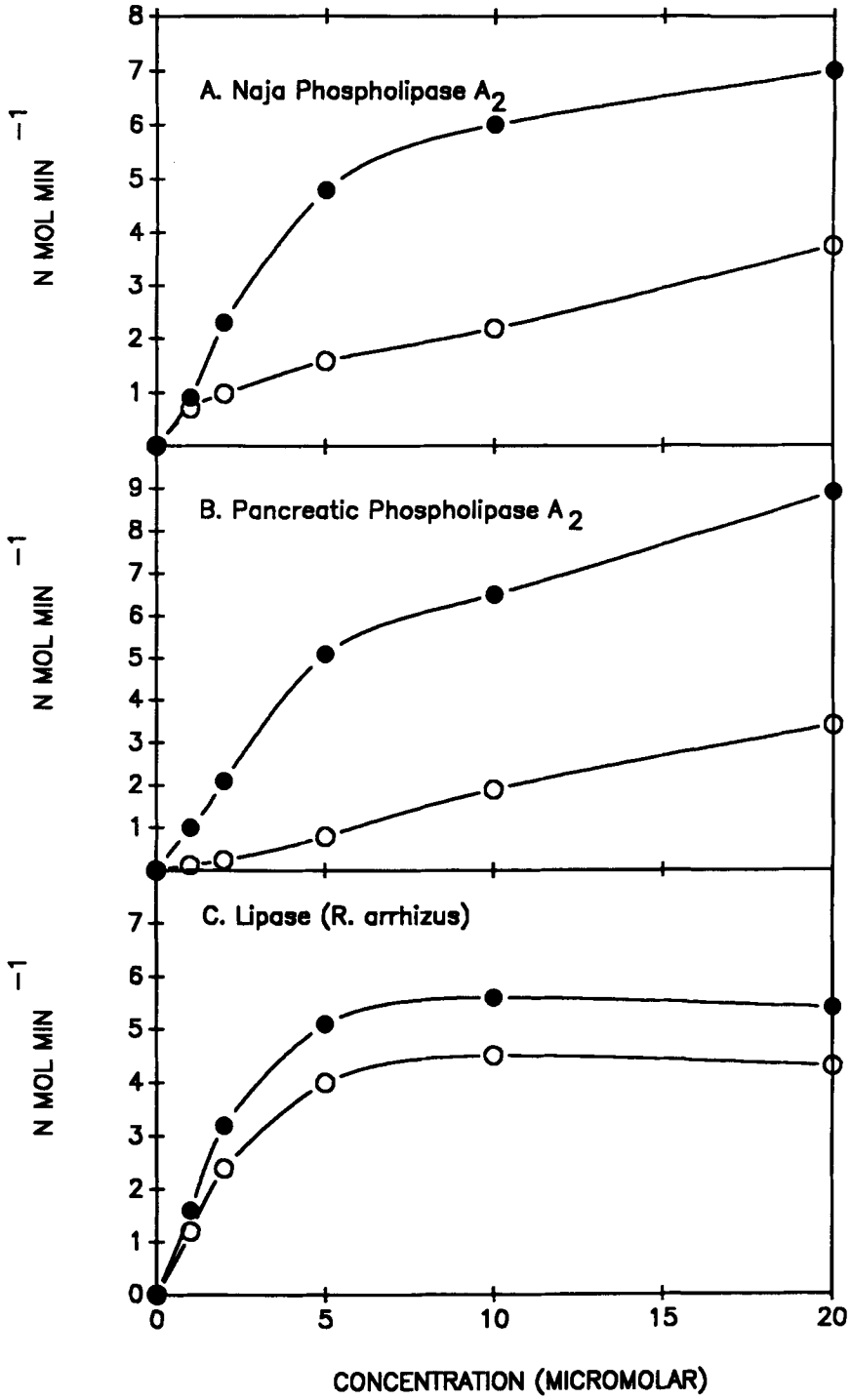

FIG. 4. Effect of concentration of NBD substrate on the enzyme activity of three commercial lipolytic enzymes. The symbols correspond to C_6-NBD-PG (●) and C_6-NBD-PC (○).

indicating that the increase in fluorescence of the solution was not caused by accumulation of the free acid and must be due to some other product. The second finding was that there was a rapid accumulation of C_6-NBD-diacylglycerol (DAG), the expected product of hydrolysis by C-type phospholipases. However, the accumulation of DAG began earlier and proceeded at a much faster rate than the increase in fluorescence of the entire aqueous solution. This experiment indicates that although NBD lipids may be useful for detecting phospholipase C activity, the rates of increase in fluorescence of the reaction mixture may not be an accurate indication of the actual rates of formation of product. This experiment also indicates that one limitation of this assay technique is that it is incapable of distinguishing between phospholipase A_2 and C-type activities. These results differ from those

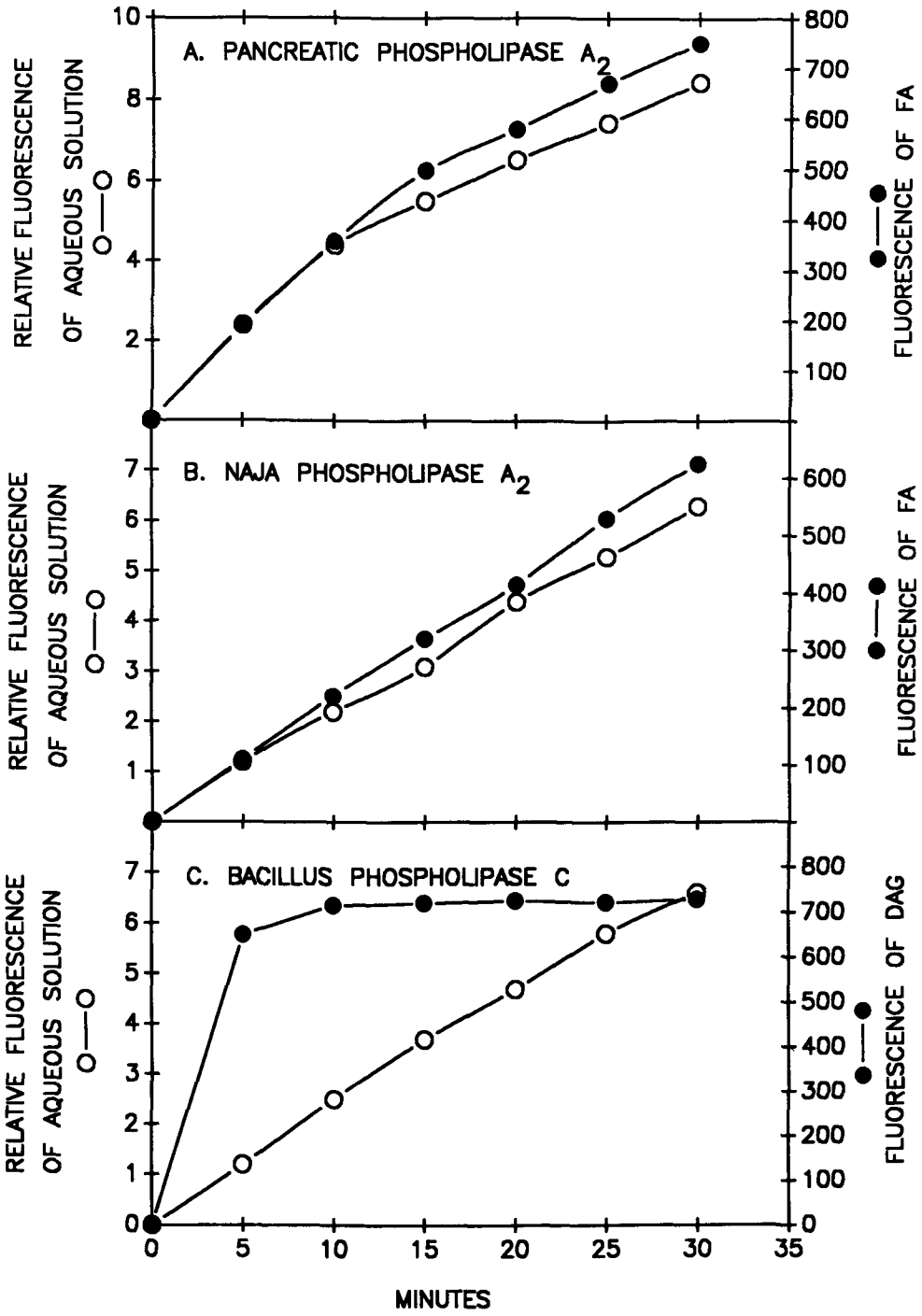

FIG. 5. Time-course study of the fluorescence of NBD-products (fatty acid [FA] or diacylglycerol [DAG]) measured after separation by thin layer chromatography, and the change in relative fluorescence of the aqueous reaction mixture.

of Wittenauer *et al.* (4) who reported that phospholipase C did not catalyze an increase in fluorescence when exposed to C_6-NBD-PC. Since they did not report the source of their phospholipase C it is possible that not all C-type phospholipases are capable of hydrolyzing NBD phospholipids.

With any new fluorometric assay technique there should be concern about the aggregation state of the substrate and product and possible binding of released product by proteins. The data presented in Figure 5 indicate that at least for the two phospholipase A_2 enzymes, these two potential problems did not occur. However, these problems may have accounted for the inaccurate detection of the phospholipase C activity. When applying this technique to a new enzyme or a crude enzyme mixture, it would be advisable to determine the products of hydrolysis chromatographically and correlate their rate of appearance with the rate of increase in fluorescence as was reported in this study.

CONCLUSIONS

The results of this study reveal that NBD phospholipids offer good potential as substrates for the assay of enzymes exhibiting phospholipase A_2 activity. They offer several advantages over more common methods for detectng this type of enzyme activity: 1) The assay technique described by Wittenauer *et al.* (4) and evaluated in this study is as sensitive as common techniques of measuring the production of the product of lipolytic reactions by radioisotopic or other analytical techniques (i.e., chemical analysis of fatty acid, choline, or glycerol production). 2) This assay technique is rapid and does not require any lipid extractions. Because each enzyme sample requires only 2-5 min per analysis, this technique is able to detect the types of rapid changes in enzyme activity that occur after treatment with various environmental or chemical stimuli. In two previous studies (5,7) we were able to employ the C_6-NBD-PC assay to measure the rapid and transient stimulation of a plant phospholipase activity upon treatment with calmodulin, protein kinases, and proteases. 3) This technique does not require sophisticated or expensive instrumentation. It can be performed using a simple and relatively inexpensive filter fluorometer. The instrument employed in this study is quite attractive because it uses a quartz halogen bulb which gives off very little heat. Therefore, the cuvette chamber does not need to contain a temperature control such as is required with fluorometers which utilize other types of bulbs. Although this study revealed that a filter fluorometer performed very nicely in the routine assays described in this study, it was unable to detect the small differences in the excitation or emission maxima which may have occurred in some of the experiments in this study. It is possible that the detergent effects which were observed in Figure 3 could be better explained by using a fluorometer with scanning capabilities. We have obained very reproducible results using inexpensive disposable flint glass test tubes as fluorometric cuvettes. This is to be expected since both the excitation and emission wavelengths are in the visible spectrum. 4) All five NBD lipids used in this study are commercially available and reasonably priced. Since each assay only requires about 10 nmol of substrate, 10 mg of substrate is enough for about 1000 assays. 5) An advantage which was previously mentioned is that this continuous assay can be conducted at any pH between at least 5 and 9 using the same standard curve (Fig. 2).

There are disadvantages to this assay technique, and it is important to be aware of the following: 1) For the two phospholipase A_2 enzymes in this study, the rates of hydrolysis of NBD phospholipids are several hundred-fold lower than the rates of hydrolysis of natural phospholipids. This could be a serious disadvantage for some applications. However, because the sensitivity of this assay (ability to detect minute levels of product formed) is several hundred-fold higher than that of most other techniques, this disadvantage may be cancelled out by its high degree of sensitivity. This technique may be especially useful for applications such as monitoring phospholipase A_2 activity during enzyme purifications. 2) As mentioned previously, this study revealed that this assay technique may not be able to distinguish between A_2 and C-type phospholipases. In this study, Ca^{2+} stimulated the A_2-type phospholipases but not the C-type. If this generalization can be shown to apply to other A_2 and C-type phospholipases, stimulation by Ca^{2+} may be a means of identifying the phospholipase A_2 activities. 3) Although C_6-NBD-PC is supposed to be supplied from the manufacturer with all of the C_6-NBD on the *sn*-2 position, a small amount of it appears to be esterified to the *sn*-1 position, as previously discussed. This problem of isomeric impurities needs to be acknowledged and may limit the usefulness of this lipid as a fluorometric substrate in certain applications.

Even though each of the lipases was capable of hydrolyzing C_6-NBD-PC (Table 1), there are at least two reasons why NBD-lipids are not the preferred substrate for studies with true lipases. First, each of the four lipases in this study hydrolyzed 4MUL at a high rate, suggesting that it is a better substrate for routine studies. Second, since the purity of these commercially-prepared lipases is questionable, we do not know whether their ability to hydrolyze NBD lipids is due to a broad specificity of the actual lipase molecule or is due to contamination by phospholipases(s).

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Suppression of Growth in a Leukemic T Cell Line by n-3 and n-6 Polyunsaturated Fatty Acids

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Proliferation in a leukemic T cell line (Jurkat) was suppressed in a dose dependent manner by n-6 and n-3 polyunsaturated fatty acids (PUFA) added to the culture medium. At high concentrations, PUFA have a cytotoxic effect on Jurkat cells. The inhibitory effect of the PUFA was not due to production of prostaglandins, and lipid peroxidation was only partly responsible. In addition to production of peroxides and aldehydes, lipid peroxidation also reduced the plasmalogen levels in these cells. The antioxidant α-tocopherol blocked lipid peroxidation and restored the plasmalogen levels to normal. α-Tocopherol did not totally restore cell proliferation although the MDA-like products in these cultures (supplemented with PUFA) were reduced to control level. Cultures supplemented with n-6 PUFA seemed to respond better to α-tocopherol than n-3 PUFA. This suggests that n-6 PUFA may exert their growth inhibitory effect predominantly via lipid peroxidation while different mechanisms might be operating for the n-3 PUFA.
Lipids 24, 700-704 (1989).

Our present understanding of the regulatory process that controls normal and tumor cell growth is still unclear. Most studies have focused mainly on growth factors—hormones and genes that promote cell division. However, there is growing evidence that polyunsaturated fatty acids (PUFA) and their peroxidation products may play a major role in regulation of cell growth (1).

Long chain PUFA are known to arrest cell proliferation and exert toxic effect on several tumor cell lines in culture (1-6). Different mechanisms are possible. PUFA readily undergo lipid peroxidation with free oxygen producing lipid hydroperoxides, which, in turn, propagate the peroxidation process to lipid peroxyl and alkoxyl-radicals (7,8). The latter compounds may break down to cyclic endoperoxides and malondialdehyde (MDA)-like products. These compounds can cause membrane damage and affect cell metabolism (2,5,9).

Another possible mechanism may involve the eicosanoids produced by the cells after metabolizing the n-6 and n-3 fatty acids (1,6). Prostaglandins (PG) like PGE_1 and PGE_2 have been reported to suppress cell growth in smooth muscle cells (10-14). The role of PG in growth regulation is complicated and unclear. In some studies PGE_1 and PGE_2 were found to stimulate cell growth at low concentrations and inhibit cell proliferation at high doses (14). These substances have also been shown to enhance tumor growth in a dose dependent manner (14,15).

In transplantation studies PUFA have been shown to have immunosuppressive effects (16). This could be due to both structural and functional modulation of macrophages, T and B cells (17,18). PUFA may also influence the production of PG, especially PGE, which plays an important role in the down regulation of macrophage effector function (19).

In this study attempts were made to evaluate the relative importance of lipid peroxidation and prostaglandins in PUFA suppressed growth of a leukemic T cell (Jurkat).

MATERIALS AND METHODS

Chemicals. Linoleic acid (LA), α-linolenic acid (ALA), oleic acid (OA), arachidonic acid (AA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DCHA), α-tocopherol, thiobarbituric acid and 1,1,3,3-tetraethoxypropane were purchased from Sigma (St. Louis, MO). Indomethacin was purchased from Dumex (Stockholm, Sweden) as a sodium salt (Confortid).

Cell line. Jurkat cells (a T cell leukemia cell line) were maintained in continuous *in vitro* culture in RPMI 1640 medium supplemented with 5% fetal calf serum (FCS) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin).

Cell culture with free fatty acids (FFA). Jurkat cells were seeded at 0.25×10^6 cells/ml in 20 ml RPMI 1640 medium with 5% FCS and antibiotics in 50 ml tissue culture flask (Falcon). FFA and α-tocopherol were diluted in 95% ethanol and added to cultures at a final concentration of 15 μg/ml and 30 μM, respectively. Control cells have equivalent amounts of 95% ethanol (between 0.02-0.08%). The cells were incubated at 37°C under an atmosphere of 5% CO_2:95% air. Cell density and viability were assessed every 24 hr by haemocytometer counting (trypan blue exclusion). After 72 hr, the cells were washed twice with phosphate buffer saline (PBS) and total lipids were extracted or assayed for lipid peroxidation products.

Lipid peroxidation assay. Lipid peroxidation was assayed using thiobarbituric acid reagent for MDA-like products (20,21). Cells from each culture were suspended in 2.5 ml PBS. Fifty μl of the cell suspension were taken for protein assay and 2 ml were transferred to glass tubes with screw caps. One ml of trichloroacetic acid (20%, w/v) was added to lyse the cells and precipitate proteins. Thiobarbituric acid (2 ml of 0.67%, w/v) was added and the mixture heated in a boiling water bath for 20 min with caps screwed on. The tubes were cooled and the precipitate centrifuged off. The absorbance of the supernatant containing the MDA-thiobarbituric complex were measured at wavelength 535 nm using a spectrophotometer (Ultrospec 4050, LKB). Standards ranging from 2 to 12 nM of 1,1,3,3-tetraethoxypropane were used for the calibration.

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Abbreviations: PUFA, polyunsaturated fatty acid; LT, leukotriene; PG, prostaglandin; ALA, α-linolenic acid; LA, linoleic acid; OA, oleic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DCHA, docosahexaenoic acid; MDA, malondialdehyde; FCS, fetal calf serum; PBS, phosphate balance saline; DMA, dimethyl acetal; BHT, butyl-hydroxytoluene.

Protein assay. Lowry's method was used after solubilizing the cells with 20 μl of 2 M NaOH and neutralized with HCL. BSA was used as standard.

Extraction and methylation of lipids. Jurkat cells from each culture were resuspended in 1.6 ml PBS and transferred to glass tubes with teflon seal screw caps. Lipids were extracted using a modified method of Bligh and Dyer (22). Methanol (4 ml) and chloroform (2 ml) were added to the cell suspension and the mixture was mixed. A further 2 ml each of chloroform and water were added, and the two phases were separated by centrifugation. The lower phase was collected, and both the upper phase and interphase were re-extracted with 4 ml of chloroform. The total lower phase was blown dry with a stream of nitrogen. Methylation of the lipids was carried out by adding 200 μl methanolic sodium methoxide (0.5 M) to the dried lipids and heated at 56°C in a shaking water bath for 15 min (23). This was followed by addition of 200 μl of methanolic HCl and the mixture was heated again at 56°C for 15 min. The mixture was then diluted by adding 0.8 ml distilled water, and the methyl esters were extracted three times, each with 2 ml hexane. After evaporation of hexane by a stream of nitrogen, the methyl esters were resuspended in 200 μl of hexane with BHT (1 μg/ml). Plasmalogens in the total lipid extracts are converted to dimethyl acetals (DMA) using this methylation process and together with methyl esters were quantitated using gas chromatography.

Gas chromatography. For gas chromatographic separation of methyl esters and DMA, a Hewlett-Packard 5710A gas chromatograph with a modified solventless injector (falling needle) was used (24). The column was a fused silica column (50 m × 0.32 mm i.d.) coated with CP-Sil 88 (Chromapack, Middleburg, Holland). It was cleaned before use by passing 10 ml dichloromethane (HPLC grade) through it, and recoated with 1.2 mg/ml Silar 5CP (Supelco, Bellafonte, PA) solution using dichloromethane as solvent. Recognition and integration of peaks were determined using data handling capabilities for external analog signals provided by a Hewlett-Packard 5880A integrator coupled to an ABC B06 computer (Luxor, Motala, Sweden).

Thymidine incorporation assay. Aliquots of 200 μl Jurkat cell suspension (5×10^4 cells in RPMI 1640 medium plus appropriate FFA) were transferred into wells of a 96 well plate (flat bottom). The plate was incubated at 37°C in an incubator for 24 hr, and the last 6 hr were pulsed with 1 μCi [^{3}H]thymidine (NEM, USA) per well. The incubation was terminated by harvesting the cells from the wells onto glass fiber filters using a cell harvester (Skartron AS, Norway). The glass fiber filters were oven dried and the radioactivity determined using Highsafe 3′ scintillant (Pharmacia, Sweden) in a beta counter (LKB).

RESULTS

Suppression of cell growth by PUFA. Cell proliferation was suppressed by both the n-6 and n-3 PUFA, but not by the n-9 mono-unsaturated fatty acid (Fig. 1). The suppression was dose dependent and little difference was observed between the PUFA at 5 μg/ml. When the concentration was increased, the differential effect in suppression due to each individual PUFA became more

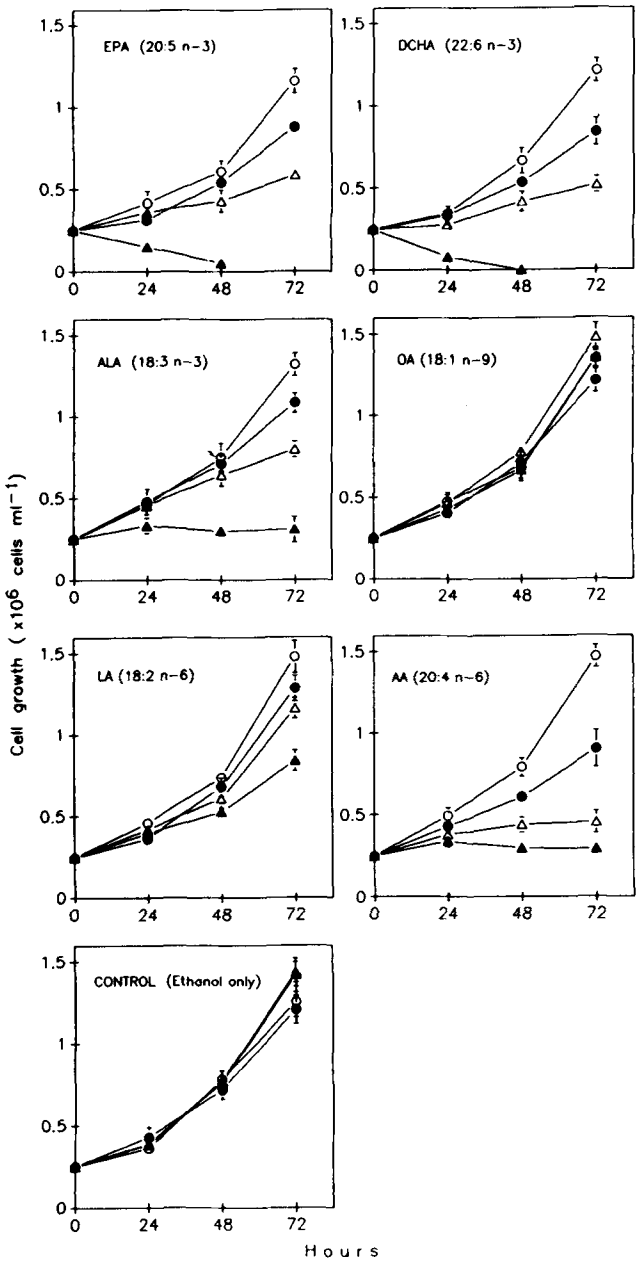

FIG. 1. Suppression of Jurkat cell proliferation by FFA. Jurkat cells were cultured in medium supplemented with different FFA for 72 hr. Concentration of FFA used were 5 (○), 10 (●), 15 (△) and 20 μg/ml (▲). Results were means from six different cultures with SEM.

apparent. Both EPA and DCHA were found to be toxic at 20 μg/ml. At 15 μg/ml, AA inhibited cell growth completely, but ALA, on the other hand, seemed to abolish cell proliferation at higher concentration (20 μg/ml). The results in Figure 1 indicate that both n-6 and n-3 PUFA were equally effective in inhibiting cell growth. Toxicity exerted by AA was found to be greater than LA in the n-6 PUFA. EPA and DCHA (n-3 PUFA) were found to be equally effective, but more potent than ALA in suppressing cell growth.

Effect of α-tocopherol on lipid peroxidation and cell proliferation. Because the inhibitory effect on cell growth was restricted to fatty acids that readily undergo

TABLE 1

Effect of α-Tocopherol on Lipid Peroxidation of PUFA in Jurkat Cells

Fatty acids		Lipid peroxidation (formation of MDA products, nmol/mg protein)	
		− α-Tocopherol	+ α-Tocopherol
Control		0.01	N.D.
OA	18:1 (n-9)	0.17	N.D.
LA	18:2 (n-6)	0.45	0.01
AA	20:4 (n-6)	0.89	0.02
ALA	18:3 (n-3)	0.48	0.02
EPA	20:5 (n-3)	0.59	0.01
DCHA	22:6 (n-3)	0.59	0.02

Jurkat cells were cultured in RPMI medium supplemented with different free fatty acids (15 μg/ml) together with or without α-tocopherol (30 μM). After 72 hr, the cells were washed and lipid peroxidation assay performed as outlined in the Methods section. The results are expressed as means from three separate cultures with SEM less than 5%. N.D., not detected.

peroxidation (n-6 and n-3), attempts were made to evaluate the role of lipid peroxidation. α-Tocopherol (30 μM) was added to the cell cultures together with FFA. As expected, the anti-oxidant reduced the MDA-like products in the cells to control levels as assayed by the thiobarbituric assay (Table 1). α-Tocopherol in itself had no effect on cell growth. All the n-6 and n-3 PUFA peroxidized readily with the exception of OA, the n-9 monounsaturated fatty acid. The highest level of peroxidation was seen with AA and the lowest with OA (n-9), 0.89 and 0.17 nmol/mg protein, respectively. The levels of MDA-like products from EPA, DCHA (0.59 nmol/mg protein each), ALA and LA (0.48 and 0.45 nmol/mg protein, respectively) were much lower than those of AA. Suppression on cell growth by these FFA was markedly reduced by the α-tocopherol added, especially for the n-6 PUFA (Fig. 2), although less reduction was observed with the n-3 PUFA. This differential effect between the two groups of PUFA was highly significant ($p < 0.001$). α-Tocopherol has no significant effect on DCHA. Together with OA, it seemed to have a slight stimulatory effect on cell proliferation.

Effect of cycloxygenase inhibitor on cell proliferation. To rule out the possible involvement of prostaglandin production in the suppressive activity of the FFA, indomethacin (a cyclooxygenase inhibitor) was added to the cell cultures together with α-tocopherol and FFA. At 10 μM, indomethacin had no effect at all on cell proliferation (Fig. 3) as measured by [^{3}H]thymidine incorporation into the cell DNA after 24 hr.

Incorporation of free fatty acids in Jurkat cells. Gas chromatographic analysis of the total lipid extracts from different cultures showed that the supplemented FFA in the culture medium were readily incorporated into the membrane lipids (Table 2). The long chain fatty acids of n-6 PUFA, AA (20:4) and docosatetraenoic acid (22:4) were suppressed when the cultures were supplemented with n-3 PUFA. All unsaturated fatty acids and OA levels (essential fatty acid supplemented only) were greatly reduced.

Effect of supplemented FFA in Jurkat cell plasmalogen levels. There was a general reduction of plasmalogen

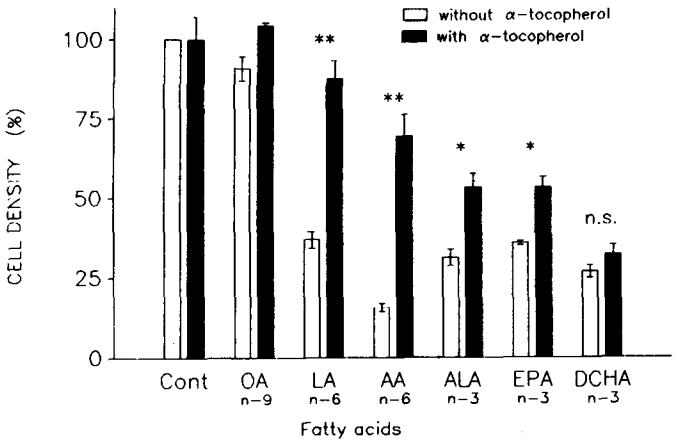

FIG. 2. The effect of lipid peroxidation by FFA on the suppression of Jurkat cell proliferation. α-Tocopherol was added to cell cultures at a final concentration of 30 μM plus 15 μg/ml FFA. After 72 hr, cell density was accessed by counting on a haemocytometer as outlined in the Methods section. Differential effect on n-6 vs n-3 FFA in the presence of α-tocopherol is significant ($p < 0.001$). Results are means from three separate cultures with SEM (N.S., not significant, *,$p < 0.01$, **, $p < 0.001$).

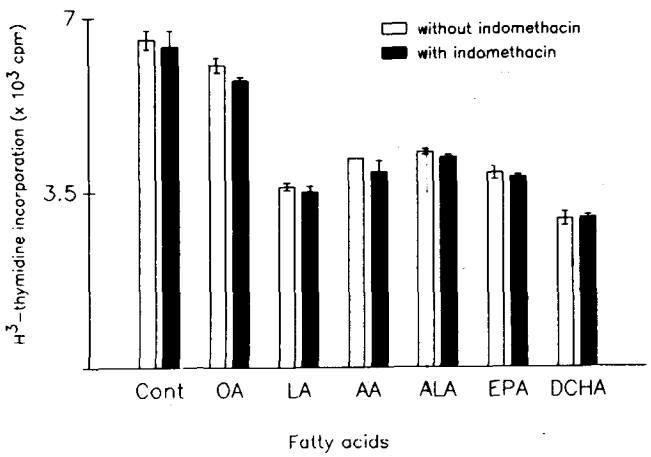

FIG. 3. The effect of indomethacin on the suppression of Jurkat cell proliferation by FFA. Indomethacin was added to cell cultures at a final concentration of 10 μM with FFA (15 μg/ml) and α-tocopherol (30 μM). Tritiated thymidine was added 6 hr prior to harvesting, as outlined in the Methods section. Results were means of quaduplicate samples with SEM from one typical experiment out of three.

levels (both 16:0 and 18:0) as a consequence of the supplemented fatty acids (Fig. 4a and b). The reduction in 16:0 DMA seemed to have little specifity with respect to which FFA were added to the cultures. On the other hand, PUFA seem to be the only FFA that induced a loss in 18:0 DMA, since OA actually increased the level of this plasmalogen. The reduction in plasmalogen levels was restored when lipid peroxidation in the cultures was inhibited by α-tocopherol.

DISCUSSION

This study supports the hypotheses and observations of several workers (1-6,9) that PUFA are cytotoxic at high

TABLE 2

Fatty Acid Composition of Total Lipids from Jurkat Cells After Being Cultured in Medium Supplemented with Different Free Fatty Acids

Fatty acids	Fatty acids supplemented in culture medium						
	Control	OA	LA	AA	ALA	EPA	DCHA
16:0	18.53	5.96	6.86	12.74	10.09	10.00	12.32
17:0	0.53	0.33	0.31	0.33	0.34	0.38	0.41
18:0	11.17	7.77	5.69	6.76	5.85	5.05	6.52
18:1 (n-9)	27.64	40.45	8.26	10.29	9.34	10.85	14.07
18:2 (n-9)	0.16	0.10	0.14	0.16	0.16	0.08	0.08
18:2 (n-6)	1.66	0.91	26.66	1.34	1.76	2.77	4.09
18:3 (n-6)	0.06	0.10	0.10	0.21	0.08	0.19	0.46
18:3 (n-3)	0.68	0.11	0.21	0.18	23.18	0.30	0.56
20:0	0.27	0.12	0.11	0.15	0.13	0.17	0.14
20:2 (n-9)	0.44	0.65	0.10	0.13	0.14	0.11	0.12
20:2 (n-6)	0.39	0.20	6.70	0.20	0.36	0.25	0.51
20:3 (n-9)	0.66	0.58	0.11	0.24	0.13	0.09	0.12
20:3 (n-6)	1.66	1.64	2.01	3.20	1.67	1.60	1.26
20:4 (n-6)	7.65	8.15	2.18	16.36	2.80	2.93	3.45
20:5 (n-3)	0.33	0.44	0.32	0.22	0.98	19.91	0.74
22:4 (n-6)	4.28	5.57	2.11	26.06	1.73	1.34	2.14
22:5 (n-6)	0.53	0.27	0.04	0.57	1.02	0.07	0.43
22:5 (n-3)	2.67	3.49	3.19	2.66	5.17	23.31	3.44
22:6 (n-3)	2.03	3.02	2.36	2.04	1.96	1.62	31.33

Total lipids were extracted from Jurkat cells that have been cultured for 72 hr in RPMI medium supplemented with different free fatty acids (15 μg/ml) and α-tocopherol (30 μM). Transmethylation and GC analysis were carried out as outlined in the Methods section. The results are the means from three separate cultures, except for the control where n = 6. SEM of the results were less than 15%.

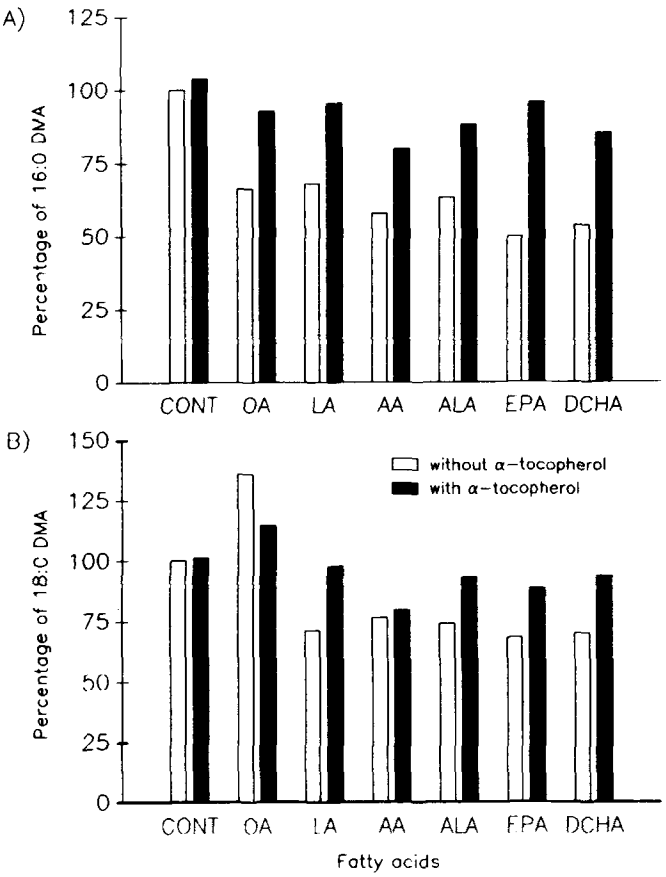

FIG. 4. Effect of FFA on the plasmalogen levels in Jurkat cells. A) Plasmalogens with 16:0 and B) 18:0. Lipids were extracted from Jurkat cells that have been cultured for 72 hr in medium supplemented with different FFA (15 μg/ml) with or without α-tocopherol (30 μM). Methylation of lipids and DMA separation was carried out as outlined in the Methods section. Results were the means from two separate cell cultures.

concentration and suppress proliferation at lower concentration in maglinant cell lines. For the Jurkat cells used in this study, the FFA most sensitive to lipid peroxidation (n-3 and n-6 series) were the most potent suppressors (Fig. 1).

Conversion of PUFA to prostaglandins were found to be of little importance for the suppressive effects. Indomethacin had no effect in low doses (10 μM). At high doses (200 μM), indomethacin was found to suppress cell growth (results not shown). This is probably due to a direct toxicity. Furthermore, PGE_2 had no affect on Jurkat cell proliferation (unpublished observation).

The antioxidant α-tocopherol was able to restore part of the suppressive effects exerted by the FFA on Jurkat cell proliferation (Fig. 2). OA, a monounsaturated fatty acid, was found to have little effect on cell proliferation and generated low level of MDA-like products. In the presence of α-tocopherol, no peroxidation was detected with OA and the Jurkat cells were proliferating better than control cells. Hence, the inability of this monounsaturated fatty acid to produce marked suppressive effect like other PUFA could be due to the degree of peroxidation associated with this fatty acid. Other PUFA (n-3 and n-6) which generated less MDA-like products in the presence of α-tocopherol (similar to control levels) were unable to bring cell proliferation back to normal (Table 1 and Fig. 2). This was particularly evident for the n-3 PUFA supplemented cultures.

These results suggest that lipid peroxidation was only partly responsible. This differs from some previous findings (2,5) that formation of MDA-like products from PUFA were the major cause of cell growth suppression. Begin *et al.* (4) reported that DCHA with 6 double bonds was less effective in suppressing cell growth than those PUFA with 3, 4 and 5 double bonds, although DCHA is the most readily peroxidized PUFA. Our results showed that AA with 4 double bonds generated much more MDA-like products compared with EPA and DCHA (5 and 6 double bonds), although little difference was seen in their suppressive effects (Table 1 and Fig. 1). This suggests that increase in double bonds does not necessarily lead to an increase in lipid peroxidation within cells.

Furthermore, a general loss of plasmalogens was observed in Jurkat cells when cultured in medium supplemented with FFA. Previous studies by Snyder and Wood (25) and Howard *et al.* (26) showed that highly proliferative tumor and neoplastic cell lines seem to have a high level of plasmalogens, implicating a relationship between growth and the ether lipids. By preventing lipid peroxidation using α-tocopherol, the level of plasmalogens was restored (Table 1 and Fig. 4). This finding suggests that the reduction in plasmalogens is somewhat related to the increase in lipid peroxidation. This is in agreement with the findings of Yavin and Gatt (27) on the decrease

in plasmalogen level with increase in peroxidation. Recent work by Zoeller *et al.* (28) and Morand *et al.* (29) characterized this relationship further and proposed that plasmalogens may actually function as scavengers for reactive oxygen species under certain oxidative stresses, hence protecting other targets which are difficult to replace. Our observations that α-tocopherol prevents the loss of plasmalogens in Jurkat cells is in line with their hypothesis.

In summary, our results suggest that n-6 PUFA may exert its suppressive effects predominantly via lipid peroxidation while different mechanisms may be operating for the n-3 PUFA.

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Effects of Cholestyramine and Squalene Feeding on Hepatic and Serum Plant Sterols in the Rat

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Hepatic and serum phytosterol concentrations were compared in the rat under basal conditions and during activated cholesterol and bile acid production due to squalene and cholestyramine feeding. Both treatments consistently decreased hepatic and serum levels of sitosterol and campesterol and, unlike esterified cholesterol, esterified plant sterols were not increased in liver during squalene feeding. Serum levels of phytosterols were decreased quite proportionately to those in the liver. The hepatic levels of sitosterol and campesterol closely correlated with each other, but not with cholesterol levels. The percentage esterification of both phytosterols was lower than that of cholesterol. The results indicate that activation of hepatic sterol production leads to depletion of hepatic plant sterols. It is suggested that poor esterification of plant sterols may contribute to this decrease. *Lipids 24,* **705-708 (1989).**

Despite their poor absorption (5%) from the gastrointestinal tract (1,2), low concentrations of phytosterols (e.g., sitosterol and campesterol), are consistently found in most tissues and in serum of man. The serum concentrations depend on the absorption efficiency of dietary cholesterol (3-5). Absorbed phytosterols are excreted by the liver into the bile as such or even faster than cholesterol, as acidic sterols (2,6). Accordingly, biliary sterol secretion may regulate plant sterol concentration in the liver and possibly in serum.

Both cholestyramine treatment and squalene feeding increase hepatic cholesterol and bile acid production (7-9). Cholestyramine causes mainly enhanced bile acid elimination while squalene increases cholesterol output also. One might expect that plant sterol content is reduced in the liver in squalene-induced activation of hepatic cholesterol synthesis, which actually may enhance biliary cholesterol secretion-associated biliary plant sterol output (10). Because plant sterols are hardly converted to normal bile acids (6), newly-synthesized cholesterol may not compete with plant sterols as a substrate for bile acid formation during bile acid malabsorption. Cholestyramine treatment may affect plant sterol concentrations in the body through inhibition of sterol absorption (11,12). In order to test these hypotheses, we have compared the concentration of hepatic and serum phytosterols during cholestyramine and squalene induced increase in cholesterol production.

MATERIALS AND METHODS

Animals. Male rats of the Sprague-Dawley strain (Orion, Finland) were kept at a reverse lighting cycle. The rats were given ground chow (Hankkija, Finland). The same batch of chow was used throughout the study. The chow contained 44% (w/w) carbohydrate, 28% digestible protein, and 3.5% fat. Its contents of cholesterol, plant sterols, and squalene were 0.5 mg, 0.4 mg, and 0.008 mg/g, respectively. The sitosterol/campesterol ratio was 3:1 in the chow. In the squalene group, squalene was dissolved in olive oil so that the final concentrations in the chow were 1% (w/w) and 5%, respectively. The cholestyramine (5%, w/w, Lääkefarmos, Finland) and control groups received the same amount of olive oil without added squalene. Tap water was given *ad libitum* and the treatment period was five days. Food consumption and weight gain were kept similar and, except for loose stools in the cholestyramine group, no ill effects were observed during this short-term study. Both squalene feeding (9) and cholestyramine treatment (13) are known to increase fecal sterol output (especially bile acids) in the rat.

Analysis of lipids. Rats were decapitated in the middle of the dark period. After exsanguination under diethyl-ether anesthesia, serum was separated, the liver was removed, and weighed portions were homogenized in chloroform-methanol (2:1, v/v). The chloroform-methanol extracts of hepatic and serum samples (14) were analyzed for different sterols as described earlier (15). In brief, hepatic free and esterified cholesterol and plant sterols (sitosterol and campesterol) were separated by thin-layer chromatography (TLC). After saponification of the ester fraction, the nonsaponifiable material was rechromatographed for isolation of esterified sterols. The sterol fractions were quantitated as trimethylsilyl derivatives by gas-liquid chromatography (GLC) on a capillary column (SE-30) using 5α-cholestane as internal standard. In serum samples, total cholesterol, plant sterols, and cholestanol were quantitated by GLC.

Statistical methods. Statistical analyses were performed using a microcomputer and the Epistat statistical package. T-test and one-way analysis of variance were used to compare the groups, and Pearson's correlation coefficient was calculated to assess the relationships between sterol concentrations. P values of 0.05 or less were considered statistically significant.

RESULTS

Hepatic lipids. In the control animals, the degree of esterification of cholesterol was significantly higher than that of the two plant sterols campesterol and sitosterol. Hepatic concentration of free cholesterol remained unchanged by the treatment. However, esterified cholesterol was elevated three-fold by squalene feeding and decreased by 40% during cholestyramine treatment (Table 1). Total campesterol and sitosterol concentrations were decreased by 20 and 65% during the squalene and cholestyramine treatment, respectively. In the squalene group, this lowering was due to the decreased free plant sterols levels because, in contrast to the enhanced esterified cholesterol level, esterified plant sterol content

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Abbreviations: ACAT, acyl coenzyme A:cholesterol acyltransferase; campesterol (cholest-5-en-24 ξ-methyl-3β-ol); cholestanol (5α-cholestan-3β-ol); cholesterol (cholest-5-en-3β-ol); GLC, gas-liquid chromatography; sitosterol (cholest-5-en-24 ξ-ethyl-3β-ol); TLC, thin-layer chromatography.

TABLE 1

Concentrations of Cholesterol, Cholestanol and Plant Sterols in Liver and Serum of Rats on Different Diets

Lipid	Control n = 5	Squalene n = 5	Cholestyramine n = 5
		Liver (mg/g)	
Cholesterol			
Free	1.83 ± 0.03	1.90 ± 0.09	1.82 ± 0.05
Ester	0.18 ± 0.02	0.52 ± 0.10[a]	0.11 ± 0.02[a,b]
Ester %	9 ± 1	22 ± 4[a]	6 ± 1[a,b]
		μg/g (μg/mg cholesterol in parenthesis)	
Campesterol			
Free	47.54 ± 3.26 (25.91 ± 1.57)	37.05 ± 1.67[a] (19.73 ± 1.24)[a]	15.44 ± 0.50[a,b] (8.51 ± 0.07)[a,b]
Ester	1.95 ± 0.23 (11.28 ± 0.73)	3.12 ± 0.58 (5.92 ± 0.18?[a]	0.88 ± 0.13 (10.15 ± 2.48)
Ester %	4 ± 1[c]	8 ± 2[c]	5 ± 1
Sitosterol			
Free	45.67 ± 3.29 (24.90 ± 1.61)	35.05 ± 1.32[a] (18.71 ± 1.23)[a]	14.90 ± 0.70[a,b] (8.19 ± 0.16[a,b]
Ester	2.44 ± 0.24 (14.20 ± 1.07)	2.19 ± 0.15 (4.55 ± 0.47)[a]	1.73 ± 0.10[a,b] (17.87 ± 2.46)[b]
Ester %	5 ± 1[c]	6 ± 1[c]	10 ± 1[a,b,c]
		Serum (mg/dl)	
Total cholesterol	67.3 ± 2.0	63.4 ± 3.0	54.6 ± 2.6[a,b]
		μg/mg cholesterol	
Total campesterol	24.6 ± 1.4	18.1 ± 1.6[a]	9.5 ± 0.2[a,b]
Total sitosterol	30.3 ± 1.9[d]	21.2 ± 1.8[a]	11.3 ± 0.2[a,b,d]
Total cholestanol	3.1 ± 0.2	3.3 ± 0.2	1.8 ± 0.04[a,b]

Values are mean ± S.E.

[a] $p < 0.05$, or less vs controls.

[b] $p < 0.05$, or less vs squalene.

[c] $p < 0.05$, or less vs cholesterol.

[d] $p < 0.05$, or less vs campesterol.

remained at control levels. Thus, in terms of μg/mg cholesterol, plant sterols were also decreased in the ester fraction. In the cholestyramine group, both free and esterified plant sterol concentrations were decreased, but in relation to cholesterol the esterified plant sterol fractions were unchanged (Table 1).

Serum sterols. Serum cholesterol levels were decreased by cholestyramine treatment, but were unchanged in the squalene group. Total campesterol and sitosterol concentrations were significantly decreased by both treatments, even in relation to cholesterol (Table 1). The values fell to almost one third in the cholestyramine group. Analysis of one serum sample per group, using TLC to separate esterified sterols (data not shown), suggested that both free and especially esterified plant sterols were decreased to a similar extent. Cholestanol levels were decreased by cholestyramine treatment, but were unaffected by squalene feeding (Table 1).

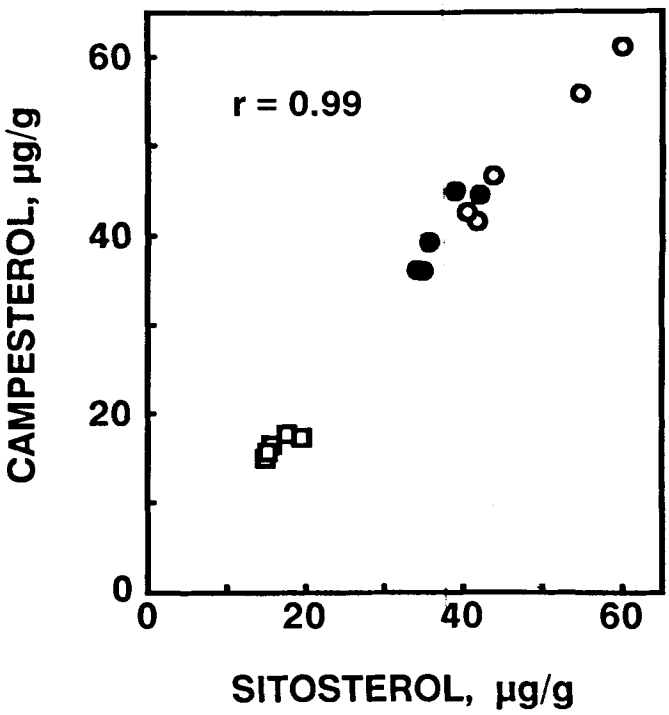

FIG. 1. Relationships between the concentrations of hepatic phytosterols. (○) Control, (●) squalene, (□) cholestyramine group.

Relationships between serum and liver. Absolute concentrations of sterols were used in these calculations. The hepatic levels of campesterol and sitosterol closely correlated with each other (r = 0.99) (Fig. 1), but not with the levels of cholesterol (r = 0.32, N.S.). The hepatic and serum concentrations of sitosterol (r = 0.96), and also those of campesterol (r = 0.94), correlated positively (Fig. 2). In serum, the concentrations of sitosterol were constantly higher than those of campesterol, whereas in the liver, their concentrations were similar.

DISCUSSION

This study shows a close correlation of phytosterols in serum and liver during various manipulations of cholesterol synthesis. We also show an ability of rat liver to diminish plant sterol content during both a positive and a negative cholesterol balance. Unlike studies carried out in man (3,5,16) and in primates (17), and consistent with data by Kuksis *et al.* (18), the levels of serum sitosterol were constantly higher than those of campesterol. However, the hepatic concentrations of these two phytosterols were similar. They also show different distributions between the microsomes and the cytosol of rat liver (19). Thus, preferential uptake of campesterol from serum is possible or, under the present experimental conditions, campesterol is secreted less efficiently into bile. Intestinal absorption studies have shown that campesterol is absorbed more readily than sitosterol in different species, including the rat (20). Thus, these two plant sterols may be handled in a reciprocal fashion in the intestine and liver of the rat. It is known that plant sterol levels in the body depend on the efficiency of sterol absorption from the gut (3), but also on the sterol excretion into the bile (2,10). The treatments

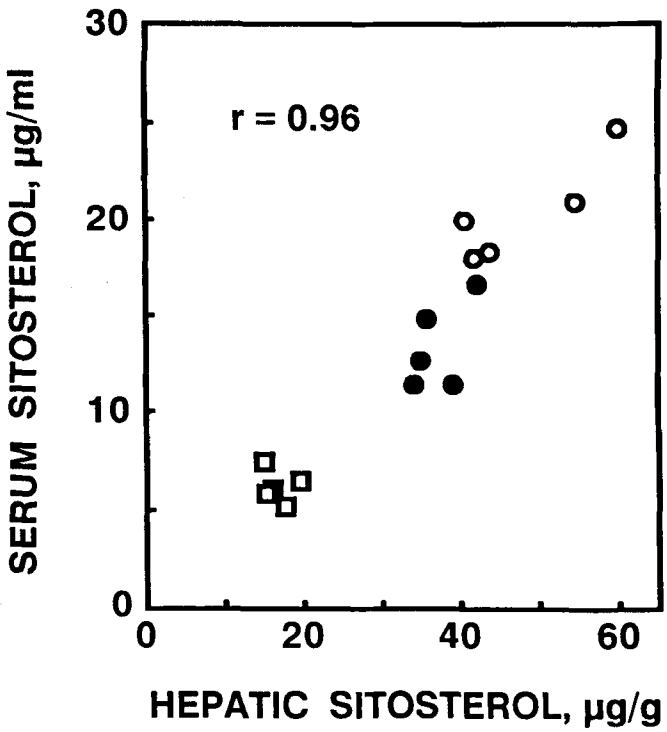

FIG. 2. Relationships between the concentrations of serum and hepatic sitosterol. (○) Control, (●) squalene, (□) cholestyramine group.

used in the present study may affect these mechanisms quite differently.

Cholestyramine interferes with the absorption of bile acids and cholesterol (11,12,21), and increases their production in the liver. If conversion of plant sterols to bile acids is also enhanced by cholestyramine, this and an inhibition of phytosterol absorption could partly explain the lowered serum and liver concentrations. Although esterified cholesterol was clearly diminished, the activity of ACAT does not seem to be affected by cholestyramine (22).

Ileal exclusion (also causing a bile acid malabsorption) used in the treatment of hypercholesterolemia has been reported to increase serum plant sterol content (15,16). This seems to contradict the present results on cholestyramine. One explanation is that unlike cholestyramine administered with food (as in the present study) (12), ileal by-pass does not significantly reduce either cholesterol or plant sterol absorption (16). Furthermore, it is unclear whether cholestyramine interferes more with plant sterol than with cholesterol absorption.

The effects of dietary squalene on cholesterol absorption are unknown thus far. In the liver of the rat, squalene feeding stimulates cholesterol production and the activity of ACAT (9,23), and it increases hepatic cholesterol ester content three-fold. Fecal secretion of sterols and of bile acids, in particular, is increased (9). Esterified hepatic plant sterol concentrations, however, remained at control levels during squalene feeding, probably reflecting low plant sterol content and the fact that phytosterols are poor substrates of ACAT in the liver (24) and intestine (25).

There is evidence that hepatic sterol output in bile vs sterol secretion in lipoproteins are related in a reciprocal manner and that ACAT would have a central role in these processes (26). Thus, the reduction of hepatic plant sterol levels during both cholestyramine and squalene feeding could be explained by the limited esterification and, consequently, better accessibility to biliary secretion (26). Differences of esterification could also explain the different levels of serum and hepatic campesterol and sitosterol. In the intestine (20) and liver (24), campesterol is esterified more efficiently than is sitosterol. In accordance, campesterol uptake is significantly higher than uptake of sitosterol (20). Lower excretion from liver might explain the present results.

In addition, serum cholestanol levels were lowered by cholestyramine treatment only (albeit relatively less than phytosterols). Cholestanol is quite efficiently esterified by ACAT (24), and thus would not be as amenable to biliary secretion as plant sterols during squalene feeding would be. Unfortunately, we were not able to analyze hepatic levels of cholestanol and the interpretation of the mechanisms by serum levels alone has its limitations.

As mentioned, the greater reduction of phytosterols during cholestyramine treatment might be partly explained by reduced absorption from the gut and/or efficient conversion to bile acids. Like ACAT, 7-α-hydroxylase can apparently distinguish between plant sterols and cholesterol (27), but substantial excretion of plant sterols as acidic sterols—20% of total in man (2)—suggests that their side chains are possibly modified before oxidation (2). The significance of bile acid formation regulating hepatic plant sterol levels may be indirectly supported by the finding of increased hepatic plant sterols during treatment with ketoconazole (28), which inhibits P450-dependent enzymes. This finding is further emphasized if not only cholesterol (29), but also plant sterol absorption, were reduced by ketoconazole.

In conclusion, the present short-term study showed the lowering of plant sterols during increased cholesterol and bile acid production induced by two different mechanisms. Whether poor esterification of plant sterols is a contributing factor may warrant further studies, especially as deranged esterification of sterols has recently been suggested to be a pathogenetic mechanism in the lipid storage disease sitosterolemia and xanthomatosis (30).

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Effect of Hyperpnea on Enzymes of the CDPcholine Path for Phosphatidylcholine Synthesis in Rat Lung

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We have examined the activity of three enzymes in pulmonary surfactant phosphatidylcholine synthesis following the hyperpnea induced by having rats either inspire 5%CO_2/13%O_2/82%N_2 for 24 hr or swim in thermoneutral water for 30 min. Both stimuli markedly increase frequency and tidal volume of breathing and promote the release of surfactant. Lungs were perfused to remove blood, lavaged, and then homogenized in 1 mM Hepes, 0.15M KCl at pH 7.0. The homogenate was centrifuged at 9,000 g (av) for 10 min to sediment the mitochondria and lamellar bodies and at 100,000 g (av) for 60 min to obtain the microsomal and cytosol fractions. Incubations were carried out under determined optimal conditions and zero order kinetics. Choline kinase (CK), cholinephosphate cytidylyltransferase (CP-cyT) and choline phosphotransferase (CPT) were assayed by the incorporation of [*methyl*-^{14}C]choline chloride into phosphocholine, [*methyl*-^{14}C]phosphocholine into CDPcholine, and [^{14}C]CDPcholine into phosphatidylcholine, respectively. The incubation products were separated by thin-layer chromatography. Whereas both forms of hyperpnea increased the activity of CP-cyT in the microsomal fraction, they had no effect on the activity of either cytosolic CP-cyT and CK, or microsomal CPT. A similar increase in tidal volume in an isolated perfused rat lung had no effect. We conclude that, *in vivo,* hyperpnea increases the activity of CP-cyT, the rate-limiting enzyme in phosphatidylcholine synthesis. Whether this is due to an increase in the amount of enzyme, or of a cofactor, is unknown.
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The gas-liquid interface in pulmonary alveoli is lined with a complex mixture of lipids and proteins known as surfactant. This specialized lining is thought to stabilize alveoli by both reducing surface tension and allowing surface tension to vary directly with the radius of curvature. Surfactant is synthesized in the endoplasmic reticulum of the alveolar type II cell and stored in organelles known as lamellar bodies. In response to a number of stimuli, these are released into the alveolar hypophase, where the surfactant forms a monolayer at the interface.

The time-constant of turnover of alveolar surfactant is very short and, in rats, is of the order of only a few hours (1). However, the absolute rate of synthesis is unknown, and we have little insight into the relative contributions of recycled and newly synthesized surfactant to that which is subsequently released. The picture is further complicated in that surfactant is a complex mixture of phospholipids, neutral lipids and specific proteins; hence, full understanding of control of synthesis must involve knowledge of all three. At present most of our knowledge is of the phospholipids, in particular, phosphatidylcholine (PC) and more specifically dipalmitoyl phosphatidylcholine (DPPC).

Convincing evidence exists that cholinephosphate cytidylyltransferase (CP-cyT) is the rate-limiting enzyme in surfactant PC synthesis in the fetus. Much work has demonstrated the ability of glucocorticoids (2,3), thyroxine (4), and 17β-estradiol (5,6) to enhance the activity of CP-cyT in the fetal lung and to increase the incorporation of choline into PC. However, very little is known of the physiological control of synthesis in the adult lung. We have previously shown that when rats inspire 5%CO_2/13%O_2/82%N_2 for 24 hr, tidal volume (Vt) and frequency of breathing (f) almost double, and there are marked increases in the amount of PC in the lamellar bodies and alveoli (7). If, after 24 hr exposure, the rats are infused with [^{3}H]choline and the exposure continued, the subsequent phospholipid specific activity-time curve for the lamellar body fraction broadens markedly, but with no apparent increase in peak specific activity above control; there is a marked increase in the amount of radioactivity present in the alveolar phospholipid fraction (8). Swimming also increases alveolar phospholipids in rats, but in contrast, there is a concomitant decrease in the phospholipid content of the lamellar body fraction with this acute stimulus (7). Swimming appears to shift the specific activity-time curve for lamellar body DPPC to the left. Swimming increases Vt and f by approximately 250 and 60%, respectively.

Our finding that 24 hr exposure to the hyperpea-inducing gas mixture increased the total radiolabeling and the amount of DPPC in both the lamellar body and microsomal fractions is consistent with an increase in synthesis of DPPC. Likewise, the change with swimming of the specific activity-time curve of DPPC in the lamellar body fraction (7) is consistent with an increase in synthesis. In the present experiments we have further explored the possible effects of hyperpnea on synthesis by investigating the activity of the three enzymes in the CDPcholine or Kennedy pathway for the *de novo* synthesis of pulmonary PC: choline kinase (CK) (EC 2.7.1.32), CP-cyT (EC 2.7.7.15) and cholinephosphotransferase (CPT) (EC 2.7.8.2), following either 30 min swimming or exposure to the above gas mixture for 24 hr.

MATERIALS AND METHODS

Swimming experiments. Male Porton rats (210-260 g) were placed in a thermoneutral (33 ± 1°C) water bath (60 × 60 × 48 cm) filled to a depth of 37 cm (9). The rats were allowed to swim for 30 min, after which they were removed, immediately injected with intraperitoneal methohexital sodium (100 mg·kg^{-1}), and placed in a warm cage until heavily anesthetized (9).

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Abbreviations: CK, choline kinase; CP-cyT, cholinephosphate cytidylyltransferase; CPT, choline phosphotransferase; DMA, dimethyllacetal; DPPC, dipalmitoyl phosphatidylcholine; EDTA, ethylenediaminetetraacetic; f, frequency of breathing; PC, phosphatidylcholine; Vt, tidal volume.

Gas-hyperpnea experiments. Rats were placed in a Plexiglas® chamber (45 × 29 × 15 cm) divided into six compartments by coarse wire mesh, through which humidified gas (5%CO_2/13%O_2/82%N_2) was flushed at 5 l/min. This gas mixture approximately doubles Vt and f (7). A parallel series of control rats were placed in an identical chamber, through which humidified medical grade air was circulated. The chambers were maintained at 22°C (7,8). After 24 hr the rats were removed from the chamber and heavily anesthetized, as above.

In all cases a tracheal catheter was then inserted and the lung ventilated at 60 breaths/min with air (Vt: 2.5 ml; end expired pressure 2 cm H_2O). The lungs were perfused via the main pulmonary artery with the plasma substitute Hemaccel (Behring Institute, Marburg, FRG) for about 3 min to flush blood from the vascular bed, they were then removed as previously described (9), degassed for 1 min at 0.5 atmospheres and lavaged at 2°C with 10 ml of 0.15 M saline, each volume being instilled and withdrawn three times.

Preparation of microsomal and cytosolic fractions. The lungs were finely minced with scissors and homogenized in 2 ml of buffer (1 mM Hepes, 0.15 M KCl at pH 7.0) at 2°C with an Ultra-Turrax (Model TP18-10, Staufen im Breisgau, FRG). The homogenate was centrifuged at 1,000 g (av) for 5 min at 4°C to pellet nuclei and cellular debris, and the supernatant was centrifuged at 9,000 g (av) for 10 min at 4°C to sediment the mitochondrial fraction. Finally, the supernatant was centrifuged at 100,000 g (av) for 60 min at 4°C in a Beckman SW27.1 rotor and a Beckman L5-50 ultracentrifuge to obtain the microsome and cytosol fractions (10). The microsomal pellet was resuspended in 1 ml of buffer and both fractions were assayed for protein using the Bradford method (11).

Purity of fractions. The microsome and cytosol fractions were assayed for β-glycerophosphatase in order to check for contamination with lamellar bodies (12). Lamellar bodies are particularly rich in this lysosomal enzyme (13).

Isolated perfused lung was prepared as previously described (14). Briefly, the lung was ventilated with positive pressure at 60 breaths/min, with Vt of 2.5 ml and end expired pressure of 2 cm H_2O, and reperfused at 10 ml/min with Krebs bicarbonate containing 4.5% bovine serum albumin through a 50 ml reservoir. The lung was hyperventilated by increasing the peak inspired pressure from 10 cm to 20 cm H_2O (approximately a doubling of Vt), and decreasing the end expired pressure from 2 to 0 cm H_2O for 30 min.

Analysis of fractions. Lipids were extracted from the microsome and cytosol fractions by the method of Bligh and Dyer (15), and the phosphorus content of each fraction measured by the method of Bartlett (16). The disaturated phospholipids were extracted by the method of Mason *et al.* (17). Phosphatidylglycerol was separated from the other phospholipids by thin-layer chromatography on Whatman LK5D silica gel plates using the solvent system of Touchstone *et al.* (18).

Choline kinase was assayed by a modification of the method of Ulane *et al.* (19). The incubations were carried out under determined optimal conditions, which were as follows: Tris Cl (133 mM) pH 8.5, dithiothreitol (2 mM), $MgCl_2$ (10 mM), ATP (20 mM). [*methyl*-^{14}C]choline chloride (15 mM) (0.5 μCi/μmol), with 50–100 μg protein (total volume 60 μl); samples were incubated at 37°C for 30 min. In the experiments designed to characterize the enzyme, the following variables and ranges were examined: protein concentration (50–120 μg/60 μl), period of incubation (10–40 min), choline chloride (2–20 mM) and $MgCl_2$ (5–40 mM). The effects of the pH (6–9) and ATP (5–30 mM) were also investigated. The reaction was started by adding the enzyme preparation and stopped by placing the tubes in boiling water for 5 min. Blank tubes were placed in the boiling water immediately after the addition of the enzyme. The tubes were then centrifuged in a Biofuge A (Heraeus-Christ, FRG) at 13,000 g (av) for 5 min, and the reaction products separated on Whatman LK5D silica gel thin-layer chromatography plates, using 0.9% NaCl:methanol:conc. NH_3 (v/v/v, 25/25/2.5). Unlabeled phosphocholine was spotted as a carrier and detected under iodine vapor. The silica gel spots were scraped directly into counting vials, 500 μl of water added, and then 10 ml of ACS (Amersham Australia, Sydney). Radioactivity was measured in a Searle Nuclear Chicago Mark III Scintillation Counter (Illinois).

Cholinephosphate cytidylyltransferase was assayed according to a modification of the method of Radika and Possmayer (20). The incubations were carried out under determined optimal conditions and the final mixture, with a total volume of 100 μl, was as follows: Hepes buffer (50 mM) pH 7.0, dithiothreitol (3 mM), EDTA (1 mM), CTP (5 mM), and 50–100 μg protein. In the case of the cytosolic fraction: magnesium acetate (6 mM) and [*methyl*-^{14}C]phosphocholine (2 mM) (1 μCi/μmol) were used. In the case of the microsomal fraction: magnesium acetate (18 mM) and [*methyl*-^{14}C]phosphocholine (1 mM) (1 μCi/μmol) were used. Incubations were carried out at 37°C for 20 min. In experiments designed to characterize the enzyme, the following variables and ranges were examined: protein concentration (40–200 μg/100 μl), time of incubation (10–60 min), phosphocholine concentration (0.2–2.0 mM) and magnesium acetate (1.5–24 mM). We also investigated the effect of varying pH (6–9) and CTP concentration (2–15 mM). The reaction was started and stopped as for CK, and the samples treated in the same way except that the unlabeled carrier in this case was CDPcholine.

Choline phosphotransferase was assayed by a modification of the method of Miller and Weinhold (21). The incubations were carried out under determined optimal conditions; the final reaction mixture, in a total volume of 100 μl, was as follows: Tris buffer (50 mM) pH 8.2, Tween 20 0.1 mg/ml, 1,2-dioleoylglycerol (1.0 mM), phosphatidylglycerol (0.8 mM), dithiothreitol (5 mM), EDTA (5 mM), $MgCl_2$ (10 mM), CDP[*methyl*-^{14}C]choline (0.5 mM) (0.5 μCi/μmol); microsomal preparation 50–100 μg protein. Incubations were carried out at 37°C for 5 min. The first four components were sonicated (B12, Branson Sonic Power Company, Danbury, CT) together for 3 min on ice. In experiments designed to characterize the enzyme, the following variables and ranges were investigated: protein (25–160 μg/100 μl), time of incubation (10–30 min), CDPcholine (0.1–0.8 mM) and $MgCl_2$ (2–10 mM). We also investigated pH (6–9) and the concentration of 1,2-dioleoylglycerol (0.2–2.0 mM). In these latter experiments the ratio of dioleoylglycerol:phosphatidylglycerol was kept constant at (5:4). The reaction was

stopped by the addition of 3 ml of methanol:chloroform (2:1) and the extraction of PC was completed by the addition of 1.0 ml chloroform and 1.7 ml water. The two phases were separated by centrifugation and the lower chloroform phase was washed once with 2.0 ml of methanol:water (1:1). The chloroform extract was evaporated to dryness and an aliquot spotted on Whatman LK5D silica gel thin-layer chromatography plates; the developing solvent system was chloroform:ethanol:triethylamine:water (30/34/35/8) (18). The plate was exposed to iodine vapor after development and the spot scraped into a scintillation vial, 500 μl of water added and then 10 ml of ACS.

When testing any of the different conditions (e.g., the effect of hyperpnea) the incubations, extractions, and chromatography for the test and control groups were performed at the same time.

Fatty acid analysis was performed by first preparing the methyl esters by transmethylation in 1% (vol/vol) H_2SO_4 in methanol. The esters were then analyzed by gas-liquid chromatography using twin columns of SP2310 (Supelco Inc., PA) in a Hewlett Packard 5840A gas chromatograph programmed for 125-225°C at 4°C/min.

Chemicals. [*Methyl*-^{14}C]choline chloride (specific activity 60 mCi/mmol^{-1}), cytidine 5′diphospho[*methyl*-^{14}C]-choline (specific activity: 60 mCi/mmol^{-1} and [*methyl*-^{14}C]phosphocholine (specific activity: 60 mCi/mmol^{-1}) were obtained from Amersham Australia (Sydney).

Statistical analysis. In each case the test group was compared with its immediate control group using a Student's t-test. Comparisons were not made between different test or different control groups, as experiments were often carried out months apart.

RESULTS

Purity of fractions. In order to assess possible contamination of our fractions by lamellar bodies, we assayed β-glycerophosphatase, the results were as follows: cytosol fraction 1.86 ± 0.16 (5 lungs), microsomal fraction 7.17 ± 0.63 (4 lungs), lamellar body fraction 147.6 ± 9.82 (5 lungs), expressed as mean ± S.E.M. in nmol/min per mg of protein.

Effect of swimming-induced hyperpnea. Swimming rats for 30 min did not affect the activity of cytosolic CK or CP-cyT, or of microsomal CPT (Tables 1 and 2). In contrast, there was a 66% increase in the activity of CP-cyT in the microsomal fraction (Table 1). Two groups of controls were used in these experiments. The first group remained in the cage for the full period, whereas the second group was immersed in thermoneutral water sufficiently shallow to permit them to stand on the bottom. There was no difference in the activity of CK or CP-cyT between these two groups (Tables 1 and 2).

Effect of gas-induced hyperpnea. Exposing rats to 5%CO_2/13%O_2/82%N_2 for 24 hr did not affect the activity of either cytosolic CK and CP-cyT, or microsomal CPT (Tables 1 and 2). However, there was a 28% increase in activity of microsomal CP-cyT. There was no difference in the activity of CK or CP-cyT in the lungs of control rats left in the normal rat cage from those in the six compartment chamber exposed to medical grade air.

Effect of added phospholipids. Either phosphatidylglycerol or lysophosphatidylcholine were sonicated in 1 ml of 50 mM Hepes buffer at pH 7.0 for 30 sec to a final

TABLE 1

The Effects of Physiologic Maneuvers on the Activity of Cholinephosphate Cytidylyltransferase in the Adult Rat Lung

Treatment	Cholinephosphate cytidylytransferase	
	Microsome	Cytosol
Control[a]	6.48 ± 0.31 (5)	1.80 ± 0.15 (6)
Shallow water[b]	6.41 ± 0.35 (6)	1.65 ± 0.15 (6)
Control[a]	4.47 ± 0.36 (5)	2.67 ± 0.11 (5)
Swim[c]	7.40 ± 0.65 (5)[j]	3.05 ± 0.26 (6)
Control[a]	4.94 ± 0.28 (5)	1.70 ± 0.14 (6)
Chamber-air[d]	5.32 ± 0.14 (5)	1.77 ± 0.08 (6)
Chamber-air[d]	3.85 ± 0.22 (8)	1.49 ± 0.11 (6)
Chamber-gas[e]	4.95 ± 0.30 (9)[i]	1.48 ± 0.14 (6)
Control	5.18 ± 0.32 (5)	1.30 ± 0.07 (6)
+ phosphatidylglycerol[f]	5.20 ± 0.37 (5)	2.02 ± 0.11 (6)[k]
Control	6.34 ± 0.41 (5)	2.36 ± 0.25 (5)
+ lysophosphatidylcholine[g]	4.97 ± 0.28 (5)[h]	2.21 ± 0.15 (6)

Results expressed as mean ± S.E.M. in nmol/min per mg of protein. Number of lungs in parentheses. The lung was lavaged, perfused and homogenized, and then centrifuged at 1,000 g (av) × 5 min to pellet nuclei and cellular debris, then at 9,000 g (av) × 10 min to pellet the mitochondrial and lamellar body fractions, and finally at 100,000 g (av) × 60 min to separate the microsome and cytosol fractions. The following incubations were carried out under optimal conditions and zero order kinetics.

[a] Rats left in normal rat cage.

[b] Rats in thermoneutral water sufficiently shallow so that they can stand on the bottom with their head above the surface.

[c] Rats allowed to swim in thermoneutral water for 30 min.

[d] Rats placed in six compartment chamber through which humidified medical grade air was passed.

[e] Rats placed in six compartment chamber through which humidified 5%CO_2/13%O_2/82%N_2 was passed.

[f] 0.3 mM phosphatidylglycerol present in the incubate.

[g] 0.3 mM lysophosphatidylcholine present in the incubate.

[h] $p < 0.05$.

[i] $p < 0.025$.

[j] $p < 0.01$.

[k] $p < 0.001$.

TABLE 2

The Effects of Physiologic Maneuvers on the Activity of Choline Kinase and Cholinephosphotransferase in the Adult Rat Lung

Treatment	Choline kinase	Cholinephosphotransferase
	Cytosol	Microsome
Control[a]	4.39 ± 0.21 (5)	
Shallow water[b]	4.00 ± 0.17 (5)	
Control[a]	5.15 ± 0.35 (5)	
Swim[c]	5.47 ± 0.33 (6)	
Shallow water[b]		1.54 ± 0.09 (9)
Swim[c]		1.25 ± 0.09 (9)
Control[a]	4.74 ± 0.17 (5)	
Chamber-air[d]	4.40 ± 0.09 (5)	
Chamber-air[d]	5.40 ± 0.25 (12)	1.37 ± 0.12 (7)
Chamber-gas[e]	5.10 ± 0.25 (11)	1.51 ± 0.17 (7)

Results expressed as mean ± S.E.M. in nmol/min per mg protein. Number of lungs in parentheses. Legend as for Table 1.

concentration of 0.3 mM in the incubation mixture. Whereas phosphatidylglycerol had no effect on the activity of microsomal CP-cyT, it increased the activity of cytosolic CP-cyT by 77% ($p < 0.001$) (Table 1). In contrast, whereas lysophosphatidylcholine had no effect on the CP-cyT activity when added to the cytosolic fraction, it depressed the activity when added to the microsomal fraction (Table 1) ($p < 0.05$).

Effect of hyperventilating the isolated perfused lung. Hyperventilating the isolated perfused lung for 30 min had no effect on the activity of CP-cyT in either the microsome (control: 5.71 ± 0.45, 5 rats; hyperventilated: 4.61 ± 0.31, 5 rats, mean ± S.E.M. in nmol/min per mg of protein) or the cytosol fraction (control: 1.16 ± 0.09, 6 rats; hyperventilated: 1.34 ± 0.19, 6 rats). Likewise, it had no effect on the activity of CK (control: 4.31 ± 0.34, 6 rats; hyperventilated: 4.15 ± 0.31, 6 rats).

Phospholipid content of fractions. Whereas exposure to gas elevated the phospholipid content of the cytosol fraction, the phospholipid content of the microsomal fraction was not significantly altered (Table 3). Thirty minutes swimming had no effect on the phospholipid content of either fraction. The ratio of DPPC to phosphatidylglycerol in the microsomal fraction was not affected by either swimming (Experiment 1) or exposure to gas (Experiment 2): (mean ± S.E.M.).

Experiment 1: control 4.83 ± 0.32 (4), swim 4.72 ± 0.60 (4).
Experiment 2: control 7.09 ± 0.74 (4), gas 6.16 ± 0.33 (5).

Hence, neither stimulus altered the concentration of phosphatidylglycerol in the microsomal fraction.

Fatty acid content of the microsome and cytosol fractions. Inducing hyperpnea by swimming rats for 30 min did not affect the fatty acid composition of either the microsome or the cytosol fraction (Table 4).

TABLE 3

The Total Phospholipid Content of Cytosol and Microsome Fractions in the Adult Rat Lung Following Hyperpnea

Treatment	Cytosol	Microsome
Control	59.0 ± 5.01 (5)	1445 ± 76 (6)
Swim	77.5 ± 5.95 (4)	1432 ± 69 (6)
Control	58.6 ± 3.06 (6)	1432 ± 52 (4)
Gas	73.1 ± 3.85 (5)[a]	1455 ± 100 (6)

Results are expressed as mean ± S.E.M. in μg phospholipid/ml fraction. Number of lungs in parentheses.
[a] $p < 0.05$.

Protein content of fractions. Inducing hyperpnea by either swimming rats or by exposing them to 5%CO_2/13%O_2/82%N_2 had no effect on the protein content of the microsomal fraction (Table 5). Likewise, hyperventilating the isolated perfused rat lung had no effect on the protein content of the microsomal fraction (Table 5).

DISCUSSION

We have shown that hyperpnea induced in rats by either swimming or by exposing them to 5%CO_2/13%O_2/82%N_2 resulted in a significant increase in activity of microsomal CP-cyT, the rate-limiting enzyme in pulmonary surfactant PC synthesis. In the former case this increase was apparent within 30 min.

Validation of methods. CP-cyT is a notoriously difficult enzyme to assay, with considerable variation apparent within assays, as evidence by the large standard errors quoted (20,22). We found that part of this variation was

TABLE 4

The Effect of 30 Minute Swimming in Thermoneutral Water on the Fatty Acids of the Total Lipid Fraction Extracted from the Microsomal and Cytosol Fractions Prepared from Rat Lung

	Microsomes		Cytosol	
	Controls	Swimming	Controls	Swimming
14:0	1.89 ± .09	1.72 ± .09	2.46 ± .11	2.21 ± .20
DMA16:0	2.55 ± .07	2.70 ± .15	0.49 ± .15	0.64 ± .15
16:0	31.80 ± .38	30.41 ± .38	32.25 ± 2.98	31.44 ± .93
16:1ω9	2.26 ± .11	2.06 ± .14	1.72 ± .99	1.65 ± .50
16:1ω7	2.96 ± .35	3.47 ± .45	5.57 ± 1.45	6.38 ± 1.55
DMA18:0	1.55 ± .08	1.54 ± .15	0.26 ± .08	0.34 ± .12
18:1ω9	17.95 ± .76	18.40 ± .97	30.66 ± 4.43	29.69 ± 2.61
18:1ω7	1.94 ± .07	2.06 ± .12	2.19 ± .27	2.29 ± .30
20:4ω6	10.42 ± .58	10.25 ± .47	3.45 ± 1.31	4.62 ± 1.66
22:4ω6	1.83 ± .12	1.92 ± .17	0.46 ± .14	0.74 ± .36
22:6ω3	1.88 ± .08	1.85 ± .07	0.80 ± .31	0.91 ± .16
Total saturated	49.59 ± .34	48.46 ± .27	42.56 ± 1.64	42.01 ± .31
Total monoenoic	26.50 ± .97	27.44 ± 1.29	41.35 ± 2.50	41.10 ± 1.72
Number of rats	4	4	4	4

Values are mean ± SEM. The values are expressed as percentages of the total amount of fatty acids present. However, in most cases only those comprising more than 1.5% are included in the table. DMA refers to the dimethylacetal derivatives.

TABLE 5

The Protein Content of the Microsomal Fraction of the Adult Rat Lung Following Hyperpnea

Treatment	
In vivo	
Control	4.99 ± 0.28
Swim	4.94 ± 0.49
Control	5.13 ± 0.54
Shallow water	5.42 ± 0.27
Control	5.36 ± 0.30
Gas	5.17 ± 0.24
Control	5.46 ± 0.29
Air chamber	5.58 ± 0.29
In vitro	
Control	5.53 ± 0.47
Hyperventilated	6.31 ± 0.38

Results expressed as mean ± SEM in mg protein/ml. In each group there were six lungs. The *in vitro* results were obtained using the isolated perfused lung preparation of the rat. Other details as described in the legend for Table 1.

attributable to differences between different batches of [*methyl*-^{14}C]phosphocholine, and between batches from different companies. In order to test this we performed assays using identical reagents but with different batches of [^{14}C]phosphocholine. In light of this finding we subsequently completed the entire assay for any one experiment, including all test and control samples, on the same day, under the same conditions and with the same reagents. As different experiments were sometimes conducted months apart, it is not valid to compare their control groups. For example, controls used for the addition of lysophosphatidylcholine differ from those involving air exposure in the chamber (Table 1). In all cases we have only compared the test results with their immediate controls, and for this reason we have used a Student's t-test rather than an analysis of variance.

Whereas the activities of CPT and CP-cyT were similar to those reported by others, our activity of CK was 5 to 10 times higher. The apparent difference was that we used a predetermined optimal concentration of 15 mM choline, which was 50 times higher than that commonly used. In fact, the only other group to use a comparable concentration (5 mM) reported a similar CP-cyT activity in primate lung (19).

We have expressed our results as per mg of protein, hence any change in the recovery of protein from our fractions would influence the results. In fact, we found no significant difference between test and control groups in the amount of protein recovered from our microsomal fraction.

Surfactant-type phospholipids, in particular phosphatidylglycerol and lysophosphatidylcholine, affect activity of CP-cyT (23). As hyperpnea markedly increases the amount of alveolar surfactant, and hence the amounts of both phosphatidylglycerol and lysophosphatidylcholine that could associate with CP-cyT during subsequent processing, it was particularly important that we lavaged the lungs. We have previously shown that by lavaging the lungs with three separate volumes of 0.15 M NaCl, each volume instilled and withdrawn three times, we remove over 90% of the surfactant phospholipids that could be removed by eight such volumes (24). Based on previous results when we hyperventilated the isolated perfused lung at different temperatures (24), we think it very unlikely that we would release lamellar bodies by lavaging the lungs at 2°C. We removed the lamellar body fraction, another rich source of phosphatidylglycerol, during the preparation of the microsome and cytosol fractions. Subsequently we found that the activity of β-glycerophosphatase, an enzyme which is abundant in the lamellar body fraction (13), was only present in low amounts in our microsome and cytosol fractions, suggesting minimal contamination. In fact, we found no change in either the phospholipid content or the DPPC/phosphatidylglycerol ratio of the microsome fraction on exposure to gas or swimming. Phosphatidylglycerol was still capable of enhancing the activity of our cytosolic CP-cyT. Feldman *et al.* (25) had reported that lavaging adult rabbit lungs led to a reduction in the proportion of CP-cyT in the high molecular weight-form. Our results are consistent with those of Chu and Rooney (23) who found that phosphatidylglycerol markedly enhanced activity in adult rabbit lung if the cytosol was first delipidated with acetone/butanol. Our present results are also consistent with those of Choy and Vance (26) who found that lysophosphatidylcholine inhibited CP-cyT activity in rat liver.

The actual stress of the rat being submerged up to the neck in thermoneutral water did not affect the activities of the enzymes. This is consistent with our previous finding that it also did not alter in the amount of phospholipid that could be lavaged from the alveolar compartment (9).

The mechanism of the increases in activity of CP-cyT with hyperpnea is unclear. Feldman *et al.* (27) reported that CP-cyT in fetal rat lung exists in two cytosolic forms: one of low molecular weight (L-form: 190kD) and relatively inactive, and the other of high molecular weight (H-form: 5,000–50,000kD). The addition of fatty acids or phosphatidylglycerol results in a polymerisation or aggregation of the L-form, with possible enhanced binding to the microsomes. This results in the formation of the active H-form of the enzyme. In fact, in adult rat lung, 70–80% of the enzyme activity in the cytosol is already in the H-form. As the overall activity falls and the H:L ratio of activities reverses following lung lavage (24), the enzyme may have been binding to some component of alveolar surfactant, such as phosphatidylglycerol. Although the present increase in activity of CP-cyT in the microsome fraction could reflect such a translocation, there was no concomitant fall in activity in the cytosol, and there was no increase in the phosphatidylglycerol content of the microsomal fraction.

Another possible explanation for the increase in activity of microsomal CP-cyT is an increase in free fatty acids in that fraction. Weinhold *et al.* (28) had reported that a number of saturated and unsaturated fatty acids both promoted translocation of inactive forms of CP-cyT to microsomes and stimulated the activity of the microsomal bound enzyme. We could find no change in the fatty acid composition of either the microsome or cytosol fractions following 30 min swimming. In order to further clarify the mechanism of increase of CP-cyT activity in the microsomes, it will be necessary to repeat the experients, but with optimal concentrations of lipids in the incubate.

Given the effects of phospholipids and free fatty acids on the activity of CP-cyT, one approach to these experiments would be to delipidate, and then add predetermined amounts of these factors. However, delipidation would tend to denature the enzymes, making quantitative results difficult. A further point is that we are concerned with surfactant-associated phospholipids, and hence the alveolar type II cell. By homogenizing whole lung, we are harvesting from as many as forty different cell types. A better way would be to isolate the alveolar type II cells and then prepare the fractions. The potential drawback of this approach is that the effects of previous physiologic maneuvers might be lost during processing.

The increase in activity of CP-cyT in the present experiments is modest and gives no indication of the rate of synthesis of PC. In fact, the increase may merely be compensating for a decrease in one of the essential cofactors or substrates of the CDP-choline pathway which may also occur with hyperpnea. However, we suggest that when the present results are considered together with our previous findings showing increases in PC in lamellar body and alveolar compartments with gas hyperpnea, and changes in incorporation of [^{3}H]choline with gas hyperpnea and swimming (7,8), this increase in enzyme activity is certainly consistent with an increase in rate of synthesis. Whereas a doubling of Vt with swimming increased CP-cyT activity within 30 min, a similar increase in Vt in the isolated perfused lung had no such effect. Although there are such differences between the two preparations as positive vs negative pressure breathing, the most likely difference is the removal of all neurohumoral factors with the isolated lung. Our results are consistent with the increase in CP-cyT activity being triggered by extrapulmonary factors, which are as yet unknown. Likewise, our results give no indication whether the increase in CP-cyT activity is due to enzyme induction or to increased amounts of a cofactor or substrate.

In conclusion, this is the first demonstration that the activity of the rate-limiting enzyme in surfactant PC synthesis can be enhanced in the adult lung by a physiologic stimulus to breathing *in vivo*. This enhancement appears very rapid, suggesting that the rate of *de novo* synthesis is very sensitive to demand.

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Acyl Lipid Metabolism in the Oleaginous Yeast *Rhodotorula gracilis* (CBS 3043)

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The effects of culture conditions on acyl lipid metabolism in the oleaginous yeast *Rhodotorula gracilis* (CBS 3043) have been investigated. Growth of *R. gracilis* under conditions of nitrogen-limitation resulted in the accumulation of large quantities of triacylglycerols. Thin layer and gas chromatographic analysis of total lipid extracts revealed that the majority of this storage lipid was produced by stationary-phase cells. In contrast, no such increase in triacylglycerol biosynthesis could be detected in carbon-limited cells. Freeze-fracture electron microscopy evidence supported these findings. Growth medium composition was found to have little effect on the relative abundance of the primary phospholipid classes present in *R. gracilis*. The acyl compositions of triacylglycerols were similarly unchanged by alterations in the composition of the growth medium. In contrast, the degree of unsaturation exhibited by the phospholipid fractions appeared to be particularly sensitive to this external parameter. Acyl quality of triacylglycerol pools extracted from nitrogen-limited cells were observed to become increasingly saturated as cultures increased in age.

Growth of nitrogen-limited cells at a lower growth temperature was observed to have little effect on triacylglycerol accumulation. However, both triacylglycerol and phospholipid fractions extracted from these cultures were found to contain increased proportions of the polyunsaturated fatty acid, α-linolenate.
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Over the last 50 years, the world demand for naturally occurring fats and oils has increased dramatically. In some cases, supplies of certain specialized oils have become erratic or inadequate. Consequently, in order to assure future availability, the commercial production of microbial lipid has been extensively investigated with respect to several fat-accumulating (oleaginous) yeast, bacterial and algal species (1,2). The carotenoid pigmented *Saccharomycetale, Rhodotorula gracilis* is a typical example of such oleaginous micro-organisms.

The amount of lipid accumulated by oleaginous organisms is determined by the culture conditions. The main determinant is an excess of carbon over some other limiting nutrient. Limitation of nitrogen (3) has been shown to be a most effective trigger for triacylglycerol accumulation in yeast. Further studies by Ratledge and co-workers (4,5) have also shown that triacylglycerol accumulation under such nutritionally limited culture conditions may be attributed to the possession of a highly active form of the cytosolic enzyme ATP:citrate lyase.

Previous studies performed on *R. gracilis* have concentrated on total lipid production under conditions of nitrogen-limitation in conjunction with changes in cultivation temperature (6), growth medium pH (7), and oxygen availability (8). In addition, the effects of other culture conditions (for example, batch culture vs fermentation) on triacylglycerol accumulation have also been reported (9,10). It was from an analysis of these studies that *R. gracilis* appeared to be a suitable organism for the study of lipid desaturation in oleaginous yeasts. As a first step in this study we have examined the effects that growth media composition and cultivation temperature have on acyl lipid biosynthesis and, in particular, triacylglycerol accumulation. A preliminary report of some of the data has been made (11).

MATERIALS AND METHODS

Experimental cultures. Rhodotorula gracilis (CBS 3043) was maintained on slopes of 3.5% yeast morphology agar (Difco Laboratories, Detroit, MI) as outlined by Whickerham (12). Cultures were grown in baffled shake flasks in an orbital incubator at 150 r.p.m. in either of the media listed below. Unless otherwise stated, cells were cultivated at 30°C. Cells were harvested by centrifugation at 2000 g for 5 min, washed once with distilled water and freeze-dried overnight.

Media. Nitrogen-limiting media comprised of the following (g/l): KH_2PO_4, 7.0; Na_2HPO_4, 2.0; $MgSO_4 \cdot 7H_2O$, 1.5; $CaCl_2 \cdot 2H_2O$, 0.1; $FeCl_3 \cdot 6H_2O$, 0.08; $ZnSO_4 \cdot 7H_2O$, 0.001; yeast extract, 1.5; NH_4Cl, 0.5; glucose, 30.0.

Two types of carbon-limiting media were utilized. These included (a) defined—compositionally identical to the nitrogen-limiting medium, with the exception of the concentrations of glucose (10 g/l) and NH_4Cl (3.0 g/l), and (b) non-defined—yeast extract, 10.0 g/l^{-1}; peptone, 20.0 g/l; glucose 20.0 g/l.

Lipid analysis. Lipids were extracted from freeze-dried material by the method of Moreton (13). Individual lipids were separated from the resulting chloroform extract by TLC on plates of silica gel (0.25 mm thick) utilizing a solvent system of petroleum ether/diethyl ether/acetic acid (80:20:2, v/v/v) for the separation of neutral lipids and one composed of chloroform/methanol/acetic acid/water (170:30:20:7, v/v/v/v) for the isolation of individual phospholipid classes. Individual lipids were identified by cochromatography with authentic standards and by use of specific spray reagents (14). Bands of individual acyl lipids were removed from t.l.c. plates and methyl esters were prepared by transmethylation with a 2 ml aliquot of 2.5% H_2SO_4 in anhydrous methanol for 2 hr at 70°C. After the addition of 5 ml 5% (w/v) NaCl, fatty acid methyl esters were extracted in three aliquots of 3 ml redistilled petroleum ether (60–80°C fraction). Individual

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Abbreviations: DPG, diphosphatidylglycerol; GLC, gas-liquid chromatography; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; TAG/PL, triacylglycerol/phospholipid; TLC, thin-layer chromatography.

fatty acid methyl esters were separated in a 15% (w/w) EGSS-X on Chromosorb (100–120 mesh) column isothermally at 190°C on a Perkin-Elmer F33 gas chromatograph. Data were quantified by use of heneicosanoic acid as an internal standard and fatty acid compositions were calculated by the method of Carroll (15). Identification of individual fatty acids was performed by comparison of retention times with those of commercial standards on different GLC stationary-phases and by determination of double bond positions in purified polyunsaturated fatty acids determined by periodate-permanganate oxidation according to the methodology outlined by Kates (14).

Electron microscopy. Freeze-fracture electron micrographs were prepared of late stationary-phase (72 hr) cultures of *R. gracilis* grown in either the nitrogen- or the non-defined carbon-limiting medium. A Balzers BAF 400 D freeze-etching unit was used for the preparation of carbon/platinum replicas, which were then released from the fracture surfaces by successive washes in household bleach and supersaturated sodium hydroxide. The replicas were examined using a Joel 1200 EX Transmission Electron Microscope. Intracellular lipid storage vacuoles were then identified by comparison with freeze-fracture electron micrographs of authentic triacylglycerols.

RESULTS AND DISCUSSION

Identification of the major acyl lipids present in R. gracilis (CBS 3043). When batch-cultured in either carbon- or nitrogen-limiting media, *Rhodotorula gracilis* (CBS 3043) was found to contain the following acyl lipids; phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, diphosphatidylglycerol, tri-, di- and monoacylglycerols, sterol esters and unesterified fatty acids. The triacylglycerol pools, together with the phospholipids, were found to account for approximately 85% of the total acyl lipids present.

Furthermore, analysis of acyl moieties by gas chromatography and permanganate-periodate treatment revealed the major fatty acids present in the acyl lipids to be myristate (14:0), palmitate (16:0), stearate (18:0), oleate (18:1), linoleate (18:2) and α-linolenate (18:3). Fatty acids present in minor quantities were identified as palmitoleic (16:1), hexadecadienoic (16:2) and arachidic (20:0) acids. In contrast to the results of Yoon *et al.* (9), who studied a different *Rhodotorula gracilis* strain, the presence of behenic (22:0) and lignoceric (24:0) acids was not detected in *Rhodotorula gracilis* (CBS 3043).

Effects of growth medium composition on triacylglycerol accumulation in R. gracilis (CBS 3043). Acyl lipid biosynthesis in *R. gracilis* cultures grown at 30°C under conditions of either carbon- or nitrogen-limitation was monitored with time (Table 1). Under conditions of carbon-limitation the total amount of acyl lipid present in the cell was observed to rise and fall with culture age. The TAG/PL ratios expressed in these cultures were found to generally agree with this trend. Such decreases in the acyl lipid content of carbon-limited, late stationary-phase cultures may be accounted for by the mobilization of the cell's carbon reserves. In sharp contrast, the acyl lipid content of cultures grown in the nitrogen-limiting medium increased dramatically with culture age. This trend was also reflected in the TAG/PL ratios exhibited by these cultures.

One of the major problems usually incurred during metabolic studies on batch grown microbial cultures is that of pH regulation. Previous studies reported by

TABLE 1

Effect of Growth Medium Composition on Lipid Accumulation in *R. gracilis* (CBS 3043) Grown at 30°C

Growth medium	Age of culture		
	Mid-log (15 hr)	Early stationary (24 hr)	Late stationary (72 hr)
Undefined C-limiting (C/N[a] = 2.2)			
Mass acyl chains/0.1 g dry weight (mg)[b]	0.8	0.9	0.2
TAG/PL ratio (wt/wt acyl chains)[c]	0.2 ± 0.0	0.7 ± 0.0	0.4 ± 0.3
Defined C-limiting (C/N = 3.5)			
Mass acyl chains/0.1 g dry weight (mg)	1.1	1.5	0.4
TAG/PL ratio (wt/wt acyl chains)	0.4 ± 0.1	0.4 ± 0.3	0.1 ± 0.1
Defined N-limiting (C/N = 34.3)			
Mass acyl chains/0.1 g dry weight (mg)	0.8	11.0	24.0
TAG/PL ratio (wt/wt acyl chains)	0.9 ± 0.4	8.0 ± 0.8	19.1 ± 5.1

[a] C/N = carbon:nitrogen ratio (wt/wt) in growth medium.

[b] Results presented are for single measurements only. The experiment, however, was repeated and gave similar results.

[c] TAG/PL ratio = Mass of acyl moieties in the triacylglycerol fraction/mass acyl moieties in the phospholipid fraction. Results given as means ± S.D. for three independent experiments.

Masses of fatty acids accumulated (ng)/ml of growth medium were as follows: Undefined C-limiting—15 hr, 9; 24 hr, 49; 72 hr, 25; Defined C-limiting—15 hr, 16; 24 hr, 42; 72 hr; 16; Defined N-limiting—15 hr, 12; 24 hr, 378; 72 hr, 2040.

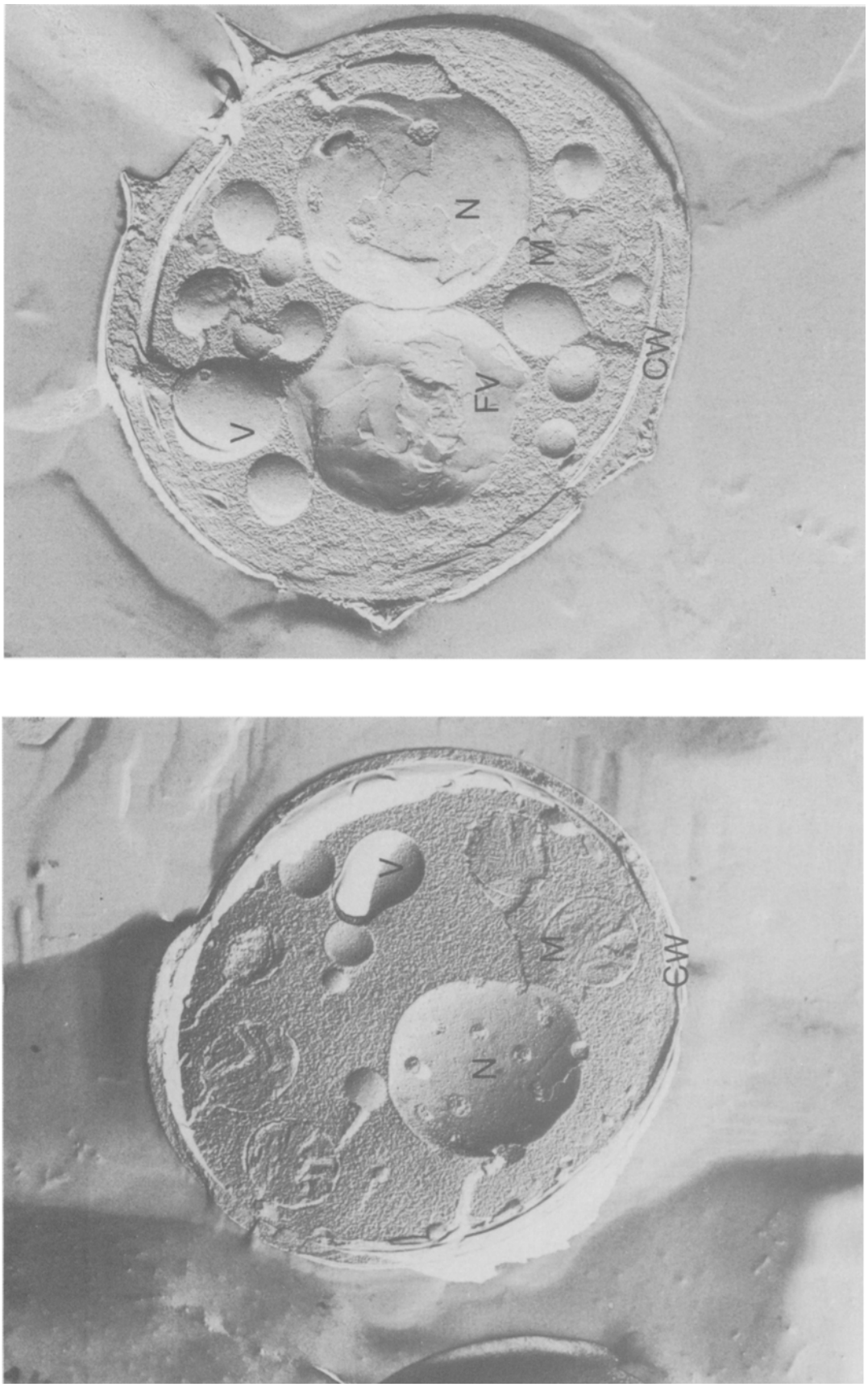

FIG. 1 (A) Freeze-fracture electron micrograph of a late stationary-phase (72 hr) cell of *R. gracilis* grown in a non-defined carbon-limiting medium at 30°C. Cells were grown in a carbon-limiting medium for 72 hr, after which time they were harvested and freeze-fracture electron micrographs prepared as described in the text. CW, cell wall; M, mitochondrion; N, nucleus; V, intracellular vesicle. Scale 1.5 cm:500 nm. (B) Freeze-fracture electron micrograph of a late stationary-phase cell of *R. gracilis* grown in a defined nitrogen-limiting medium at 30°C. Cells were grown in a nitrogen-limiting medium for 72 hr, after which time, they were harvested and freeze-fracture electron micrographs prepared as described in the text. CW, cell wall; FV, fat vacuole; M, mitochondrion; N, nucleus; V, intracellular vesicle. Scale: 2.0 cm:500 nm.

Kessell (7) have shown that triacylglycerol accumulation in *R. gracilis* may be triggered by low media pH. However, we feel that the above observation does not apply to our studies since the buffering capacity of the phosphate ions present in the growth medium was found to be sufficient to maintain the culture pH at a value between 5.0–6.0 throughout a four-day culture period (16).

Freeze-fracture electron microscopy was also employed to monitor triacylglycerol accumulation. Figure 1A shows a mid stationary-phase (72 hr) cell of *R. gracilis* grown in the non-defined carbon-limiting medium. The cell pictured contains a prominent nucleus, several mitochondria and intracellular vesicles. Figure 1B shows an equivalent mid stationary-phase (72 hr) cell of nitrogen-limited *R. gracilis*. The most characteristic feature of this cell is the possession of a large intracellular lipid storage vacuole. Intracellular storage vacuoles were found to be absent in all of the carbon-limited cells studied and, in general, carbon-limited cells tended to possess a slightly thinner cell wall than cells grown under conditions of nitrogen-limitation. Such observations were to be expected, since in a carbon-deficient medium very little carbon would be available for storage lipid production or for cell wall thickening. In addition, nitrogen-depleted cells were found to possess a higher number of non-lipid containing intracellular vesicles than carbon-limited cultures.

Effects of growth medium composition on phospholipid and triacylglycerol metabolism in 30°C grown cultures of R. gracilis. The acyl compositions of triacylglycerol and phospholipid fractions extracted from early stationary-phase (24 hr) cultures of *R. gracilis* (CBS 3043) grown in either nitrogen- or carbon-limiting growth media are given in Table 2. In all cases, the major fatty acids present in these two acyl lipid fractions were found to be palmitate, stearate, oleate, linoleate and α-linolenate. Irrespective of the growth media composition, phospholipids were found to contain high (>35%) proportions of C_{18} polyunsaturated acyl chains, while triacylglycerol fractions were characterized by a high (approx. 40–60%) oleic acid content. Growth media composition was found to have significant effects on the relative proportions of C_{18} unsaturated fatty acids present in phospholipid fractions. However, as different trends were detected in both non-defined vs defined and carbon- vs nitrogen-limiting media, very few overall trends may be discerned. In contrast, the acyl compositions of the triacylglycerol fractions appeared to remain relatively constant irrespective of growth medium composition. Similarly, growth media composition had a generally minimal effect on the relative proportions of the major phospholipid classes found in log-phase (15 hr) cultures of this oleaginous yeast species (Table 3). Under all growth conditions, phosphatidylcholine and phosphatidylethanolamine were found to be the most abundant phospholipid classes present in *R. gracilis* (CBS 3043). These results are very different to those reported by Yoon and Rhee (10), who performed similar studies on both batch-cultured and fermenter grown *R. gracilis* (NRRL Y-1091), where, in contrast to the general data available for *Saccharomycetales* yeasts (1), phosphatidylserine was observed to be the major component of the extracted phosphoglycerides. In addition, the presence of glycolipids was also not detected in *R. gracilis* (CBS 3043).

Effect of culture age on phospholipid and triacylglycerol acyl compositions. The effects of culture age on the acyl compositions of phospholipid and triacylglycerol fractions present in *R. gracilis* (CBS 3043) grown in a nitrogen-limiting medium are depicted in Figure 2A and B, respectively. The most obvious changes during the experimental period were the initial decline in the percentage of polyunsaturated fatty acids in triacylglycerols as observed at the beginning of stationary-phase. This decline was presumably caused by the synthesis of molecular species containing higher proportions of palmitate and oleate during lipid accumulation in stationary-phase. Similar changes in the relative proportions of oleate, linoleate and α-linolenate were also seen in the phospholipid fraction at the onset of stationary-phase (Fig. 2A) but, in this case,

TABLE 2

Acyl Compositions of Phospholipid and Triacylglycerol Fractions from Early Stationary-Phase (24 hr) Cultures of *R. gracilis* (CBS 3043) Grown in Different Growth Media at 30°C

	% Fatty acid composition				
	16:0	18:0	18:1	18:2	18:3
Phospholipids					
C′[a]	10.7 ± 1.6	1.1 ± 0.3	50.6 ± 7.6	26.2 ± 6.7	9.9 ± 1.7
C″[b]	11.9 ± 1.9	2.6 ± 0.6[d]	27.0 ± 3.2[d]	37.5 ± 0.9[d]	18.6 ± 2.4[d]
N[c]	15.4 ± 2.5[d]	2.3 ± 1.6	35.8 ± 2.3[d,e]	29.6 ± 7.8[e]	12.9 ± 2.2[e]
Triacylglycerols					
C′[a]	14.7 ± 2.6	7.5 ± 2.1	56.7 ± 9.4	13.1 ± 5.7	4.0 ± 1.5
C″[b]	15.2 ± 8.0	7.2 ± 1.9	38.5 ± 7.6	25.6 ± 9.4	9.4 ± 4.0
N[c]	18.6 ± 3.0[d]	8.0 ± 0.6	50.0 ± 6.2	16.2 ± 7.1	4.6 ± 3.0

[a]C′ = non-defined carbon-limiting medium.

[b]C″ = defined carbon-limiting medium.

[c]N = defined nitrogen-limiting medium.

[d]$p < 0.05$ where N and C″ are compared with C′ and

[e]$p < 0.05$ where N is compared with C″.

Results given as means ± S.D. for three independent experiments. Statistical analysis by Student's t-test. Other fatty acids present in minor quantities included 14:0, 16:1 and 20:0.

TABLE 3

Relative Proportions of Individual Phospholipid Classes Present in Log-Phase Cultures of *R. gracilis* (CBS 3043) When Grown in Different Growth Media at 30°C

Growth medium	Phospholipid distribution (% major classes)				
	PC	PE	PS	PI	DPG
C′	57.9 ± 4.1	21.7 ± 11.5	7.7 ± 3.3	6.1 ± 0.6	6.5 ± 3.2
C″	35.2 ± 0.5[a]	39.1 ± 2.3	12.6 ± 4.2	4.4 ± 2.3	8.8 ± 3.7
N	47.3 ± 3.8[b]	26.1 ± 0.5	8.4 ± 2.1	10.5 ± 7.9	7.7 ± 1.3

[a] $p < 0.05$ where N and C″ are compared with C′ and

[b] $p < 0.05$ where N is compared with C″.

Results given as means ± S.D. for two independent experiments. Percentage compositions derived from acyl chain (wt/wt) analysis. Statistical analysis by Student's t-test. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; DPG, diphosphatidylglycerol; C′, non-defined carbon-limiting medium; C″, defined carbon-limiting medium; N, defined nitrogen-limiting medium.

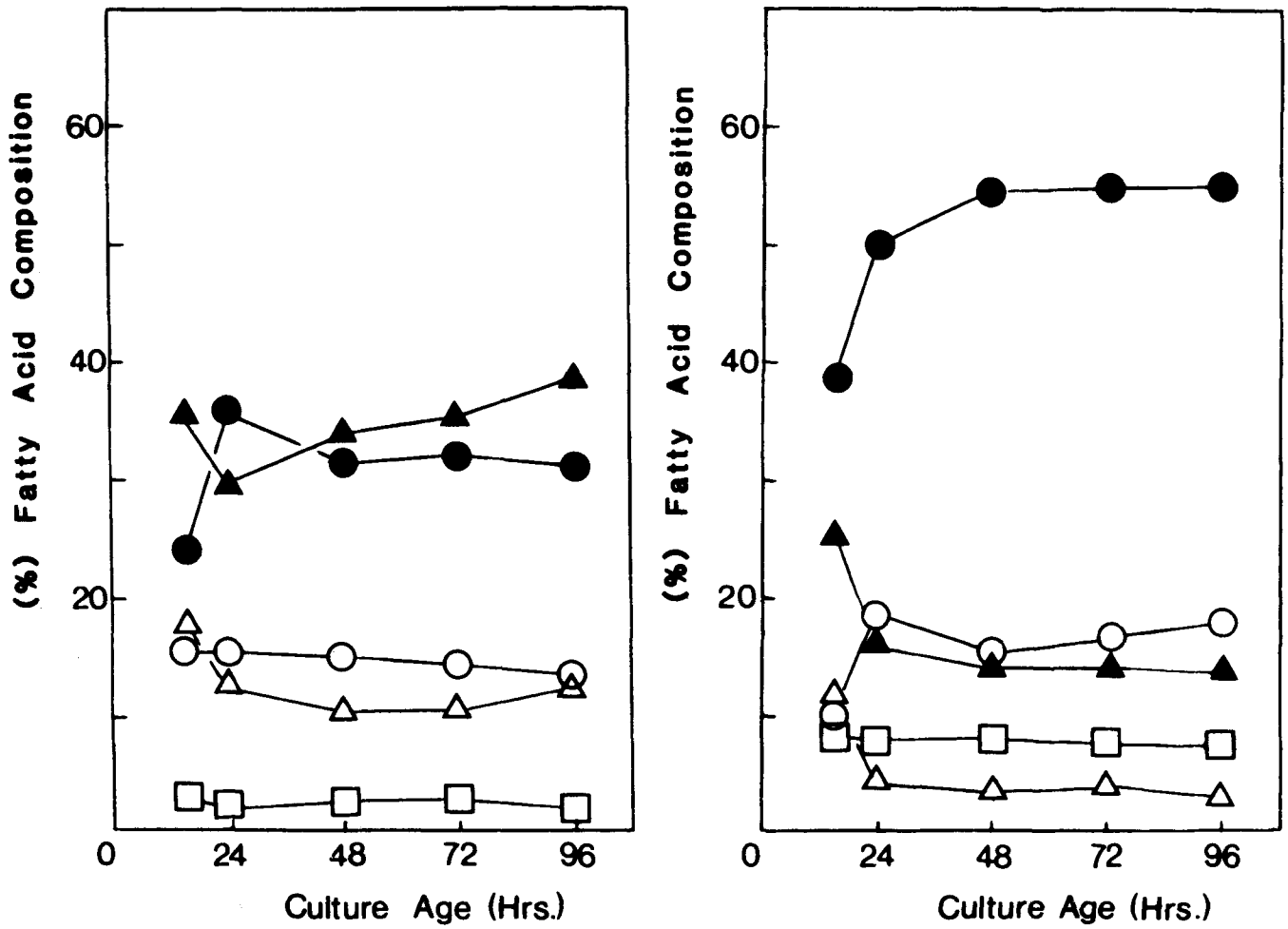

FIG. 2. (A) Effect of culture age on the acyl composition of total phospholipid fractions present in *R. gracilis* grown in a defined nitrogen-limiting medium at 30°C. Lipids were extracted from freeze-dried N-limited cultures in various stages of growth. Phospholipid fractions were then prepared and analyzed by TLC and GLC, respectively, as described in the text. Data points are for single measurements only. The experiment, however, was repeated and gave similar results. ○, 16:0; □, 18:0; ●, 18:1; ▲, 18:2; △, 18:3. (B) Effect of culture age on the acyl composition of total triacylglycerol fractions present in *R. gracilis* grown in a defined nitrogen-limiting medium at 30°C. Lipids were extracted from freeze-dried N-limited cultures in various stages of growth. Triacylglycerol fractions were then prepared and analyzed by TLC and GLC, respectively, as described in the text. Data points are for single measurements only. The experiment, however, was repeated and gave similar results. ○, 16:0; □, 18:0; ●, 18:1; ▲, 18:2; △, 18:3.

TABLE 4

Effect of Growth Temperature on the Acyl Composition of Phospholipid and Triacylglycerol Fractions Present in *R. gracilis* Grown in a Nitrogen-Limiting Medium

	% Fatty acid composition				
	16:0	18:0	18:1	18:2	18:3
Phospholipids					
15°C[a]	10.3 ± 1.5	2.7 ± 0.6	28.9 ± 1.4	32.3 ± 2.1	19.7 ± 4.9
30°C	14.7 ± 2.0	1.8 ± 0.3	35.4 ± 3.5	33.8 ± 2.1	9.8 ± 5.0
Level of significance	$p < 0.001$	$p < 0.01$	$p < 0.05$	n.s.	$p < 0.005$
Triacylglycerols					
15°C[a]	18.5 ± 2.5	7.2 ± 0.7	46.4 ± 2.0	16.2 ± 3.1	7.5 ± 1.5
30°C	18.2 ± 1.3	6.7 ± 2.5	52.8 ± 8.1	13.9 ± 3.5	3.9 ± 2.4
Level of significance	n.s.	n.s.	$p < 0.1$	$p < 0.1$	$p < 0.005$

Results given as means ± S.D., where n = 7 or [a]n = 6.

Statistical analysis by Student's t-test; n.s., not significant.

there was a recovery in the percentage of polyunsaturates during the latter stages of this growth phase.

Effect of growth temperature on acyl lipid metabolism. Although growth of *R. gracilis* (CBS 3043) at a lower growth temperature of 15°C rather than that of 30°C resulted in an approximate doubling of the growth cycle time, it was found to have little effect on the triacylglycerol/phospholipid ratios exhibited by the nitrogen-limiting cultures (16). However, changes in cultivation temperature resulted in similar changes in the relative proportions of the total fatty acids present in *R. gracilis* (CBS 3043) to those reported by Enebo and Iwamoto (6), with cultures grown at temperatures less than 20°C containing significantly higher proportions of polyunsaturated fatty acids than those grown at temperatures greater than 25°C. An increase in unsaturation at lower temperatures is the most characteristic lipid change which may be involved in the adaptation and the maintenance of membrane function (17).

Analysis of lipid (Table 4) also revealed that both phospholipid and triacylglycerol fractions extracted from 15°C grown cultures contained approximately twice as much α-linolenate than the respective fractions extracted from 30°C grown cells. The absolute increase in α-linolenate was much greater in the phospholipid fraction (due to the higher content of polyunsaturates) and was accompanied by significant decreases in palmitate and oleate. It is reasonable to assume that the large increase in α-linolenate proportions in the phospholipid fraction may be related to adaptive regulation of membrane fluidity as a result of lower growth temperature (18,19). However, the statistically significant rise in this fatty acid in the triacylglycerol fraction (Table 2) also suggests that it may merely reflect a response but not an adaptation —possibly because of increased oxygen availability for the Δ12 and Δ15 desaturations at lower temperatures (17).

In conclusion, the results presented in this report have shown the extent to which acyl lipid metabolism in batch-cultured *R. gracilis* (CBS 3043) can be altered by changes in media composition and cultivation temperature. The ability to modify various acyl lipid biosynthetic pathways by such external parameters can be used to evaluate such aspects as the control of both triacylglycerol accumulation and acyl chain desaturation in this oleaginous organism.

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Microsomal Lipid Peroxidation: Effect of Vitamin E and Its Functional Interaction with Phospholipid Hydroperoxide Glutathione Peroxidase

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The role of vitamin E in the protection against iron dependent lipid peroxidation was studied in rat liver microsomes and Triton-dispersed microsomal lipid micelles. In these systems, an antioxidant effect of vitamin E at a physiological ratio to phospholipids could be observed only in the presence of phospholipid hydroperoxide glutathione peroxidase (PHGPX) and glutathione. The rationale of this cooperation is discussed on the basis of the hydroperoxyl radical scavenging capacity of vitamin E and the reduction of membrane hydroperoxides by PHGPX. The scavenging of lipid hydroperoxyl radicals by vitamin E, although inhibiting propagation of the peroxidative chain, produces lipid hydroperoxides from which ferrous iron generates alkoxyl radicals that react with vitamin E almost as fast as with fatty acids. Therefore, only if membrane hydroperoxides are continuously reduced by this specific peroxidase does the scavenging of hydroperoxyl radicals by vitamin E lead to an effective inhibition of lipid peroxidation.
Lipids 24, 721-726 (1989).

The first event of NAD(P)H and ascorbate dependent microsomal lipid peroxidation is the reduction of a suitable iron complex (1-3). The reduced iron complex then reacts with oxygen to generate an oxidant able to abstract a hydrogen atom from a methylene group of a polyunsaturated fatty acid (LH), giving rise to a carbon centered radical (initiation). Although the exact nature of this oxidant is still uncertain, the most probable species seems to be the perferryl ion, Fe^{3+}-O_2^- (2-4). Following hydrogen abstraction, the lipid carbon centered radical ($L^\bullet$) reacts reversibly (5) with oxygen, giving rise to a hydroperoxyl radical ($LOO^\bullet$). This may start a chain reaction by abstracting hydrogen atoms from another fatty acid giving rise to a lipid hydroperoxide (LOOH) and a new carbon centered radical (propagation). The chain is terminated by radical-radical or radical-scavenger interactions (6).

Lipid hydroperoxides play a dual role in peroxidation, since they are both products of peroxidation and substrates for further initiation reactions (4). The cleavage of the O-O bond of lipid peroxides by ferrous iron in a Fenton-like reaction generates alkoxyl radicals ($LO^\bullet$) (7) which are able to start new peroxidative chains. In fact, following the formation of some hydroperoxides, peroxidation proceeds at a faster rate by "secondary initiations" or "branching," as long as iron, reducing equivalents, oxygen and polyunsaturated lipids are available. These chain reactions then subside via several termination reactions (6). During our studies on the physiological mechanisms of protection against microsomal lipid peroxidation we isolated and purified from different mammalian organs a new Se-dependent glutathione peroxidase active on membranes, the phospholipid hydroperoxide glutathione peroxidase (PHGPX) (8).

The inhibition of lipid peroxidation by PHGPX and glutathione was complete when free radicals were generated in liposomes by lipid hydroperoxides and a ferric iron complex. In that peroxidation system the antiperoxidant effect of PHGPX was apparently due to the reduction of hydroperoxides from which peroxidizing free radicals were generated by the iron complex.

However, when lipid peroxidation was induced in liposomes (9) or liver microsomes (10) by ascorbate and iron ADP, the inhibition by PHGPX and glutathione was observed only if vitamin E was also present (9). These results suggest a cooperation between the peroxidase and the free radical scavenger, and so the question arises on what the mechanism of the cooperation and the role of vitamin E actually are.

Vitamin E is the most active natural antioxidant present in plasma and tissues (11,12). This antioxidant capacity is apparently due to the fact that vitamin E reacts with hydroperoxyl radicals several orders of magnitude faster than lipids, the rate constants for the reaction of $LOO^\bullet$ being 2.4×10^6 M^{-1} sec^{-1} and 62 M^{-1} sec^{-1} for the reaction with vitamin E (11) and lipids (13) in homogeneous solution, respectively.

This paper reports a plausible explanation for the cooperation between vitamin E and PHGPX in the inhibition of ferrous iron dependent lipid peroxidation. The antioxidant effect of vitamin E, that is related to the previous reaction, is fully expressed, under these conditions, when the peroxidase, by reducing lipid hydroperoxides, prevents the formation of alkoxyl radicals with which lipids reacts almost equally as fast as vitamin E.

MATERIALS AND METHODS

Rat liver microsomes were prepared as previously described (9). Vitamin E enriched microsomes were prepared from rats treated 15 hours before sacrifice with a large dose (100 mg/kg) of vitamin E. Rats were injected intraperitoneally with a vitamin E dispersion prepared as follows: vitamin E was dissolved in one volume of ethanol and then diluted with nine volumes of 16% (v/v) Tween 80 in saline. The injected volume was usually less than 1 ml. Rat liver microsomes (1 mg/ml), were peroxidized in the presence of 0.3 mM NADPH, 2 μM $FeCl_3$, 0.2 mM ADP in 2.5 ml of 0.1 M Tris-HCl buffer pH 7.4 at 30°C. In experiments where microsomal lipids were used, these were extracted with chloroform: methanol (2:1), the solvent evaporated and Triton X-100 and buffer added to the residue to form mixed micelles. Care was taken to keep all solvents and lipid dispersions under argon to prevent autoxidation. For peroxidation experiments lipid dispersions were diluted up to twenty times with buffer and vortexed to eliminate argon. Peroxidation was

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Abbreviations: PHGPX, phospholipid hydroperoxide glutathione peroxidase; $L^\bullet$, lipid carbon centered radical; $LOO^\bullet$, lipid hydroperoxyl radical, $LO^\bullet$, lipid alkoxyl radical; LOOH, lipid hydroperoxide; MDA malondialdehyde; LH, polyunsaturated fatty acid; ADP, adenosine diphosphate.

induced by 2 μM $FeCl_3$, 0.2 mM ADP, 0.2 mM ascorbate in 2.5 ml of 0.1 M Tris HCl, 0.15 M KCl pH 7.4. The amount of extracted lipids used corresponded to 3.25 μmol of phospholipids. In all experiments peroxidation was measured both as oxygen consumption using a Clark electrode, and as malondialdehyde production (14). Pig heart PHGPX was purified as previously described (8,15). Vitamin E was measured by HPLC with fluorescence detection following saponification of the sample in the presence of ascorbate as described (16). Tocopherylquinone was measured by HPLC as described (17). Phosphatidylcholine hydroperoxide was prepared (8) and measured (18) as previously described. Phospholipids were measured according to (19). Peroxide free Triton X-100 was from Boehringer, Mannheim, DL-α-tocopherol was from Merck, soybean phosphatidylcholine was from Sigma.

RESULTS

To get a better understanding of the cooperation between the peroxidase active on membrane hydroperoxides (PHGPX) and vitamin E in the control of microsomal lipid peroxidation, the role of vitamin E in the absence of the peroxidase in our peroxidation systems first had to be characterized.

Vitamin E disappears during microsomal lipid peroxidation induced by iron ADP and NADPH when lipid hydroperoxides and MDA are produced and oxygen is consumed (Fig. 1). The same results were observed when 0.3 mM ascorbate was substituted for NADPH. Under these conditions the consumption of vitamin E is apparently due to free radical reactions. To validate this we measured by HPLC the production of tocopherylquinone, one of the oxidation products of tocopherol, and we

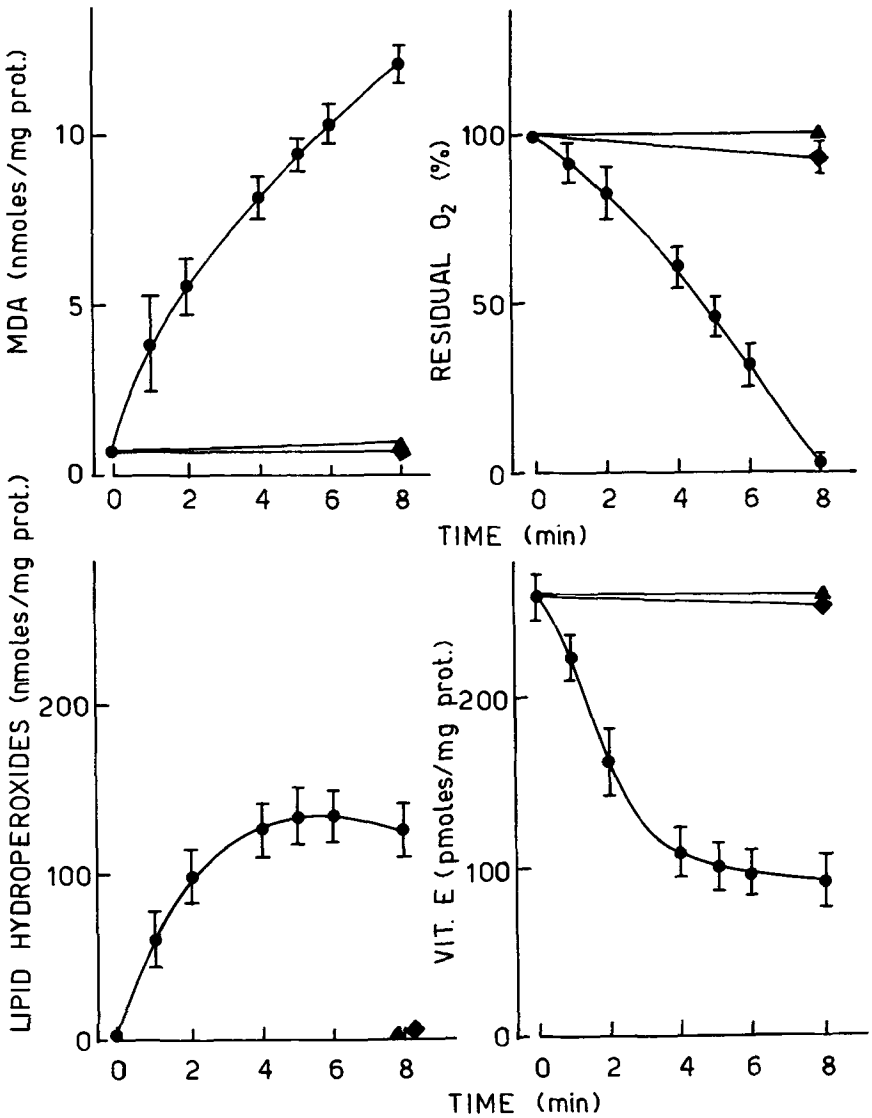

FIG. 1. Time course of oxygen consumption, MDA and lipid hydroperoxides production and vitamin E disappearance during microsomal lipid peroxidation. Incubation mixture contained in 2.5 ml of 0.1 M Tris-HCl buffer pH 7.4 at 30°C: 1 mg/ml rat liver microsomes, 2 μM $FeCl_3$, 0.2 mM ADP and 0.3 mM NADPH. Complete reaction mixture (●); reaction mixture without NADPH (▲); reaction mixture without $FeCl_3$ ( ). The experiments were carried out in the oxygraph chamber, and each point represents the mean values and SE of three independent experiments.

observed that the production of this accounts, under the conditions of Figure 1, for 25–35% of vitamin E disappearance.

The evidence that vitamin E is not consumed in the absence of either NADPH or iron, as well as the previous report that vitamin E under similar conditions is not consumed in the absence of polyunsaturated fatty acids (20), indicates that vitamin E: i) is not oxidized by iron-ADP under these conditions and ii) is competent for interacting with oxidizing species, possibly radicals, generated during peroxidation.

However, in order to know if this consumption of vitamin E is actually related to an inhibition of lipid peroxidation, an increase or an inhibition of peroxidation rate by decreasing or increasing the vitamin E content in membranes, respectively, had to be observed.

In our experimental conditions, depletion of vitamin E in liver microsomes, obtained by dietary manipulation (21) or by acetone extraction (10) did not significantly increase the rate of peroxidation induced by NADPH or ascorbate and iron ADP. That is why we focused on the effect of an increased concentration in the following experiments.

Since the insertion of vitamin E into the correct sites of membranes is actually difficult and poorly reproducible *in vitro*, we resorted to using two different peroxidable substrates: i) microsomes from rats treated *in vivo* with a supraphysiological amount of vitamin E and ii) microsomal lipids in a mixed micellar form with Triton X-100 to which vitamin E was directly added.

By injecting rats intraperitoneally with 100 mg/kg vitamin E in Tween-80 the vitamin E content in microsomal membranes was increased more than ten times. In these microsomes lipid peroxidation rate is slower than in control microsomes (Fig. 2—note the different time and vitamin E scales), while in both cases the consumption of vitamin E parallels peroxidation rate measured as oxygen consumption (Fig. 2). Malondialdehyde production was consistent with oxygen consumption and, when all the oxygen was consumed—i.e., after 10 min when vitamin E was low, and 35 min when vitamin E was high—MDA production was 13 ± 2 and 11 ± 3 nmol/mg of protein, respectively. Control experiments without NADPH or without iron showed that under these conditions there is neither lipid peroxidation nor vitamin E consumption (Fig. 1).

When Triton X-100 dispersed microsomal lipids were used as peroxidable substrates, vitamin E was added in a small volume of ethanol (10 μl), as this amount of ethanol was ineffective as antioxidant in this peroxidation system. In these experiments (Table 1) the peroxidation

TABLE 1

Effect of Vitamin E on Iron Ascorbate Induced Lipid Peroxidation Measured as Time Integrated Oxygen Consumption in Microsomal Lipids Dispersed with Different Amounts of Triton X-100[a]

	Time (2 min) integrated oxygen consumption (M · min)[b]
Lipid dispersion in 0.04% Triton	$0.54 \pm 0.08 \times 10^{-3}$
+ E (1.5 nmol)	$0.54 \pm 0.07 \times 10^{-3}$
+ E (15 nmol)	$0.53 \pm 0.09 \times 10^{-3}$
Lipid dispersion in 0.075% Triton	$0.32 \pm 0.06 \times 10^{-3}$
+ E (1.5 nmol)	$0.30 \pm 0.06 \times 10^{-3}$
+ E (15 nmol)	$0.25 \pm 0.06 \times 10^{-3}$
Lipid dispersion in 0.1% Triton	$0.28 \pm 0.03 \times 10^{-3}$
+ (1.5 nmol)	$0.18 \pm 0.02 \times 10^{-3}$[c]
+ E (15 nmol)	$0.06 \pm 0.02 \times 10^{-3}$[c]

[a] Peroxidation was induced by 2 μM $FeCl_3$, 0.2 mM ADP, 0.2 mM ascorbate in 2.5 ml of 0.1 M Tris HCl, 0.15 M KCl, pH 7.4. The amount of extracted microsomal lipids used, corresponded to 3.25 μmol of phospholipids. Mean values ±SD of five experiments are reported.

[b] Oxygen consumption rate due to ascorbate autoxidation, in the absence of peroxidable substrate was less than 5% of the consumption due to peroxidation.

[c] Significantly different ($P < 0.001$) than in the absence of vitamin E.

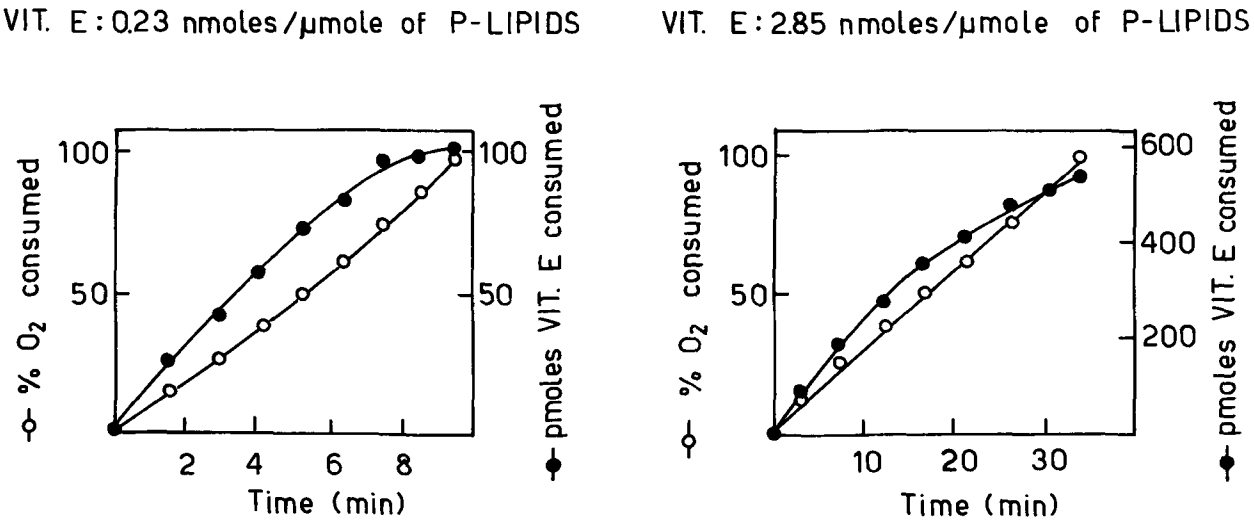

FIG. 2. Relationship between vitamin E disappearance and lipid peroxidation in native and vitamin E loaded microsomes. Microsomes (1 mg/ml), were peroxidized at 30°C in 2.5 ml of 0.1 M Tris-HCl, pH 7.4, 0.3 mM NADPH, 2 μM $FeCl_3$, and 0.2 mM ADP. The complete experiment was repeated three times and a typical experiment is reported. Since the experiments were carried out in the oxygraph chamber and the complete reaction mixture is consumed for vitamin E measurement, each point actually represents a distinct experiment.

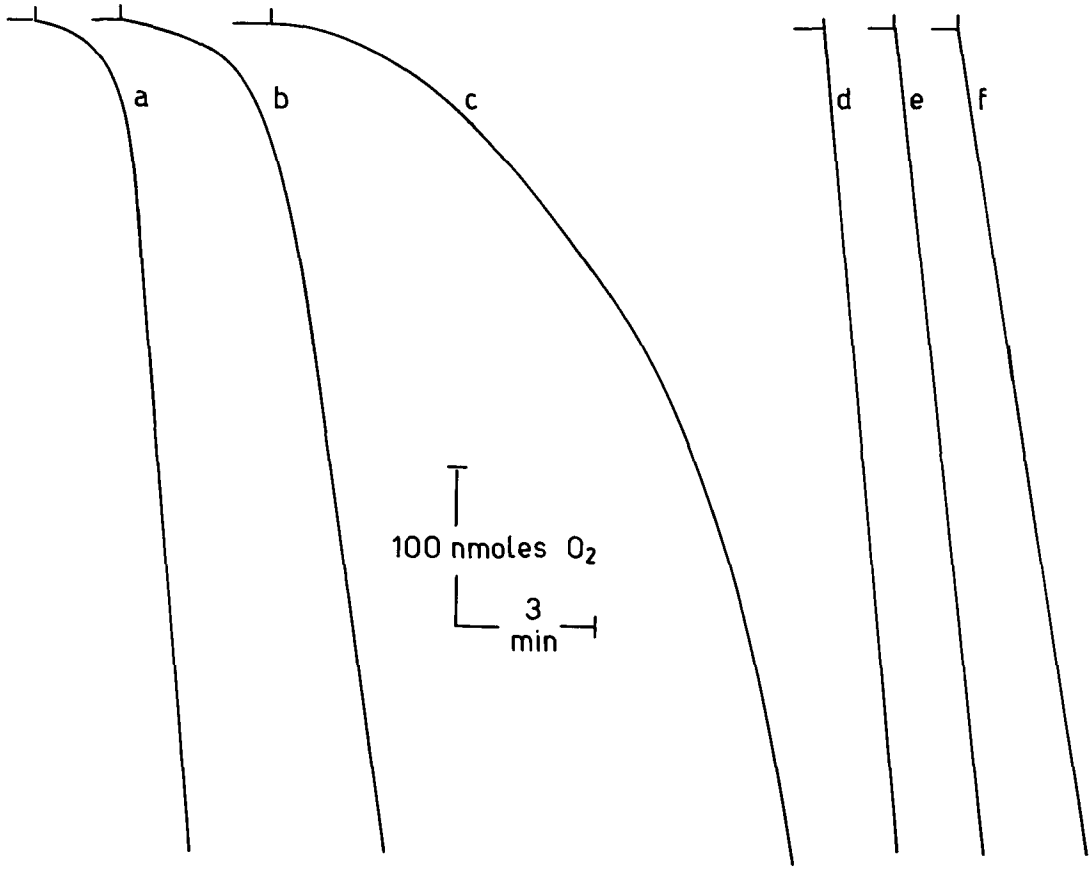

FIG. 3. Effect of vitamin E and phospholipid hydroperoxides on peroxidation of microsomal lipid micelles. Peroxidation was induced in 2.5 ml of 0.1 M Tris HCl, 0.15 M KCl pH 7.4, 2 μM $FeCl_3$, 0.2 mM ADP, and 0.2 mM ascorbate. The amount of extracted lipids used, corresponded to 3.25 μmol of phospholipids. Triton X-100 was 0.075%. Vitamin E was 0, trace a and d; 1 nmol, trace b and e; 25 nmol, trace c; 50 nmol, trace f. Five nmoles of phosphatidylcholine hydroperoxide—prepared as described in ref. 8 and measured as described in ref. 18—were added to mixed micelles in d, e, and f. The typical experiment reported was repeated three times and was highly reproducible. MDA production at the end of the experiments was: 43 $\pm$ 3, 42 $\pm$ 2, 44 $\pm$ 3, 45 $\pm$ 4, 43 $\pm$ 4 and 44 $\pm$ 5 nmol in a, b, c, d, e, and f, respectively.

rate was measured as time integrated oxygen consumption because the consumption rate was not linear but progressively increasing (Fig. 3). As in microsomes also in micelles of detergent solubilized microsomal lipids (Table 1), vitamin E inhibits peroxidation rate, although at a ratio to phospholipid higher than physiological. However, the antioxidant effect of vitamin E is apparent only at high detergent concentration. It is noteworthy that the detergent itself reduces peroxidation rates and also that lower concentrations of detergent are sufficient to introduce vitamin E into mixed micelles (vitamin E is indeed oxidized under these conditions as in Figs. 1 and 2).

Although these results are highly reproducible, a different peroxidation pattern can also be observed if lipid dispersions are not prepared and stored under argon. In this case a faster peroxidation rate and a complete disappearance of the initial accelerating phase can be observed. This is apparently due to minute amounts of lipid hydroperoxides that are probably produced during either preparation of microsomes or lipid extraction and storage of dispersions. To verify the role of lipid hydroperoxides, we incorporated enzymatically generated phosphatidylcholine hydroperoxides into microsomal lipid dispersions, and we observed that in the presence of as little as 0.15% of phospholipids in peroxide form, the initial accelerating phase completely disappeared (Fig. 3). Under these conditions vitamin E fails to inhibit lipid peroxidation significantly.

A variable initial acceleration of peroxidation rate due to the presence of minute amounts of lipid hydroperoxides can also be observed in microsomes as indicated by the higher variability in the early phase (Fig. 1) and also in microsomes the addition of phospholipid hydroperoxides completely abolished the initial acceleration (10).

The results reported in Table 1 and Figure 3 suggest that vitamin E is not a good antioxidant when free radicals are generated by ferrous iron catalyzed decomposition of lipid hydroperoxides. On the other hand, vitamin E seems active in delaying the shift from primary to secondary initiations (Fig. 3).

The results reported in Figure 4 show that vitamin E, at a physiological ratio to phospholipids, efficiently inhibits iron-ascorbate induced lipid peroxidation in the presence of PHGPX and glutathione. In these experiments we used the same peroxidation system as in Table 1 and Figure 3, and a Triton concentration at which no significant effect of vitamin E alone was apparent. It is noteworthy that the 50% inhibition observed in the

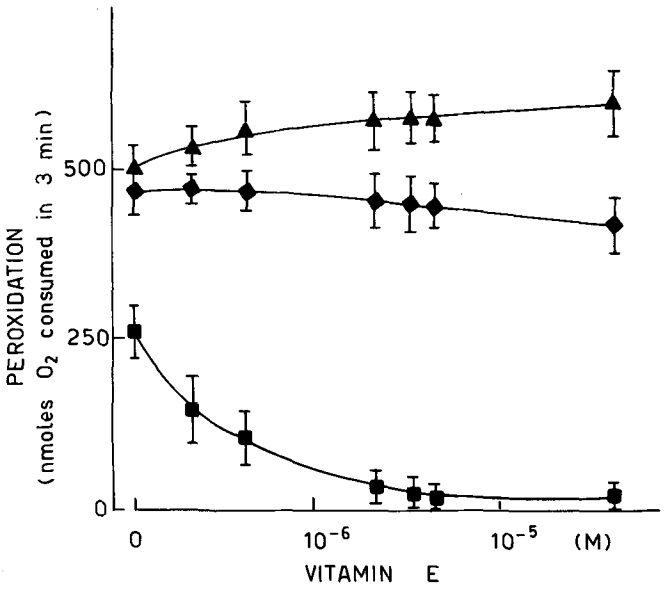

FIG. 4. Antixodant effect of vitamin E, PHGPX and glutathione in mixed micelles of extracted microsomal lipids and Triton X-100. Triton X-100 was 0.05%, peroxidation was induced in 2.5 ml of 0.1 M Tris HCl, 0.15 M KCl, pH 7.4, 2 μM $FeCl_3$, 0.2 mM ADP, and 0.2 mM ascorbate. The amount of extracted microsomal lipids used corresponded to 3.25 μmol of phospholipids. Basic system ( ); basic system plus 3 mM glutathione (▲); basic system plus 3 mM glutahione and 2 μg/ml PHGPX (■). The mean values of three separate experiments and SE are reported.

presence of PHGPX and glutathione, but without any added vitamin E, may also be partially due to the minute amount of vitamin E, already present in the lipid extract.

DISCUSSION

In our experimental conditions, during ferrous iron dependent microsomal lipid peroxidation, vitamin E is cooxidized with lipids (Figs. 1 and 2) and inhibits peroxidation rate only at supraphysiological concentrations. It is furthermore noteworthy that, in this peroxidation system vitamin E does not induce a lag in peroxidation during which the antioxidant is consumed. A progressive rise of peroxidation rate similar to a lag can actually be observed during ferrous iron dependent lipid peroxidation of microsomes (Fig. 1 and experiments in ref. 2 and 9) or microsomal lipid micelles (Fig. 3). This is probably due to the shift from hydroperoxide independent to hydroperoxide dependent initiations (4), i.e., from primary to secondary initiations since it is completely abolished by adding minute amounts of lipid hydroperoxides. From this point of view, ferrous iron dependent microsomal lipid peroxidation is completely different from both autoxidation of lipids or peroxidation induced by thermal decomposition of diazocompounds, a typical model for the study of the chemistry of vitamin E as an antioxidant. In these cases, the initiating species are peroxyl radicals (11,22), and vitamin E completely inhibits peroxidation by reacting with these several orders of magnitude faster than polyunsaturated fatty acids. Therefore, when these initiation systems are involved, vitamin E induces a lag of peroxidation that is proportional to the amount of available vitamin E (22).

On the other hand, during ferrous iron dependent microsomal lipid peroxidation, vitamin E does not react with the initiating free radical. Vitamin E indeed is not oxidized if polyunsaturated fatty acids are not present (20). Moreover, since the reaction of vitamin E with carbon centered radicals seems improbable (23), and since the addition of oxygen to these is a diffusion limited reaction, the first free radical with which vitamin E can react is LOO•, also in this peroxidation system. Therefore, vitamin E in microsomes, challenged by iron and reducing agents, is active as a chain breaking antioxidant, and not as a primary antioxidant. The observed higher consumption of vitamin E per unit of oxygen consumed when membranes were loaded with vitamin E (Fig. 2) is in agreement with this.

If vitamin E is active as a chain breaking antioxidant, its actual antioxidant capacity must be related to the length of peroxidative chains that in turn depends on the facility of interactions between LOO• and LH. Actually the rate of this reaction is determined by the rate constant and by the concentration of LH, i.e., the vicinity of unsaturation centers in membranes. If we consider that, while 10 to 100 propagation cycles have been measured in methyl linoleate dispersions (24), microsomal lipid peroxidation is not autocatalytic, giving rise to no more than 2-3 cycles following stoppage of initiations (2) this could account for the weak antioxidant effect of vitamin E observed in Figure 2, as well as, possibly, the relatively high free radical generation rate.

By reacting with lipid hydroperoxyl radicals vitamin E prevents propagation, but this antioxidant reaction is tempered in this peroxidation system by several factors: i) propagation is *per se* limited in microsomes; ii) vitamin E actually promotes the formation of a hydroperoxide by reacting with LOO•; and iii) from these hydroperoxides new radicals are generated, against which vitamin E is not a good antioxidant. In fact, following the formation of lipid hydroperoxides, lipid peroxidation proceeds by ferrous iron catalyzed formation of lipid alkoxyl radicals (LO•). Against these radicals the antioxidant effect of vitamin E seems dramatically limited (Fig. 3), apparently because the rate constants of the reaction of LO• with vitamin E and lipids (25) are not so different as the rate constants between LOO• and vitamin E and lipids (11,12). Therefore, when LO• are generated, an amount of vitamin E much larger than the amount actually present in the membranes would be necessary to protect lipids and only a cooxidation of vitamin E and lipids can be observed.

If PHGPX and glutathione are present both in lipid dispersions (Fig. 4) and membranes (9), lipid hydroperoxides are continuously reduced and the shift from primary to secondary-hydroperoxide dependent-initiations is prevented, as well as the production of alkoxyl radicals. Therefore, in the presence of PHGPX and glutathione vitamin E is spared and can exert its chain breaking activity by interacting with lipid hydroperoxyl radicals.

The overall scheme for the mechanisms of both microsomal lipid peroxidation and protection is presented in Figure 5.

According to this scheme, during iron dependent lipid peroxidation in the absence of PHGPX, vitamin E is consumed, without playing a dramatic antioxidant effect.

In conclusion, in microsomal membranes challenged by iron and a reducing system (ascorbate or microsomal

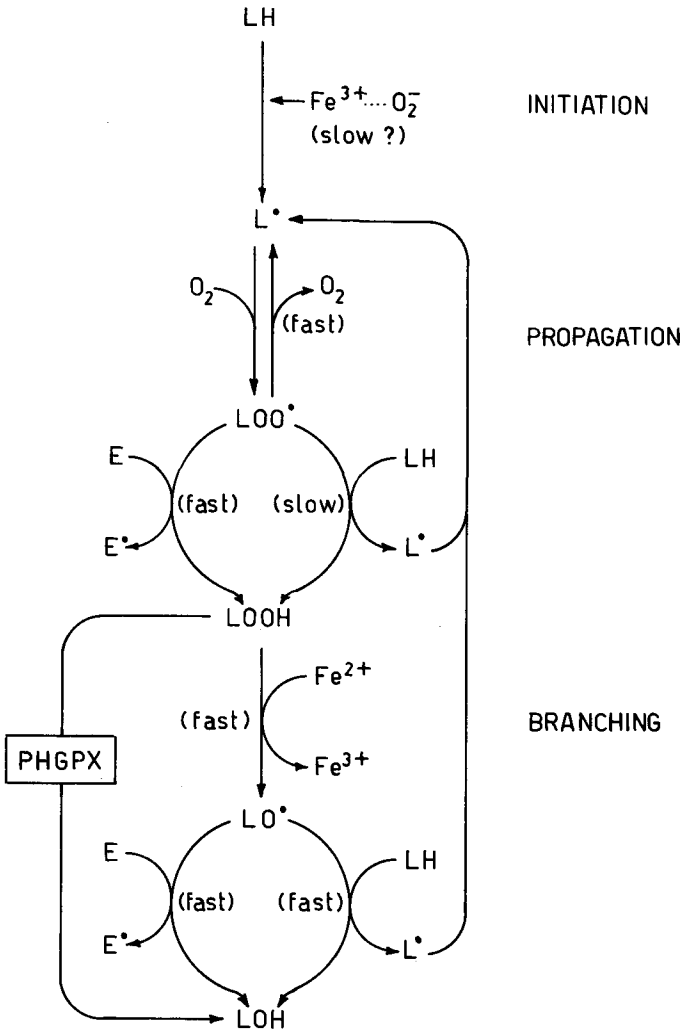

FIG. 5. Scheme of microsomal lipid peroxidation and protection mechanisms. (See text for details.)

electron transport system plus NADPH), vitamin E expresses an effective antioxidant effect only if PHGPX and glutathione are present, basically because the enzyme prevents the formation of alkoxyl radicals against which vitamin E is a relatively weak antioxidant. This cooperative effect accounts for the increased turnover of vitamin E during selenium deficiency (26), as well as for the synergistic effect between selenium and vitamin E (27).

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Effect of Magnesium Deficiency on Δ6 Desaturase Activity and Fatty Acid Composition of Rat Liver Microsomes

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Experimental Mg^{2+} deficiency was induced in a group of rats by feeding them a Mg^{2+}-deficient diet for 23 days. They were pair-fed to compare with a control group of rats fed a Mg^{2+}-sufficient diet. In the Mg^{2+}-deficient group the plasma total cholesterol and triglyceride levels were increased while HDL-cholesterol was decreased. In the Mg^{2+}-deficient group the plasma level of thiobarbituric acid reacting substances (TBARS) used as a measure for lipid peroxidation was increased. The increase was attributed to the increased cytosolic Ca^{2+} in Mg^{2+}-deficiency which can cause: 1) increase of hydro and endoperoxide levels as a consequence of the increase of arachidonic acid release and eicosanoid synthesis in Mg^{2+}-deficiency, and 2) inhibition of the mitochondrial respiratory activity and activation of Ca^{2+}-dependent proteases which may activate the conversion of xanthine dehydrogenase to xanthine oxidase which generates active O_2 species. In the Mg^{2+}-deficient group, the fatty acid composition of the liver microsomes indicated a slower rate of conversion of linoleic acid to arachidonic acid which was consistent with the decrease of Δ6 desaturase activity in liver microsomes of Mg^{2+}-deficient rats as measured *in vitro*. The decrease of Δ6 desaturase activity was attributed to the lower concentration of actual enzyme molecules as a result of the decreased rate of protein synthesis in Mg^{2+}-deficiency. The possible effects of the increased catecholamine release in Mg^{2+}-deficiency are discussed.
***Lipids* 24, 727-732 (1989).**

Several studies provided ample evidence that Mg^{2+}-deficiency affects lipid metabolism. Mg^{2+}-deficiency produced hypercholesterolemia, hypertriglyceridemia and dyslipoproteinemia characterized by an increase of VLDL and LDL and a decrease of HDL (1). In Mg^{2+}-deficient rats an increase of plasma-free cholesterol and a decrease of esterified cholesterol as a result of the reduced lecithin-cholesterol acyltransferase activity (LCAT) were observed (1). Changes in plasma fatty acids were also reported in Mg^{2+}-deficient rats. These changes were characterized by a decrease of stearic and arachidonic acids and an increase of oleic and linoleic acids (2). They were related to the hypertriglyceridemia and the increase of VLDL and LDL and decrease of HDL since a notable alteration of fatty acids can occur as the density of the lipoprotein increases (2). In patients with latent tetany, a disease characterized by a magnesium deficiency (3), the plasma phospholipid fatty acids showed an increase in linoleic acid and a decrease in dihomo-(gamma)-linolenic (20:3ω6) and arachidonic (20:4ω6) acids (4).

Many cellular functions and responses are affected when the membrane lipid or fatty acid unsaturation is modified. These include carrier-mediated transport, membrane-bound enzymes and receptor properties (5). The present experiment was conducted to determine the origin of the fatty acid modification in Mg^{2+}-deficiency. We studied the effect of Mg^{2+}-deficiency on Δ6 desaturase, the enzyme which regulates the biosynthesis of essential polyunsaturated fatty acids (such as 20:4ω6 and 22:6ω3) derived from essential fatty acids. We also studied the changes in membrane fatty acids in Mg^{2+}-deficiency using liver microsomal membrane as a model.

MATERIALS AND METHODS

Chemicals and reagents. [1-^{14}C]Linoleic acid (sp. act. 58.7 mCi/mmol) was purchased from Amersham Corporation (Arlington Heights, IL); [1-^{14}C]linoleoyl-coenzyme A (sp. act. 54.4 mCi/mmol) was purchased from New England Nuclear (Boston, MA). Linoleoyl-coenzyme A, ATP, COA (lithium salt), NADH, bovine serum albumin V fraction (essentially free of fatty acid), thiobarbituric acid (TBA), 1,1,3,3-tetramethoxypropane, and the diagnostic kits for the enzymatic assay of total cholesterol, HDL-cholesterol, and triglycerides in plasma were purchased from Sigma Chemical Company (St. Louis, MO). Linoleic acid and methyl esters of linoleic and γ-linolenic acids were purchased from Nu-Chek Prep. Inc. (Elysian, MN).

Animals and diets. Male Sprague-Dawley weanling rats weighing about 30 grams were housed individually in suspended cages with wire-mesh bottoms. They were subjected to alternating 12 hr periods of light and darkness and were divided randomly to provide a control (12 animals) and an experimental magnesium-deficient group (30 animals). The rats were pair-fed fresh food daily with a modified AIN-76 semipurified diet (Table 1). The magnesium content determined by analysis was a 45 mg/kg diet for the deficient diet group and this was adjusted to a 960 mg/kg diet for the control group by addition of magnesium oxide. Distilled water was available *ad libitum*. The rats were fed their respective diets for 23 days before blood and tissue were collected.

Analysis. Blood was collected by cardiac puncture after ether anaesthesia at the end of the dark period after 12 hr fasting. A single blood sample was taken from each rat. Twelve rats from the control and 20 rats from the deficient groups were used. Plasma from heparinized blood was obtained by immediate centrifugation at 2000 *g* for 15 minutes at 4°C and then frozen immediately for subsequent lipid and mineral analyses.

The enzymatic methods for total cholesterol (6), HDL-cholesterol (7) and triglyceride (8) determination in plasma were used according to the instruction manuals accompanying the diagnostic kits obtained from

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Abbreviations: ATP, adenosine triphosphate; COA, coenzyme A; FFA, free fatty acid; HDL, high density lipoprotein; HCl, hydrochloric acid; LCAT, lecithin cholesterol acyltransferase activity; LDL, low density lipoprotein; MDA, malondialdehyde; NADH, nicotinamide adenine dinucleotide; TBARS, thiobarbituric acid reacting substances. TBA, thiobarbituric acid; VLDL, very low density protein.

TABLE 1

Composition of Semipurified Magnesium-Deficient Diet[a]

Component	gm/100 gm
Casein	20.0
DL-methionine	0.3
Corn starch	15.0
Sucrose	50.0
Fiber	5.0
Corn oil	5.0
Choline bitartrate	0.2
AIN mineral mix (omitting Mg^{2+})	3.5
AIN vitamin mix	1.0
Mg^{2+}	Varied

[a]Modified AIN-76 semipurified diet from which magnesium was omitted. The magnesium level of this diet was 45 mg magnesium/kg diet (deficient diet). The magnesium hydroxide was added to reach the level of 960 mg magnesium/kg diet which represents the control diet in this experiment. The modified AIN-76 semipurified diet from which magnesium omitted was obtained from ICN-Biochemical, Cleveland, OH.

Sigma Co. Lipid oxidation was estimated by assaying thiobarbituric acid reactive substances (TBARS) using the fluorometric method of Yagi (9). Fluorometric measurements were made at 553 nm with 515 nm excitation. The amount of malondialydehyde (MDA) equivalent was determined against a standard curve obtained by using freshly diluted 1,1,3,3-tetramethoxypropane. The degree of lipid oxidation was expressed in nmoles of MDA equivalent per 100 ml plasma.

Magnesium was estimated by atomic absorption flame spectrophotometry (Thermal Jarell Ash Video 12-E) and was carried out at the Atomic Absorption Center, Rodger Adams Laboratory, University of Illinois at Urbana. The plasma samples were diluted 1:50 with a $LaCl_3$ solution (0.1% lanthanum). Microsomes were dried at 105°C for 24 hr and ashed at 550°C for 24 hr. The ashed residue was dissolved in dilute HCl before dilution with the lanthanum solution.

Incubation conditions and assay of desaturase. For microsomal preparation the rats were killed by decapitation after which the abdomen was opened and livers removed. Livers were washed three times in fresh, ice-cold, homogenization solution and immediately processed to obtain the microsomal preparation. The livers of two rats from the same group were pooled to yield enough microsomal preparation for fatty acid analysis and desaturase assay. The rats were not fasted before being killed so that the maximal activities of the liver desaturase could be measured (10).

The liver microsomes were assayed for Δ6 desaturase activity as previously described (11). Each incubation in 1 ml of 0.15 M KCl-0.25 M sucrose solution contained (in μmoles): ATP, 5.0; CoA, 0.25; NADH, 1.0; $MgCl_2$, 5.0; glutathine, 1.5; NaF, 45.0; nicotinamide, 0.5; phosphate buffer (pH 7.0), 100.0; and 2 mg protein of a microsomal suspension. The microsomal protein concentration was measured according to Lowry *et al.* (12). The microsomes were separated by centrifugation at 105,000 × *g* for 2 hr (13). One hundred nmol (containing an amount of ^{14}C-labeled acid equivalent to 0.1 μCi) of linoleic acid in the form of the sodium salt bovine albumin complex (1 μg free fatty acid/11.5 μg bovine serum albumin) were used as substrate. Under these conditions, the enzyme was saturated by the substrate. The incubations were carried out for 20 min in a Dubnoff metabolic shaker at 37°C. The products of the reaction under the assay conditions as described were proportional to the 2 mg protein concentration and the 20 min reaction time.

The reaction was terminated by the addition of 5% HCl in methanol, and the lipids were extracted with chloroform/methanol (2:1, v/v). The extract was dried under a stream of N_2 and transesterified with 3 N methanolic-HCl (Supelco, Inc., Bellefonte, PA) at 70°C for 2 hr (14). The HCl-methanol was evaporated under N_2, and the methyl esters were dissolved in petroleum ether. A mixture of unlabeled carriers of methyl esters of 18:2 + 18:3 was added to the incubation products. The esters were separated on 10% $AgNO_3$ Silica Gel GHL plates (Analtech, Newark, DE) as previously described (15). The separated bands were scraped into scintillation vials, 10 ml of scintillation fluid (Beckman, Ready Solv) were added, and the activity was counted in a Beckman LS 3801 Scintillation Counter. The percentage of desaturation was calculated as the ratio of the counts in the desaturated products to the sum of the counts in the substrate plus product, corrected for background. The nmol of the product was then calculated. The recovery of the radioactivity was more than 85% of the amount used.

Extraction of lipids and analysis of fatty acids. In order to study the effect of magnesium deficiency on the fatty acid composition of the liver microsomal fraction, the microsomes were extracted by the method of Folch *et al.* (16). The methyl esters of fatty acids were prepared from the microsomal lipids following saponification and methylation (17). All operations were carried out under N_2. A Packard model 428 gas chromatograph (Hewlett-Packard Instrument Co., Inc., Chicago, IL), equipped with an all-glass injection splitter and flame ionization detector (FID), was used to separate the methyl esters on a Supelcowax-10, 30 m × 0.25 mm ID fused silica column. The oven temperature was programmed from 160–220° at 1°C/min; the injector and detector temperatures were 280° and 300°C, respectively. The N_2 flow rate was 0.7 ml/min with a split ratio of 110:1. Retention time, peak areas and peak relative area percentages were determined electronically using a Hewlett-Packard Model 3390 A Reporting Integrator. The mol% of fatty acids was then calculated. Identification of methyl esters of fatty acids was accomplished by comparing relative retention time with authentic standards (Nu-Chek Prep Inc.), Elysian, MN, and Supelco, Inc., Bellefonte, PA).

Statistical analysis. Results were expressed as means with their standard deviations. The statistical significance of differences between means was assessed by Student's t-test using group analysis.

RESULTS AND DISCUSSION

The effectiveness of the diet (Table 1) used to induce Mg^{2+} deficiency was clearly shown by the usual depression in plasma Mg^{2+} (Table 2) as well as the clinical manifestations of the syndrome (18). The Mg^{2+}-deficient diet resulted in a decreased efficiency of food utilization and a lower body weight of Mg^{2+}-deficient

TABLE 2

Magnesium Concentrations in Plasma and Liver Microsomal Fraction of Rats Fed on a Control or Magnesium-Deficient Diet[a]

	Control	Mg-deficient	P
Plasma (mg Mg^{2+}/L)	20.10 ± 1.56 (n=10)	4.60 ± 1.71 (n=19)	<0.001
Microsomes (μg Mg^{2+}/mg Protein)	521.00 ± 35.00 (n=5)	389.00 ± 29.00 (n=8)	<0.020

[a]Number in parenthesis represents the number of samples analyzed. In case of microsomes each sample represents microsomes obtained from two pooled livers. Values are given as means ± standard deviations. Statistical analysis was by Student t-test.

rats compared with pair-fed controls. Clinical symptoms of Mg^{2+} deficiency such as hyperaemia of the ears (manifested during the first two weeks), alopecia and hypoxia were also observed: Mg^{2+}-deficient rats were notably excitable. It was not possible to follow their weight increase after 10 days on the deficient diet because once they were touched or disturbed they started convulsing and died within one or two minutes. The mortality rate was 33% after 23 days on the Mg^{2+}-deficient diet.

In Mg^{2+}-deficient rats the decrease of Mg^{2+} level was more severe in plasma than in the microsomal fraction (Table 2). The decrease in plasma Mg^{2+} level agrees with previous reports (19,20). In a previous study (21) a decrease in Mg^{2+} concentration of the liver was reported while Mg^{2+} levels remained unchanged in the liver microsomes of Mg^{2+}-deficient rats. In the latter study, however, adult rats which weighed 100 g were used; while in the present experiment, we used weanling rats weighing only 30 g. This may indicate that the response of adult rats to Mg^{2+}-deficient diets may differ from the weanling rats which are more severely affected by Mg^{2+} deprivation as previously reported (1).

Plasma total cholesterol and triglyceride concentrations were significantly elevated in Mg^{2+}-deficient rats and the increase in triglycerides was much higher than the increase in total cholesterol (Table 3). The total cholesterol/HDL cholesterol ratio was significantly increased in Mg^{2+}-deficient rats, while HDL cholesterol was decreased which indicates that total cholesterol in the lower density lipoproteins such as VLDL and LDL was increased. The increase of plasma triglycerides and decrease of HDL-cholesterol in Mg^{2+}-deficient rats are in agreement with the previous reports (22,23). The significant increase of plasma total cholesterol of Mg^{2+}-deficient rats in our experiment is in agreement with that reported by Jaya and Kurup (22) while it disagrees with that reported by Rayssiguier and Gueux (24) who observed no change in total serum cholesterol of Mg^{2+}-deficient rats. This could be due to the short duration of their experiment which continued for only 8 days on the Mg^{2+}-deficient diet.

The elevated level of plasma-total cholesterol in Mg^{2+}-deficient rats was mainly attributed to: 1) increased activity of hepatic HMG-CoA reductase and the increased incorporation of [^{14}C]acetate into the cholesterol of liver and intestine (22) and 2) a decrease in the degradation of cholesterol to bile acids (22).

An elevated plasma triglyceride level in Mg^{2+}-deficient rats may arise either from an increased synthesis of triglycerides in the liver and increased secretion of VLDL particles, from a decreased removal of lipids from the blood, or from a combination of both. Previous studies (22,23) showed that the triglyceride accumulation in plasma of Mg^{2+}-deficient rats was mainly due to the decreased uptake of triglyceride lipoproteins from the circulation by extrahepatic tissues (heart and adipose tissues) because of the decrease in the lipoprotein lipase activity in these extrahepatic tissues of Mg^{2+}-deficient rats. After intravenous heparin administration, a significant reduction in plasma post-heparin lipolytic activity in Mg^{2+}-deficient rats was observed (1).

Another possible mechanism may involve hypomagnesaemia increasing the catecholamines released (25) with activation of adenylate cyclase through their β-adrenergic effect and enhancing the synthesis of cAMP. Elevated cAMP activates triglyceride lipase of adipose tissue, lipolysis is thus increased, and free fatty acids (FFA) are formed. The FFA enter the blood stream where they are transported to the liver and enhance lipogenesis (26,27). This mechanism is supported by the results of Itokawa *et al.* (28) who demonstrated an acceleration of the incorporation of [^{14}C]acetate into the hepatic lipids in Mg^{2+}-deficient rats. Thus, the increase in plasma cholesterol and triglycerides observed in Mg^{2+}-deficient rats may be the result of increased hepatic synthesis, increased release of lipoproteins into the circulation and their decreased uptake from circulation by the extrahepatic tissues.

The thiobarbituric acid reacting substances (TBARS) used as a measure for the lipid peroxide levels in plasma were significantly increased in Mg^{2+}-deficient rats (Table 3). It is not known if these lipid peroxidation products originated within the vascular compartment of the body or were released from other organs or tissues.

In magnesium deficiency, an increase in cell membrane permeability produces a decrease in cytosolic [K^+] and [Mg^{2+}] and an increase in cytosolic [Na^+] and [Ca^{2+}]. The increased cytosolic [Na^+] induces a release of mitochondrial Ca^{2+} (29,30) and a further elevation of cytosolic [Ca^{2+}]. The calcium-dependent activation of phospholipases with the subsequent release of free fatty acids from membrane phospholipids and activation of the arachidonic acid cascade then occur. This will result in an increase of eicosanoid synthesis as previously reported in different organs and tissues of Mg^{2+}-deficient rats (31). Therefore, higher levels of the

TABLE 3

Plasma Lipids and Lipid Peroxidation Levels in Rats Fed on a Control or Magnesium-Deficient Diet for 23 Days[a]

	Control	Mg-deficient	P
Total cholesterol (mg/100 ml)	125.20 ± 17.80 (9)	178.50 ± 23.44 (20)	<0.001
HDL-cholesterol (mg/100 ml)	51.20 ± 3.71 (8)	45.60 ± 6.24 (16)	<0.05
$\frac{\text{Total cholesterol}}{\text{HDL-cholesterol}}$ %	2.45 ± 0.20 (8)	3.96 ± 0.31 (16)	<0.001
Triglycerides (mg/100 ml)	46.00 ± 3.80 (9)	189.20 ± 50.50 (17)	<0.001
TBARS (nmol MDA/100 ml)	559.00 ± 52.00 (9)	935.00 ± 120.00 (20)	<0.001

[a]Number in parenthesis indicates number of animals used for each assay. Values are given as means ± standard deviations. Statistical analysis was by Student t-test.

hydro-, and endoperoxides should be produced from arachidonic acid through action of lipoxygenase and cyclooxygenase within the tissues of Mg^{2+}-deficient rats. These lipid peroxides are both intermediates in and regulators of prostaglandin synthesis: they have a short lifetime. The increased levels of these peroxides above a certain level within the cell may have an initiating effect on lipid peroxidation if the glutathione peroxidase and glutathione transferase enzyme levels are not enough to protect against their peroxidative effect. The increase of intracellular $[Ca^{2+}]_i$ also may inhibit the mitochondrial respiratory activity and activate Ca^{2+}-dependent proteases which may catalyze conversion of xanthine dehydrogenase to xanthine oxidase (32) and lead to the increase of lipid peroxidation through the generation of active O_2 species. A similar effect can be produced also as a result of the stress accompanying Mg^{2+} deficiency. This stress can increase xanthine and hypoxanthine concentrations within the cells which result in a greater production of H_2O_2 and O_2 (33).

In comparison to the control group, a significant decrease in microsomal arachidonic (20:4ω6) and 22:4ω6 acids and an increase in 20:3ω6 and 18:2ω6 acids were noted in the Mg^{2+}-deficient rats (Table 4). These changes are not fully in agreement with the changes previously reported in other studies (34,35) regarding the effect of Mg^{2+} deficiency on fatty acid composition. These studies (34,35) reported an increase of arachidonic acid in the triglyceride fraction and an increase of 22:4ω6 and 22:5ω3 acids in the triglycerides and phospholipid fraction of the arterial bed of the magnesium-deficient rats as compared to the controls. These discrepancies between our results and the previously reported results (34,35) could be attributed to the difference in the degree of Mg^{2+} deficiency. In the present study, Mg-deficient rats were fed for only 23 days a diet which contained 45 mg Mg/kg diet; while in the other studies (34,35), the rats were fed a diet containing 120 mg Mg^{2+}/Kg diet for 14 weeks.

The increase of 18:2ω6 and the decrease of 20:4ω6 in Mg^{2+}-deficient rats may possibly indicate a decrease in the rate of conversion of 18:2ω6 to 20:4ω6. Another possible reason for the decrease of arachidonic acid could be the increased rate of eicosanoid synthesis in the tissues and organs of Mg^{2+}-deficient rats (31) which can increase 20:4ω6 utilization in these tissues. In addition, the increased rate of lipid peroxidation in Mg deficiency could contribute to the decrease of 20:4ω6 acid since it is one of the highly susceptible acids to oxidation.

Since Δ6 desaturase is the key enzyme for the conversion of 18:2ω6 acid to any of ω6-metabolites, the activity of this enzyme then can be measured by the ratio of total ω6 metabolites total ω6 acids (36). From Table 4 it is clear that this ratio was significantly decreased in Mg^{2+}-deficient rats. These changes in fatty acids are consistent with the lower Δ6 desaturase activity of liver microsomes derived from Mg^{2+}-deficient rats, as measured *in vitro* (Table 5).

The true substrates for the desaturase enzymes *in vivo* are the Coenzyme-A (CoA) esters of fatty acids which require Mg^{2+}, ATP and CoA for their formation. A low cellular Mg^{2+} level could decrease the cellular ATP level (37,38) or directly affect the rate of Acyl-CoA formation. This activation step may become a rate-limiting step in the fatty acid desaturation reactions in Mg^{2+} deficiency which could explain the lower conversion rate of 18:2ω6 to 20:4ω6 in the liver microsomes of Mg^{2+}-deficient rats *in vivo*. However, when the liver microsomes of Mg^{2+}-deficient rats were incubated *in vitro* with 18:2ω6 acid as substrate in the presence of $MgCl_2$, CoA and ATP as cofactors, the Δ6 desaturase activity was not restored but remained significantly lower than that of controls (Table 5). Similarly, when linoleoyl-CoA was used as substrate instead of linoleic acid the Δ6 desaturase activity also remained lower in Mg^{2+}-deficient rats than in the controls. These results indicate that the activation step of the fatty acids to their CoA-ester is not the contributing factor for the lower microsomal Δ6 desaturase activity in Mg^{2+} deficiency. It is possible that Mg^{2+} deficiency affects the concentration of actual enzyme molecules since a decreased rate of liver protein synthesis and hypoproteinemia are early symptoms of Mg^{2+} deficiency (39).

As previously indicated a reduced plasma Mg^{2+} concentration released increased catecholamines (40). In previous studies it was shown that epinephrine regulates the biosynthesis of polyunsaturated fatty acids in rat liver microsomes (41). A single dose of this hormone (1 mg/kg body wt) administered to normal

TABLE 4

Fatty Acid Composition of Liver Microsomal Lipids of Rats Fed on a Control or Magnesium-Deficient Diet[a]

Fatty acid	Control (n=6) mol%	Mg^{2+}-deficient (n=6) mol %	P
14:0	1.00 ± 0.2	0.96 ± 0.2	N.S.[b]
16:0	18.30 ± 2.1	18.24 ± 0.7	N.S.
16:1	3.00 ± 0.7	3.20 ± 0.2	N.S.
18:0	6.80 ± 0.9	5.40 ± 0.6	<0.05
18:1ω9	8.10 ± 1.3	6.70 ± 0.5	N.S.
18:1ω7	2.00 ± 0.2	2.26 ± 0.3	N.S.
18:2ω6	16.40 ± 0.7	20.44 ± 1.3	<0.01
18:3ω6	0.60 ± 0.1	0.52 ± 0.1	N.S.
20:3ω6	0.80 ± 0.3	1.20 ± 0.2	<0.02
20:4ω6	31.60 ± 1.9	28.48 ± 0.9	<0.02
22:4ω6	1.20 ± 0.2	0.85 ± 0.1	<0.05
22:5ω6	3.30 ± 0.3	3.20 ± 0.6	N.S.
22:5ω3	1.10 ± 0.2	0.86 ± 0.2	N.S.
22:6ω3	6.20 ± 0.7	5.48 ± 0.8	N.S.
20:4ω6 / 18:2ω6	1.93 ± 0.1	1.40 ± 0.1	<0.001
Total ω6 acids	53.88 ± 1.7	55.08 ± 1.4	N.S.
Total ω6 metabolites	37.51 ± 1.3	34.64 ± 0.5	<0.01
ω6 Metabolites[c] / ω6 Acids	0.70	0.63	<0.001

[a]Results are expressed as means ± standard deviations. Statistical significant differences were calculated by Student t-test.

[b]N.S. - not significant.

[c]ω6 Metabolites are total ω6 acids other than 18:2ω6 acid.

TABLE 5

Liver Microsomal Δ6 Desaturase Activity in Rats Fed on a Control or Magnesium-Deficient Diet[a]

Substrate	Controls (n=6)	Mg-deficient (n=8)	P
Linoleic acid +Cofactors nmol 18:3 produced/ min/mg microsomal protein	0.238 ± 0.018	0.192 ± 0.44	<0.01
Linoleoyl-CoA nmol 18:3 produced/ min/mg microsomal protein	0.282 ± 0.018	0.245 ± 0.025	<0.01

[a]Results are expressed as mean ± standard deviation. Values are given as nmol of 18:3ω6 produced/min/mg microsomal protein. Statistical analysis was by Student t-test.

rats produced a significant decrease in Δ6 and Δ5 desaturase activities in rat liver microsomes. In the liver, this action was attributed to an increase in the intracellular level of cAMP (39) and operated through a β-adrenergic mechanism (42). The increase of cAMP was followed by a glycogen breakdown in liver which led to an increase in blood glucose and a decrease in Δ6 desaturase activity (41). Since epinephrine injection promoted only a modification of the V_{max} of linoleic acid desaturation while K_m remained constant, it was concluded that the inhibitory effect of epinephrine is evoked through a decrease in the amount of active enzyme (41) which could be evoked through an inhibition of the synthesis of Δ6 desaturase.

The results of the present study confirmed the previous findings that Mg^{2+} deficiency increases the plasma total cholesterol and triglyceride levels while decreasing the HDL-cholesterol. It also shows, to the best of our knowledge, for the first time that Mg^{2+} deficiency increases the plasma lipid peroxidation products' level. In addition, it modifies the membrane fatty

acid composition due to the decrease of Δ6 desaturase activity.

The impairment of essential fatty acid metabolism could have an effect on the polyunsaturated essential fatty acid content in the cell membranes this can affect the cellular membrane fluidity and permeability. A known relationship exists between thrombotic-induced aggregation and fatty acid composition of the platelets (43). The possibility exists that Mg^{2+} deficiency may have an affective role on platelet functions. All these changes favor increased risk of atherosclerosis.

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Activation of Rat Liver Cholesterol Ester Hydrolase by cAMP-Dependent Protein Kinase and Protein Kinase C[1]

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Short term regulation of hepatic cholesterol ester hydrolase by reversible phosphorylation is described. Two different kinase systems seem to be involved in this regulation. The addition of ATP, cyclic AMP and Mg^{2+} to rat liver 104,000 × g supernatant (S104) produced a 100–140% increase in cholesterol ester hydrolase activity. This stimulation was abolished when protein kinase inhibitor was added prior to the addition of ATP, cyclic AMP and Mg^{2+}. Cholesterol ester hydrolase activity was also stimulated when calcium ions, phosphatidylserine, and diolein were added to S104 along with ATP and Mg^{2+}. Diolein in this reaction could be substituted by phorbol 12-myristate 13-acetate. Preincubation of S104 with alkaline phosphatase resulted in a deactivation of cholesterol ester hydrolase. The addition of increasing concentrations of Mg^{2+} to S104 produced increasing inhibition of cholesterol ester hydrolase activity, and this effect was blocked by NaF.

It is suggested that rat liver cholesterol ester hydrolase is activated by cyclic AMP dependent protein kinase and protein kinase C. Deactivation is accomplished by dephosphorylation catalyzed by a phosphoprotein phosphatase, dependent on Mg^{2+}.
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Cholesterol ester hydrolase catalyzes the release of free cholesterol from stores of cholesterol esters present as lipid droplets in the cytoplasm. In liver, cholesterol esters are primarily derived from the uptake and degradation of plasma lipoprotein and, following hydrolysis, the resulting free cholesterol as well as the endogenous cholesterol is either reesterified and stored, or catabolized to form bile acids. The total amount of cholesterol in the hepatic cell changes in response to changes in dietary cholesterol content and to other manipulations that affect the rate of endogenous cholesterol biosynthesis. However, the concentration of unesterified cholesterol present in the liver remains within a relatively narrow range, whereas the amount of esterified cholesterol can be markedly increased (1–3).

Hepatic levels of cholesterol are maintained by a precise balance between reactions catalyzed by HMGCoA reductase, 7α-hydroxylase, acylCoA:-cholesterol acyltransferase, (ACAT) and cholesterol ester hydrolase. HMGCoA reductase and 7α-hydroxylase are the rate limiting enzymes in cholesterol and bile acid synthesis, respectively. ACAT catalyzes the esterification of cholesterol whereas cholesterol ester hydrolase releases free cholesterol from the stored cholesterol esters. Phosphorylation/dephosphorylation has been implicated as a common mechanism for the short term regulation of HMGCoA reductase, ACAT and 7α-hydroxylase in liver (4). Much evidence has accumulated supporting the concept that cholesterol ester hydrolase from non-hepatic tissues is regulated by reversible phosphorylation by cAMP-dependent protein kinase. The activity of cholesterol ester hydrolase in adrenal gland (5), corpus luteum (6), adipose tissue (7) and ovaries (8,9) is modulated by hormones which are thought to act via cAMP. The activity of partially purified preparations of the enzyme from rat testis (10) and adrenal cortex (11) is elevated by incubation with cAMP dependent protein kinase and ATP-Mg^{2+}. The activation of purified enzyme from corpus luteum closely correlates with the phosphorylation of the protein (12), and phosphoamino acid analysis showed that only one serine residue is phosphorylated.

Although liver is the principal organ for the catabolism of the steroid nucleus, no reports are available regarding the regulation of cholesterol ester hydrolase in this organ. In the present paper we present evidence to suggest the activation of the enzyme in rat liver by cAMP-dependent protein kinase and also by Ca^{2+}-phospholipid dependent protein kinase (protein kinase C). Our results indicate that in rat liver, cholesterol ester hydrolase is regulated by reversible phosphorylation, the phosphorylated form of the enzyme being the more active form.

MATERIALS

[1-^{14}C]Cholesterol oleate (56.6 mCi/mmol) was purchased from New England Nuclear (Boston, MA). All solvents were purchased from Fischer Scientific (Columbia, MD). ATP disodium salt, adenosine 3′,5′-cyclic monophosphate (sodium salt), alkaline phosphatase (Type IX from bovine liver), rabbit skeletal muscle protein kinase inhibitor, phorbol 12-myristate 13-acetate, phosphatidylserine, and diolein were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were of analytical grade.

METHODS

Preparation of rat liver cytosol. Adult male Sprague-Dawley rats (Flow Laboratories) were used for the experiments. They had free access to food and water and were kept at 24°C on a cycle of 12 hr light/12 hr darkness. Rat liver was processed as previously described (13), with minor modifications. Rats weighing 200–300 g were sacrificed by decapitation and the liver

[1]This work was presented at the Twenty-Third Southeastern Regional Lipid Conference, held October 26–28, in Cashiers, North Carolina.

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Abbreviations: ACAT, acylCoA:cholesterol acyltransferase; AMP, adenosine monophosphate; ATP, adenosine triphosphate; cAMP, cyclic 3′,5′-adenosine monophosphate; CEH, cholesterol ester hydrolase; CEH-P, phosphorylated form of cholesterol ester hydrolase; DG, diacylglycerol; PI, phosphatidylinositol; PKI, protein kinase inhibitor; TCA, trichloracetic acid; PMA, phorbol 12-myristate 13-acetate; S104, the 104,000 × g supernatant.

removed and washed in 20 mM Tris HCl buffer pH 7.5, containing 5 mM 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 minutes, 10000 × g for 30 minutes and 104000 × g for 90 minutes to yield a high speed supernatant (S104). In some experiments S104 was brought to 40% saturation with ammonium sulfate. The precipitated proteins were collected by centrifugation and used in the assays.

Cholesterol ester hydrolase assay. Cholesterol ester hydrolase activity was measured by the method described by Chen *et al.* (14). The substrate, cholesterol oleate, was prepared as previously described (15). In brief, the assay mixture contained 100 mM Tris HCl buffer pH 7.5 containing 5 mM 2-mercaptoethanol and 300–500 μg enzyme protein in a final volume of 500 μl. The reaction was started by the addition of 75 μM [1-^{14}C]cholesterol oleate (40000–50000 dpm) and incubated for 30 minutes at 37°C. It was terminated by the addition of 3.25 ml of methanol/chloroform/heptane (3.85:3.42:2.73 v/v/v) and 50 μl of 1M NaOH. Each tube was vortexed immediately and the phases were separated by centrifugation for 10 minutes at 2000 rpm. 1 ml of the upper phase was removed and transferred into scintillation vials. 5 ml of Aquasol-2 was added and the associated radioactivity determined. The reaction rate was linear within the time of incubation employed.

Activation of cholesterol ester hydrolase by cofactors of protein kinases. Activation by cAMP-dependent protein kinase was determined using the same reaction mixture described above with the addition of 1 mM $MgCl_2$, 5 mM ATP and 100 μM cAMP. The enzyme was preincubated with the co-factors for 1–2 minutes before the addition of the substrate, and the incubation was continued as described above. Similarly, activation by protein kinase C 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. The phosphatidylserine and diolein solutions were prepared as described by Beg *et al.* (16).

Protein determination. Since the homogenizing buffer contained mercaptoethanol, protein was determined by BCA procedure following TCA precipitation (17).

RESULTS

Activation by cyclic AMP-dependent protein kinase. Cholesterol ester hydrolase activity in S104 was increased by 100–140% when assayed in the presence of cyclic 3′,5′-adenosine monophosphate (cAMP) and Mg^{2+}-ATP, suggesting activation by a cAMP-dependent protein kinase (Fig. 1). Maximum activation was obtained with a two minute preincubation, whereas longer preincubations resulted in a gradual decline in activation (data not shown). Addition of skeletal muscle protein kinase inhibitor decreased the activation by 25%, providing additional evidence for mediation by cAMP-dependent protein kinase (Fig. 1).

Activation by protein kinase C. Cholesterol ester hydrolase activity of S104 was increased 50–100% when assayed in the presence of Mg^{2+}-ATP, $CaCl_2$, phosphatidylserine and diolein (data not shown), indicating a role of protein kinase C in the activation process. However, absolute dependence of the activation process on phosphatidylserine and diolein could not be shown in S104, probably due to the presence of endogenous lipids. Consequently, proteins precipitated between 0–40% ammonium sulfate saturation, which have been reported to include both cholesterol ester hydrolase and protein kinase C (6,11,18), were used as the enzyme source. In the current study, 95–100% of cholesterol ester hydrolase activity was recovered in the ammonium sulfate precipitate. Maximum activity was observed in the presence of all the required co-factors (Fig. 2), indicating the involvement of protein kinase C. Diolein could be substituted by phorbol ester (PMA), providing additional evidence for mediation by protein kinase C.

Effect of Alkaline Phosphatase and Mg^{2+} ions. S104 was preincubated in the absence or presence of 0.1 units of alkaline phosphatase and then assayed for cholesterol ester hydrolase. Only 31% activity was recovered following alkaline phosphatase treatment. This decrease in activity by 69% suggested an inactivation of the enzyme following dephosphorylation. Addition of Mg^{2+} to S104 also produced a significant decrease in cholesterol ester hydrolase activity (Fig. 3). This inhibition increased with increasing Mg^{2+} concentration. At 10 mM $MgCl_2$ cholesterol ester hydrolase activity was only 15% of the control value. Inactivation of cholesterol ester hydrolase by Mg^{2+} could be partially blocked by NaF, a phosphatase inhibitor. This suggests a presence of the Mg^{2+} dependent phosphatase in the S104 catalyzing the dephosphorylation and thus the inactivation of cholesterol ester hydrolase.

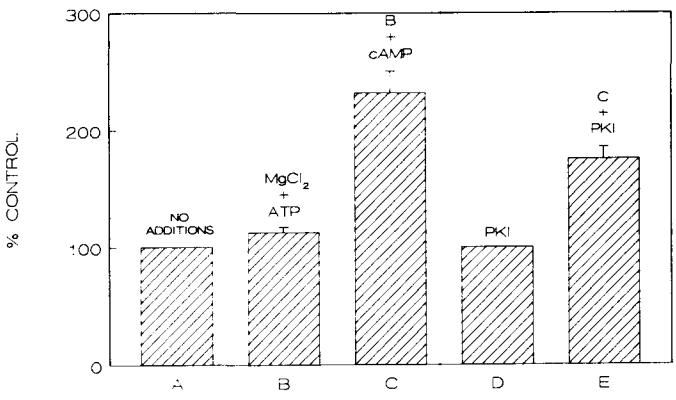

FIG. 1. Activation of cholesterol ester hydrolase by cAMP dependent protein kinase. Incubations were carried out in a final volume of 500 μl for 30 min at 37°C as described in Methods. S104 (600 μg protein) was used as the enzyme source, and cholesterol ester hydrolase activity was assayed in the presence of the following additions. A, no additions; B, 1 mM $MgCl_2$ + 5 mM ATP; C, B + 100 μM cAMP; D, 100 μg protein kinase inhibitor; E, C + protein kinase inhibitor (PKI). Values shown are mean + S.D. of triplicates. 100% control was 0.27 nmoles/hr/mg protein. The experiment was repeated (n=3) with similar results. The specific activity varied from 0.27 to 0.81 nmoles/hr/mg protein. 54–140% activation was observed in the presence of Mg^{2+}, ATP and cAMP.

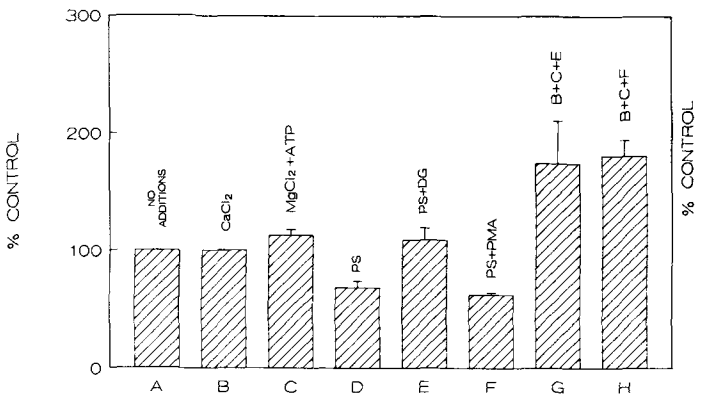

FIG. 2. Activation of cholesterol ester hydrolase by protein kinase C. Cholesterol ester hydrolase was assayed as described in Methods, using ammonium sulfate precipitate (270 μg protein) as the enzyme source and in the presence of the following: A, no additions; B, 1 mM $CaCl_2$; C, 1 mM $MgCl_2$ + 5 mM ATP; D, 20 μg/ml phosphatidylserine, E, 20 μg/ml phosphatidylserine + 4 μg/ml diolein; F, 20 μg/ml phosphatidylserine + 50 ng/ml PMA; G, 1 mM $CaCl_2$ + 1 mM $MgCl_2$ + 5 mM ATP + 20 μg/ml phosphatidylserine + 4 μg/ml diolein; H, 1 mM $CaCl_2$ + 1 mM $MgCl_2$ + 5 mM ATP + 20 μg/ml phosphatidylserine + 50 ng/ml PMA. Values shown are mean + S.D. of triplicates. 100% control was 0.47 nmoles/hr/mg protein. The experiment was repeated (n=3) with similar results. The specific activity varied from 0.47–1.16 nmoles/hr/mg protein. 74–162% activation was observed in the presence of Mg^{2+}, ATP, Ca^{2+}, phosphatidylserine and diolein.

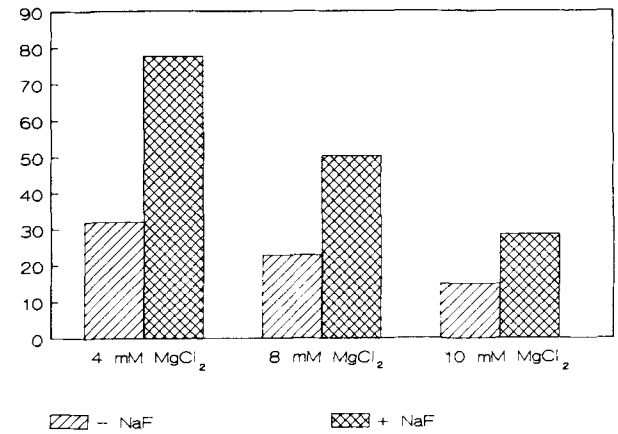

FIG. 3. Effect of $MgCl_2$ on cholesterol ester hydrolase activity. Cholesterol ester hydrolase activity was determined in the presence of 4 mM, 8 mM and 10 mM $MgCl_2$, with or without the addition of 40 mM NaF. The different additives were present in the reaction mixture for the entire length of incubation. S104 (570 μg protein) was used as the enzyme source. Values shown are mean of triplicates. 100% control was 0.365 nmoles/hr/mg protein. The experiment was repeated with similar results.

DISCUSSION

These results provide evidence for the short term regulation of hepatic cholesterol ester hydrolase by protein kinases. Cholesterol ester hydrolase activity was increased 100–140% by Mg^{2+}-ATP and cAMP. Moreover, this activation of cholesterol ester hydrolase was partially abolished by rabbit skeletal muscle protein kinase inhibitor indicating the involvement of a cAMP-dependent protein kinase. Presence of a cytosolic cAMP-dependent protein kinase has been demonstrated in rat liver (18). In the current study, cholesterol ester hydrolase was also activated by the cofactors for protein kinase C, Mg-ATP, Ca^{2+}, phosphatidyl-serine and diolein. In this activation process, diolein could be substituted by phorbol ester, a known activator of protein kinase C, providing additional evidence for the involvement of protein kinase C. Such short term regulation by multiple kinases has been well documented for hepatic HMGCoA reductase, the rate limiting enzyme in cholesterol biosynthesis (4).

Increasing concentration of Mg^{2+} inactivated the cholesterol ester hydrolase, indicating the presence of a Mg^{2+} dependent phosphatase as reported earlier by Trzeciak and Boyd in adrenal cortex (19), and by Bailey and Grogan (10) in rat testis. NaF, a known protein phosphatase inhibitor, partially blocked the inhibitory effect of Mg^{2+} on cholesterol ester hydrolase activity in S104. At higher Mg^{2+} concentration (10 mM) and, presumably, higher phosphatase activity, NaF was not very effective. The inability of NaF or even phosphate (a competitive inhibitor of phosphatase) to effectively block higher phosphatase activity has also been observed by others (20, 21).

Deactivation of cholesterol ester hydrolase with the addition of alkaline phosphatase provides further evidence to support the view that cholesterol ester hydrolase is deactivated by dephosphorylation. Merlvede and Riley (22) reported a Mg^{2+} dependent phosphorylase phosphatase in adrenal cortex which is responsible for the dephosphorylation and deactivation of phosphorylasea. A similar mechanism of activation/deactivation of hormone sensitive lipase in adipose tissue has also been suggested (23–27). It is possible that a similar phosphatase is responsible for deactivation of cholesterol ester hydrolase in rat liver.

We propose the following mechanism for the regulation of cholesterol ester hydrolase in rat liver (Fig. 4). Under conditions of increased cholesterol need, there is activation of adenylate cyclase to increase the intracellular levels of cAMP and also increased phosphatidylinositol turnover to increase intracellular diacylglycerol (DG) and Ca^{2+}. This activation could be hormone mediated, analogous to regulation of carbohydrate metabolism in liver, by hormones acting via cAMP, and also by hormones which increase intracellular Ca^{2+} (28–32). cAMP and Ca^{2+}-DG would then activate cAMP-dependent protein kinase and protein kinase C, respectively. The active protein kinases would, in turn, phosphorylate and activate the inactive or less active cholesterol ester hydrolase to an active form. The active cholesterol ester hydrolase would then hydrolyze cholesterol ester stores to maintain free cholesterol for bile acid synthesis and maintenence of membrane cholesterol.

In the regulation of cholesterol metabolism, the esterification of free cholesterol is catalyzed by microsomal acylCoA:cholesterol acyltransferase (ACAT), the conversion of cholesterol to bile acids by 7α-hydroxylase, the hydrolysis of esterified cholesterol to free cholesterol by cholesterol ester hydrolase, and *de novo* synthesis of cholesterol by HMGCoA reductase. There

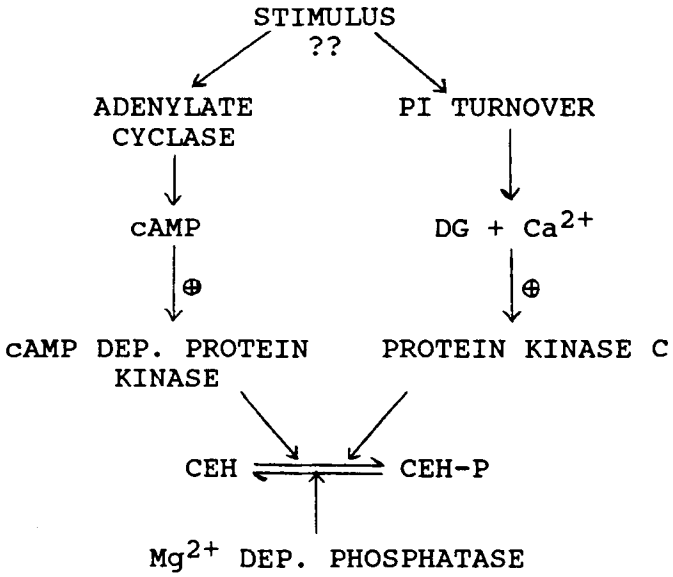

FIG. 4. Proposed mechanism for the regulation of cholesterol ester hydrolase in rat liver. PI, phosphatidylinositol; DG, Diacylglycerol; CEH, cholesterol ester hydrolase; CEH-P, phosphorylated form of CEH.

is substantial evidence for short term regulation of ACAT, 7α-hydroxylase and HMGCoA reductase by reversible phosphorylation (20, 33, 4). In contrast to HMGCoA reductase, which is inhibited by phosphorylation and activated by dephosphorylation, the activities of ACAT and 7α-hydroxylase are increased by *in vitro* phosphorylation, whereas treatment with a cytosolic fraction containing phosphatase is associated with a decline in enzyme activities (20, 33). Our results provide evidence for the activation of cholesterol ester hydrolase by protein kinase A and protein kinase C and deactivation by Mg^{2+} dependent phosphatase. This implies that cholesterol ester hydrolase is regulated by reversible phosphorylation and that the phosphorylated form is the active form. Based on these observations it appears that there is a coordinated control of the enzymes involved in cholesterol metabolism, by reversible phosphorylation. Under conditions of increased phosphorylation in the cell, HMGCoA reductase would be inhibited and the free cholesterol pools would be depleted due to increased activities of ACAT and 7α-hydroxylase. Cholesterol ester hydrolase, which is more active in the phosphorylated state, would then be important in maintaining the free cholesterol level in the cell. However, the mechanism by which cellular cholesterol levels might affect the phosphorylation status of the various enzymes has not been demonstrated. It is possible that this mode of regulation responds to the nutritional status or other humoral factors affecting hormone levels, rather than to cholesterol levels *per se.*

ACKNOWLEDGMENTS

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Dietary Fish Oil Augments the Function and Fluidity of the Intestinal Brush-border Membrane of the Carp

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Two groups of carps were raised on a commercial nutritionally complete diet: one was the control group, the other was fed the same diet enriched with 7% fish oil. The experiment lasted seven months (July through February), during which time the environmental temperature dropped gradually from 25°C to 13°C. Intestinal microvillus membranes were isolated after four and seven month feeding and examined for fluidity by fluorescence polarization with 1,6-diphenyl-1,3,5-hexatriene. The functionality of the membrane was assessed by the activity of the intrinsic enzyme alkaline phosphatase.

The experimental group exhibited increased membrane fluidity and elevated enzyme activity only when the environmental temperature decreased to 13°C. These changes in the membrane properties seemed to correlate with alterations in the fatty acid profile of the membrane phospholipids. Whereas the control group showed some increase in the n-3 C20:5, C22:5, and C22:6 fatty acids most likely due to cold adaptation, the membranes isolated from the group fed fish oil showed a considerably higher level of these fatty acids reflecting the combined effect of the dietary manipulation and cold adaptation.
Lipids 24, 737-742 (1989).

It is now widely accepted that the function of membrane proteins depends on the membrane fluidity (1). The fluidity, in turn, is largely affected by the temperature. Poikilotherms such as fish may develop severe malfunction in winter, below their optimal existence temperature (2). Thus, it is known from common practice that fish often cease eating below a certain water temperature characteristic of the fish species. It is conceivable that rigidification of the membranes at low environmental temperatures contributes to the ill effects by impairing membrane permeability and function of membrane-bound proteins (2).

It was shown that fish can adapt to cold temperatures by preferentially synthesizing long-chain polyunsaturated fatty acids, especially docosahexaenoic acid (3, 4). Another study demonstrated the importance of dietary polyunsaturated fatty acids in maintaining the resistance of the carp to cold temperatures (5).

The intestinal brush-border membrane specializes in digestion and absorption of nutrients, and is rich in a large number of functional proteins, including bound enzymes and transport proteins (6). It was therefore of interest to evaluate at the membrane level the effect of dietary polyunsaturated fatty acids on the structure, dynamics and function of this membrane in the carp under decreasing environmental temperatures.

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Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; PUFA, polyunsaturated fatty acids.

MATERIALS AND METHODS

Animals and diets. Two groups of carp *(Cyprinus carpio)* having individual initial weights of 80 g were grown in plastic cages of $1m^3$ suspended in a fish pond. One group was fed a commercial pelleted diet (control). The second group was fed the same pellets coated with 7% fish oil (experimental diet). The fatty acid profile of the fish oil is given in Table 2. The feeding started in July and continued until the end of February the following year.

Membrane isolation. Fish taken from each group in November and in February were killed by a blow to the head. Mucosa scrapings of the upper half of the small intestine of five fish were used for microvillus membrane preparation. Prior experiments (Behar, D., unpublished data) showed that the lower half of the small intestine of carp possesses poor alkaline phosphatase activity. Membranes were isolated essentially as described by Schmitz *et al.* (7) and Brasitus *et al.* (8), except that the initial homogenization step was longer, using a Waring Blender for 2.5 min at medium speed followed by 2 min at full speed. The purity of the isolated membranes relative to the crude mucosa homogenate was assessed by estimating the specific activity of alkaline phosphatase. The various preparations were purified 8-11-fold.

Fluorescence studies. The fludity of the membrane preparation was assessed by steady state fluorescence polarization measurements using 1,6-diphenyl-1,3,5-hexatriene (DPH) as the fluorescent probe (9). The polarization of fluorescence was expressed as the fluorescence anisotropy, r, and the anisotropy parameter, $[(r_o/r)-1]^{-1}$, was calculated using a limiting anisotropy value of DPH of $r_o=0.362$. The anisotropy parameter is inversely related to the membrane lipid fluidity (9) and was presented as Arrhenius plot of log $[(r_o/r)-1]^{-1}$ vs 1/T.

Analytical determinations. Protein was quantified by the method of Lowry *et al.* (10). Alkaline phosphatase was assayed according to the method described by Brasitus *et al.* (8), except for the elimination of zinc ions. Lipids were extracted from the microvillus membrane according to Folch *et al.* (11). The fatty acid profile of the membrane lipids and that of the fish oil was determined after methylation with boron trifluoride according to Miller (12), using a Model 3700 Varian gas chromatograph equipped with a flame ionization detector and Varian Model CDS 1112 integrator. The methyl esters were resolved on a column packed with GP 10% SP 2330 on chromosorb WAW (Supelco). Individual fatty acids were identified with a fatty acyl methyl ester standard from a marine source (PUFA-1 Supelco). The temperature of the injection port and detector was

TABLE 1

Fluoresence Anisotropy Parameter and Alkaline Phosphatase Activity in Brush-Border Membranes Isolated from Rats Fed Fish Oil and Control Diet at the End (February) of 7 Month Feeding[a]

	Anisotropy Parameter $[(r_0/r)-1]^{-1}$		Alkaline Phosphatase Activity (μmole•min^{-1}•mg protein^{-1})	
Diet type	10°C	25°C	10°C	25°C
Control	3.92±0.15	2.28±0.14	0.024±0.005	0.062±0.008
Fish-oil	2.84±0.18[c]	1.88±0.10[b]	0.056±0.007[c]	0.157±0.007[c]

[a]Values are mean ± SEM for 4 different preparations.

Significantly different from the respective control.
[b]$p<0.05$.
[c]$p<0.01$.

230°C and that of the column was 210°C. Unidentified peaks amounting to less than 0.5% of the total area were disregarded.

Statistical analysis was carried out using Student's t-test.

RESULTS

During the growth period of seven months, both the experimental and the control fish exhibited normal development and weight gain.

The temperature dependence of the fluorescence anisotropy parameter $[(r_0/r)-1]^{-1}$ of DPH in the intestinal brush-border membrane preparations of both carp groups, following four months of feeding, is illustrated by Arrhenius plots in Figure 1. At this stage of development with a relatively high environmental temperature of about 20°C, typical of the month of November, the dietary fish oil had no effect on the membrane fluidity. Similiarly, an essentially identical alkaline phosphatase activity was observed over the range of 1-38°C, as shown in Figure 2. The Arrhenius plots of the enzymatic activity at this high environmental temperature were characterized by a transition temperature of 18°C for both the control and the experimental groups. With the advancement of the season, the water temperature declined and reached 13°C in February. Typical Arrhenius plots (Fig. 3) as well as values at 10°C and 25°C (Table 1) of the fluorescence anisotropy parameter at this stage of the experiment show that the membranes of the carps fed fish oil became considerably more fluid as compared to the membranes of the control group. The effect of the polyunsaturated fatty acids was also manifested in the activity of the mem-

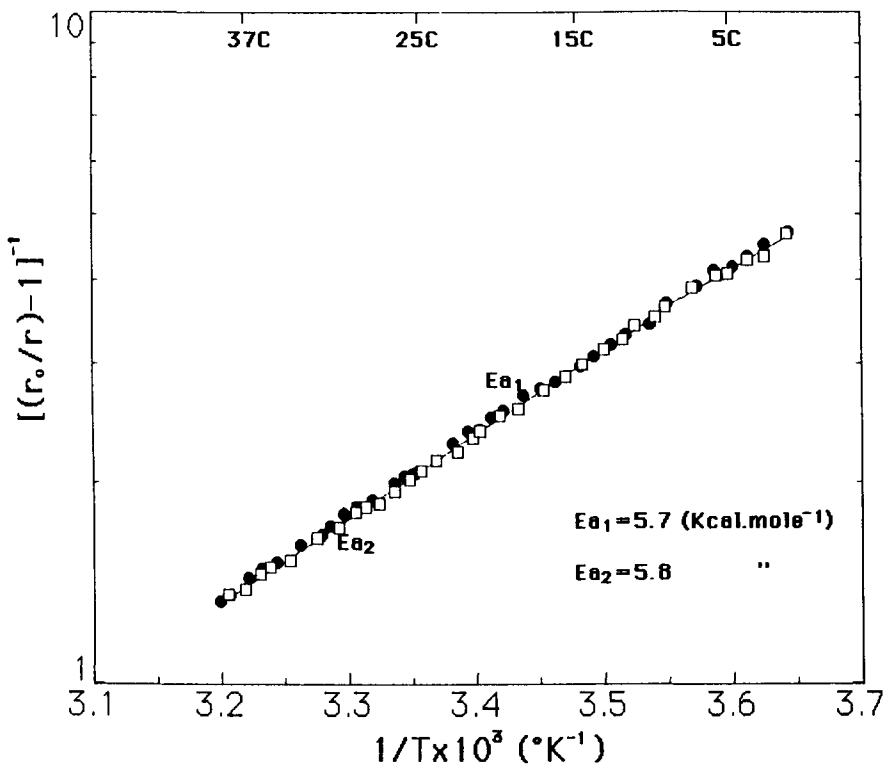

FIG. 1. Arrhenius plots of the temperature dependence of the diphenylhexatriene anisotropy parameter $[(r_0/r)-1]^{-1}$ of the intestinal microvillus membrane of carps fed a control diet (●) and a fish oil enriched diet (□) following 4 month feeding. Ea, energy of activation. Values are for one pair of preparations and are representative of four such comparisons.

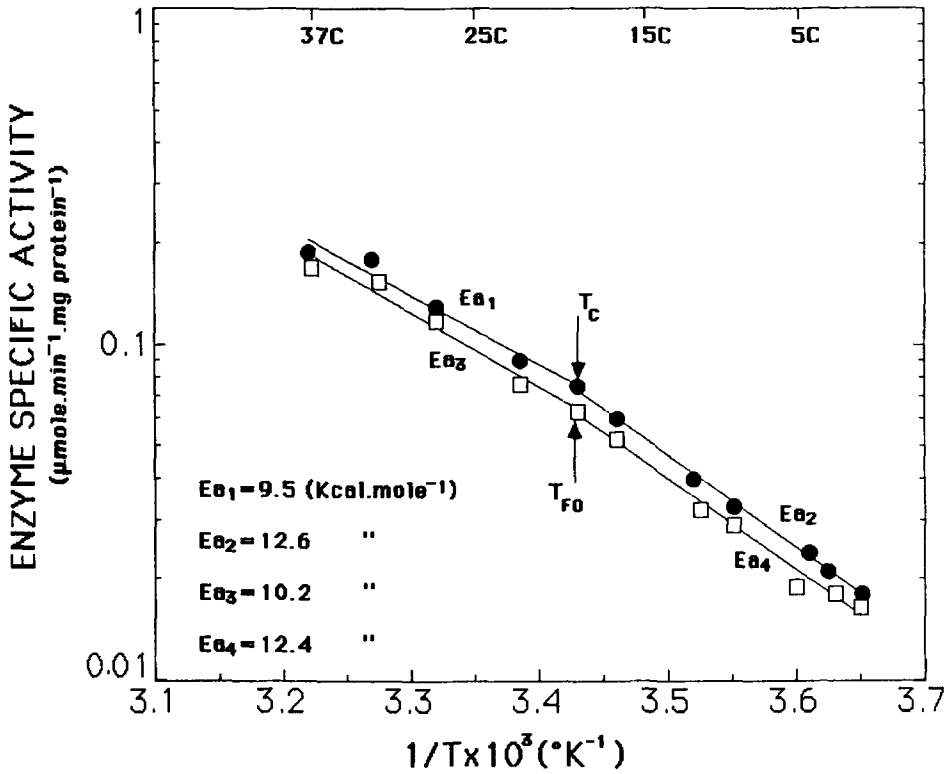

FIG. 2. Arrhenius plots of the temperature dependence of alkaline phosphatase activity in intestinal microvillus membranes of carps fed a control diet (●) and a fish oil enriched diet (□) following 4 month feeding. Ea, energy of activation; T_c and T_{fo} thermotropic transition temperatures of the membranes derived from fish fed control and experimental diets, respectively. Values are for one pair of preparations and are representative of four such comparisons.

brane bound alkaline phosphatase (Fig. 4 and Table 1). Thus, the observed specific activity of this enzyme was about 2-3-fold higher in the intestinal membranes prepared from the carp fed fish oil as compared to the respective activity in the membranes of the control group. No change was observed in the breakpoint of the Arrhenius plot of the enzymatic activity, which remained 18°C at a water temperature of 13°C. The energy of activation of the alkaline phosphatase activity and of the anisotropy parameter were not affected by the dietary lipids throughout the experiment.

The fatty acid profile of the intestinal brush-border membrane lipids of both groups of fish following four and seven month feeding is presented in Table 2. Feeding the fish oil for four months resulted in an increase in the content of the fatty acids which are typical for this oil, namely, the C20:5 and C22:6 acids. Thus, the amount of these n-3 fatty acids found in the membranes derived from the animals fed fish oil rose to 14.8%, and was significantly higher than the respective value of 10.1% observed for the membranes of the control group. Throughout this first phase of the experiment the environmental temperatures were 25-20°C. During the following three month feeding, in a period in which the environmental temperature dropped to 13°C, the n-3 fatty acid content of the membranes separated from the experimental carps increased to 21.4%, which was significantly higher than the amount of 16.0% observed for the control group.

DISCUSSION

Membrane dynamics which controls various membrane functions is determined by membrane composition and structure (1). In the present study membrane dynamics was assessed in terms of membrane fluidity, and alkaline phosphatase activity was used to evaluate membrane function. Alkaline phosphatase is known to be intimately associated with the hydrophobic core of the membrane (8). Such an intrinsic enzyme may be more sensitive to minor changes in the membrane ultrastructure when compared to extrinsic enzymes.

As long as the environmental temperature remained high, above or in the vicinity of 20°C, feeding of fish oil for a period of four months had no effect on membrane fluidity and on the enzymatic activity of the intrinsic protein alkaline phosphatase. Continuation of the feeding for an additional three months during which the temperature declined to 13°C resulted in marked changes in the properties of the brush-border membranes, namely elevated fluidity and enzyme activity in the membranes of the experimental group relative to the control.

Brasitus *et al.* (13) showed that membrane fluidity in rats can be manipulated by dietary lipids. Cossins *et al.* (14) showed that the fluidity of goldfish synaptosomal membranes increased as the water temperature decreased and attributed it to cold adaptation. In experiments with carps, Farkas *et al.* (3) observed that fish exposed to cold temperatures synthesized unsatu-

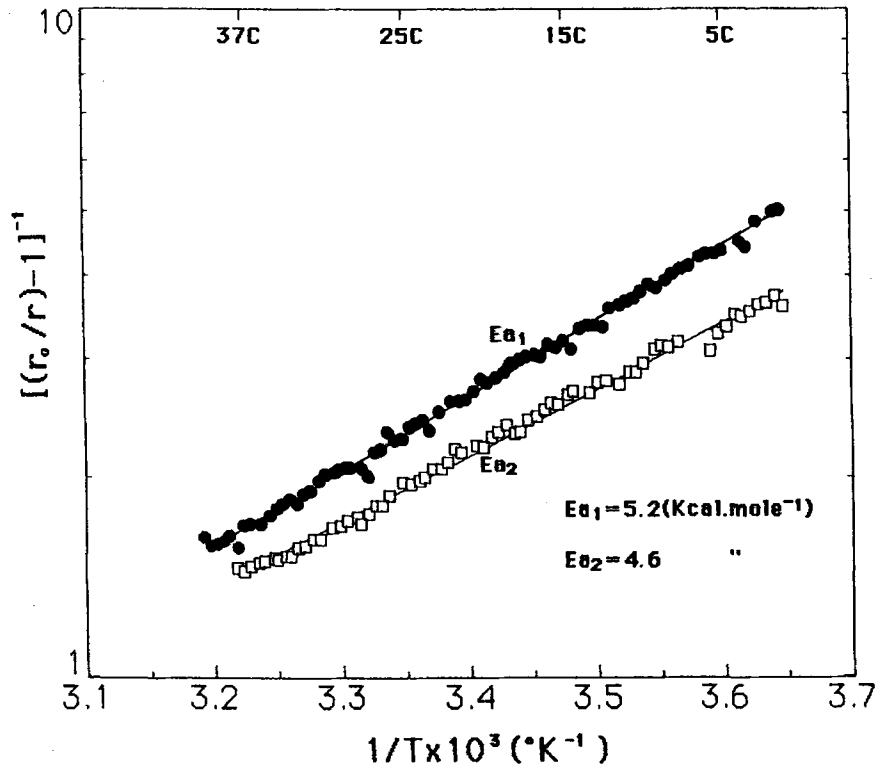

FIG. 3. Arrhenius plots of the temperature dependence of the diphenylhexatriene anisotropy parameter $[(r_o/r)-1]^{-1}$ of the intestinal microvillus membrane of carps fed a control diet (●) and a fish oil enriched diet (□) following 7 month feeding. Ea, energy of activation. Values are for one pair of preparations and are representative of four such comparisons.

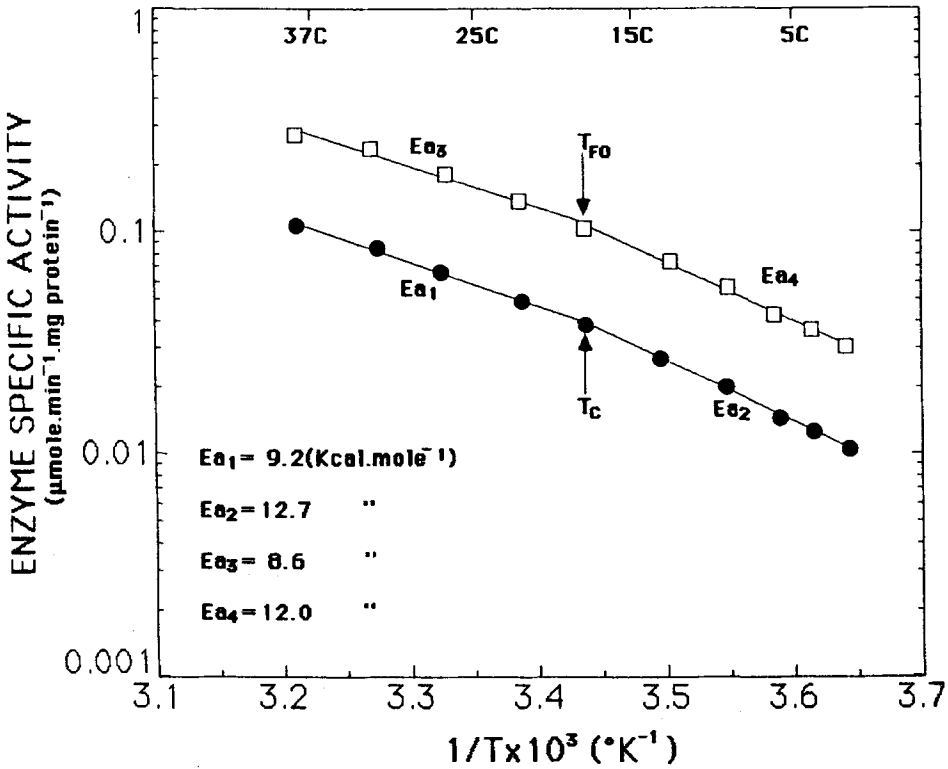

FIG. 4. Arrhenius plots of the temperature dependence of alkaline phosphatase activity in intestinal microvillus membranes of carps fed a control diet (●) and a fish oil enriched diet (□) following 7 month feeding. Ea, energy of activation; T_c and T_{fo} thermotropic transition temperatures of the membranes derived from fish fed control and experimental diets, respectively. Values are for one pair of preparations and are representative of four such comparisons.

TABLE 2

Fatty Acid Composition of the Dietary Fish Oil (Experimental) and of the Intestinal Microvillus Membranes after 4 (November) and 7 (February) Month Feeding (% of total fatty acids)[a]

Fatty acids	Fish oil	November		February	
		Control	Experimental	Control	Experimental
14:0	6.4	1.8±0.3	1.7±0.5	1.0±0.3	1.4±0.3
15:0	—	5.7±0.5	5.2±0.2	3.9±0.7	3.6±0.7
16:0	10.8	19.6±1.3	21.8±0.6	22.2±1.1	21.7±0.6
16:1	10.4	7.4±0.8	8.1±0.7	5.3±0.6	5.6±0.9
18:0	1.4	5.8±0.6	5.4±0.6	4.0±0.5	3.2±0.6
18:1	12.7	19.6±0.2	19.9±1.0	18.7±1.0	17.1±0.6
18:2	1.8	8.1±1.2	5.4±0.8	10.0±0.5	8.5±1.4
18:3	1.0	4.7±0.5	4.5±0.2	7.9±0.4	3.0±0.6
20:1	14.4	4.5±0.7	6.4±0.7	3.5±0.5	6.2±1.0
18:4	4.1	—	—	—	—
20:3(n3)	—	3.5±0.4	1.9±0.4	2.5±0.3	1.9±0.5
22:1	15.2	2.6±0.6	2.3±0.4	1.0±0.4	3.4±0.2
20:4	—	1.2±0.3	0.9±0.3	0.8±0.2	0.9±0.1
20:5	8.8	0.9±0.2	3.5±0.6	1.5±0.2	4.1±0.3
22:5	1.5	0.2±0.1	0.7±0.3	0.7±0.2	1.0±0.3
22:6	6.1	9.2±0.7	11.3±0.2	14.5±0.8	17.3±1.2
Σ(20:5,22:6)		10.1±0.9	14.8±0.8[b]	16.0±1.0	21.4±1.5[c]

[a]Values are means ± SEM for 4 different preparations.

Significantly different from the respective control.
[b]$p<0.01$.
[c]$p<0.05$.

rated fatty acids in the liver more than those exposed to warm water. These investigators related the increase in unsaturated fatty acid synthesis in the cold to the mechanism by which the organism regulated its membrane fluidity. Wodtke (15) who worked with mitochondrial membranes of carp also suggested that such fatty acid substitution in the membrane phospholipids represents a temperature induced fluidity adaptation. Nonetheless, direct fluidity measurements were not performed in these last two studies.

In our study the fatty acyl composition of the brush border membrane phospholipids of the experimental as well as the control group changed throughout the experiment. Such a change can conceivably result from reduction in the environmental temperatures (16-18) and from effects of dietary lipids (3). The changes in the fatty acid pattern of the control group following the seven month feeding period reflect cold adaptation, whereas the changes in the fatty acid pattern of the experimental group reflects both cold adaptation as well as dietary manipulation.

As far as the membrane lipid fluidity and the function of the intrinsic enzyme alkaline phosphatase are concerned, the dietary manipulation of the membrane phospholipid fatty acids was effective only at reduced environmental temperatures. It is conceivable that as long as the environmental temperature was high (above 20°C), the manipulation of the membrane fatty acids by dietary means was accompanied by changes in other membrane lipids so as to maintain homeoviscosity (17).

It is well known that marine fish oil is particularly rich in n-3 20:5 and 22:6 fatty acids. Some investigators (19, 20) have shown that one of the major changes in fatty acid composition of fish lipids at low temperatures is an increase in the level of the 22:6 fatty acid. A similar effect was observed in our study with the intestinal microvillus membrane. With regard to 20:5, the level of this acid in the membrane phospholipids also increased throughout the experiment, but to a lesser extent. Thus, the content of these two fatty acids in the membranes rose upon cold adaptation to 16.0% and increased more markedly upon cold adaptation and dietary manipulation to 21.4%.

It was suggested that the 22:6 acid plays an important role in raising membrane fluidity (20). It is also worth noting that the 20:5 acid is known to have the lowest melting point of any PUFA (2). Smith *et al.* (21) offered an explanation for the possible role of long chain polyunsaturated fatty acids, with 20 or more carbon atoms, in membrane fluidization. They suggested that these fatty acids are likely to introduce a disordering effect also in the region near the membrane phospholipid headgroups, since double bonds in these acids are located close to this region.

In conclusion, the present study suggests that the carp possesses only a limited capability to incorporate the long chain n-3 fatty acids into the membrane lipids during cold adaptation; the cold adaptation can be enhanced by the inclusion of these fatty acids in the diet, as evidenced by the assessment of the dynamics and function of the intestinal microvillus membrane.

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METHODS

Thiol Esters of 2-Mercaptoethanol and 3-Mercapto-1,2-Propanediol

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2-Mercaptoethanol and 3-mercapto-1,2-propanediol are selectively S-acylated by fatty acids with dicyclohexylcarbodiimide (DCC) catalyzed by 4-dimethylaminopyridine. Since reaction of such thiols with acid halides produces mixtures of S- and O-acylated materials, the procedure using DCC is more efficient. Such thiol esters are valuable as substrates in the study of lipase activity, using Ellman's Reagent in a continuous spectrophotometric assay. This selective synthesis will facilitate the study of fatty acid residues that are less available. Rearrangement of S- to O-acylated compounds occurs only slowly under the conditions of the lipase assay and should not significantly affect activity measurements. Using DCC, it is possible to sequentially esterify 2-mercaptoethanol to form unsymmetrical diesters.

Lipids 24, 743-745 (1989).

There is continuing interest in the chemistry and characterization of lipases. Jensen recently critically reviewed the methods for detecting and determining the triacylglycerol hydrolases (EC 3.1.1.3) (1). In 1976, Aarsman *et al.* reported the use of thiol esters as substrates for spectrophotometric assays of thiolate ion (2). This technique relies upon the nucleophilicity of thiol released, for example, by hydrolysis toward Ellman's Reagent (5,5'-dithio-bis[2-nitrobenzoic] acid) to produce a chromophore with λ_{max} = 410. By employing solutions of hexamethylphosphoric triamide (HMPT), the relatively insoluble thiol esters of long chain fatty acids, such as 2-mercaptoethanol palmitate, can be dissolved, thereby extending the use of Ellman's Reagent to evaluate lipase activity in a homogeneous medium (3). The utility of this assay method would be extended greatly if the preparation of the thiol esters—that also contain one or more hydroxyl groups—could be improved. The published procedure involves treating ethereal solutions of the mercaptoalcohols with the required acid halide and pyridine. The hydroxyl and thiol groups are competitively acylated, and the resulting mixture is separated into the component monoacylated adducts and the diester by column chromatography. In order to facilitate the study of candidate substrates, the acid residues, which are themselves the products of synthesis, a more efficient procedure seemed desirable. Recently, glycidol esters have been converted to 1-S analogs of 1,3-diacylglycerols using thiolacids (4); the chemistry reported here offers a complementary approach with broader applicability.

In addition, although the ease of transacylation in the case of vic-diols is well understood (5), and studies of the related N-O rearrangements also have been discussed recently (6), only limited information about analogous S-O migrations have been reported, at least in simple aliphatic systems (4). Such transacylations in the case of β-hydroxythiol esters during lipase assays could cause erroneously elevated levels of lipase activity. In any case, it seemed useful to establish the stability of such thiol esters toward the usual conditions for preparing and purifying organic compounds.

MATERIALS AND METHODS

Nuclear magnetic resonance spectra were obtained (^{13}C NMR) with a Bruker 300 MHz spectrometer; only diagnostically useful signals are recorded below. Infrared spectra (IR) were obtained on a Perkin-Elmer 1310 Spectrophotometer (Norwalk, CT) using 3% solutions in carbon tetrachloride. Gas liquid chromatography (GLC) was accomplished using a Shimadzu GC-Mini 2 instrument (Columbia, MD) using either an SP-2340 or SPB-1, column (0.25 mm i.d. × 30 m) with flame ion detection, and He as carrier gas with a 50:1 split ratio. Mass spectra (GC-MS) were obtained with a Hewlett-Packard 5995 GC-MS system (Avondale, PA) that was interfaced with an OV-1 column (0.25 mm × 12 m). Chemical analyses were accomplished by Microanalysis Inc. (Wilmington, DE). Thin layer chromatography (TLC) was done using silica gel G (0.25 mm) from Analabs Inc. (Radnor, PA).

All organic solvents were reagent grade or better. The fatty acids employed were obtained from Aldrich Chem. Co. (Milwaukee, WI) and were >96% pure; the oleic acid was a gift of Nippon Oil Co. (Nagoya, Japan) and was 99% pure. The mercaptoalcohols, dicyclohexylcarbodiimide (DCC), and 4-dimethylaminopyridine (DMAP) were also purchased from Aldrich Chem. Co., and were employed directly.

2-Mercaptoethanol S-alkanoates (1). The alkanoic acid (10 mmol) was added to a solution of DCC (2.18 g, 10.5 mmol), 2-mercaptoethanol (0.75 ml, 10.7 mmol), and DMAP (0.12 g, 1.0 mmol) in 100 ml of methylene chloride that was stirred in an ice bath. The mixture was stirred for 2 hr without the cooling bath. The reaction mixture was suction filtered, and the filtrate was shaken with 100 ml of 2N HCl and separated. The organic phase was thoroughly washed with water, dried ($MgSO_4$), and then stripped of solvent. The crude product was taken up in hot hexane (about 50 ml per 2 g of crude product) and filtered to remove residual urea. The solution was cooled (−20°C) and suction filtered to obtain the crystallized thioalcohol. In this manner, the following was obtained:

S-Palmitate (**1a**; 72.5%): mp 57-59°C; IR 3460, 3400 (br), 1690, 1050 cm^{-1}; ^{13}C NMR 200.1 (C=O), 61.94 (CH_2H), and 44.15 (CH_2SC=O) ppm GLC (SPB-1, 260°C) k′ = 2.28 (O-ester k′ = 0.84); TLC (90:10 $CHCl_3$-

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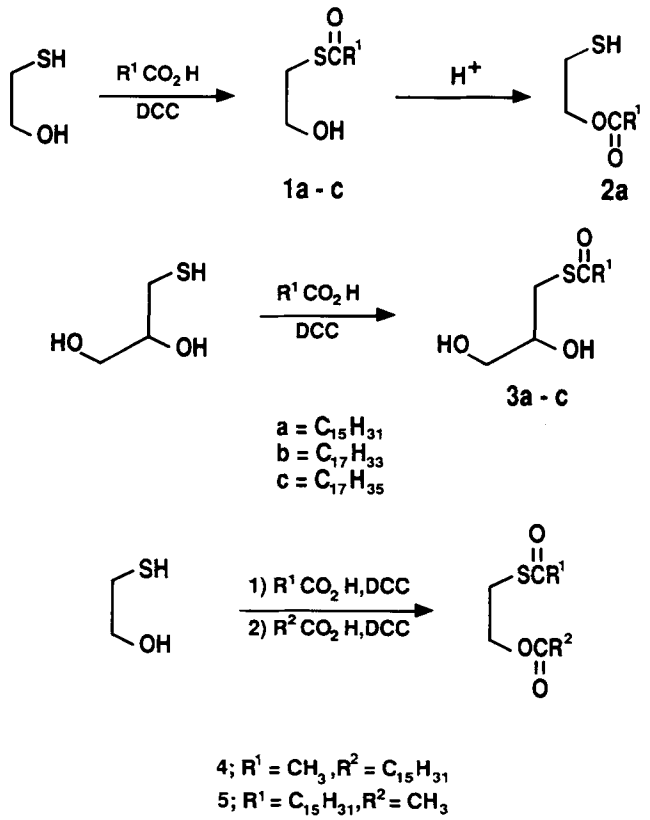
Abbreviations: DCC, dicyclohexylcarbodiimide; DMAP, dimethylaminopyridine; GC-MS, gas chromatography-mass spectra; GLC, gas liquid chromatography; HMPT, hexamethylphosphoric triamide; i.d., inner diameter; IR, infrared spectra; TLC, thin-layer chromatography; UV, ultraviolet.



acetone) R_f = 0.36 (O-ester R_f = 0.78). Anal. Calcd. for $C_{18}H_{36}O_2S$: C, 68.30; H, 11.46; S, 10.13. Found: C, 68.57; H, 11.80; S, 10.32.

S-Oleate (**1b**; 90%): Liquid at 25°C; spectral and TLC data as for the palmitate; and additional signals in the ^{13}C NMR for the alkene carbons at 129.6 and 129.9 ppm; GLC (SPB-1, 260°C) k′ = 3.7 (O-ester k′ = 1.4).

S-Stearate (**1c**; 67%): mp 63.5–64.5°C; spectral and TLC data as for the palmitate; GLC (SPB-1, 260°C) k′ = 4.0 (O-ester k′ = 1.5).

3-Mercapto-1,2-propanediol S-alkanoates (*3*). These S-esters were prepared in the same manner as the S-esters of 2-mercaptoethanol.

S-Palmitate (**3a**; 52%): mp 76–77°C (methanol); IR 3640, 3400 (br), 1690, 1050 cm^{-1}; ^{13}C NMR 201.04 (C=O), 71.28 and 64.63 (C-O), and 44.11 (C-S) ppm; TLC (90:10 $CHCl_3$-acetone) R_f = 0.2 (the O-ester isomers are 0.58 for secondary alcohol ester and 0.83 for the primary ester).

S-Oleate (**3b**; 60%): mp 48–50°C; spectral and chromatographic data as for the palmitate and additional signals in the ^{13}C NMR for the alkene carbons at 129.6 and 129.9 ppm.

S-Stearate (**3c**; 63%): mp 78.5–81°C; spectral and chromatographic data as for the palmitate.

*2-Mercaptoethanol O-palmitate (***2a***).* The S-palmitate ester (0.24 g, 0.76 mmol) was warmed under gentle reflux in benzene (20 ml) containing 30 mg of *p*-toluene-sulfonic acid (anhydrous) for 16 hr. The solvent was removed, and the crude product was recrystallized from hexane with filtration of the hot solution to give the O-ester: 0.16 g, 67%, mp 38–39°C; IR 1740, 1170 cm^{-1}; ^{13}C NMR 173.54 (C=O), 65.48 (C-O), and 34.18 (C-S) ppm; TLC and GLC as stated above.

2-Mercaptoethanol O-palmitate S-acetate (4). A solution of 2-mercaptoethanol (0.70 ml, 10 mmol), acetic acid (0.57 ml, 10 mmol), and DCC (2.08 g, 10 mmol) in 100 ml of methylene chloride was prepared at 0–5°C and allowed to stir for 2 hr attaining room temperature. Palmitic acid (2.56 g, 10 mmol) and DCC (2.08 g, 10 mmol) were added along with 200 ml of the solvent, and the resulting mixture was stirred for 16 hr. The mixture was suction filtered, the solvent was removed from the filtrate, and the crude product was then recrystallized from hexane with filtration. A recrystallization from methanol gave 1.54 g (44.3%) of the mixed diester: mp 47–49°C IR 1740, 1690, 1050 cm^{-1}; ^{13}C NMR 195.0 (SC=O), 173.56 (OC=O), 62.51 (C-O), 44.11 (C-S), and 34.18 (S[C=O]CH_3) ppm; GC-MS 239 ($C_{15}H_{31}$ C=O)$^+$.

*2-Mercaptoethanol O-acetate S-palmitate (***5***).* The S-palmitate (1.00 g, 3.16 mmol) was allowed to react with an equivalent amount each of DCC and acetic acid as above. After the usual workup procedure, the crude product was recrystallized from hexane, giving 1.01 g (89.4%) of the mixed diester isomeric with **4**: mp 33°C; IR as above; ^{13}C NMR 198.69 (SC=O), 170.76 (OC=O), 62.90 (C-O), 44.11 (C-S), and 20.04 (O[C=O]CH_3) ppm; GC-MS 239 ($C_{15}H_{31}$ C=O)$^+$.

RESULTS AND DISCUSSION

Evaluation of the ratio of O- to S-acylation was obtained most easily from a set of IR spectral standards in $CHCl_3$ with the pure O- and S-palmitates of 2-mercaptoethanol. Although separation of the two monoacylated, and the diacylated esters was easily achieved by HPLC, a solvent gradient was required, which relegated detection to UV. However, the extinction coefficient of the O-ester at 230 nm, for example, was more than thirty times that of the S-ester. This made estimation of the ratio of the products difficult when the S-ester was the minor component. We were unable to separate the monoesters by reverse phase HPLC (the increased solvent polarity that was required to affect separation, namely mixtures of acetonitrile and methylene chloride, caused the material to deposit in the column), and the monoesters rearranged in the injection port during gas chromatography. The IR analysis, fortunately, allowed reasonably accurate determination of the relative amounts of O- and S-ester (diester) present—ca. 2%.

The procedure for esterification described by Renard *et al.* (3) was repeated with palmitoyl chloride and gave a crude product that was 75% O-acylated and contained about 10% of the diester. Changing the reaction conditions (temperature and time) had no significant effect on this ratio. Reaction of palmitic acid with dicyclohexylcarbodiimide (DCC) in methylene chloride (Fig. 1), however, produced >97% S-acylation with the diester as the only contaminant detectable by TLC. Evidently the greater nucleophilicity of the thiol group is manifest in the absence of base under conditions whereby neutral oxygen and sulfur compete for the acylated imide (or palmitic

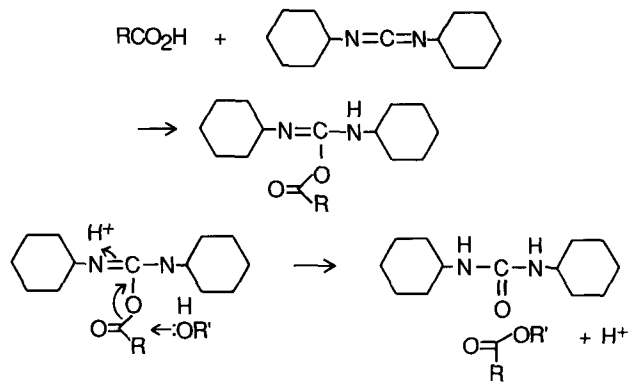


FIG. 1. Syntheses of S- and O-esters of mercaptoalcohols.

anhydride). When this procedure was employed using stearic acid, the relatively insoluble stearic anhydride precipitated, and longer reaction times were required. The addition of a catalytic amount of 4-dimethylaminopyridine (DMAP), facilitated the reaction, and was thereafter routinely employed for the sake of a uniform procedure. The combination of DMAP and DCC to secure thiol esters from thiols has been previously reported, although it was not ascertained that the reaction would be selective for thiols in the presence of alcohols (7). Interestingly, the alkylation of 2-mercaptoethanol with one equivalent of butyllithium and palmitoyl chloride in tetrahydrofuran at −78°C produced a crude material that was 79% O-acylated, while the same reaction conducted with the addition of two equivalents of hexamethylphosphoric triamide (sufficient to tie up the lithium cation, presumably) produced material that was 94% S-acylated. Since the procedure with DCC was easier to conduct and employed the organic acid itself as the reagent, it appears to be the method of choice.

We then turned our attention to the problem of transacylation. Exposure of 2-mercaptoethanol S-palmitate to the assay conditions of Renard *et al.* (3), namely 20% HMPT in water at pH 8.0 and at 30°C, led to 5% transacylation in 18 hr. Since the assay is conducted during the course of a few minutes, migration of acyl groups from S to O would not affect the assay significantly. Simple stirring of a solution of the S-ester in either HMPT or pyridine for 24 hr at 30°C similarly produced no more than 5% of O-ester. However, exposure of that ester to an equal weight of silica gel as a suspension in ether for 16 hr gave 70% O-ester, while Florisil produced 80% rearranged ester under the same conditions. Evidently, column chromatography on silica gel of S-esters that feature a vicinal hydroxyl group exposes the compound to potential isomerization. Treatment in ether with an equivalent of *p*-toluenesulfonic acid hydrate, *p*-TsOH.H_2O, yielded predominantly O-ester (93%), and much of the remaining product was the free acid. Rearrangement to the O-ester, **2a**, for synthetic purposes can be accomplished by warming the S-ester in benzene with a catalytic amount of anhydrous *p*-TsOH (Fig. 1).

Selectively diacylated materials can be prepared easily from 2-mercaptoethanol, using the selectivity of the DCC-promoted acylation, and then depending on a relatively fast O-acylation that employs a different acid and is not complicated by S to O migration. In this manner, the isomeric acetate-palmitates, **4** and **5**, were prepared, the ^{13}C NMR spectra of which were distinguishable and clean.

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The Simultaneous Separation and Quantitation of Human Milk Lipids[1]

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A protocol using a dry column method was modified for the extraction of total lipids and the simultaneous separation and quantitation of neutral and polar lipids in human milk. The triacylglycerol, cholesterol, phospholipid and vitamin E contents of the lipid extracts were determined and compared with lipids extracted using a modified Folch procedure. Good precision for the extraction of neutral, polar and total lipids, as well as the different lipid classes, was demonstrated. No significant differences were found between the two methods with respect to the amount of cholesterol, phospholipid, total lipid or vitamin E extracted, thus validating the method as an extraction technique. We discuss the relationship between vitamin E and the three major milk lipids as an indicator of the vitamin's place of origin in the mammary gland. Our findings do not support the idea that vitamin E in mature milk has its original location in the apical membrane.
Lipids 24, 746-749 (1989).

Data on the lipid composition of human milk tend to be variable, partly due to the wide array of methods used for determining the amount of lipids in human milk. Although several reliable solvent extraction techniques are available, nine different procedures have been utilized to measure total lipid content of human milk (1). Most short procedures have not been validated for small sample size. This should be a consideration, as the collection of breast milk should generally be limited to a small volume for ethical reasons.

The two methods most commonly employed for the isolation of human milk lipids are the Roese-Gottlieb method (2) and the modified Folch procedure (3, 4). Problems are encountered using the Roese-Gottlieb method, however, as phospholipids are not totally recovered (5). Although lipid recovery is quantitative with the Folch procedure, the formation of troublesome emulsions has been reported (5).

The dry column method (6), a rapid extraction procedure which utilizes a solid support, has been used for the analysis of bovine milk. This method involves the absorption of the milk into dry sodium sulfate-Celite columns, followed by the elution of the fat with a 9:1 mixture of dichloromethane and methanol. By changing the sequence of solvents used to elute the lipid, neutral and polar lipids may be separated on one column. The separation of human milk using this method has not been assessed. Further, fractionation of the total lipids into neutral and polar components is useful for effectively determining fatty acid composition of phospholipids. Because phospholipids in milk are present in extremely low concentrations as compared to the neutral lipids, separation and concentration is desirable for compositional studies (7).

The purpose of this investigation was to modify the dry column method and develop a protocol for the simultaneous separation and quantitation of neutral and polar lipids from a 1 ml sample of human milk. The method was analyzed for its completeness of extraction of triacylglycerol, cholesterol, and phospholipids in the extracted lipid by comparison to the amounts extracted using the modified Folch procedure. The precision and variation of the dry column method in extracting total lipid for α-tocopherol analysis were also assessed.

Using dry column methodology, the relationship between vitamin E and the three major milk lipids—triacylglycerol, cholesterol and phospholipid—as an indicator of the vitamin's place of origin in the mammary gland, was examined.

MATERIALS AND METHODS

Milk samples were collected with an Egnell electric breast pump (Egnell/Ameda, Cary, IL) or by hand pump expression from mothers with their informed consent. Milk was collected from one breast of each mother at the second nursing of the day, and completeness of breast emptying was assessed according to the procedure of Ferris and Jensen (1). Milk from three women was pooled as described by Moffatt *et al.* (8). One milliliter samples of the pooled milk were extracted using the dry column single (n=9) and sequential (n=9) elution techniques, and the modified Folch procedure (n=9). The following analyses were made on the lipid extracts from each procedure: vitamin E analysis by HPLC (on total lipid extracts from dry column single extraction and Folch extractions), cholesterol analysis (on neutral lipids from dry column sequential extraction and total lipids from Folch extraction), triacylglycerol analysis (on neutral lipids from dry column sequential extraction and total lipids from Folch extraction) and phospholipid analysis (on polar lipids from dry column sequential extraction and total lipids from Folch extraction) (Fig. 1).

To study fresh vs frozen milk and correlations between α-tocopherol and milk lipids, milk from six women was collected as described above and placed on dry ice after collection. Upon delivery to the laboratory, the milk was mixed in the collection bottle and two 1 ml samples were removed for immediate dry column single and sequential lipid extraction. The remainder of the sample was stored at −70°C for approximately two weeks. The cholesterol and triacylglycerol contents of the polar extracts and vitamin E content of the total lipid extracts were determined as described below.

Extraction and separation of neutral and polar lipids. Prior to all lipid extractions, milk samples were warmed to 38°C and swirled to allow for complete lipid mixing.

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METHODS

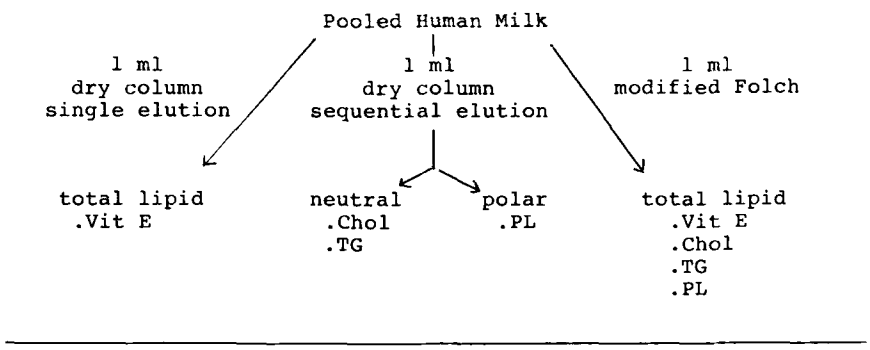

FIG. 1. Extractions and Analyses of Pooled Human Milk. Vit E = vitamin E; Chol = cholesterol; TG = triacylglycerol; PL = phospholipid.

A 1 ml sample of milk was combined with 4 g of granular anhydrous sodium sulfate in a glass beaker and mixed thoroughly. 3 g of Celite 545 (Fisher Scientific, Inc.) were added and quickly ground until a uniform powder was formed. This mixture was then packed into a glass chromatography column (22 mm × 30 cm with sealed-in coarse fritted disc) above 2 g of firmly packed calcium phosphate/Celite 545 (1:9 g/g). A tared flask was placed under the column drip tip to collect the eluent. The column was eluted with 100 ml of distilled dichloromethane. When the solvent level reached the top of the column bed, the receiving flask (containing the neutral lipids) was removed and a second tared flask was placed under the column drip tip. The column was then eluted with 50 ml of distilled dichloromethane/methanol (9:1 v/v), and allowed to drip until the column bed was dry (30-45 min). This step extracted the polar lipids. The solvent was removed by evaporation under reduced pressure, and the amounts of neutral and polar lipids were determined gravimetrically.

Total lipid extraction. The dry column procedure used to extract total lipid was similar to that used for neutral and polar lipid isolation, with the exception that only one solvent system was required. 50 ml of distilled dichloromethane/methanol (9:1 v/v) was used to elute the total lipid from the column. Also, all solid materials used in making the column (granular anhydrous sodium sulfate, Celite 545 and calcium phosphate) were washed with a 9:1 mixture of distilled dichloromethane/methanol (9:1 v/v) and allowed to dry prior to column analysis. This washing step was necessary to eliminate substances that absorbed in the UV detection range of α-tocopherol.

For comparative purposes, total lipid was extracted from 1 ml aliquots of the pooled milk, using the Folch procedure (3), as modified by Clark *et al.* (4).

Cholesterol determination. Lipid extracts were dissolved in 1 ml of hexane and duplicate 200 μl aliquots were transferred to centrifuge tubes for cholesterol analysis. The o-phthaladehyde assay, as described by Bachman *et al.* (9) was utilized for total cholesterol determination. The remainder of the lipid was aliquoted for triacylglycerol analysis.

Triacylglycerol determination. An enzymatic test kit (Sigma Chemicals, Triglyceride [INT] 20, Cat. No. 336-20) was used for the analysis of triacylglycerol in the extracted milk lipid. The kit was originally designed for analysis of serum triacylglycerol, but was modified for use with extracted milk lipid. In the modified procedure, a 100 μl aliquot of the lipid remaining from the cholesterol analysis was added to a sample tube, dried down under nitrogen, and diluted with 2 ml of Triton-X 100 (1:100 v/v). The sample was homogenized for two minutes with a sonicator and allowed to sit overnight (4°C). The enzymatic test kit procedure was then followed.

To validate the described method for the analysis of milk triacylglycerol, human milk and extracted milk lipid from milk with lipid concentrations of 2, 3, 4, 5, and 6% were analyzed for triacylglycerol using the modified enzyme assay. Mean triacylglycerol (% of total lipid) was 99.0 and 92.8% for milk and extracted lipid, respectively. There was no difference between those mean values, and the correlation between triacylglycerol and lipid content of milk was r=0.994, and between triacylglycerol and extracted lipid, r=0.99. Thus, the method described was accurate for quantitating milk triacylglycerol and could be used for determinations on either whole milk or extracted lipid.

Phospholipid determination. The polar lipid extracts obtained by the dry column separation technique and lipid from the Folch extractions were analyzed for organic phosphorus according to the colorimetric procedure described by Hundrieser *et al.* (10). The amount of phospholipid was determined by multiplying the amount of phosphorus present by the factor 25, as the average phosphorus content of phospholipid is 4% (11).

Vitamin E determination. Total lipid was dissolved in hexane and analyzed for α-tocopherol according to the HPLC methodology described by Lammi-Keefe (12). The HPLC operating conditions used were: instrument, Varian Model 5000 liquid chromatograph; column, Alltech-NH_2 (25 cm × 4.6 mm); solvents, hexane:2-propanol (98:2); flow rate, 1.4 ml/min; detector, 290nm; and absorbance range, 0.05.

RESULTS AND DISCUSSION

To evaluate the applicability of the dry column method for extracting human milk lipids, the precision of the method for isolating total, neutral and polar lipids from a pooled milk sample was determined. At a mean of 4.69 g/dl milk, the precision for total lipid extraction in duplicate was 98.7%. The precision of the dry col-

umn sequential elution method for separating and extracting neutral and polar lipids was 96.5% and 83.3%, respectively, when extractions were made in duplicate. Although the neutral fraction collected was free of polar lipids, a small amount of neutral lipids was consistently eluted with the polar fraction. This carryover of neutral lipids into the polar fraction was also observed by Maxwell *et al.* in the analysis of bovine milk (6). The inability of the dry column method to elute all of the nonpolar lipids in the first elution step was thought by Marmer and Maxwell to be the result of an affinity of the Celite reagent for nonpolar lipids (13). In the current investigation, a mean of 10.8% of the neutral lipids was carried over into the polar fraction in the sequential elution of the milk. Variability in the amount of neutral lipid carryover would contribute to the lower precision for the extraction of polar lipids.

The sum of the weights of the neutral and polar lipid fractions obtained by the dry column sequential extraction procedure was compared to the amount of total lipid extracted by the dry column single elution procedure (Table 1). There was no difference between the two elution procedures with respect to the amount of total lipid extracted. Both elution procedures would thus be suitable for extracting the total lipid from human milk.

The triacylglycerol and cholesterol contents of the neutral lipids, phospholipid content of the polar lipids and α-tocopherol content of the total lipids extracted by the dry column procedure were also determined. The "components of variance" (14) technique was utilized to characterize the sources of variation in these experimental procedures.

The triacylglycerol, cholesterol and phospholipid analyses were partitioned according to the source of variance—lipid extraction and method of lipid determination. The largest source of variance in the triacylglycerol and cholesterol analyses was the method of lipid determination. In the phospholipid analysis, the largest source of variance was the method of lipid extraction. Duplicate extractions and determinations significantly reduced the standard error of the mean for each of the milk lipids: 59% in the triacylglycerol determination, 40% in the cholesterol determination, and 37% in the phospholipid determination.

In the α-tocopherol analysis, variation was partitioned according to its sources—lipid extraction and injection onto the HPLC column. The greatest source of variance in this procedure was attributed to the extraction technique. If duplicate extractions with five injections per extraction were made, the error in α-tocopherol determination was reduced by 41%.

TABLE 1

Comparison of Single vs Sequential Dry Column Elution Procedures for Total Lipid

	Total lipid (g/dl)[a]	C.V.[b]
Single elution	4.69 ± 0.06	1.22
Sequential elution	4.73 ± 0.25	5.20

[a]Mean ± SD, n=9.
[b]Coefficient of variance (%).

The precision of the α-tocopherol, triacylglycerol, cholesterol, and phospholipid analyses, when lipids were extracted in duplicate using dry column methodology and analyzed in duplicate (quintuplicate for α-tocopherol), was also calculated. At a mean of 350.2 μg/dl milk, the precision for α-tocopherol determination was 96.7%. The precision for the triacylglycerol, cholesterol and phospholipid determinations was 96.3% ($\bar{x}$=3936 mg/dl), 92.7% ($\bar{x}$=19.81 mg/dl milk) and 97.4% ($\bar{x}$=15.73 mg/dl milk), respectively. There were no differences in the amounts of lipid or α-tocopherol extracted from fresh vs frozen milk using the dry column method (Table 2).

In the final analysis of the dry column method, the amounts of the individual lipids were compared with the amounts analyzed when the lipid was extracted by the modified Folch procedure (Table 3).

All cholesterol and triacylglycerol values determined from neutral extracts obtained by the dry column sequential elution procedure were corrected for the amount of lipids carried over into the polar fraction. There were no differences, by Student's t-test, between the two methods with respect to total lipids, cholesterol or phosopholipid. Significantly more triacylglycerol was recovered by the dry column sequential elution procedure, possibly due to a greater efficiency of extraction of triacylglycerol containing medium chain fatty acids.

The suitability of the dry column single elution procedure as a technique for extracting total lipid for vitamin E analysis was also determined. There was no difference between the dry column and modified Folch methods with respect to α-tocopherol (271.0 and 304.3 μg/dl milk, respectively).

The results of these investigations not only support the use of the methods chosen in this study for the analysis of phospholipid, cholesterol, triacylglycerol, and vitamin E, but also validate the use of the dry column procedure as a technique for isolating total, neutral, and polar lipids from human milk. Because a small amount of neutral lipids was consistently carried over with the polar lipids, the real value of the sequential elution technique may lie in the quantitative separation and concentration of the polar lipids for the determination of phospholipids.

To assess the relationship between vitamin E and the three major milk lipids as an indicator of the vitamin's place of origin in the mammary gland, those lipids were measured in milk from six women. Total lipid was extracted using the dry column procedure, and α-tocopherol, triacylglycerol, cholesterol, and phospholipid were analyzed (Table 4).

From these vitamin E and lipid data, Pearson's correlation coefficients were derived for α-tocopherol and the three milk lipids, triacylglycerol, cholesterol and phospholipid. α-tocopherol was significantly correlated with both cholesterol and triacylglycerol, but not with phospholipid (Table 5). Based on the close correlation between vitamin E and cholesterol, and vitamin E and triacylglycerol, it seems likely that, in mature milk, the secretion of at least part of the vitamin E is dependent on the secretion of triacylglycerol and cholesterol, and independent of phospholipid secretion. These findings do not support the hypothesis (15) that the apical membrane is the place of origin for vitamin

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TABLE 2

α-Tocopherol and Extracted Lipid from Fresh and Frozen Milk by the Dry Column Method

	Fresh (n=6)	Frozen (n=6)
Total lipid (g/dl)	3.88 ± 2.05[a]	3.93 ± 1.89
α-Tocopherol (μg/dl)	342.42 ± 161.80	328.86 ± 160.60
Neutral lipid (g/dl)	3.55 ± 1.71	3.56 ± 1.71
Polar lipid (g/dl)	0.32 ± 0.20	0.32 ± 0.10
Phospholipid (mg/dl)	21.03 ± 4.79	21.55 ± 4.99
Triacylglycerol (mg/dl)	3169.00 ± 543.70	3707.00 ± 1461.80
Cholesterol (mg/dl)	10.58 ± 4.59	10.53 ± 4.57

[a]Means ± SD.

TABLE 3

Analysis of Lipids in Extracts by the Dry Column and Modified Folch Procedures

	Dry column (Sequential elution)	Modified Folch
Total lipid (g/dl)	4.73 ± 0.32[a,b]	4.62 ± 0.11
Triacylglycerol (mg/dl)[c]	4332.00 ± 393.80[d]	3916.00 ± 101.80
Cholesterol (mg/dl)[c]	21.22 ± 1.91	22.08 ± 1.92
Phospholipid (mg/dl)	15.64 ± 1.08	16.04 ± 1.13

[a]Total lipid = neutral lipid + polar lipid.
[b]Means ± SD.
[c]Value corrected for amount carried over into polar fraction. See text.
[d]Significant difference by Student's t-test ($p<.05$).

TABLE 4

α-Tocopherol, Triacylglycerol, Cholesterol and Phospholipid Contents of Milk

	Mean ± SD (n=6)	C.V.[a]
α-Tocopherol (μg/dl)	342.42 ± 169.20	46.3
Triacylglycerol (mg/dl)	3769.00 ± 1618.00	47.6
Cholesterol (mg/dl)	10.58 ± 4.81	50.9
Phospholipid (mg/dl)	21.03 ± 4.99	21.6

[a]Coefficient of variance (%).

E in mature milk. If this were the case, a correlation between vitamin E and the membrane phospholipid would be expected.

TABLE 5

Correlation Analysis of α-Tocopherol and Human Milk Lipids

Correlation between	Pearson correlation coefficient
α-Tocopherol (μg/dl) and triacylglaycerol (mg/dl)	.94[a]
α-Tocopherol (μg/dl) and cholesterol (mg/dl)	.73[a]
α-Tocopherol (μg/dl) and phospholipid (mg/dl)	.40

[a]Significant correlation ($p<.05$).

ACKNOWLEDGMENT

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COMMUNICATIONS

α-Oxidation of α-Hydroxyfatty Acids in Rat Brain. Possible Involvement of an α-Peroxylactone

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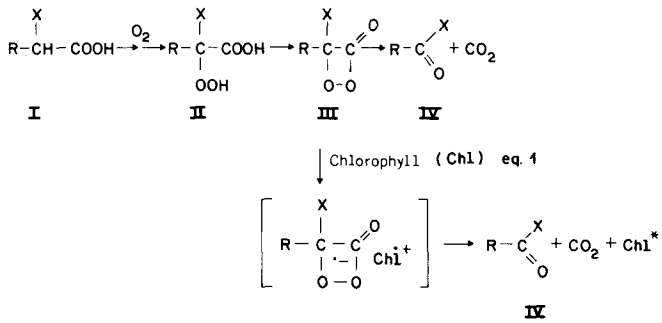
Combined—but not individual—microsomal and supernatant fractions obtained from rat brains not only consume oxygen but also provoke emission from added chlorophyll. These results are consistent with literature data (Levis and Mead, *J. Biol. Chem. 239,* 77 [1964]) for trapping of radioactive $^{14}CO_2$ following addition of α-hydroxy-[1-^{14}C]stearic acid. The most plausible explanation for emission is the interaction of chlorophyll with an α-peroxylactone. An intermediary α-peroxylactone in α-oxidation is consistent with other available data (Salim-Hanna, Campa and Cilento, *Photochem. Photobiol. 45, 849* [1987]; Campa, Salim-Hanna and Cilento, *Photochem. Photobiol. 49,* 349 [1989]) and, on chemical grounds, provides a feasible route to the final products.
Lipids 24, 750–752 (1989).

The α-oxidase preparation of higher plants converts a long-chain fatty acid (I; X=H) into the next lower aldehyde (IV, X=H) (1,2). When preparations from *Pisum sativum,* which contain broken chloroplasts, are used one observes excitation of chlorophyll, as indicated by red emission (3). The use of cucumber preparations and of long-chain fatty aldehydes as substrates suggested that chlorophyll is likely to be excited by a CIEEL ("chemically initiated electron exchange luminescence") process involving an α-peroxylactone (III; X=H) intermediate (4) (Scheme 1):



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This inference was based on the facts that (i) on chemical grounds an α-peroxylactone is the most likely immediate precursor of the products; it might be formed from the α-hydroperoxide (II; X=H), an intermediate suggested by Shine and Stumpf (2) and supported by recent data (3,4); (ii) chlorophyll is extremely efficient in CIEEL processes with α-peroxylactones (5), and could not be replaced by other emitters unless the substrate was the aldehyde (4).

Systems which carry out α-oxdation also occur in mammalian cells (6). The α-oxidation of α-hydroxyfatty acids (I; X=OH) in rat brain microsomes suggests the possible intermediacy of an α-hydroxy-α-peroxylactone (III; X=OH). A further motive for our interest in extending our studies to rat brain preparations was that these preparations do not act upon unfunctionalized acids (I; X=H). Since the product is an acid (IV; X=OH) rather than an aldehyde, and chemiexcitation of the acid is energetically prohibitive, rat brain preparations provide a crucial test for the CIEEL mechanism. Finally, such studies might also be of interest with regard to the conversion of α-hydroxyphytanic acid to pristanic acid (7).

MATERIALS AND METHODS

Palmitic acid, chlorophyll-a, ascorbic acid, hydrogen peroxide (Sigma Chemical Co., St. Louis, MO), imidazole (Calbiochem, Los Angeles, CA), and EDTA (Reagen Quimbras, Ind. Quim, Rio de Janeiro, Brazil) were commercially available.

Chlorophyll-a stock solutions were prepared by dissolving in ethanol. The concentration was determined spectrophotometrically at 665 nm ($\varepsilon = 6.94 \times 10^4\ M^{-1}\ cm^{-1}$) (8); the final concentration in the reaction mixture was always 6 μM.

Light emission was measured with a red-sensitive Thorn EMI (9658 (AM) photomultiplier tube cooled at −20°C by a Thorn EMI (Model Fact 50 MK III) thermoelectric cooler. The phototube output was connected to an amplifier-discriminator (Model 1121 A, Princeton Applied Research, Princeton, NJ). Oxygen consumption was followed on a Yellow Springs Instruments Model 53 oxygen monitor. Spectrophotometric determinations were carried out on a Zeiss DMR-10 spectrophotometer.

Enzymatic preparations (6). Three-month-old male Wistar rats were sacrificed by decapitation, and the whole brain was removed. Most of the white matter was removed by dissection and discarded. The remaining tissue consisted mainly of neurons and part of the glia, and was kept on ice until use. All subsequent operations were carried out at 0–4°C. The brains were homogenized with three volumes of 0.25 M sucrose solution, pH 7.5, in a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 2000 × *g* for 15 minutes and the residue discarded. The supernatant was centrifuged at 18000 × *g*

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Abbreviations: ATP, adenosine triphosphate; CIEEL, chemically initiated electron exchange luminescence; EDTA, ethylenediamine tetracetic aicd.

for 20 minutes to obtain the mitochondrial and supernatant fractions. The supernatant (S_A) was fractionated into microsomal and supernatant (S_B) fractions by centrifugation at 100,000 × *g* for 60 minutes. The microsomal fraction was suspended in a small volume of phosphate buffer, pH 7.5. Protein was determined by the biuret method, using bovine serum albumin as standard.

Unless otherwise stated, the standard reaction mixture was prepared as follows: chlorophyll-a was vortexed with the microsomal fraction for 30 seconds, and 1.8 ml of the supernatant (S_B) and 0.2 M phosphate buffer, pH 7.5, were added. The final volume was 3 ml and the temperature was 34°C. The amount of microsome employed corresponded to 1.4 mg protein. The concentration of the long chain fatty acid, when present, was 2.0×10^{-4} M.

RESULTS AND DISCUSSION

While microsomes or supernatant fractions alone consume little, if any, oxygen, the combined fractions show a remarkable oxygen uptake (Fig. 1), even in the absence of added substrate. No light emission whatsoever is observed from microsomes, supernatant, or their mixture. Likewise, when microsomes were vortexed with chlorophyll, little or no emission developed; however, the addition of the supernatant to chlorophyll-containing microsomes elicited significant emission (Fig. 2). The presence of chlorophyll had no significant effect on the rate of oxygen uptake. Clearly, then both oxygen uptake and chlorophyll emission indicate that oxidation of an endogenous substrate takes place when microsomes and supernatant fractions are combined. This result is fundamentally analogous to that reported by Levis and Mead (6) when α-hydroxy-[1-^{14}C]stearic acid was added to the fraction(s) and oxidation was measured by trapping the radioactive CO_2 (Table 1). These facts and the failure of these preparations to act upon stearic and palmitic acid—confirmed in this work—strongly indicate that the endogenous substrate in our system is an α-hydroxyfatty acid; in this regard, we note that rat brain is notoriously rich in these acids and that the enzymatic preparation is specific for α-hydroxyfatty acids (6). Moreover, ATP—a required cofactor (6)—markedly enhanced the emission (Fig. 2).

The effect of chlorophyll appears to be specific, as chlorophyll could not be replaced by other common sensitizers such as xanthene dyes, flavins, or the 9,10-dibromoanthracene-2-sulfonate and 9,10-diphenylanthracene-2-sulfonate ions (9). Presumably partly due to increased turbidity, the chlorophyll emission increased only slightly upon increasing the amount of supernatant (microsomes constant) or vice versa.

The sensitized chlorophyll emission, though relatively weak, is remarkable. Thus, if we consider the rate of

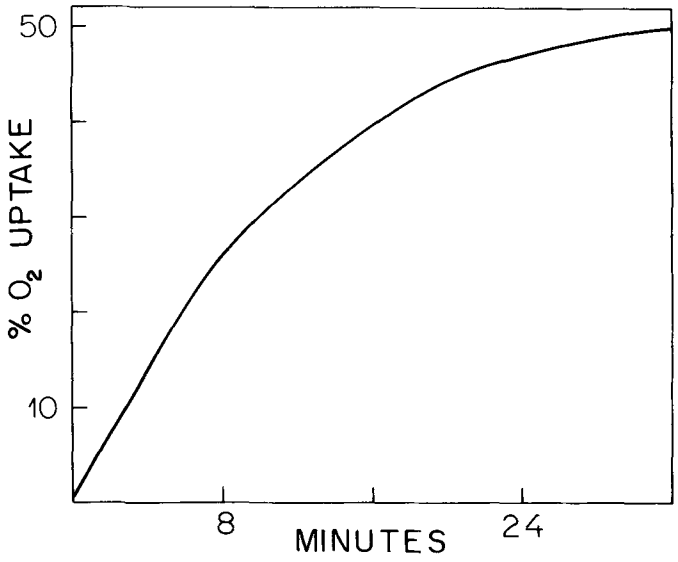

FIG. 1. Oxygen uptake by microsomes plus supernatant. There is no consumption by the microsomes or supernatant alone.

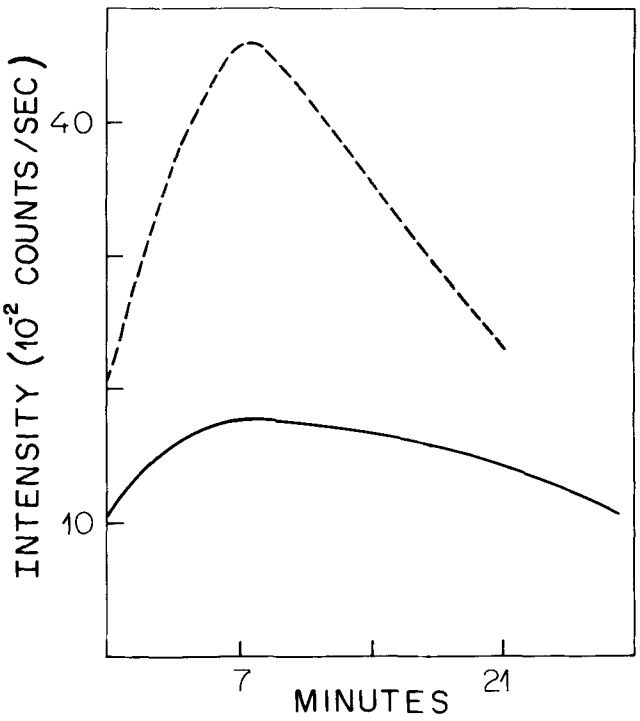

FIG. 2. Emission by the chlorophyll-containing enzymatic preparation (microsomes plus supernatant) (——). There is no significant emission by microsomes or supernatant fractions. The curve (— — —) denotes the effect of 1 mM ATP upon this emission. There is no emission in the presence of EDTA.

TABLE 1

α-Oxidation of α-Hydroxyfatty Acids by Rat Brain Preparations as Monitored by $^{14}CO_2$ Release and by Chlorophyll Elicited Emission[a]

Fractions	Radioactivity $Ba^{14}CO_3$[b] (relative values)	Chlorophyll emission
Microsomes	0.006	Hardly detectable
Supernatant	0.012	Hardly detectable
Microsomes plus supernatant	1.00	Detectable

[a]This table has only comparative value as it refers to different experiments.

[b]Adapted from Levis and Mead (6).

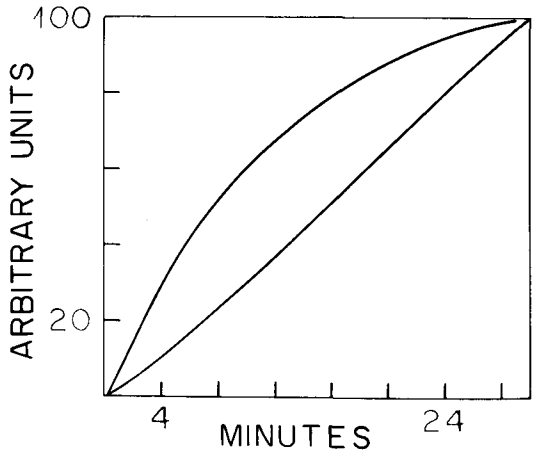

FIG. 3. Lack of correlation between endogenous oxygen consumption (upper curve) and integrated light emission.

oxygen uptake (Fig. 1), we can infer that the overall chemiexcitation yield (number of chlorophylls in the fluorescent state/molecule of O_2 consumed) is 1.3×10^{-6}, in spite of the very low chlorophyll concentration (6 μM). For this calculation the quantum yield of chlorophyll fluorescence was assumed to be 0.2. The most ready explanation for emission is interaction of chlorophyll with the α-peroxylactone (see below).

Examination of the light kinetics—a slow increase followed by a flat maximum and slow decrease (Fig. 2)—suggests the presence of a long-lived intermediate. This can also be inferred from the fact that, in certain cases, the emission continues even when oxygen is no longer being consumed. Figure 3 presents the correlation between O_2 uptake and integrated photon emission. The fact that O_2 uptake is faster is in keeping with the accumulation of an oxygen-containing intermediate. Since CIEEL processes are very fast, it is likely that in the presence of chlorophyll, the only intermediate which accumulates is the precursor of the α-peroxylactone, i.e., the α-hydroperoxide. It is interesting to note that intermediates accumulate in rat brain homogenates undergoing autoxidation (10).

Concluding remarks. Spontaneous thermal cleavage of peroxylactones bearing appropriate simple substituents typically leads to an excited triplet carbonyl (11). In the present case, however, the product is an acid, whose chemiexcitation is energetically prohibitive. Since excitation of chlorophyll cannot be expected to occur by energy transfer, chlorophyll must be excited by a CIEEL process with an α-hydroxy-α-peroxylactone.

Despite the fact that, on chemical/enzymatic grounds, the α-hydroperoxide (II) and the α-peroxylactone (III) are the most likely intermediates in the α-oxidation of fatty acids and of α-hydroxyfatty acids, an interemediary α-peroxylactone has not been previously postulated. Here, as in earlier works (3,4), chemiexcitation provides a clue to the mechanism.

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Feeding Pure Docosahexaenoate or Arachidonate Decreases Plasma Triacylglycerol Secretion in Rats

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Essential fatty acid (EFA)-deficient rats were fed highly purified methyl esters of docosahexaenoate (22:6n-3), arachidonate (20:4n-6), alpha-linolenate (18:3n-3) or oleate (18:1n-9) (100 mg/day, tube fed for 3–10 days), and their plasma triacylglycerol (TG) secretion rates were measured. Secretion rates of TG into plasma were reduced by tube-feeding 22:6n-3, 20:4n-6, 18:3n-3, but not 18:1n-9, to EFA-deficient rats. A significant reduction occurred after feeding 22:6n-3 for only three days. Feeding 22:6n-3 or 18:3n-3 to EFA-deficient rats for three days also reduced the activities of liver lipogenic enzymes and sharply increased the proportions of 22:6n-3 and 20:5n-3 in liver phospholipid fractions. Mechanisms by which these EFA may reduce lipogenesis are discussed.
Lipids 24, 753–758 (1989).

Dietary fish oils rich in eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3) reduce the plasma concentrations of TG and VLDL-TG in experimental animals and humans, in comparison with safflower or corn oils, which supply mainly C_{18}-unsaturated fatty acids. These effects of fish oils could result from decreases in hepatic fatty acid synthesis and/or in hepatic TG synthesis and secretion by fish oil-fed animals in comparison with those fed vegetable oils (1–7). These changes have been attributed to the high content of C_{20}- and C_{22}-polyunsaturated fatty acids of the n-3 series in fish oil.

C_{20}-polyunsaturated fatty acids and 22:6n-3 are stronger inhibitors of hepatic fatty acid synthesis than are C_{18}-polyunsaturated fatty acids (8–12) for both short-term and long-term (adaptive) regulation of hepatic fatty acid synthesis. Fatty acids synthesized *de novo* in the liver contribute significantly to the fatty acids of TG secreted by the liver in rats fed low-fat, high carbohydrate diets (13–15). Consequently, reduced plasma TG levels in rats fed fish oil could be due, at least in part, to reduced hepatic TG formation because of the inhibitory effects of n-3 polyunsaturated fatty acids on hepatic fatty acid synthesis. If this were so, then feeding individual pure fatty acids should show which of these is able to reduce the rate of hepatic TG secretion as indicated by plasma TG secretion. To our knowledge, the ability of highly purified C_{20}- and C_{22}-polyunsaturated fatty acids to decrease plasma TG secretion *in vivo* has not been tested.

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Abbreviations: EFA, essential fatty acid(s); FAS, fatty acid synthetase; FCR, fractional catabolic rate; G6PD, glucose-6-phosphate dehydrogenase; GLC, gas-liquid chromatography; HCNO, hydrogenated coconut oil; 6PGD, 6-phosphogluconate dehydrogenase; PUFA, polyunsaturated fatty acids; SAFF, safflower oil; TG, triacylglycerol; TLC, thin-layer chromatography; VLDL, very low density lipoprotein.

Regulation of hepatic lipogenesis in rats is most often studied in rats made hyperlipogenic by feeding purified high carbohydrate, low-fat or fat-free diets (4,10–12). Indices of hyperlipogenesis include increased activities of lipogenic enzymes, increased fatty acid synthesis *in vitro* and *in vivo,* and increased plasma TG secretion (8–10, 12–16). Hyperlipogenesis develops after a few days when such diets are fed to previously fasted rats (10,16–18), and after a week when non-fasted rats are used, i.e., rats in the initial phases of EFA depletion (9,11). With both sets of conditions, feeding polyunsaturated fatty acids (PUFA) decreases lipogenesis. The extent and rapidity of the decrease depend on the type and amount fed; saturated or monoenoic acids have little or no effect (4,9,16,19).

We have previously shown that rats fed an EFA-deficient diet *ad libitum* have increased rates of plasma TG secretion (20), as well as increased hepatic lipogenesis *in vitro* (8) and increased activities of hepatic lipogenic enzymes (9). Therefore, we have used EFA-deficient rats as a system for testing the ability of pure methyl esters of 22:6n-3, arachidonic acid (20:4n-6) or alpha-linolenic acid (18:3n-3) to reduce the rates of TG secretion into the plasma. We also measured the activities of hepatic lipogenic enzymes and fatty acids of liver phospholipids in EFA-deficient rats treated with 18:3n-3 or 22:6n-3, in comparison with untreated rats.

MATERIALS AND METHODS

Rats and diets. Male Sprague Dawley rats, 45–50 g (about 21 days old) were used in all experiments. Simonsen Laboratories (Gilroy, CA) supplied the rats used in the experiments in which lipogenic enzymes activities and liver phospholipid fatty acids were measured. Bantin-Kingman (Fremont, CA) supplied rats used in measuring plasma TG secretion rates. The rats were housed individually in suspended, wire-mesh-bottom galvanized cages, with tap water and diet. The animal room was kept at 22°C with lighting from 7:00 to 19:00. Rats were assigned to diet groups by body weight, so that the average starting weights in all groups in an experiment were equal.

Diet compositions are shown in Table 1. For measurement of hepatic lipogenic enzyme activities, rats were fed a fat-free diet for five weeks before supplementation with methyl esters (100 mg/day by stomach tube) for three days. Livers from these rats also were analyzed for phospholipid fatty acids. Diet was available to these rats until they were sacrificed (9:00–10:00) by decapitation without anesthesia.

For measurement of plasma TG secretion rates, rats were fed an EFA-deficient diet containing 5% HCNO for eight weeks. A non-deficient reference group was fed the 5% HCNO diet supplemented with 1% SAFF. After eght weeks, some of the EFA-deficient rats were supplemented with the methyl esters (100 mg/day by stomach tube) for three or five days for measurement of TG secretion rate the morning after the last dose of methyl ester. Methyl

TABLE 1

Diet Composition

Diets[a]	Components, g/100 g of diet	
	Fat	Sucrose[b]
EFA-deficient		
0% fat	0	74.5
5% HCNO[c]	5	69.5
EFA-adequate		
1% SAFF[d] + 5% HCNO[c]	6	68.5

[a] All diets included (g/100 g diet): 20.0 vitamin-free casein (91.0% crude protein, Teklad, Madison, WI); 1.0 B-vitamin mix in sucrose (21) (vitamins from Sigma, St. Louis, MO); 1.0 vitamin A, D, E mix in sucrose (21) (vitamins from Sigma); 3.5 mineral mix UCB-1Rb (21); plus Na_2SeO_3 and $KCr(SO_4)_2 \cdot 12\ H_2O$ added separately in sucrose to give 0.1 mg Se and 2 mg Cr/kg diet (Mallinckrodt, Paris, KY); 0.18 choline bitartrate (Sigma).

[b] Powdered sucrose, C & H Sugar Co. (San Francisco, CA).

[c] Hydrogenated coconut oil, California Fats and Oils (Richmond, CA). HCNO fatty acids, % by weight: 8:0, 4%; 10:0, 6%; 12:0, 49%; 14:0, 18%; 16:0, 10%; 18:0, 12%.

[d] Safflower oil, Saffola (Los Angeles, CA). SAFF fatty acids, % by weight: 16:0, 7%; 18:0, 3%; 18:1n-9, 12%; 18:2n-6, 77%; 18:3n-3, 0.5%.

esters of 22:6n-3, 20:4n-6, 18:3n-3 and 18:1n-9 (99% purity as confirmed by GLC for all experiments) were from Nuchek Prep (Elysian, MN). The methyl esters were given daily between 15:00 and 16:00, and diet was available until the rats were anesthetized at 8:00–9:00.

Plasma TG secretion rates were also measured in another experiment in which methyl esters were mixed into the diet, for comparison with methyl esters given by stomach tube. In these experiments, animals were fed the 5% HCNO diet or the 1% SAFF plus 5% HCNO diet for eight weeks. Then 100 mg of methyl ester was mixed into 20 g (ordinary daily consumption) of fresh EFA-deficient diet each day for five or ten days and given to the rats at 16:00. This amount of diet was usually consumed by the time of the next feeding. Other rats in this experiment were given 100 mg/day of 18:3n-3 methyl ester daily by stomach tube, as described above. The level of 100 mg/day is about 1.1% of the dietary kcal, a little greater than the minimum requirement for n-6 fatty acids (22) for 250 g rats eating an average of 20 g/day of a purified, low-fat, high-carbohydrate diet.

Rats were made EFA-deficient for five weeks for enzyme and liver fatty acid measurements, and for eight weeks for measurements of TG secretion rates to make it easier to compare these observations with those in the literature (9,23–26). Both the fat-free and 5% HCNO diets gave similar growth rates and liver fatty acid compositions (21,26). The largest changes in lipogenic enzyme activities and liver phospholipid fatty acids in EFA-depleted rats occurred by four weeks of EFA-depletion, with relatively little additional change from 4 to 8 weeks (21).

Enzyme activities. The activities of fatty acid synthetase (EC 2.3.1.85, FAS), glucose-6-phosphate dehydrogenase (EC 1.1.1.49, G6PD), 6-phosphogluconate dehydrogenase (EC 1.1.1.43, 6PGD), malic enzyme (EC 1.1.1.40) and ATP-citrate lyase (EC 4.1.3.8) were measured as previously described (19).

Analysis of liver phospholipid classes and phospholipid fatty acids. These were separated by TLC and analyzed by GLC as previously described (20,27).

Measurement of triglyceride secretion rate. The right external jugular vein and the left carotid artery were cannulated (20) in rats anesthetized by intraperitoneal injection with Nembutal, 50 mg/kg body weight (Abbott Laboratories, North Chicago, IL).

Blood samples, 0.3 ml, were drawn from the carotid cannula (20) immediately before and 10, 30, 50, 70 and 90 minutes after injection of Triton WR-1339 into the jugular cannula (1 ml of 10% Triton WR-1339 [Tyloxapol, Sigma, St. Louis, MO] in 0.9% saline). Lipids were extracted from 0.12 ml plasma and fractionated by TLC to isolate TG, and the amount of TG in each sample was measured spectrophotometrically as before (20). A measured amount of ^{14}C-glycerol trioleate (Amersham, Arlington Heights, IL) was added to the plasma before extraction; recovery of radioactivity in individual TG samples ranged from 80–95%, and individual sample values were corrected for losses.

The rate of TG secretion for each rat can be calculated from the plasma TG concentrations observed at intervals after administration of Triton WR-1339, which blocks removal of TG from the plasma (28–30). The slope (rate) and correlation coefficient of TG accumulation were calculated from TG concentrations measured at six time points in each rat. The fractional catabolic rate, min^{-1} (FCR or k) is rate (mg $\times$ min^{-1} $\times$ ml^{-1}) divided by TG concentration (mg $\times$ ml^{-1}) (13).

Statistics. Student's unpaired t-test (31) was used to compare the mean values for EFA-deficient rats supplemented with methyl esters with EFA-deficient rats or with rats fed the 1% SAFF plus 5% HCNO diet.

RESULTS

Activities of liver lipogenic enzymes (Table 2). Feeding 18:3n-3 or 22:6n-3 for three days reduced the activities

TABLE 2

Body Weights, Food Intakes and Activities of Hepatic Lipogenic Enzymes of EFA-deficient Rats Fed Methyl Esters of 18:3n-3 and 22:6n-3 (Mean ± SD)

Body weight and food intake	Diet		
	Fat-free	Fat-free + 18:3n-3	Fat-free + 22:6n-3
Initial wt., g	234 ± 7	233 ± 3	234 ± 3
Gain, g	9 ± 2	10 ± 2	8 ± 3
Food intake, g	18.8 ± 1.2	19.4 ± 0.4	19.5 ± 0.3
Enzyme activities[a]			
FAS	90 ± 24	69 ± 12	65 ± 15[b]
G6PD	567 ± 150	412 ± 49[b]	348 ± 64[b]
6PGD	174 ± 56	163 ± 44	150 ± 42
ATP-citrate lyase	91 ± 20	69 ± 10[b]	59 ± 2[b]
Malic enzyme	296 ± 46	241 ± 44[b]	218 ± 42[b]

n = 6 in all groups. The fatty acid methyl esters (100 mg) were given by stomach tube for three days.

[a] Milliunit/mg protein.

[b] Significantly different from the fat-free group, $P \leq 0.05$.

TABLE 3

Fatty Acids of Liver Phosphatidylcholine, Phosphatidylethanolamine, Phosphatidylserine and Phosphatidylinositol Fractions in EFA-deficient Rats Fed 100 mg 18:3n-3 or 22:6n-3[a]

Diets	Fatty acids, % of total methyl esters[b]										
	16:0	18:0	18:1n-9	18:2n-6	18:3n-3	20:3n-9	20:4n-6	20:5n-3	22:5n-6	22:5n-3	22:6n-3
						Phosphatidylcholine					
Fat-free	21.5	21.6	24.7	2.3	1.1	20.7	5.8	nd	1.1	nd	0.8
Fat-free + 18:3n-3	21.3	22.3	20.4	1.8	0.4	15.2	5.9	3.5	0.9	0.9	7.0
Fat-free + 22:6n-3	22.8	21.0	17.4	1.5	0.3	9.6	5.9	1.8	0.9	nd	18.6
Pooled SD	1.2	1.4	2.2	0.5	0.9	1.3	0.8	0.8	0.4	0.2	1.6
						Phosphatidylethanolamine					
Fat-free	14.6	22.8	15.3	1.1	0.75	18.7	22.0	0.5	2.2	nd	2.3
Fat-free + 18:3n-3	16.2	23.2	9.9	0.33	0.2	8.9	13.8	4.8	1.9	1.9	18.7
Fat-free + 22:6n-3	19.5	21.2	7.4	nd	0.1	4.9	11.1	1.4	0.8	nd	33.7
Pooled SD	1.6	1.8	0.7	0.2	0.1	0.7	1.5	0.9	0.9	0.6	2.6
						Phosphatidylinositol					
Fat-free	3.9	46.4	4.0	0.9	nd	28.7	10.5	0.8	1.3	nd	nd
Fat-free + 18:3n-3	5.5	44.1	3.9	0.6	0.8	26.4	10.1	1.6	nd	1.0	5.2
Fat-free + 22:6n-3	4.4	47.0	2.8	0.3	nd	17.6	9.5	1.1	1.7	nd	15.4
Pooled SD	1.2	3.2	1.8	0.4	0.4	4.9	1.1	0.9	0.1	0.7	4.0
						Phosphatidylserine					
Fat-free	15.6	27.8	15.7	1.2	0.7	14.6	18.9	nd	2.1	nd	2.8
Fat-free + 18:3n-3	16.9	28.1	11.3	0.8	0.2	7.4	11.8	3.2	1.1	2.4	16.5
Fat-free + 22:6n-3	23.4	21.9	8.8	0.4	nd	5.4	10.2	1.1	nd	nd	28.9
Pooled SD	3.0	4.8	2.1	0.6	0.1	1.0	1.1	1.7	0.7	1.4	1.6

[a]n = 3 in all groups. The fatty acid methyl esters (100 mg) were given by stomach tube daily for three days.

[b]Other minor components, not greater than 1% of total fatty acids, have been omitted; nd = not detected.

of hepatic lipogenic enzymes, in comparison with the EFA-deficient rats. The reductions with 22:6n-3 were somewhat greater than with 18:3n-3. We have previously compared 18:3n-3 and 20:4n-6 (9) and found that both polyunsaturated fatty acids reduced the activities of these enzymes, with a more rapid decrease with 20:4n-6. We also found (Y. T. Yang and M. A. Williams, unpublished data) that 22:6n-3 and 18:3n-3 reduced the activities of hepatic lipogenic enzymes in fasted-refed rats treated according to the procedure of Muto and Gibson (10).

Fatty acid composition of liver phospholipids (Table 3). Feeding 22:6n-3 for three days produced high levels of this fatty acid in all phospholipid fractions, but caused only small increases in 20:5n-3. The highest level of 22:6n-3 was in the phosphatidylethanolamine fraction and the lowest in the phosphatidylinositol fraction. Feeding 18:3n-3 also increased the proportions of 22:6n-3 in liver phospholipids, but less so than feeding 22:6n-3. However, feeding 18:3n-3 caused greater increases in 20:5n-3. The incorporation of 22:6n-3 and 20:5n-3 decreased the proportions of 20:3n-9 and 18:1n-9, so that the ratio of saturated to unsaturated fatty acids showed little change. These patterns are similar to the fatty acid patterns in lipids of liver mitochondria of EFA-deficient rats supplemented in the same manner with 18:3n-3 or 22:6n-3 (23).

Plasma triglyceride secretion by rats fed methyl esters of 22:6n-3, 20:4n-6, 18:3n-3 or 18:1n-9. Feeding 22:6n-3 or 20:4n-6 for five days by stomach tube significantly lowered plasma TG secretion rates, in comparison with the EFA-deficient rats (Table 4). A significant decrease occurred after feeding 22:6n-3 for only three days. Feeding 18:1n-9 reduced the secretion rate slightly, but not significantly. Both 22:6n-3 and 18:1n-9 reduced plasma TG concentration. FCR values wre not significantly reduced by the methyl ester supplementation.

Feeding 18:3n-3 for ten days by stomach tube (Table 5) significantly lowered plasma TG secretion, in comparison with the EFA-deficient rats. However, the same amount of 18:3n-3 was less effective when incorporated into the diet (0.5% by weight). A higher level of 18:3n-3 (2% by weight) did reduce lipogenic enzyme activities in three days in our earlier experiments (19). Both 18:3n-3 and 18:1n-9 tended to reduce plasma TG concentrations below those in the untreated deficient rats, but did not reduce FCR values.

DISCUSSION

Fatty acids synthesized by the liver contribute significantly to the TG secreted by the liver in rats fed low-fat, high carbohydrate diets containing little or no EFA (13–15). Therefore, it is reasonable to expect that hepatic TG secretion might be reduced when fatty acid synthesis is suppressed by the feeding of polyunsaturated fatty acids. We have shown in this report (Table 2) and earlier (9) that feeding of 18:3n-3, 22:6n-3 or 20:4n-6 reduces the activity of lipogenic enzymes in livers of EFA-deficient rats. These decreases can occur after only three days' dosage of methyl esters by stomach tube. Rat liver phospholipids also respond quickly to dietary fatty acids, as shown by the large changes in 20:3n-9, 20:4n-6, 20:5n-3 and 22:6n-3 after only three days' treatment with 18:3n-3 or 22:6n-3 (Table 3).

In the present experiments we show that feeding pure 22:6n-3 or 20:4n-6 at low levels (100 mg per day or 1.1% of kcal) for a short time (3–5 days) can reduce the rate

TABLE 4

Plasma TG Secretion Rates in EFA-deficient Rats and EFA-deficient Rats Given Methyl Esters of 22:6n-3, 20:4n-6 or 18:1n-9 by Stomach Tube for Three or Five Days (Mean ± SD)

Diet	BW g	n	Secretion rate mg/min/ml	Plasma TG mg/ml	FCR min^{-1}
−EFA (5% HCNO)	326 ± 20	5	0.088 ± 0.012	0.90 ± 0.42	0.108 ± 0.030
−EFA + 22:6n-3					
3 days	311 ± 28	5	0.054 ± 0.016[a]	0.80 ± 0.31	0.076 ± 0.039
5 days	316 ± 38	5	0.060 ± 0.016[a]	0.63 ± 0.22	0.109 ± 0.067
−EFA + 20:4n-6					
3 days	310 ± 26	5	0.063 ± 0.022	0.78 ± 0.29	0.084 ± 0.031
5 days	318 ± 46	4	0.068 ± 0.006[a]	0.93 ± 0.37	0.084 ± 0.039
−EFA + 18:1n-9					
3 days	307 ± 36	4	0.078 ± 0.008	0.68 ± 0.27	0.125 ± 0.035
5 days	313 ± 25	5	0.079 ± 0.020	0.64 ± 0.20[b]	0.129 ± 0.028
+EFA (1% SAFF + 5% HCNO)	384 ± 36	5	0.060 ± 0.021[a]	1.03 ± 0.26	0.062 ± 0.023[a]

[a]Statistically significant difference from the EFA-deficient group, $P \leq 0.05$.

[b]Statistically significant difference from the group fed the 1% SAFF + 5% HCNO diet, $P \leq 0.05$.

TABLE 5

Effect of Feeding EFA-Deficient Rats 18:3n-3 by Stomach Tube or Incorporated in the Diet on Plasma TG Secretion Rates (Mean ± SD)

Diet	BW g	n	Secretion rate mg/min/ml	Plasma TG mg/ml	FCR min^{-1}
−EFA (5% HCNO)	303 ± 30	5	0.086 ± 0.021	1.23 ± 0.55	0.081 ± 0.038
−EFA + 18:3n-3 stomach tube					
5 days	300 ± 11	5	0.064 ± 0.023	1.08 ± 0.32[b]	0.064 ± 0.028
10 days	322 ± 32	5	0.054 ± 0.013[a]	0.74 ± 0.24[b]	0.076 ± 0.019[b]
−EFA + 18:3n-3 in diet					
5 days	300 ± 87	5	0.071 ± 0.021	0.79 ± 0.48[b]	0.119 ± 0.060[b]
10 days	323 ± 35	5	0.077 ± 0.015	0.92 ± 0.22[b]	0.089 ± 0.030[b]
−EFA + 18:1n-9 in diet					
5 days	314 ± 32	5	0.086 ± 0.013	1.36 ± 0.28	0.065 ± 0.011[b]
10 days	325 ± 31	3	0.081 ± 0.018	0.61 ± 0.18[b]	0.149 ± 0.085[b]
+EFA (1% SAFF + 5% HCNO)	380 ± 24	5	0.058 ± 0.012[a]	1.68 ± 0.44	0.038 ± 0.020

[a]Statistically significant difference from EFA-deficient group, $P \leq 0.05$.

[b]Statistically significant difference from the group fed the 1% SAFF + 5% HCNO diet, $P \leq 0.05$.

of plasma TG secretion in EFA-deficient rats fed diets containing 5% HCNO. Each fatty acid can reduce hepatic TG secretion, and the amount and length of time needed are less than one might expect. Our daily dose of 22:6n-3 methyl ester is similar to the amount contained in 10 g of the fish oil diet in the experiments of Wong *et al.* (1,2). We used methyl esters because they have been used extensively as sources of pure EFA in rat studies (22). An even lower intake of 22:6n-3 or 20:4n-6 might be effective if supplied as unesterified fatty acids or as TG, rather than as methyl esters, because intestinal hydrolysis of these esters and absorption of their fatty acids may be less efficient (32,33). Differences in the metabolism of methyl esters and TG might also be expected because of the observed differences in apoprotein/TG ratio and particle size between the TG-rich lipoproteins formed when corn oil fatty acids were fed as methyl esters and the lipoproteins formed when the same amounts of these fatty acids were supplied as TG (34).

Feeding 18:3n-3 by stomach tube for ten days, but not five days, also decreased plasma TG secretion. The longer period may have been needed to produce sufficiently high levels of 20:5n-3 and/or 22:6n-3, if these should be the functional "regulatory compounds." The slower response of plasma TG secretion to 18:3n-3 was consistent with the smaller changes in the activities of hepatic lipogenic enzymes (Table 2). There is no obvious explanation for the greater effectiveness of 18:3n-3, when given by stomach tube, rather than when incorporated into the diet. However, it is quite likely that the 18:3n-3 containing lipoproteins formed in the presence of dietary fat will differ in composition and in metabolism from those formed when 18:3n-3 is absorbed at a time (16:00–19:00, late light period) when the rat is not actively eating. We

have previously found that 18:3n-3, as 2% (w/w) of the diet, significantly reduced the activities of hepatic lipogenic enzymes in EFA-deficient rats (19).

Mechanisms by which 22:6n-3 and 20:4n-6 could decrease the rate of hepatic TG secretion. There are several ways to do this, including: (a) reducing the supply of fatty acids available for hepatic TG synthesis by reducing hepatic fatty acid synthesis and/or increasing fatty acid oxidation; (b) interfering with the esterification of fatty acids into glycerides; and/or (c) interfering with the secretion of TG-rich lipoproteins. There is evidence for each of these possibilities (1-4,35).

The long-term (adaptive) inhibition of hepatic fatty acid synthesis by polyunsaturated fatty acids is caused by decreases in the levels of acetyl-CoA carboxylase and other lipogenic enzymes as a result of inhibition of gene transcription and/or decreased mRNA stability (11,36-38). The rapid, short-term inhibition of hepatic fatty acid synthesis by polyunsaturated fatty acids (8) could result if these fatty acids or their CoA esters inhibited a crucial enzyme in *de novo* fatty acid synthesis, such as acetyl CoA carboxylase. Direct inhibition is unlikely to be the major mechanism, since it has been previously shown that the CoA esters of saturated fatty acids, particularly stearoyl-CoA, are more potent inhibitors of acetyl CoA carboxylase than are the CoA esters of unsaturated fatty acids (39-41). Although the CoA esters of 20:5n-3 and 22:6n-3 do not appear to have been tested as inhibitors of this enzyme, the available evidence indicates that the inhibitory potency of fatty acids decreases as the degree of unsaturation of the acyl CoA increases (41).

Nevertheless, polyunsaturated fatty acids (including 22:6n-3 and 20:4n-6) may reduce hepatic fatty acid synthesis indirectly by inhibiting the desaturation of $C_{18:0}$ and monounsaturated fatty acids (42-48), causing stearoyl CoA to accumulate and thus to inhibit acetyl CoA carboxylase. Alternatively, the cellular concentration of stearoyl CoA could also be increased if 22:6n-3 and 20:4n-6 inhibited fatty acid oxidation. This possibility has been suggested by recent studies on mitochondrial oxidation of C_{20}- and C_{22}-polyunsaturated fatty acids with double bonds at the C-4 or C-5 position (49-55). These fatty acids are poor substrates for mitochondrial or peroxisomal β-oxidation. Accumulation of their oxidation intermediates inhibits β-oxidation of oleate and saturated fatty acids.

This proposed mechanism for inhibition of *de novo* fatty synthesis could still function even when oxidation of C_{20}- and C_{22}-fatty acids is increased, e.g., when 20:5n-3 and 22:6n-3 (as fish oils) are fed (1,2,54). Despite the increased oxidation, elevated tissue levels of these fatty acids are still maintained, as indicated by the fatty acid compositions of phospholipids (2,56,57).

Another possible explanation for our results with 22:6n-3 is that 22:6n-3 and 20:5n-3 (which could be formed from 22:6n-3) may interfere with fatty acid esterification and/or TG secretion. Wong *et al.* (1,2) observed that in hepatocytes isolated from chow-fed rats, the addition of 20:5n-3 or 22:6n-3 inhibited esterification, even when 18:1n-9 was added to stimulate TG formation. In contrast, 18:3n-3 and 20:4n-6 had no effect (2). The inhibitory effects of 20:5n-3 and 22:6n-3 appeared and disappeared rapidly when these fatty acids were added to or removed from the medium (2,3). Wong and Marsh (58) reported that 22:6n-3 was more potent than 20:5n-3 and 18:1n-9 in reducing secretion of TG and apo B by Hep G2 cells. In addition, Strum-Odin *et al.* (4) observed that TG secretion by cultured rat hepatocytes was reduced more by 20:5n-3 than by 20:4n-6 or 18:2n-6.

In addition, there is evidence that 20:5n-3 and 22:6n-3 inhibit diglyceride production and conversion of diglycerides to TG. The activity of microsomal phosphatidate phosphohydrolase, a key enzyme in diglyceride production, was decreased in rat livers perfused with either 20:5n-3 or 22:6n-3, in comparison with livers perfused with 18:1n-9 or without fatty acids (58). In cultured cells, TG synthesis from diglycerides was inhibited by 20:5n-3, but not by other polyunsaturated fatty acids, including 18:3n-3 and 20:4n-6 (4,59). Hepatic cholesterol esterification was also inhibited by 20:5n-3 (60). These same effects could also be produced by 20:5n-3 formed from 22:6n-3, even if 22:6n-3 itself were ineffective.

In conclusion, our results demonstrate the efficacy of feeding low levels of pure 22:6n-3 and 20:4n-6 in reducing hepatic TG secretion *in vivo*. Although the precise biochemical mechanism(s) involved are still unclear, it is probable that a reduction of hepatic fatty acid synthesis is an important factor.

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Inhibition and Induction of Bile Acid Synthesis by Ketoconazole. Effects on Bile Formation in the Rat

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The effects of ketoconazole, an antimycotic agent, and metyrapone, an inhibitor of mixed function oxidases, on bile acid synthesis were compared in the rat both *in vitro* and *in vivo*. In rat liver microsomes, ketoconazole was much more potent than metyrapone in inhibiting the activity of cholesterol 7α-hydroxylase, the rate-limiting enzyme in the synthesis of bile acids. The I_{50} values were 0.42 μM and 0.91 mM for ketoconazole and metyrapone, respectively. Intraduodenal administration of ketoconazole caused a rapid, dose-dependent reduction of bile acid synthesis in eight-day bile diverted rats. A single dose of 50 mg/kg reduced bile acid synthesis to 5% of control value; the same dose of metyrapone caused a reduction to only 85%. Inhibition of bile acid synthesis by ketoconazole was followed by a marked overshoot. At 28 hr after injection of 50 mg/kg of the drug, formation of bile acids was stimulated maximally by 45% compared to control value and remained elevated for more than 20 hr thereafter. Synthesis of all primary bile acids was affected to the same extent. Cholesterol 7α-hydroxylase activity in livers of ketoconazole treated (30 mg/kg) rats with an intact enterohepatic circulation was increased by 70% at 16 hr after i.p. injection of the drug. During the very large decrease of biliary bile acid output with ketoconazole, bile flow rate was relatively increased, due to stimulation of the bile acid-independent fraction of bile flow. The latter effect can probably be explained as caused by biliary secretion of osmotically active metabolites of ketoconazole.
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The imidazole derivative ketoconazole is a potent broad-spectrum antifungal agent (1). Its action is based on inhibition of the cytochrome-P450 mediated C_{14}-demethylation of lanosterol in ergosterol synthesis in fungi. The drug has been shown to affect a number of cytochrome-P450 dependent reactions in mammalian systems, including steps in the synthesis of cholesterol (2-6) and of other steroids (7,8), as well as hepatic drug-metabolizing activities (9,10).

In a recent paper (11) we reported on the effects of ketoconazole on bile acid synthesis, a process involving a number of cytochrome-P450 dependent enzymes. It appeared that the drug very potently inhibits bile acid synthesis, both in cultured hepatocytes and *in vivo* in the rat, by blocking the rate-limiting enzyme cholesterol 7α-hydroxylase.

In the present study, we have further examined the influence of ketoconazole on bile acid synthesis and its effects on bile formation. First, we compared acute effects of ketoconazole on bile acid synthesis and cholesterol 7α-hydroxylase activity with that of metyrapone, a well-established inhibitor of oxidative drug metabolism (12), including the activity of cholesterol 7α-hydroxylase in rat liver microsomes (13). Secondly, since imidazoles are known to induce a number of cytochrome P450-dependent drug metabolizing reactions at prolonged time intervals after treatment (10,14,15), we studied the effects of ketoconazole on bile acid synthesis after the inhibition phase had elapsed. Thirdly, biliary bile acid secretion is the main driving force for bile formation (16,17). Because ketoconazole strongly interferes with bile acid secretion *in vivo* (11), we analyzed the consequences of ketoconazole treatment upon the process of bile formation.

METHODS

Materials. Ketoconazole and metyrapone were obtained from Janssen Life Sciences Products (Beerse, Belgium) and Sigma Chemical Co. (St. Louis, MO), respectively. Reagents, solvents and standards were purchased from previously described sources (11,18).

Animals. Male Wistar rats (280-320 g) were used throughout this study. The animals were maintained on standard lab chow (Hope Farms, Woerden, The Netherlands) in a temperature- and light-controlled room (temperature 20°C; lights on from 6 a.m. to 6 p.m.).

For experiments on bile acid synthesis, rats were equipped with permanent catheters in bile duct and duodenum (18), which were connected to each other to maintain an intact enterohepatic circulation. After a four-day recovery period, the enterohepatic circulation was interrupted, and bile was diverted for a period of eight days to establish a new steady state in bile acid synthesis (18). Under conditions of long-term bile diversion, biliary bile acid output directly reflects hepatic synthesis rate (18). The rats were housed in individual plexiglass cages; bile was led outside the animals' cages by polyethylene tubing using a swivel-joint, to permit free movement of the animals.

In vivo experiments. In the first set of experiments, ketoconazole (5, 10 or 50 mg/kg), metyrapone (50 mg/kg) or an equivalent amount of the solvent (50 mM HCl, 5 ml/kg) were introduced via the duodenal catheter at noon, and bile was collected continuously in tared test tubes for 8 hr in 2-hr fractions. Previous studies (11) have shown that under these conditions ketoconazole does not interfere with hepatic bile acid transport processes. Two pre-injection samples were collected to assess baseline rates of bile flow and biliary bile acid secretion.

In the second experiment, we investigated the prolonged effects of ketoconazole on bile acid synthesis. Because of the pronounced day-night variations in bile acid synthesis, which show a virtually identical amplitude in individual bile-diverted rats under *ad libitum* conditions (19), we chose to use every rat as its own control.

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Abbreviations: I_{50}, 50% inhibitory concentration; HMGCoA, 3-hydroxy-3-methylglutaryl coenzyme A; LDL, low density lipoprotein.

For this purpose, bile was collected continuously in 2-hr fractions for 72 hr, starting at noon. The samples collected during the 24 hr prior to injection of ketoconazole were used as a reference. After 24 hr, i.e., at noon on the second day, ketoconazole (50 mg/kg) was administered as described above, and bile was collected for another 48 hr. Bile flow and bile acid output after ketoconazole administration were expressed as percentage of values in the same time interval in the pre-injection period.

Analyses. Total biliary bile acids were measured enzymatically, using a commercially available kit (Nyegaard & Co., Oslo, Norway). In selected bile samples, individual bile acids were determined by capillary gas chromatography on a Hewlett-Packard 5880 gas chromatograph with a CP-Sil 19-CB column (Chrompack B.V., Middelburg, The Netherlands). For this purpose, bile acids were extracted from 50 μl of bile using Sep-Pak C_{18} cartridges (Waters Associate, Milford, MA), enzymatically hydrolyzed by cholylglycine hydrolase (Sigma) and converted to their trimethyl silyl ether derivatives (20).

Cholesterol 7α-hydroxylase assay. Cholesterol 7α-hydroxylase activity was measured exactly as previously described (11). Microsomes were prepared from rats killed between 9 and 10 a.m. Ketoconazole or metyrapone were added to microsomal incubations in varying amounts, dissolved in 50 mM HCl-1.5 mg/ml Tween 80 or water, respectively, in a volume of 20 μl.

In an additional experiment, cholesterol 7α-hydroxylase activity was measured in liver microsomes from unoperated rats, 16 hr after intraperitoneal administration of ketoconazole (30 mg/kg) or its solvent (50 mM HCl brought to pH 3.0 with 100 mM NaOH).

Statistics. Data are expressed as means ± standard error (SEM) and compared using Student's t-test: P-values of 0.05 or less were considered statistically significant.

RESULTS

Effects of ketoconazole and metyrapone on bile acid synthesis and cholesterol 7α-hydroxylase activity. Figure 1 shows the effects of intraduodenal administration of ketoconazole or metyrapone on biliary bile acid output in eight-day bile diverted rats. Bile acid output directly reflects the hepatic bile acid synthesis rate under these conditions (18). The increase in bile acid synthesis observed in control rats is due to the well-documented day-night rhythm in hepatic bile acid synthesis in rats (19). Ketozonazole caused a rapid, dose-dependent, but transient reduction of bile acid synthesis. Metyrapone, which was only administered in a relatively high dose, was less potent in this respect. Similarly, ketoconazole was far more effective than metyrapone in inhibiting cholesterol 7α-hydroxylase activity in rat liver microsomes (Fig. 2), the enzyme catalyzing the first and rate-limiting step in the conversion of cholesterol to bile acids. The I_{50} values, calculated from these data by linear regression analysis, were 0.42 μM and 0.91 mM for ketoconazole and metyrapone, respectively.

When bile acid synthesis rate was followed over a more prolonged period after ketoconazole treatment (50 mg/kg), it appeared that inhibition of this process, which lasted for approximately 22 hr, was followed by a marked overshoot (Fig. 3). Maximal induction, occurring at 24–28 hr

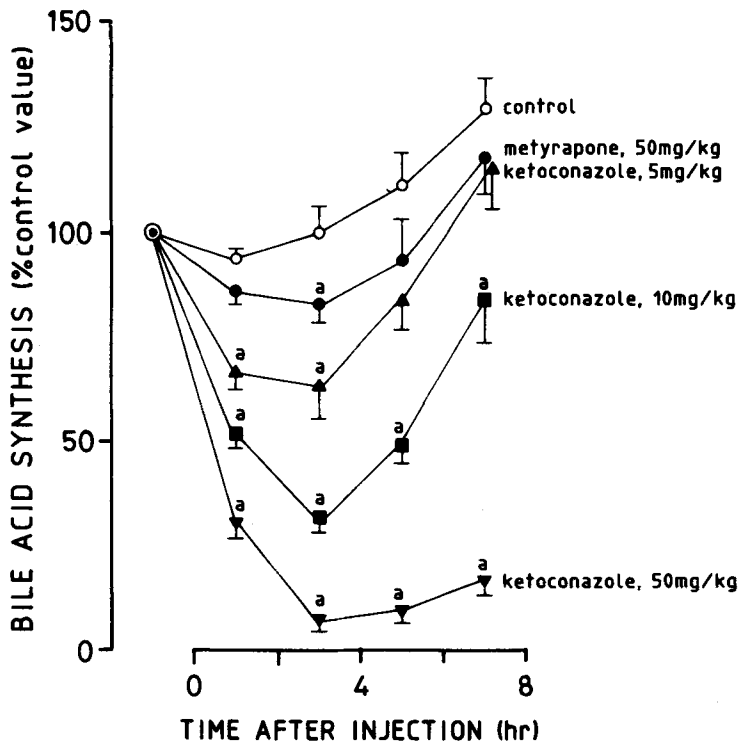

FIG. 1. Bile acid synthesis in rats after intraduodenal injection of the solvent (○), 50 mg/kg of metyrapone (●) or 5, 10, and 50 mg/kg of ketoconazole (▲, ■, ▼). Mean values ± SEM, n = 4–6. a = significantly different from control values at $p < 0.05$ level.

after injection, was 45%, compared to pre-injection control values. Bile acid synthesis was still significantly increased at 44 hr after injection. No differences in the relative contribution of individual bile acids were found in this experiment, either during inhibition or during induction of bile acid synthesis (Table 1). Cholic acid was quantitatively the predominant bile acid species during the course of the experiment.

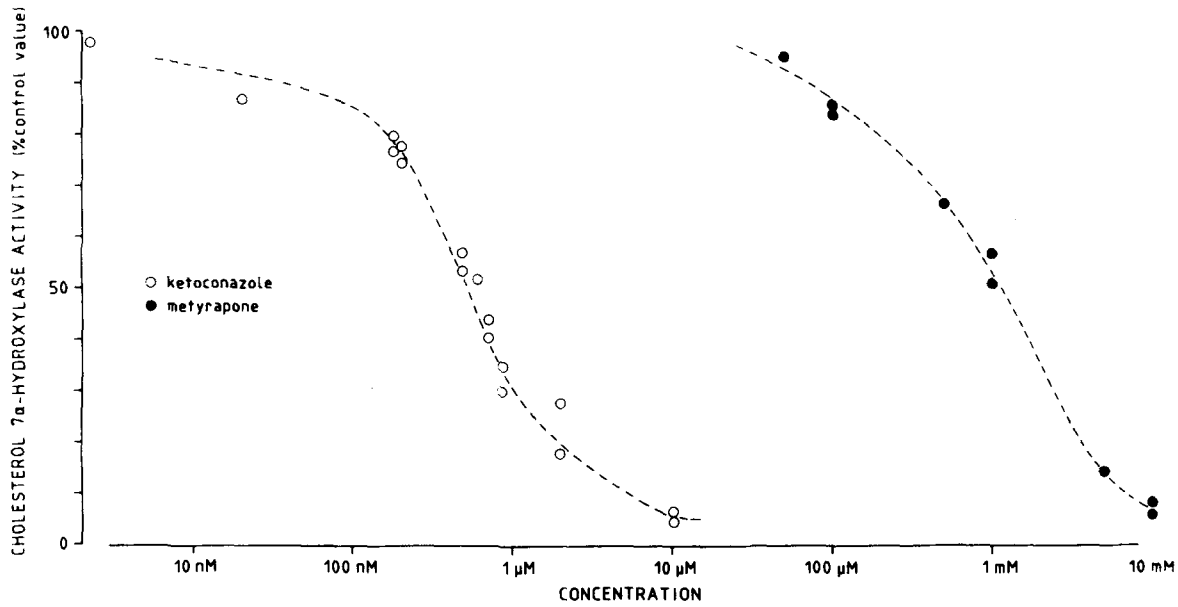

FIG. 2. Inhibition of cholesterol 7α-hydroxylase activity in rat liver microsomes by ketoconazole (○) or metyrapone (●).

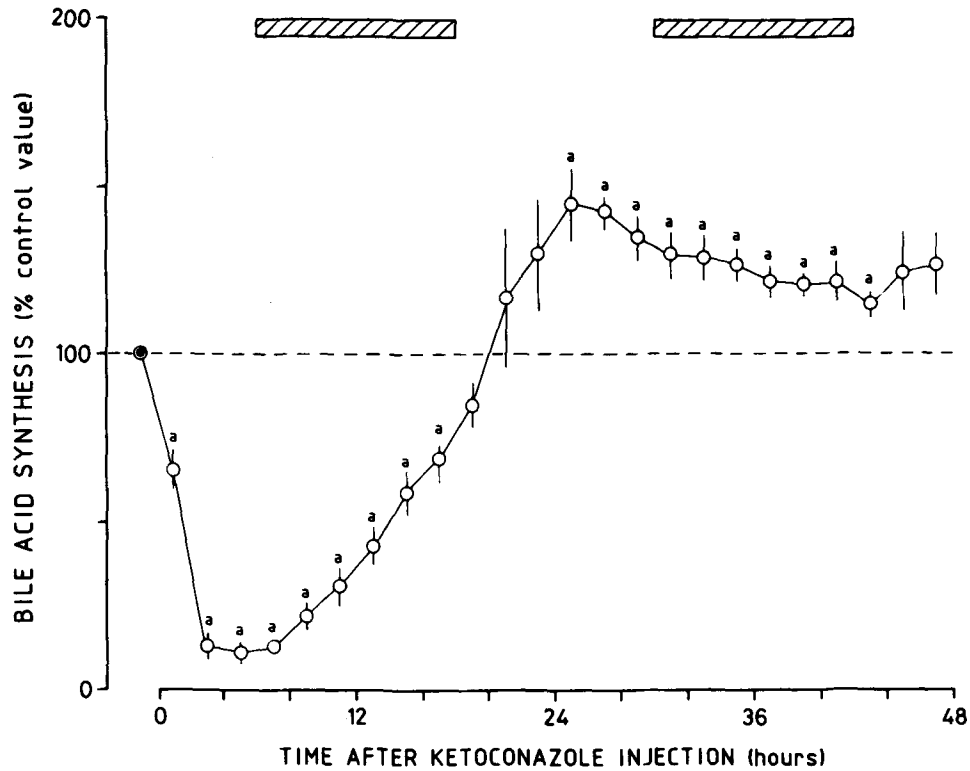

FIG. 3. Inhibition and induction of bile acid synthesis in bile-diverted (8 days) rats after administration of 50 mg/kg ketoconazole via a permanently implanted duodenal catheter. Values are expressed as percent of bile acid synthesis in the same time interval during the 24 hr control period before drug administration, to correct for day-night rhythm in bile acid synthesis. Horizonal bars indicate dark period. Mean values ± SEM, $n = 5$. a = significantly different from control values at $p < 0.05$ level.

In addition, the activity of cholesterol 7α-hydroxylase *in vivo* was also induced by ketoconazole, when measured at 16 hr after administration of the drug. Enzyme activity in microsomes isolated from rats with an intact enterohepatic circulation was increased by 72% after a single intraperitoneal ketoconazole injection (30 mg/kg), when compared to vehicle-treated controls: 2.72 ± 0.95 vs 1.58 ± 0.60 nmol/h/mg protein ($p < 0.01$, $n = 11$ in both groups).

Effects of ketoconazole on bile formation. During experiments on the *in vivo* effects of ketoconazole on bile acid synthesis, biliary bile acid output rates varied from 100 nmol/min/100 g body weight to values as low as 5 nmol/min/100 g body weight, thus approaching the situation in which bile formation is "bile acid-independent." Bile acid concentration in bile decreased to 2-3 mM in this situation, i.e., in the concentration range reported for the CMC values of conjugated bile acids (21). At maximal inhibition of bile acid output (to 5% of control values), bile flow was only reduced to 60% (data not shown). Analysis of the relation between bile flow and biliary bile acid secretion, as proposed by Berthelot *et al.* (22), revealed that during the "inhibition period" (0-24 hr after injection), bile flow was relatively increased with respect to bile acid secretion (Fig. 4). Values obtained during the "control" and "induction period" showed a

TABLE 1

Effect of Ketoconazole on Biliary Bile Acid Composition

Time after injection (h)	Bile acid output (μmol/h)	C (%)	CDC (%)	αMC (%)	βMC (%)	UDC (%)
Control	11.43 ± 0.66	69 ± 3	20 ± 2	6 ± 1	3 ± 1	2 ± 1
2-8	1.60 ± 0.25[a]	65 ± 6	24 ± 4	6 ± 3	3 ± 1	1 ± 1
26-32	15.32 ± 0.34[a]	69 ± 2	19 ± 2	7 ± 1	3 ± 1	1 ± 1

n = 5; C, cholic acid; CDC chenodeoxycholic acid; αMC, α-muricholic acid; βMC, β-muricholic acid; UDC, ursodeoxycholic acid.

[a]<0.05 or less vs control.

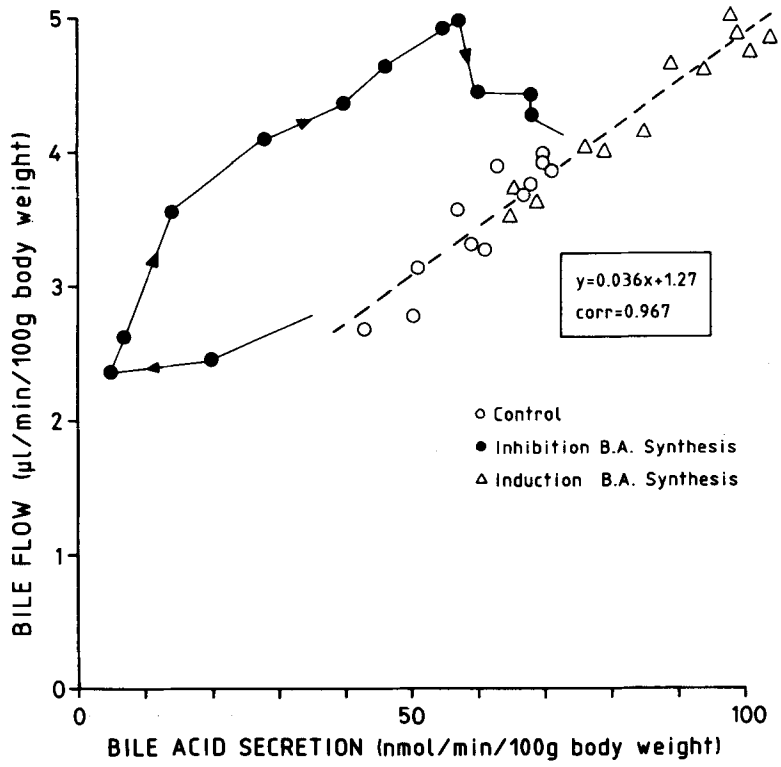

FIG. 4. Relationship between bile acid secretion and bile flow in a representative animal treated with 50 mg/kg ketoconazole in the control period (○), during inhibition of bile acid synthesis (0-24 hr after injection, ●) and during induction of bile acid synthesis (24-48 hr after injection, Δ). Regression analysis was performed on data from control and induction period. Arrowheads indicate the sequence of experimental points during the inhibition period.

strong linear relationship, with a positive intercept at the Y-axis of 1.27 μl/min/100 g body weight, representing the so-called bile acid-independent fraction of bile flow. During the inhibition period, however, this particular fraction of bile flow was very markedly increased, but returned to normal within 24 hr after ketoconazole administration.

DISCUSSION

Ketoconazole, a commonly used antimycotic drug, has been shown to affect steroid synthesis in mammalian systems. In particular, its inhibitory effects on cholesterol synthesis have recently gained wide interest. A lowering effect on plasma levels of low density lipoprotein (LDL)-cholesterol in patients treated with high doses of ketoconazole has repeatedly been described in recent literature (23–25). Kempen *et al.* (6) demonstrated an accelerated uptake and degradation of human LDL by $HepG_2$ cells incubated with ketoconazole, in accordance with up-regulation of LDL receptor activity during reduced cholesterol synthesis. Available data indicate that ketoconazole inhibits cholesterol synthesis by blocking C-14 (and to some extent C-4 [4]) demethylation processes as well as by reducing the activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMGCoA) reductase (4,6,26). The reduction of HMGCoA reductase activity may be a secondary effect, due to intracellular accumulation of metabolites of lanosterol and/or dihydrolanosterol (6).

In a recent paper (11), we demonstrated that ketoconazole also very potently inhibits synthesis of bile acids, the major catabolic products of cholesterol, in human and rat hepatocytes as well as *in vivo* in the rat. This inhibition was not caused by depletion of the precursor (cholesterol) but by non-competitive inhibition of the activity of cholesterol 7α-hydroxylase, the rate-limiting enzyme in bile acid synthesis, and occurred at concentrations easily achieved in plasma of patients treated for fungal infections (2–20 μg/ml).

In the present study, we compared the inhibitory effects of ketoconazole on bile acid synthesis with that of metyrapone, a well-known inhibitor of mono-oxygenases *in vitro* (12,27). The latter drug was shown by Schwartz and Margolis (13) to inhibit cholesterol 7α-hydroxylase activity in rat liver microsomes. The I_{50} value found for metyrapone in our study with rat liver microsomes (0.91 mM) relates very well to the 67% inhibition of cholesterol 7α-hydroxylase activity at a metyrapone concentration of 1.0 mM, reported by Schwartz and Margolis (13). Yet, ketoconazole was much more potent in this respect, with an I_{50} value of only 0.41 μM. Similarly, ketoconazole was much more effective than metyrapone in inhibiting bile acid synthesis *in vivo*. In the latter studies, we used rats with an eight days biliary drainage, in which bile acid synthesis is de-repressed owing to the absence of bile acids in the enterohepatic circulation (18). A dose of only 5 mg/kg of ketoconazole caused a stronger reduction of bile acid synthesis than 50 mg/kg of metyrapone. In our opinion these findings make it unlikely that treatment of patients with metyrapone, for instance, to manage excessive cortisol production, would significantly affect bile acid synthesis, as was suggested by Schwartz and Margolis (13). However, large doses of ketoconazole have recently been shown to decrease bile acid synthesis in patients with prostate cancer (23).

Inhibition of bile acid synthesis by ketoconazole (50 mg/kg) *in vivo* was followed by a significant, long-lasting stimulation of this process. A dose of 30 mg/kg of the drug caused a 70% increase in the activity of cholesterol 7α-hydroxylase in rats with an intact enterohepatic circulation, in which the basal activity of the enzyme was not stimulated by absence of circulating bile acids. A similar inductive effect of ketoconazole has been described for cytochrome P450-dependent drug metabolizing enzymes in rats (10,14) and for the cytochrome P450-mediated lanosterol 14α-demethylation in rats (26). From these studies it appeared that ketoconazole is a relatively specific inducer (10,14). Induction seems to take place only with those cytochrome P450 enzymes which interact strongly with this drug (14), e.g., I_{50}-values of cholesterol 7α-hydroxylase and lanosterol 14α-demethylase are only 0.42 μM (this study) and 0.40 μM (28), respectively.

Marco de la Calle *et al.* (26) recently showed that the activity of non-saponifiable lipid formation and of hepatic HMGCoA reductase in rats after intragastric administration of ketoconazole (6 mg/rat, i.e., 20–25 mg/kg) followed a similar pattern as found for bile acid synthesis and cholesterol 7α-hydroxylase in the present study: maximal inhibition between 2–12 hr after administration followed by an overshoot (60% more than control) at 24 hr. These data are in accordance with a functional coupling between the two enzyme systems. At present, we can only speculate about the mechanism(s) of induction of bile acid synthesis by ketoconazole. From our data it is clear, however, that induction can take place in situations with an initial relatively low enzyme activity as well as in situations with an increased activity, i.e., after long-term biliary drainage.

In agreement with our previous results in isolated hepatocytes (11), we observed no differences in the relative contribution of individual bile acids in bile *in vivo*, either during inhibition or induction of bile acid synthesis. Because the ratio between cholic and chenodeoxycholic acids depends on the activities of the 12α-hydroxylase and the 26-hydroxylase (29), this finding suggests that the effect of ketoconazole on cytochrome P450-mediated oxidative reactions in bile acid synthesis may be selective. However, our data contrast with those reported by Miettinen (23), who showed a very marked decrease of chenodeoxycholic acid relative to cholic acid in bile of patients with prostate cancer, treated with high doses of ketoconazole. Whether this is due to differences in regulation of bile acid biosynthetic pathways between rat and man, or to use of estrogens during or immediately before ketoconazole treatment by the patients included in the study of Miettinen, is unknown.

During the inhibition of bile acid synthesis by ketoconazole, biliary bile acid concentration approached values reported for the critical micellar concentrations of the individual conjugated bile acids (21). Thus, an increased osmotic force exerted by the biliary bile acids, due to the presnce of monomers instead of micelles, would offer an attractive explanation for the observed increase of bile flow relative to bile acid secretion, which fits in with the present concepts of hepatic bile formation (17). However, bile flow remained relatively too high during

recovery of bile acid output in comparison to control values. Therefore, we suggest that metabolites of ketoconazole, i.e., products formed after scission of the imidazole ring, the piperazine ring, the dioxolane ring and/or oxidative *O*-dealkylation, which are known to be rapidly eliminated via bile in the rat (30), exert an osmotic effect and increase the magnitude of the so-called bile acid-independent fraction of bile flow. Very similar results, i.e., a decrease in bile acid synthesis and hepatic cholesterol 7α-hydroxylase activity and a simultaneous stimulation of bile acid-independent bile flow, were reported by Le Dafniet *et al.* (31) for rats treated with the antibiotic troleandomycin.

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Effect of Eicosapentaenoic Acid Ethyl Ester on Proteinuria of Streptozotocin-induced Diabetes Mellitus in Rats

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Streptozotocin (45 mg/kg) was intravenously administered to 7-week-old Wistar rats through their tail veins. After 11 days, the rats were divided into two groups. One group was fed a lipid-free diet (90%, w/w) plus lard (8%) and safflower oil (2%) for four weeks (Diet 1 group, n = 12). The other group was fed in the same way, except that safflower oil was replaced by 90% pure eicosapentaenoic acid (EPA) ethyl ester (Diet 2 group, n = 13). Twenty-four-hour urine was collected just before the diets started and during the experiment at 7-day intervals. In the second and third week, the levels of proteinuria were significantly lower in the Diet 2 group than they were in the Diet 1 group. There was no significant difference in the levels of creatinine, urea nitrogen, or lipids in plasma or in body weights between the two groups after four weeks on the diets. Because Diet 2 reduced proteinuria of diabetic rats compared to Diet 1, an EPA-rich diet may retard the development of diabetic nephropathy.
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Since it has been shown that fish oil or eicosapentaenoic acid (EPA), a major fatty acid of fish oil, has beneficial effects in preventing atherosclerosis and thrombosis (1), several papers have appeared which report the effects of fish oil on glucose metabolism or diabetic disorders in animals (2–4), as well as in diabetic patients (5–12). These papers deal with changes in fatty acid patterns (5,6), glucose tolerance (2), *in vivo* insulin sensitivity (12), lipid metabolism (3,4,12), and with various hemostatic and rheological aspects (5–12). However, with regard to proteinuria due to diabetes, there is only one report by Haines *et al.* (10). These authors observed that a fish oil supplement with 15 g of MaxEPA per day had no significant effect on albuminuria in insulin dependent diabetics within three or six weeks, although there was a significant reduction in thromboxane production by platelets stimulated by collagen.

On the other hand, there are many reports dealing with the effects of dietary fish oil on proteinuria levels or renal function in renal diseases of nondiabetic origin (13–22). Prickett *et al.* (13) first reported the beneficial effects of fish oil on proteinuria using an autoimmune nephritis model of NZB × NZW F_1 mice. With regard to autoimmune nephritis, there are other reports supporting Prickett's finding using the same model (17,19), mercuric chloride-induced autoimmune glomerulo-nephritis (14), and MRL-lpr mice (18). Apart from autoimmune nephritis, fish oil has beneficial effects on experimental renal dysfunction, such as partial nephrectomy in rats (15), adriamycin-induced nephrosis in rats (20) and cyclosporin-induced nephrotoxicity in rats (21). Fish oil also has beneficial effects on renal functions in IgA nephropathy patients (22), and in various chronic renal diseases (16).

To investigate the effects of EPA on proteinuria and renal functions, we made rats diabetic with streptozotocin (STZ), divided them into two groups, and fed one of the groups a control diet and the other group an EPA-rich diet. The levels of proteinuria and renal functions in the two groups were then compared.

MATERIALS AND METHODS

Experimental design. Six-week-old male Wistar rats were purchased from Sankyo Labo Service (Tokyo), housed in groups of 4 to 5, and fed a standard pellet diet, F-1 (Funabashi Farms, Chiba) *ad libitum* for one week. The rats were then injected with STZ (45 mg/kg body weight) through the tail veins and fed the same diet. Blood glucose levels and body weights were checked nine days after injection of STZ. On the following day, the rats were divided into two groups, pair-matched for blood glucose levels and body weight, and were transfered to metabolic cages and housed there throughout the experiment. Twenty-four-hour urine volume and urinary protein levels were measured while both groups were still fed the same diet. Eleven days after injection, the rats were placed on the experimental diets. One group (Diet 1 group, n = 12) was fed a lipid-free powder diet (Funabashi Farms) (90%, w/w) plus lard (8%) and safflower oil (2%). The other group (Diet 2 group, n = 13) was fed the same diet as Diet 1, except that safflower oil was replaced with EPA ethyl ester, which was prepared as previously described (23). Rats were fed a measured amount of the diet (approximately 20 g) every day. The composition of the lipid-free powder diet and the fatty acid composition of EPA ethyl ester and safflower oil are shown in Tables 1 and 2, respectively. The lipid-free diet was mixed with lard and oil every other day and used on the same day or the next. If the mixed diet was not used on the same day, it was stored at −20°C for not more than one day. Lipid peroxides did not increase appreciably during storage (data not shown). Rats were weighed at the time points blood samples were taken.

Sampling procedure. Twenty-four-hour urine and venous blood from tail veins were collected at weekly

TABLE 1

Composition of the Lipid-free Powder (Weight %)

Crude protein	20.1
Crude lipids	0.1
Crude fiber	2.0
Minerals	4.0
Soluble non-nitrogenous materials	64.0
Water	9.8

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Abbreviations: EPA, eicosapentaenoic acid; STZ, streptozotocin; EDTA, ethylenediaminetetraacetate; GOT, glutamic oxaloacetic transaminase.

TABLE 2

Fatty Acid Composition (mol %) of Lipids Used for Diets 1 and 2

Fatty acids[a]	Lard	Safflower oil	EPA ethyl ester
16:0	20	6	
16:1	3		
18:0	8	1	
18:1n-9	48	7	
18:2n-6	16	84	
18:3n-3	1		
18:4n-3			2
20:4n-6			5
20:5n-3 (EPA)			87

[a]Only fatty acids which comprise more than 1% are listed. Diet 1 was a mixture of lipid-free powder diet (Table 1) (90%, w/w), lard (8%) and safflower oil (2%). In Diet 2, EPA ethyl ester was used instead of safflower oil.

TABLE 3

Changes in Body Weight (g) and Blood Glucose Levels (mg/dl) During Four Weeks on Experimental Diets

Duration on diet	Diet 1 group			Diet 2 group		
	n[a]	Body weight	Blood glucose	n[a]	Body weight	Blood glucose
Before diet	12	248 ± 24	309 ± 44	13	248 ± 18	309 ± 47
1 week	10	199 ± 22	n.d.	13	200 ± 22	n.d.
2 weeks	9	187 ± 22	248 ± 34	13	176 ± 18	263 ± 46
3 weeks	9	192 ± 24	318 ± 30	12	176 ± 20	296 ± 38
4 weeks	8	203 ± 30	257 ± 24	12	180 ± 24	250 ± 25

[a]Number of surviving rats.

n.d.: Not determined.

TABLE 4

Comparison of Urinary Volume (ml/d) and Urinary Protein (mg/d) Between the Two Groups

Duration on diet	Diet 1 group		Diet 2 group	
	Volume	Protein	Volume	Protein
Before diet	125 ± 10	27 ± 6	131 ± 17	27 ± 10
1 week	111 ± 9	50 ± 15	111 ± 18	38 ± 12
2 weeks	101 ± 11	65 ± 7	96 ± 14	49 ± 15[a]
3 weeks	161 ± 24	92 ± 15	152 ± 19	62 ± 25[a]
4 weeks	141 ± 24	83 ± 19	132 ± 19	71 ± 28

[a]$p < 0.01$, comparison was made between the two groups.

intervals. After collection of the last urine samples, the rats were sacrificed by exsanguination from aortas under ether anesthesia. Blood was anticoagulated with EDTA and centrifuged to obtain plasma. Kidneys were collected, stripped of perirenal fat, and stored at −80°C until fatty acids were analyzed. Blood glucose levels were measured by the glucose oxidase method (24), using a glucose analyzer (YSI 23A, Yellow Springs Instrument, Yellow Springs, OH). Urinary protein concentrations were measured according to Watanabe *et al.* (25). Hematuria was detected by the dip-stick method (Multistix SG, Miles Sankyo, Tokyo) (26). Frozen kidneys were thawed, minced, and homogenized with a polytron (10 sec × 3), and total lipids were extracted with chloroform/methanol (2:1) (27). Total phospholipids were isolated by thin-layer chromatography on Silica Gel 60-plates (Merck, Darmstadt, FRG) using petroleum ether/diethyl ether/acetic acid (80:20:1, by vol) as solvent. Methanolysis of phospholipids was done at 70°C for 45 min with 6% sulfuric acid in anhydrous methanol without prior extraction of the lipids from silica gel. Methylated fatty acids were analyzed by gas chromatography using a GC-14A (Shimadzu, Kyoto) equipped with a 30-m Supelcowax 10 column (Supelco, Bellfonte, PA) essentially as described by Ackman (28). Levels of total protein, glutamic oxaloacetic transaminase (GOT), urea nitrogen, creatinine, free fatty acids, triglycerides, total cholesterol and phospholipids in plasma were determined using an autoanalyzer (Hitachi 736, Hitachi-shi).

Statistical analysis. Data were analyzed by Student's t-test and are shown as means ± SD.

RESULTS

During four weeks on the experimental diets, four of the rats on Diet 1 and one of the rats on Diet 2 died of cachexia. There was no significant difference between the two groups either in body weights or in blood glucose levels throughout the experiment (Table 3). Urine volume and urinary protein levels are shown in Table 4. After the second and third week, significantly less protein was excreted into urine in the Diet 2 group than in the Diet 1 group. There was no significant difference in urinary glucose levels between the two groups at any time during the experiment (data not shown). Hematuria was not detected by the dip-stick method in any urinary samples. After having collected the last urinary samples and measured body weight, rats were sacrificed, and blood was analyzed. There was no significant difference between the two groups in plasma lipid levels and renal function as measured by plasma urea nitrogen and creatinine levels (Table 5). The fatty acid composition of total phospholipids in kidneys after four weeks on the experimental diets is shown in Table 6. In the Diet 2 group, EPA and docosapentaenoic acid were markedly elevated at the expense of arachidonic and linoleic acids. There was no difference in the levels of docosahexaenoic acid between the two groups.

DISCUSSION

The appearance of proteinuria in diabetics is an early sign of diabetic nephropathy (29). Taking into account that the leakage of protein may itself lead directly or indirectly to glomerular damage (30), the management of proteinuria is of vital importance for the prevention of diabetic nephropathy. However, means to control proteinuria are rather limited. A one-year strict control of blood glucose levels has been proven ineffective (31). The control of hypertension is of course important (32), and the administration of thromboxane synthetase inhibitor may be promising (33). In the present study we have shown that an EPA-rich diet might be another means to control proteinuria.

TABLE 5

Total Protein, Creatinine, Urea Nitrogen, Lipids and GOT in Plasma of Diabetic Rats After Four Weeks on Experimental Diets

Measured items	Diet 1 group	Diet 2 group
Total protein (g/dl)	5.4 ± 0.2	5.5 ± 0.3
Creatinine (mg/dl)	0.3 ± 0.2	0.4 ± 0.2
Urea nitrogen (mg/dl)	35 ± 10	44 ± 9
Free fatty acids (mEq/l)	1.34 ± 0.85	1.07 ± 0.58
Triglycerides (mg/dl)	231 ± 66	198 ± 49
Phospholipids (mg/dl)	270 ± 157	188 ± 94
Total cholesterol (mg/dl)	147 ± 78	130 ± 98
GOT (U)	117 ± 29	105 ± 37

TABLE 6

Fatty Acid Composition (mol %) of Total Phospholipids of the Whole Kidney After Four Weeks on Experimental Diets

Fatty acids	Diet 1 group (n = 8)	Diet 2 group (n = 11)
16:0	20.0 ± 0.9	21.2 ± 1.0[a]
18:0	21.3 ± 0.8	21.5 ± 0.6
18:1n-9	6.3 ± 0.4	7.9 ± 0.5[b]
18:2n-6	12.4 ± 0.8	9.2 ± 0.4[b]
20:4n-6	25.1 ± 1.3	19.5 ± 0.4[b]
20:5n-3 (EPA)	0.10 ± 0.02	5.4 ± 0.4[b]
22:5n-3	0.14 ± 0.05	1.3 ± 0.1[b]
22:6n-3 (DHA)	2.0 ± 0.1	1.8 ± 0.3

[a] $p < 0.05$.

[b] $p < 0.001$.

DHA, docosahexaenoic acid.

It is not clear why Diet 2 would reduce proteinuria as compared to Diet 1. However, the following actions of EPA could be considered. 1) Reduction of platelet function due to the inhibition of thromboxane formation (34). Increased platelet aggregability, which is often observed in diabetics (35), is probably related to microangiopathy due to underperfusion. 2) Hemorheological improvement. Oral administration of fish oil or EPA ethyl ester is known to increase red blood cell filterability in normals (36,37) and to increase membrane fluidity of red blood cells in diabetics (8). Poor filterability of red blood cells may be injurious to glomeruli (38) where hematocrit is already high because of ultrafiltration. 3) Reduction in blood pressure and vascular reactivity. EPA ethyl ester reduces experimental hypertension in rats (39) and vascular reactivity to angiotensin II in rabbits (40). Fish oil reduces blood pressure in humans, too (41,42). 4) Reduction in inflammatory response. Production of a proinflammatory eicosanoid, leukotriene B_4, which is derived from arachidonic acid, may be reduced by administration of fish oil (43); leukotriene B_5, which is synthesized from EPA, is much less active than leukotriene B_4 (44). This may be relevant to proteinuria.

In the present study, we used purified EPA ethyl ester rather than purified fish oil, because the fatty acid composition of fish oil proved to vary from batch to batch. The direct comparison of EPA ethyl ester with safflower oil could be questioned because one component is an ethyl ester, and the other is a glycerol triester. However, as far as the absorption by rats is concerned, EPA ethyl ester seems to be absorbed to the same extent as the triglyceride form of EPA (45).

To provide a constant amount of diet for each rat and to prevent rats from coprophagy, we housed them separately in metabolic cages throughout the experimental period. However, it has been shown that a kangaroo rat reingests its own fecal pellets (46). Whether Wistar rats behave similarly is not known.

Illman *et al.* (4) showed that fish oil-supplemented diets cause hypertriglyceridemia for up to 11 days after STZ injection into rats compared to a standard chow or a safflower oil-supplemented diet. In our study, all lipid levels measured were lower in the Diet 2 group after four weeks on the diet than in the Diet 1 group, although the difference was not significant (Table 5). This discrepancy between the two studies is probably due to the different experimental conditions.

In a preliminary study, we had found that the mortality rate of the Diet 1 group was significantly higher than in the Diet 2 group. In our present study, we found the same trend, although the difference between the two groups was not significant (Table 3). If there had been more deaths in the Diet 2 group than in the Diet 1 group, deaths from cachexia may have obscured our major finding that Diet 2 (EPA-rich) reduced proteinuria.

In the present study, we did not microscopically examine renal tissues to differentiate the cause of proteinuria. However, we checked amino acids of the third week urine and found that amino acid excretion patterns of diabetic rats were not typical of Fanconi's syndrome (47) (data not shown), which was the most common renal damage due to STZ (48). In Fanconi's syndrome the urinary excretion of almost all amino acids is reported to be increased (47). However, in our present study some amino acids were actually excreted to a smaller extent in diabetic urine than in normal urine (data not shown). Also, the absence of hematuria would rule out a diagnosis of renal tubular damage due to STZ. Consequently, it is not likely that proteinuria was directly due to effects of STZ.

The difference in levels of proteinuria became nonsignificant after four weeks on the diets. In view of the chronic course of diabetes, it is essential to investigate any long-term effect of EPA on proteinuria. We are now conducting a long-term experiment using diabetic rats in our laboratory.

Haines *et al.* (10) reported that a fish oil supplement administered for three to six weeks did not have any significant effects on albuminuria in insulin dependent diabetics. However, the duration of supplementation in their experiment seemed to be too short to fully observe the effects of fish oil in the diet.

In conclusion, because an EPA-rich diet has the effect of reducing levels of proteinuria in diabetic rats, it may retard the progress of diabetic nephropathy.

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Ozone-Induced Alterations of Lamellar Body Lipid and Protein During Alveolar Injury and Repair

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Alveolar Type II cells in the rat respond to severe, acute ozone injury (3 ppm ozone for eight hours) by increasing their intracellular pool of surfactant; however, the newly stored surfactant is abnormal in composition. Lamellar bodies isolated between 24 and 96 hours after ozone exposure contained significantly more cholesterol in relation to phosphatidylcholine than did controls. By contrast, the cholesterol content of surfactant isolated from alveolar lavage remained unchanged throughout an 8-day post-ozone period. The total protein content of lamellar bodies in relation to phosphatidylcholine was significantly decreased at 24 and 48 hours post-ozone. Analysis of lamellar body proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed that the amount of a 14 kDa proteolipid was greatly reduced at the end of the eight-hour ozone exposure and remained low for at least 48 hours. This proteolipid appeared to be a specific lamellar body component since it was not detected in extracellular surfactant. The findings indicate that oxidative alveolar stress initiates characteristic alterations in both lipid and protein constituents of stored surfactant, without perturbation in the composition of extracellular surfactant.
Lipids 24, 769–774 (1989).

Alveolar Type II cells synthesize surfactant phospholipids which are stored in lamellar bodies prior to their secretion into the alveolar space. Additional constituents of the lamellar body, a multifunctional compartmented organelle, include other lipids (1), hydrolytic enzymes (2), and surfactant-associated glycoproteins and proteolipids, which are apparently located within the peripheral compartment and multivesicular components of this organelle (3,4).

Dipalmitoyl phosphatidylcholine (DPPC), the primary functional component of alveolar surfactant, is the major component of lamellar bodies (1). The less abundant lipids, including cholesterol, and the proteins associated with intracellularly stored surfactant have not been studied adequately. In recent years it has been possible to isolate relatively pure lamellar body fractions, but the small quantities obtainable have limited the opportunity to perform detailed analyses of stored surfactant components other than phosphatidylcholine.

Characteristic compositional changes of the alveolar surfactant have been reported in hyaline membrane disease of the newborn (5), as well as in various forms of diffuse alveolar damage in both man and experimental animals (6,7). Our recent studies demonstrated that exposure of adult rats to 3 ppm ozone for 8 hours results in transient diffuse alveolar damage with well-defined stages of bronchiolo-alveolar injury and repair (8). Using this model we established that acute oxidant injury initiates progressive hypertrophy followed by hyperplasia of Type II cells, in association with expansion of the lamellar body compartment and increased storage of surfactant phosphatidylcholine.

In the present study we report that acute oxidant stress initiates additional lamellar body changes including progressive enrichment in cholesterol and a striking deficiency of a specific 14 kDa lamellar body proteolipid. Part of these results have appeared previously in abstract form (9,10).

MATERIALS AND METHODS

Adult, specific pathogen-free, male Fischer 344 rats weighing 260 ± 10 grams were used in all experiments. Groups of six rats were exposed to 3 ppm ozone for eight hours in a specially designed lucite chamber (11). Ozone was generated by passing 100% oxygen through an ozone generator, and the ozone was mixed with compressed air to produce 3.0 ± 0.2 ppm ozone. The total gas flow was maintained at 120 liters/minute to give approximately one air exchange per minute. The ozone concentration within the chamber was monitored continuously on a Mast Model 727-3 Ozone Monitor. Some groups of rats were killed immediately after the ozone exposure (0-time) while others were maintained in filtered room air for 24, 48, 96 or 192 hours. Control animals were rats from the same shipment maintained in filtered room air.

Animals were killed by a lethal intraperitoneal injection of ketamine hydrochloride. The trachea was intubated and the pulmonary vasculature was perfused with calcium and magnesium-free Hank's balanced salt solution containing 10 U/ml sodium heparin. The lungs were lavaged ten times with a total of 60 ml of cold Hank's balanced salt solution to remove extracellular surfactant. The combined lavage fluids from each rat were centrifuged at 480 × g for ten minutes to remove cells, and the supernatants were frozen until use. Extracellular surfactant was isolated from portions of the cell-free lavage fluids by upward flotation in sodium bromide density gradients as previously described (12).

Lamellar bodies were isolated from the perfused, lavaged lung by the method of Duck-Chong (13), which is based on the principle of upward flotation of lamellar bodies during centrifugation on discontinuous sucrose gradients. Briefly, the lungs were diced with a razor blade, homogenized in 1 M sucrose, filtered through gauze and layered under a sucrose gradient formed by consecutively layering 0.2–0.8 M sucrose in 0.1 M increments. Following centrifugation at 4°C for three hours in a Beckman ultracentrifuge using an SW25.2 swinging bucket rotor (90,000 × g_{max}), the lamellar body band was located at

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Abbreviations: PC, phosphatidylcholine; DPPC, dipalmitoyl phosphatidylcholine; HDCBS, sodium 2-hydroxy-3,5-dichlorobenzenesulfonate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

the interface of 0.4 and 0.5 M sucrose. The lamellar bodies were collected from the gradient, diluted with an equal volume of distilled water and pelleted by centrifugation at 4°C for 30 minutes at 14,000 × *g*. The lamellar body pellets were resuspended in a small volume of distilled water and stored at −70°C. The purity of lamellar body preparations was monitored by electron microscopy (8,14).

Lipids were extracted from lamellar bodies and extracellular surfactant with chloroform/methanol (2:1) and washed using the Folch method (15). Phosphatidylcholine (PC) was quantitated by a kinetic modification of a combined enzymatic method (16) in which the choline released from PC by phospholipase D (EC 3.1.4.4) is oxidized by choline oxidase (EC 1.1.3.17). The hydrogen peroxide generated in the enzymatic oxidation of the choline reacts with sodium 2-hydroxy-3,5-dichlorobenzenesulfonate (HDCBS) and 4-aminoantipyrine in the presence of peroxidase (EC 1.11.1.7) to produce a chromogen with maximum absorbance at 500 nm (17). Aliquots of lipid extracts dried under nitrogen were dissolved in 300 μl of an aqueous solution containing 0.2% Triton X-100 and 0.09% sodium dodecylsulfate, then 100 μl of 24 mM aminoantipyrine in 0.2 M Tris buffer, pH 7.0, were added. Phospholipase D from cabbage (Type IV, Sigma Chemical Co., St. Louis, MO), which does not hydrolyze sphingomyelin (18), was prepared in 0.2 M Tris buffer, pH 7.0, containing 0.1% bovine serum albumin to give a concentration of 100 U/ml. Fifty microliters of the phospholipase D solution and 50 μl of aqueous 0.3 M $CaCl_2$ were added and incubated at 37°C for 30 minutes. Standards were prepared containing from 2 to 100 μg of DPPC. At the end of the 30-minute incubation, 500 μl of the oxidase-peroxidase reagent were added. This reagent contained 0.2% Triton X-100, 18 mM HDCBS, 800 units of horseradish peroxidase (Type VI, Sigma) and 300 units of choline oxidase (Sigma) per 100 ml of 0.2 M Tris buffer, pH 8.0. The rate of chromogen formation was measured by continuously recording the absorbance at 500 nm for five minutes. The amount of PC in each sample was determined by comparison with the absorbance change per minute obtained with the standards of DPPC.

Free cholesterol was quantitated using a modification of an enzymatic method for determination of cholesterol in lipid extracts (19). Dried lipid extracts were dissolved in 300 μl of 0.1 M Tris buffer pH 7.0 containing 0.5% Triton X-100. Two-hundred microliters of ethanol and 100 μl of the same amino antipyrine reagent used in the PC assay were added. Oxidase-peroxidase solution was prepared as in the PC assay, but contained 1.25 U/ml cholesterol oxidase, E.C. 1.1.3.6 (Calbiochem, LaJolla, CA) instead of choline oxidase, 6 mM sodium taurocholate, and 0.5% Triton X-100. Following addition of 500 μl of the oxidase-peroxidase solution, samples and standards (2–25 μg cholesterol) were incubated at 37°C for 15 minutes and the absorbance measured at 500 nm.

Total protein content of the lamellar bodies was determined using the Markwell adaptation (20) of the Lowry method with human serum albumin as the standard. Proteins not treated with disulfide reducing agents were studied by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using the Fairbanks system (21) with slab gels containing 10% (w/v) acrylamide. Two-dimensional SDS-PAGE was done as previously described (22) using gels containing 10% acrylamide in both directions to determine which proteins are composed of disulfide-linked peptides. Gels were stained with a silver/Coomassie blue double staining technique (23). After separation by SDS-PAGE, proteins were electrophoretically transferred to nitrocellulose and immunologically detected (24) with antisera as one of the following: anti-rat lung surfactant 72 kDa dimer (22); anti-human lung surfactant proteolipids (25); or anti-rat-albumin (Organon Teknika-Cappel, Malvern, PA).

Statistical analysis was accomplished using a microcomputer and "SPSS/PC+" software (SPSS, Inc., Chicago, IL). The data in Tables 1 and 2 and the protein data in Figure 2 were analyzed using an analysis of variance (ANOVA). Prior to running the ANOVA, homogeneity of variances was tested using the Burr-Foster Q-Test. The data in Table 1 were found to have heterogeneity of variances, and a log transformation was applied. The data were found to have homogeneity of variances after rerunning the Burr-Foster Q-Test on the transformed data. The ANOVAs showed significant F values, and Duncan's multiple range test was used to test for differences among groups. The cholesterol data in Figure 2 were tested using the nonparametric Kruskal-Wallis 1-way ANOVA. A distribution-free multiple comparison based on Kruskal-Wallis rank sums (26) was used to test for differences among groups.

RESULTS

Cholesterol of lamellar bodies and lavage surfactant. The sequential compositional changes of lamellar bodies in response to acute ozone stress are presented in Table 1. The cholesterol content of the isolated lamellar body fraction was significantly elevated at the end of the 8-hour exposure period. As previously reported (14), both PC and protein contents of lamellar bodies were unchanged at this time. The cholesterol content continued to increase, and as with PC, reached maximum values 48 hours post-ozone and remained significantly elevated thereafter. Unlike the PC and protein (14), lamellar body cholesterol per 10^8 Type II cells remained 3-fold higher than controls at 96 hours (Fig. 1). In addition, significantly elevated values of cholesterol, PC and protein per lung were found 8 days (192 hours) after ozone exposure, presumably due to persistent Type II cell hyperplasia.

The changes in cholesterol and total protein relative to the PC of lamellar bodies are presented in Figure 2. Lamellar bodies of control rats contained about 4 micrograms cholesterol per 100 micrograms of PC. This value progressively increased following ozone exposure and reached a maximum value of 8 micrograms cholesterol per 100 micrograms of PC at 96 hours. The protein content of isolated lamellar bodies increased at a slower rate than did PC, resulting in lamellar bodies that were relatively deficient in protein (Fig. 2). There was a significant decrease in the protein to PC ratio at 24 and 48 hours. At recovery, 96 hours post-ozone exposure, the ratio of protein to PC had returned to control values even though the levels of both components per gram of rat lung remained elevated.

Changes in extracellular surfactant isolated from alveolar lavage fluids are shown in Table 2. The amount of surfactant PC gradually increased in lavage fluid

TABLE 1

Lamellar Body Cholesterol (Chol), Protein and Phosphatidylcholine (PC)

		Time post-ozone exposure (hours)				
	Control	0	24	48	96	192
μg Chol/lung	18 (2) N = 22	30[a] (2) N = 14	91[b] (5) N = 17	168[c] (11) N = 13	108[b] (9) N = 12	55[d] (6) N = 11
μg Protein/lung	81 (3) N = 11	73 (4) N = 7	135[d] (12) N = 9	234[c] (20) N = 8	165[d] (19) N = 7	165[d] (25) N = 3
μg PC/lung	544 (36) N = 34	627 (42) N = 20	1595[d] (111) N = 23	2641[c] (138) N = 22	1404[d] (120) N = 18	1251[d] (144) N = 9

[a]Values, Mean with SEM in parenthesis, are significantly different from Control.
[b]Significantly different from Control, 0-time and 192 hours.
[c]Significantly different from all other groups.
[d]Significantly different from Control and 0-time.

TABLE 2

Lavage Surfactant Cholesterol (Chol) and Phosphatidylcholine (PC)

		Time post-ozone exposure (hours)				
	Control	0	24	48	96	192
	N = 8	N = 5	N = 3	N = 7	N = 5	N = 3
μg Chol/lung	132 (10)	90 (7)	174[a] (30)	124 (5)	232[b] (16)	185[c] (36)
μg PC/lung	1251 (92)	981 (86)	1643[d] (233)	1203 (38)	2426[b] (201)	1917[c] (352)
μg Chol/100 μg PC	10.6 (0.4)	9.2 (0.4)	10.5 (0.6)	10.4 (0.5)	9.8 (0.9)	9.7 (0.8)

[a]Values, Mean with SEM in parentheses, are significantly different from 0-time and 48 hours.
[b]Significantly different from all other groups.
[c]Significantly different from Control, 0-time, and 48 hours.
[d]Significantly different from 0-time.

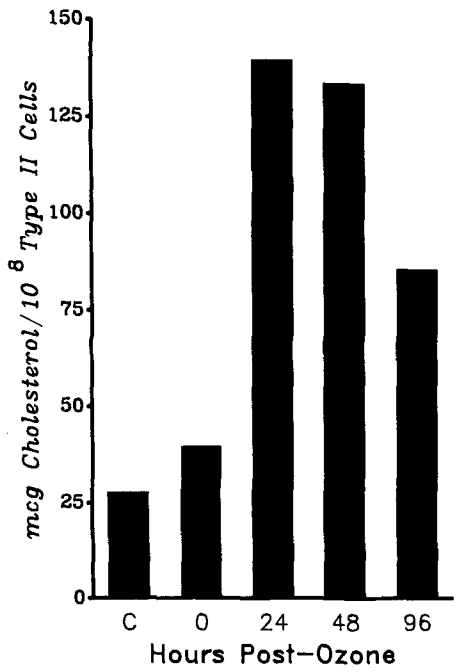

FIG. 1. Sequential changes of lamellar body cholesterol relative to the mean number of Type II cells. The data for number of Type II cells were estimated with stereologic techniques and are presented in Reference 8.

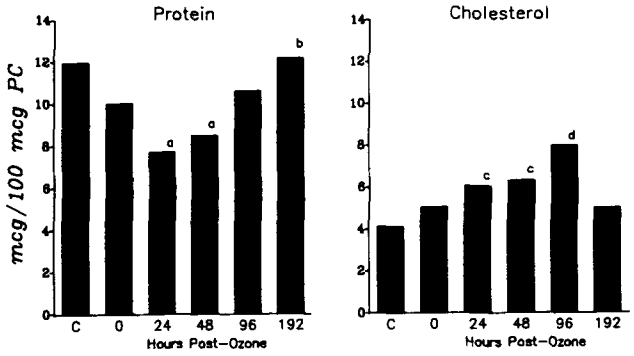

FIG. 2. Total protein and cholesterol per 100 micrograms lamellar body PC: (a) significantly different from control, 0 and 96 hours post-ozone exposure; (b) insufficient data for statistical analysis; (c) significantly different from control; (d) significantly different from all other groups.

reaching levels 2-fold greater than controls by 96 hours. In control rats the extracellular surfactant contained two and one half times as much cholesterol as did lamellar bodies, 10.6 micrograms per 100 micrograms of PC. Unlike intracellular surfactant, the ratio of cholesterol to PC did not change in response to ozone exposure.

Characterization of proteins. Investigation of the lamellar body proteins by SDS-PAGE showed a rapid

decrease in the 14 kDa component following oxidant injury (Fig. 3). This protein was markedly decreased at the end of the 8-hour ozone treatment and remained at low levels during the reparative stage. Additional studies of 1, 2 and 4 hours of ozone exposures revealed that this depletion was detectable as early as 2 to 4 hours after the onset of exposure (27). By 96 hours, the protein appeared to be present at a level similar to that of control. This 14 kDa protein was characterized as a surfactant-associated proteolipid immunochemically by its reaction with specific antiserum (Fig. 4). Two-dimensional electrophoresis (Fig. 5) showed that the migration of the 14 kDa proteolipid was not changed following the addition of disulfide reducing agents. An 18 kDa proteolipid, whose concentration changed little during recovery from ozone injury, migrated to a position consistent with its being a disulfide linked dimer and is thus tentatively identified as alveolar surfactant protein B (SP-B). Only the 18 kDa proteolipid could be detected in surfactant isolated from alveolar lavage (Fig. 4) suggesting that the 14 kDa proteolipid is a non-secreted component of the lamellar bodies. Although this protein reacts with antiserum to proteolipids of human surfactant, it does not have the characteristics of any previously defined alveolar surfactant protein.

Lamellar bodies also contained a protein of approximately 70 kDa, which increased following ozone injury (Fig. 3). This protein was initially assumed to be the dimeric form of surfactant-associated glycoprotein (SP-A), but upon reduction with disulfide reducing agents we were unable to find the typical 35 kDa monomers (Fig. 5). Using the more sensitive immunochemical methods and nonreduced samples, we were able to detect the 72 kDa dimer of SP-A, as well as smaller quantities of the monomers, in lamellar bodies (Fig. 6), indicating that this organelle does contain small amounts of surfactant-associated glycoproteins. However, no increase in the amount of these proteins was apparent by immunostaining a 24-hour post-ozone lamellar body preparation (Fig. 6). The 70 kDa protein, which increased in response to ozone stress, was subsequently identified as albumin by immunoblotting using commercially prepared anti-rat albumin sera.

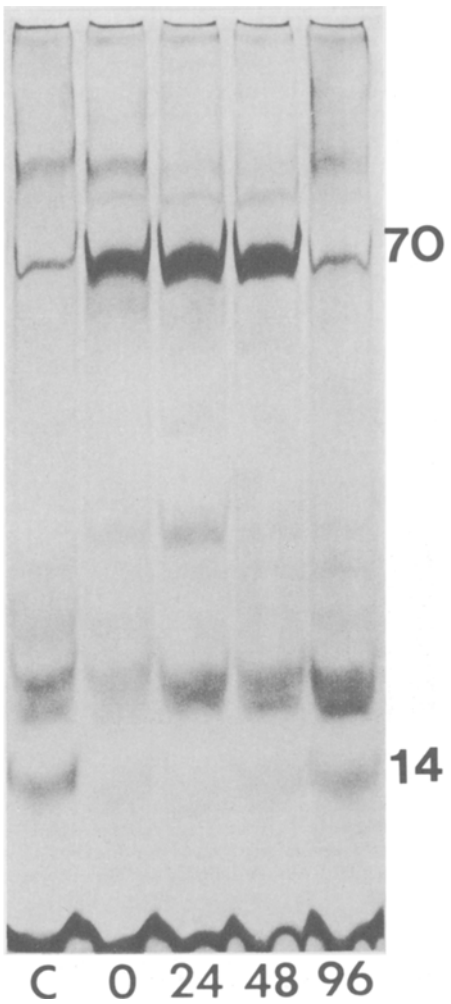

FIG. 3. A representative SDS-PAGE of lamellar bodies isolated from control and ozone-exposed rats. The sample applied to each lane contained 100 μg PC in Fairbanks (21) sample treatment buffer without disulfide reducing agents. The gels were stained with silver/Coomassie blue. The amount of the 14 kDa band associated with 100 μg lamellar body PC is obviously much lower in 0-time, 24- and 48-hour samples than in control (C) and at 96 hours.

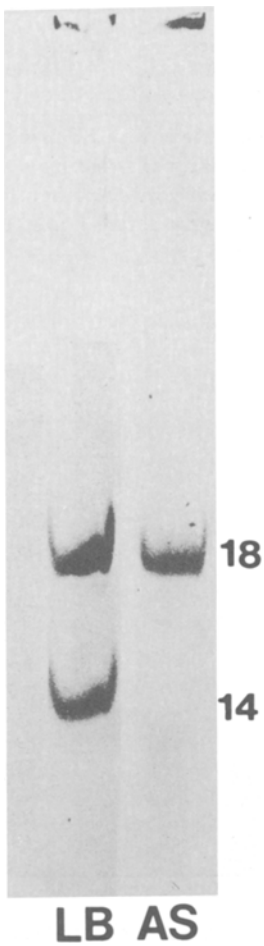

FIG. 4. Immunoblot of nonreduced lamellar body (LB) and alveolar surfactant proteins (AS) reacted with antiserum to human surfactant proteolipids. Samples containing 100 μg PC were applied to each lane.

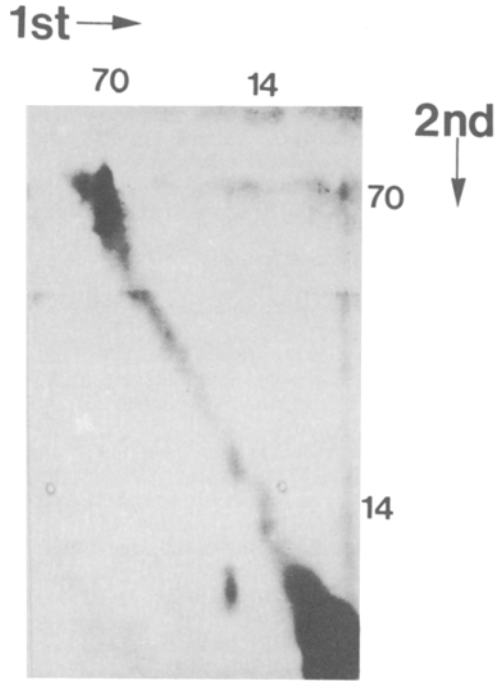

FIG. 5. Lamellar body proteins from a rat 48 hours after ozone exposure electrophoresed first in a 10% acrylamide tube gel without disulfide reducing agent then into a 10% acrylamide slab gel after passing through agarose containing dithiothreitol. The gel is stained with silver/Coomassie blue.

FIG. 6. Immunoblot of nonreduced lamellar body proteins from control (C) and 24-hour post-exposure rats with antisera to rat surfactant-associated glycoproteins. Each sample contained 100 μg lamellar body PC.

DISCUSSION

Acute ozone stress of Type II alveolar cells causes sustained changes in the lipid and protein composition of lamellar bodies. A rapid depletion of a 14 kDa, lamellar body specific, proteolipid persists during the early reparative stage, which is characterized by Type II cell hypertrophy. Concurrently, the free cholesterol content of the lamellar bodies increases relative to the PC content. The reappearance of the 14 kDa proteolipid in the lamellar body begins with the onset of Type II cell hyperplasia which heralds the recovery stage of oxidative alveolar damage while the cholesterol continues to increase. To our knowledge this is the first report indicating that injury to Type II epithelium can be detected by specific changes in components of lamellar bodies.

Phizackerley *et al.* (28) isolated lamellar bodies from pig lung and found, in addition to proteins insoluble in organic solvents, hydrophobic proteins that constituted about 40% of the total lamellar body protein and about 13% of the total protein of surfactant isolated from the lavage fluids. Our current studies of proteolipids and surfactant-associated glycoproteins in rat lung have shown that although both classes are present in extracellular as well as intracellular surfactant pools, there is a marked differential distribution. The 35 kDa glycoproteins (SP-A), found in the rat mainly as a 72 kDa dimer (22), are much more abundant in the extracellular surfactant while the proteolipids are predominantly localized within the lamellar bodies (unpublished observations).

Our results do not provide direct information regarding the etiology of proteolipid deficiency within the lamellar bodies. The possibility that this proteolipid depletion is caused by accelerated exocytosis of lamellar bodies is unlikely in view of the evidence that the 14 kDa proteolipid does not accumulate in the lavage fluid. An alternative possibility is that this proteolipid is rapidly degraded either within the secretory pathway of Type II cells or immediately following secretion. It is well-documented that lamellar bodies are not merely storage sites for surfactant constituents, but they actually represent multifunctional, compartmented organelles containing a diversity of lysosomal enzymes (2,3). Additional studies have demonstrated that the deficiency of surfactant proteolipid occurs concurrently with depletion of several lamellar body enzymes (14). Since the lysosomal system plays an important role in the degradation or post-translational modifications of proteins within the secretory pathway (29,30), it is conceivable that lamellar body enzymes regulate lamellar body proteolipids, which, in turn, modulate the accumulation of surfactant lipids within the lamellar bodies by enhancing their synthesis and reutilization. This hypothesis is consonant with the results of *in vitro* studies indicating that surfactant proteolipids (31), as well as SP-A (32), promote reuptake of surfactant phospholipids.

Oxidative stress, which results in alveolar transudation of plasma proteins, resulted in increased accumulation of albumin in lamellar bodies without increasing their total protein content. We consider it unlikely that this increase in albumin concentration is an artifact of the lamellar body isolation procedure since the lungs were perfused and lavaged before the tissue was homogenized. In addition, the level of albumin in lamellar bodies remained high at 48 hours when the concentration of protein in the lavage fluid had dropped significantly from the levels found at 0-time and 24 hours (8). There is morphological evidence that acute oxidative injury triggers accelerated emptying of lamellar bodies in association with extensive intercommunication and fusion of lamellar bodies (8). The above processes may promote internalization of albumin from the alveolar fluid to lamellar bodies.

It is also intriguing that the cholesterol content of lamellar bodies is persistently elevated throughout the recovery stage of ozone-induced alveolar damage, approaching the values found in alveolar surfactant, but the cholesterol content in alveolar surfactant remains constant. While our studies do not address the source of the increased cholesterol in the lamellar body fraction, it is tempting to speculate that it may be a result of internalization of extracellular material which accumulates in ozone-damaged lungs. Our findings do indicate that alterations of surfactant are first manifested by changes in composition of intracellular surfactant, possibly because Type II cells actively participate in the regulation of extracellular surfactant homeostasis. The necessity for maintaining well-controlled levels of cholesterol in extracellular surfactant is not surprising in view of the known detrimental effect of excess cholesterol on surfactant function (33).

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Phospholipid Content and Fatty Acid Composition of Human Heart

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Phospholipid content and fatty acid composition of human heart were determined on 36 biopsy specimens collected during open heart surgery. The main phospholipid classes, phosphatidylcholine (PC), phosphatidylethanolamine (PE), diphosphatidylglycerol (DPG), and sphingomyelin (SPH) were separated by HPLC, quantified, and converted to fatty acid methyl esters which were chromatographed on capillary GLC columns. Sex and age (mainly 40–70) of patients had no significant influence on the relative distribution of phospholipid classes and only a slight effect on fatty acid composition. Incorporation of *trans* 18:1 in phospholipid classes was low. *cis* and *trans* octadecenoic isomers seemed to be selectively incorporated, the Δ9 and Δ11 *cis* or *trans* isomers being predominant. Human and rat data were compared, and some species differences were noticed. In human PC, palmitic acid is higher and stearic acid much lower than in rat PC. Saturated dimethyl acetals (16:0 and 18:0) in PC and PE were greater for humans. Incorporation of 20:4 n-6 in human PE is higher than in rat PE.
Lipids 21, 775–780 (1989).

Phospholipid content and fatty acid composition of rat heart have been extensively studied in the last two decades. It has been shown that dietary fatty acids, mainly the unsaturated ones, greatly influence the phospholipid content and fatty acid composition of cellular membranes. The pathological and functional consequences of these changes are still being debated (1). Data for human heart phospholipids are much less abundant. In 1985, we reported the fatty acid composition of human heart total phospholipids from 53 biopsy specimens collected during open heart surgery at the Dijon University and Regional Hospital (2). It was shown that sex and age (mainly 40–70) of patients, as well as the morphological state of the organ, had no significant effect on major fatty acids. Moreover, *trans*-octadecenoic isomers were detected in all samples, but only at low levels (0.4–1.2% of the total phospholipid fatty acids) despite the fact that the diets of most of the subjects had included hydrogenated fats, which contain *trans* fatty acids. Our data confirmed that of Heckers *et al.* (3), Ohlrogge *et al.* (4) and Adlof and Emken (5), which was obtained on a more limited number of autopsy samples.

Since 1985, 36 new biopsy specimens have been collected. The present work is a more detailed investigation of individual phospholipid classes and their fatty acid composition. *trans* and *cis* 18:1 isomers were also identified in a pooled fraction of phosphatidylcholine and sphingomyelin.

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Abbreviations used: DMA, dimethyl acetals; DPG, diphosphatidylglycerol; GLC, gas-liquid chromatography; HPLC, high performance liquid chromatography; NPL, non-phospholipids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PL, phospholipids; SPH, sphingomyelin; TL, total lipids.

MATERIALS AND METHODS

Thirty six heart samples were collected from the left ventricule (papillary muscle) of 17 male and 19 female patients during mitral valve replacement. Only tissue which had to be removed during the operation was used for lipid analyses. Samples (about 1 g) were immediately placed in chloroform-methanol (2:1, v/v). Hydroquinone was added (0.01%) as an antioxidant, and the samples were stored at −20°C until analysis. Possible enzymatic or oxidative alteration of the lipids was therefore minimized. Total heart lipids were extracted from homogenized tissue following the procedure of Folch *et al.* (6). Total phospholipids were separated from total non-phospholipids on silicic acid cartridges (7). Contents of total lipids (TL), phospholipids (PL) and non-phospholipids (NPL) were obtained gravimetrically after drying. Individual phospholipid classes were quantified as previously described (8). Phospholipid classes were separated by high performance liquid chromatography (9), and their constituent fatty acids were converted to methyl esters by direct transesterification with 2 ml of 5% boron trifluoride in methanol at 95°C. Esterification time was 1 hr, 30 min for phosphatidylcholine (PC), phosphatidylethanolamine (PE), and diphosphatidylglycerol (DPG), and 12 hr for sphingomyelin (SPH). Fatty acid methyl esters were analyzed by gas-liquid chromatography on glass capillary columns as previously described (2). Identification of individual *trans* and *cis* octadecenoic isomers on a pooled fraction of PC and SPH, the major phospholipid fraction and the one which has been reported to incorporate the highest levels of *trans* fatty acids in the rat (10), was done according to Sebedio *et al.* (11). Statistics were done by analysis of variance and a Newman-Keuls comparison of means (12).

RESULTS AND DISCUSSION

Table 1 shows that age and sex of patients had no significant influence on the relative distribution of lipid classes. PL values showed about the same degree of dispersion as was found in other studies (13,14). However, the DPG level was higher: 13.6 vs 9% (13) and 7% (14). This may be due to post mortem changes, which would be found in autopsy samples, or degradation after sampling.

The data show considerable dispersion of values for human heart PL, but they are within the range of values which have been reported for rats (14–21). No species differences are thus apparent.

Tables 2–5 list the fatty acid composition of phospholipid classes and observed variations due to sex and age. The results complement and confirm previously obtained data (2). Better chromatographic conditions in this study permitted the separation of dimethyl acetals (DMA), and of 16:1 isomers and *trans* 18:1. The results differ from those of Fletcher (22), who identified only seven fatty acids (14:0, 16:0, 16:1, 18:0, 18:1, 18:2, 20:4) in PC, PE, and SPH, and did not find any C22 polyunsaturates in PC and PE or any long chain saturates (20:0–24:0) in

TABLE 1

Distribution of Major Phospholipid Classes in the Human Heart[a]

Phospholipid class	Grand mean ± SD (n = 31)	Sex		Age (years)		
		Male (n = 13)	Female (n = 18)	11-50 (n = 10)	51-60 (n = 8)	61-80 (n = 13)
PC[b]	40.9 ± 3.83	42.2	40.0	43.0	39.7	40.1
PE	20.6 ± 4.78	20.6	20.6	20.5	20.0	21.0
DPG	13.6 ± 3.09	12.5	14.5	13.7	14.9	12.8
SPH	4.7 ± 2.48	4.7	4.6	4.5	4.6	4.8
Other classes	20.3 ± 3.88	20.1	20.5	18.3	20.9	21.6

[a]Values shown are percentage of total lipid phosphorus (Mean ± Standard-Deviation, n = number of samples).
[b]PC = phosphatidylcholine, PE = phosphatidylethanolamine, DPG = diphosphatidylglycerol, SPH = sphingomyelin, other classes = phosphatidylinositol + phosphatidylserine + lysophosphatidylcholine + lysophosphatidylethanolamine.

TABLE 2

Fatty Acid Composition (%) of Human Heart Phosphatidylcholine

Fatty acid	Grand mean ± SD (n = 36)	Sex		Age (years)		
		Male (n = 17)	Female (n = 19)	11-50 (n = 13)	51-60 (n = 9)	61-80 (n = 14)
14:0	1.1 ± 0.25	1.1	1.2	1.2[a,b]	1.3[b]	1.0[a]
16:0 DMA	8.3 ± 1.04	8.2	8.3	8.4	8.1	8.2
16:0	27.0 ± 1.18	27.5[a]	26.5[b]	27.0	27.0	27.0
16:1 n-9	0.2 ± 0.11	0.2	0.3	0.2	0.3	0.2
16:1 n-7	0.8 ± 0.22	0.7	0.8	0.7	0.8	0.8
17:0	0.4 ± 0.09	0.4	0.4	0.4	0.4	0.4
18:0 DMA	1.9 ± 0.33	1.7[a]	2.0[b]	1.9[a,b]	1.6[a]	2.0[b]
18:1 n-9 DMA	1.4 ± 0.32	1.2[a]	1.6[b]	1.4	1.2	1.5
18:1 n-7 DMA	0.6 ± 0.13	0.5[a]	0.6[b]	0.6	0.5	0.6
18:0	6.0 ± 0.66	6.2	5.9	6.1	6.3	5.8
18:1 *trans*	0.5 ± 0.13	0.5	0.5	0.5	0.5	0.5
18:1 n-9	11.9 ± 1.28	11.7	12.2	11.6	12.5	11.9
18:1 n-7	1.9 ± 0.27	1.9	2.0	1.9	2.0	1.9
18:2 n-6	16.1 ± 2.84	15.7	16.5	16.0	16.2	16.2
18:3 n-3	0.2 ± 0.09	0.3	0.2	0.2	0.3	0.3
20:2 n-6	0.2 ± 0.04	0.2	0.2	0.2	0.2	0.2
20:3 n-6	0.8 ± 0.13	0.9	0.8	0.8	0.9	0.9
20:4 n-6	16.4 ± 1.97	17.0	15.9	16.8	15.8	16.4
20:5 n-3	0.4 ± 0.12	0.4	0.4	0.4	0.4	0.3
22:5 n-3	1.2 ± 0.23	1.2	1.2	1.1	1.2	1.2
22:6 n-3	2.1 ± 0.50	2.1	2.2	2.2	2.2	2.0
U.I.	1.37 ± 0.06	1.38	1.36	1.38	1.37	1.37
Saturates	34.5 ± 1.57	35.2[a]	33.9[b]	34.7	34.9	34.1
Monoenes	15.3 ± 1.55	14.9	15.7	14.9	16.0	15.4
Polyenes	37.5 ± 2.01	37.7	37.3	37.8	37.2	37.5
Polyenes/Sat.	1.1 ± 0.09	1.1	1.1	1.1	1.1	1.1
n-6/n-3	8.9 ± 1.60	8.9	8.9	9.0	8.2	9.2

DMA = dimethyl acetals.
U.I. = Unsaturation Index is the sum of the percentages of individual unsaturated fatty acid × number of double bonds/100.
[a,b]Values within a line without a common superscript are significantly different ($p < 0.05$).

SPH, which are generally thought to be characteristic fatty acids of these compounds. Also, no occurring DPG was reported (22). The quality of biopsy specimens vs autopsy samples and the performances of capillary vs packed GLC columns may explain some of these differences. Significant age and sex differences were found for some fatty acids. In females, stearic DMA, 18:1 DMA in PC, and 18:1 n-9 in SPH were higher, and 17:0, 18:0 in DPG and 16:0 in PC were lower than in males. Linoleic acid (18:2 n-6) and 20:4 n-6 in DPG were respectively higher (+6%) and lower (−11%) in females than in males. This can be of some physiological importance since

TABLE 3

Fatty Acid Composition (%) of Human Heart Phosphatidylethanolamine

Fatty acid	Grand mean ± SD (n = 36)	Sex		Age (years)		
		Male (n = 17)	Female (n = 18)	11-50 (n = 13)	51-60 (n = 9)	61-80 (n = 13)
14:0	0.4 ± 0.14	0.4	0.4	0.5	0.4	0.4
16:0 DMA	6.4 ± 0.89	6.6	6.3	6.5	6.9	6.0
16:0	3.6 ± 0.42	3.7	3.5	3.7	3.6	3.6
16:1 n-9	0.4 ± 0.22	0.4	0.4	0.5	0.4	0.4
16:1 n-7	0.3 ± 0.07	0.3	0.3	0.3	0.3	0.3
18:0 DMA	8.4 ± 1.20	8.2	8.6	8.7	8.1	8.3
18:1 n-9 DMA	2.7 ± 0.46	2.6	2.9	2.9	2.6	2.7
18:1 n-7 DMA	1.6 ± 0.30	1.5	1.6	1.6	1.6	1.6
18:0	21.1 ± 1.32	21.3	20.9	20.7	21.1	21.4
18:1 *trans*	0.6 ± 0.13	0.5	0.6	0.5	0.6	0.6
18:1 n-9	2.1 ± 0.28	2.1	2.1	2.1	2.1	2.1
18:1 n-7	1.7 ± 0.19	1.6	1.7	1.7	1.7	1.7
18:2 n-6	2.8 ± 0.55	2.7	2.9	2.7	2.9	2.8
18:3 n-3	0.5 ± 0.35	0.7	0.4	0.5	0.7	0.5
20:2 n-6	0.1 ± 0.05	0.2	0.1	0.2	0.1	0.2
20:3 n-6	0.4 ± 0.08	0.4	0.4	0.4	0.4	0.4
20:4 n-6	35.1 ± 1.47	35.3	35.0	$35.0^{a,b}$	33.9^{a}	36.2^{b}
20:5 n-3	0.7 ± 0.23	0.6	0.7	0.7	0.6	0.6
22:4 n-6	0.8 ± 0.19	0.9	0.8	0.8	0.9	0.8
22:5 n-6	0.6 ± 0.17	0.7	0.6	0.5	0.7	0.6
22:5 n-3	2.1 ± 0.31	2.1	2.0	1.9^{a}	2.2^{b}	2.1^{b}
22:6 n-3	7.2 ± 1.45	7.0	7.4	7.8	7.8	6.5
U.I.	2.17 ± 0.05	2.17	2.17	2.16	2.18	2.17
Saturates	25.1 ± 1.45	25.4	24.9	24.9	25.1	25.4
Monoenes	4.8 ± 0.52	4.7	4.8	4.8	4.7	4.8
Polyenes	50.3 ± 0.88	50.4	50.3	50.0	50.3	50.7
Polyenes/Sat.	2.0 ± 0.12	2.0	2.0	2.0	2.0	2.0
n-6/n-3	3.9 ± 0.74	4.0	3.9	$3.9^{a,b}$	3.4^{a}	4.3^{b}

See Table 2 for abbreviations.

^{a,b}Values within a line without a common superscript are significantly different ($p < 0.05$).

TABLE 4

Fatty Acid Composition (%) of Human Heart Diphosphatidylglycerol

Fatty acid	Grand mean ± SD (n = 36)	Sex		Age (years)		
		Male (n = 17)	Female (n = 19)	11-50 (n = 13)	51-60 (n = 9)	61-80 (n = 14)
14:0	1.0 ± 0.38	1.1	0.9	1.0	1.0	1.0
16:0 DMA	0.9 ± 0.17	0.9	0.8	0.9	0.9	0.8
16:0	4.0 ± 1.61	4.4	3.6	4.4	4.1	3.5
16:1 n-9	1.1 ± 0.66	1.4	1.0	1.2	1.1	1.1
16:1 n-7	1.5 ± 0.72	1.3	1.6	1.5	1.5	1.4
17:0	0.4 ± 0.17	0.4^{a}	0.3^{b}	0.4	0.5	0.3
18:0 DMA	1.0 ± 0.20	1.0	1.0	1.0	1.0	1.0
18:1 n-9 DMA	0.3 ± 0.11	0.4	0.3	0.3	0.3	0.3
18:1 n-7 DMA	0.2 ± 0.07	0.2	0.2	0.1	0.2	0.2
18:0	4.5 ± 0.98	4.9^{a}	4.2^{b}	4.7	4.8	4.2
18:1 n-9	5.1 ± 0.93	5.3	4.9	4.8	5.4	5.2
18:1 n-7	4.2 ± 1.21	4.4	4.0	3.5^{a}	5.0^{b}	$4.3^{a,b}$
18:2 n-6	66.5 ± 4.67	64.4^{a}	68.4^{b}	66.6	65.0	67.4
18:3 n-6	0.2 ± 0.14	0.3	0.2	0.2	0.3	0.3
18:3 n-3	0.5 ± 0.11	0.5	0.5	0.5	0.5	0.4
20:2 n-6	0.5 ± 0.16	0.5	0.5	0.5	0.5	0.5
20:3 n-6	0.3 ± 0.08	0.3	0.3	0.3	0.3	0.3
20:4 n-6	5.1 ± 0.73	5.4^{a}	4.8^{b}	5.1	5.2	5.0
22:5 n-3	0.5 ± 0.13	0.5	0.5	0.4	0.5	0.5
22:6 n-3	1.1 ± 0.33	1.2	1.0	1.2	1.2	1.0
24:0	0.3 ± 0.13	0.3	0.3	0.3	0.3	0.3
U.I.	1.78 ± 0.07	1.77	1.80	1.78	1.78	1.79
Saturates	10.2 ± 3.02	11.2^{a}	9.2^{b}	10.8	10.6	9.3
Monoenes	11.9 ± 1.93	12.4	11.4	11.0	13.0	12.0
Polyenes	74.7 ± 4.16	73.0^{a}	76.2^{b}	74.8	73.5	75.3
Polyenes/Sat.	8.0 ± 2.42	7.1^{a}	8.9^{b}	7.7	7.5	8.6
n-6/n-3	37.6 ± 8.09	35.5	39.4	36.4	33.6	41.1

See Table 2 for abbreviations.

^{a,b}Values within a line without a common superscript are significantly different ($p < 0.05$).

TABLE 5

Fatty Acid Composition (%) of Human Heart Sphingomyelin

Fatty acid	Grand mean ± SD (n = 36)	Sex		Age (years)		
		Male (n = 17)	Female (n = 19)	11-50 (n = 13)	51-60 (n = 9)	61-80 (n = 14)
14:0	1.7 ± 0.82	1.6	1.7	1.8	1.8	1.4
15:0	0.8 ± 0.40	0.8	0.8	0.8	0.9	0.7
16:0 DMA	0.7 ± 0.48	0.6	0.9	0.8	0.7	0.7
16:0	21.6 ± 4.43	21.3	21.9	20.6	23.4	21.4
16:1 n-7	2.1 ± 1.15	2.0	2.1	2.0	2.2	2.0
17:0	0.7 ± 0.15	0.7	0.8	0.8	0.8	0.7
18:0 DMA	0.5 ± 0.23	0.4	0.5	0.5	0.5	0.4
18:1 DMA	0.2 ± 0.11	0.2	0.2	0.2	0.2	0.2
18:0	16.0 ± 2.12	16.2	15.8	15.8	16.3	16.0
18:1 *trans*	0.2 ± 0.13	0.2	0.2	0.2	0.2	0.2
18:1 n-9	3.3 ± 1.58	2.7^a	3.9^b	3.0	3.8	3.3
18:1 n-7	0.5 ± 0.31	0.5	0.6	0.5	0.5	0.5
18:2 n-6	1.9 ± 0.99	1.7	2.1	2.0	1.8	1.9
18:3 n-3	0.3 ± 0.15	0.3	0.3	0.4	0.3	0.3
19:0	0.4 ± 0.10	0.4	0.4	0.4	0.4	0.4
20:0	4.4 ± 0.77	4.5	4.3	4.2	4.3	4.6
20:4 n-6	2.8 ± 1.22	2.7	2.9	3.4^a	$2.7^{a,b}$	2.2^b
21:0	1.8 ± 0.85	1.7	1.8	1.8	1.5	1.9
22:0	12.8 ± 4.01	13.4	12.2	12.5	12.2	13.4
23:0	3.7 ± 0.88	3.7	3.8	3.7	3.8	3.7
24:0	8.4 ± 2.24	9.0	7.9	8.5	8.1	8.5
24:1 n-9	14.6 ± 4.14	14.7	14.5	14.9	13.3	15.1
U.I.	0.36 ± 0.05	0.35	0.38	0.39	0.35	0.34
Saturates	72.1 ± 3.54	73.1	71.3	70.8	73.5	72.5
Monoenes	20.7 ± 3.86	20.0	21.3	20.6	20.0	21.2

See Table 2 for abbreviations.

^{a,b}Values within a line without a common superscript are significantly different ($p < 0.05$).

18:2 n-6 is by far the main fatty acid in heart DPG, an important mitochondrial component. Age had a significant effect on 20:4 n-6 in PE, in which it represents the major fatty acid. Its percentage was significantly lower in patients 51-60 years of age (33.9%) than it was in patients over age 60 (36.2%). It is sometimes postulated that arachidonic biosynthesis is impaired with age by a lowering of the Δ6 desaturase activity (23,24). This does not appear to be the case here since the 20:4 deposition in PE of those over age 60 is similar to that of those under age 50.

Total *trans* 18:1 in human heart PL was low. It was equally distributed in PC and PE, less in SPH, and was absent in DPG. These data are similar to those reported by Ohlrogge *et al.* (25), who found 0.77 and 0.66% of *trans* 18:1 in PC and PE, respectively. The double bond distribution of the *cis* and *trans* 18:1 positional isomers in the pooled fraction of PC and SPH is shown in Figure 1. Apart from *cis* Δ9 and *cis* Δ11 acids, which could be primarily of endogenous origin, the other *trans* and *cis* 18:1 isomers would be expected to come from hydrogenated and ruminant (dairy, meat) fats consumed by the subjects. As already observed by Ohlrogge *et al.* (4,25), the *cis* double bond distribution in human lipids, including heart PC, shows two main components: Δ9 and Δ11 acids. In addition, *cis*-Δ7 and Δ15 isomers not mentioned by Ohlrogge *et al.* were detected here. These isomers are usually absent (Δ15) or present only in trace amounts (Δ7) in margarines and butter. More striking differences from the Ohlrogge *et al.* data appear in the *trans* isomer distribution. Whereas Ohlrogge *et al.* found a rather broad distribution from Δ8 to Δ14, with Δ11 and Δ10 being predominant, our study showed Δ9 to be better incorporated than Δ11 and Δ10 and Δ8 were hardly detected. Noticeable amounts of Δ6 and Δ15, which were found only at trace levels in the above mentioned study, also occur. Non-incorporation of Δ10 is rather surprising since it is predominant in margarines (25-27). It would appear significant in this context that the Δ10 isomer was also shown to be specifically excluded from rat heart lipids (28). Also intriguing is the occurrence of Δ6 and Δ15, which are absent or present only in small amounts in margarines and butter. In the human heart samples we analyzed, a more highly selective incorporation of individual *cis* and *trans* isomers seems to have occurred. It could also be that the intake level and the fatty acid composition of the hydrogenated fats and dairy products consumed by the French patients were different from those examined by Ohlrogge *et al.* (25).

Comparison of the fatty acid compositions of major human heart phospholipids (PC, PE, DPG, SPH) with those of male rats (16-21, 29-35) shows interesting specific features. First, it appears to be clear that in human PC 16:0 is higher and 18:0 is much lower than in rat PC. A similar pattern was previously described for human mitochondrial and microsomal PC (36,37). Percentages of 16:0 DMA and 18:0 DMA in PE and PC are also greater in man than in rats (18,19,21,31,35). This is

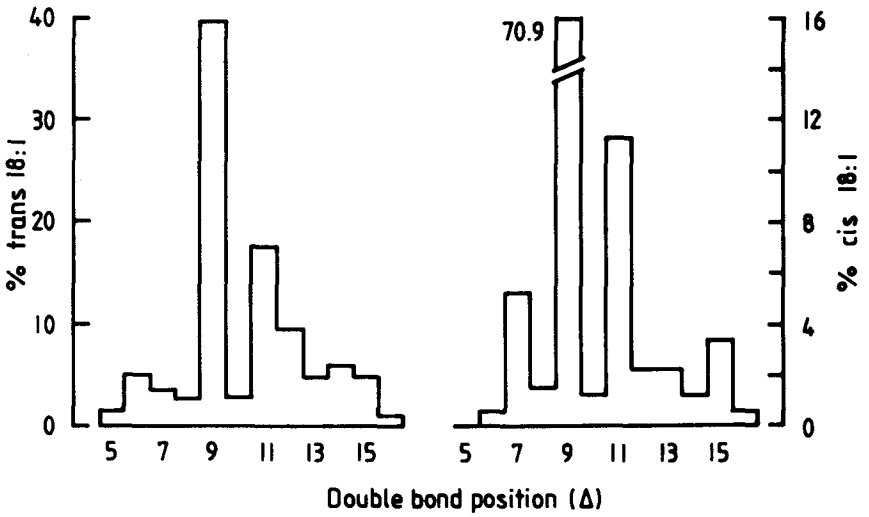

FIG. 1. Relative distribution of *trans*- and *cis*-18:1 isomers in a pooled fraction of human PC plus SPH.

particularly obvious for PC which in man contains more than 8% of 16:0 DMA as compared to less than 1% in rats. Because 16:0 and 18:0 in rat heart PL are hardly affected by dietary lipid manipulation, it can be concluded that differences are primarily species-related. This could mean that in humans, but not in rats, acylating enzymes preferentially incorporate 16:0 rather than 18:0 into the 1-position of heart PC. Thus, 16:0 PC will predominate in human heart, whereas in rat heart 18:0 PC will predominate (38). Second, the level of 20:4 found in human cardiac PE seems to be much higher than in male rats, even when they are fed diets containing amounts of 18:2 far higher than those normally found in human diets. This result supports the findings of Gloster and Harris (36,37) for mitochondrial and microsomal PE. Third, levels of 22 n-6 and n-3 polyunsaturated fatty acids in human PE appear to be close to the lowest values usually reported for rats. These 22 polyunsaturates are largely dependent on the dietary supplies of n-6 and/or n-3 essential fatty acids. Thus, 22 n-3 will increase and replace 22 n-6 when n-3 fatty acids are fed to rats. Conversely, when n-3 fatty acids are omitted, 22 n-3 decrease as 22 n-6 increase.

Therefore, the low level of 22:5 n-6 in human PE, together with the presence of 22 n-3 polyunsaturates in higher amounts, would indicate a significant intake of n-3 fatty acids. It could also be hypothesized that the conversion of 20:4 n-6 and of 18:3 n-3 to higher n-6 and n-3 metabolites, respectively, is slower in humans than in rats.

Finally, incorporation of 18:1 *trans* isomers in human heart PC, PE or SPH is much less significant than in rats fed partially hydrogenated fats (39,40) or pure elaidic acid (10,35,40). This is due, at least in part, to the amount of ingested isomeric fats, which is certainly lower in normal human diets than in those of the rats in the experiments; but the higher oxidation rate of the *trans* isomers by human hearts may also be a factor (41).

These species differences in fatty acid compositions can result in differences in total unsaturation of the phospholipid classes. Thus, the higher levels of saturated DMA in PC and PE and the lower levels of 20:4 n-6 in PC and of C22 polyunsaturates in PE in man can lead to a decreased unsaturation index of the corresponding phospholipids. This could contribute to species differences in membrane fluidity and, subsequently, in membrane function. However, lipid unsaturation is but one of several factors that can affect membrane fluidity, while the specific effect of unsaturation is still being debated (1).

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Rhizomucor miehei Triglyceride Lipase Is Processed and Secreted from Transformed *Aspergillus oryzae*

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The cDNA encoding the precursor of the *Rhizomucor miehei* triglyceride lipase was inserted in an *Aspergillus oryzae* expression vector. In this vector the expression of the lipase cDNA is under control of the *Aspergillus oryzae* α-amylase gene promoter and the *Aspergillus niger* glucoamylase gene terminator. The recombinant plasmid was introduced into *Aspergillus oryzae*, and transformed colonies were selected and screened for lipase expression. Lipase-positive transformants were grown in a small fermentor, and recombinant triglyceride lipase was purified from the culture broth. The purified enzymatically active recombinant lipase (rRML) secreted from *A. oryzae* was shown to have the same characteristics with respect to mobility on reducing SDS-gels and amino acid composition as the native enzyme. N-terminal amino acid sequencing indicated that approximately 70% of the secreted rRML had the same N-terminal sequence as the native *Rhizomucor miehei* enzyme, whereas 30% of the secreted rRML was one amino acid residue shorter in the N-terminal. The recombinant lipase precursor, which has a 70 amino acid propeptide, is thus processed in and secreted from *Aspergillus oryzae*. We have hereby demonstrated the utility of this organism as a host for the production of recombinant triglyceride lipases.
***Lipids* 24, 781–785 (1989).**

The phycomycete fungus *Rhizomucor miehei* (1) secretes a triglyceride lipase (RML, triacylglycerol acylhydrolase EC 3.1.1.3). We have previously reported on the partial purification (2) of this enzyme, and we recently isolated the lipase cDNA from a *Rhizomucor miehei* cDNA library constructed in *E. coli* (3). From the DNA sequence of the lipase cDNA clones we deduced the amino acid sequence of the 363 amino acid lipase precursor. We presented evidence that RML is synthesized as a 39.4 kD precursor with a 24 amino acid signal peptide and a 70 amino acid propeptide. The propeptide is cleaved (in one or more as yet unidentified steps) from the 269 amino acid residues of the mature enzyme through a cleavage between a methionine and a serine residue in the precursor. The enzymatic mechanism in the maturation process remains unknown.

Triglyceride lipases have a number of potential industrial applications exemplified by their use in transesterification and in household detergents. Therefore, it will be important to provide a production system that will make these enzymes available at a reasonable cost. The use of recombinant DNA technology in the production of triglyceride lipases will probably be important in achieving this goal.

We now report on the heterologous expression of the *Rhizomucor miehei* lipase in another filamentous fungus —*Aspergillus oryzae*. We have recently established an efficient expression system for heterologous proteins in this organism (4). The aspartic proteinase (5) from *Rhizomucor miehei* was secreted from a transformed strain of this organism into the growth medium in high quantities. Our present experiments present evidence that the precursor of the RML is correctly processed in *A. oryzae*, and that the mature lipase is secreted into the growth medium.

MATERIALS AND METHODS

Expression of RML in Aspergillus oryzae. The 1.2 kb RML cDNA (3) was inserted by the use of synthetic adaptors and linkers into an *A. oryzae* expression vector pBoel-777 (4) using standard recombinant DNA technology (6). In the resulting construct pRML-787 (Fig. 1), the preproRML cDNA is flanked by a *Bam*HI site (GGATCC) just 5′ to the initiating methionine codon, the actual sequence reading: GGATCCACC*ATG*. Through this *Bam*HI site the 5′ end of the preproRML encoding cDNA is joined to a *Bam*HI site previously introduced at a position 9 nucleotides 5′ to the initiating methionine codon of the α-amylase gene. The 5′ untranslated region of an RML mRNA synthesized from this construct would therefore be identical in length and sequence to the 5′ untranslated region of the α-amylase mRNA from *A. oryzae*, except for the presence of the *Bam*HI linker 5′ to the methionine codon. Site specific oligonucleotide directed mutagenesis (6) in M13 vectors (7) was used to introduce

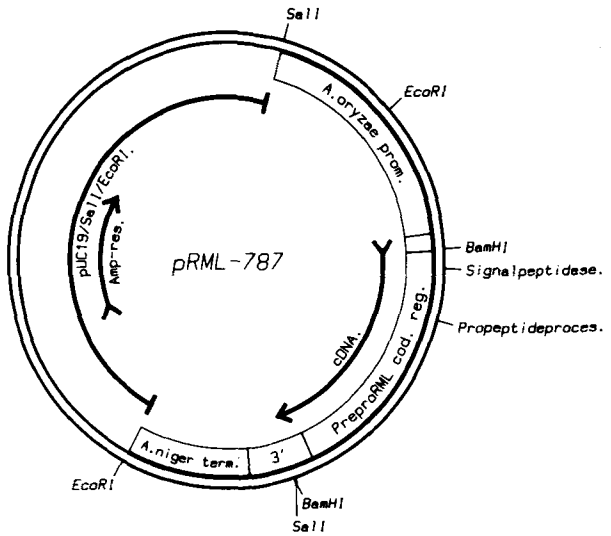

FIG. 1. The *A. oryzae* expression plasmid pRML-787 (5.7 kb) with the preproRML coding region inserted at a unique *Bam*HI cloning site between the *A. oryzae* α-amylase promoter and the *A. niger* glucoamylase terminator. "Amp-res." is the β-lactamase gene of pUC19. The position of the signalpeptide- and propeptide-processing sites in the RML precursor protein are indicated. "3′" designates a region that contains 3′ untranslated sequences from both the RML and the glucoamylase cDNA.

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Abbreviations used: bp, base pair; kb, kilobase; SDS, sodium dodecyl sulphate; LU, lipase units; RML, *Rhizomucor miehei* lipase; rRML, recombinant-RML synthesized in *A. oryzae*.

a *Bam*HI site at the 3′ end of the preproRML cDNA at a position 90 bp downstream from the terminator codon. This *Bam*HI site was ligated to a *Bam*HI linker that previously had been introduced in the *A. niger* glucoamylase gene at the *Sal*I site 5′ to the terminator codon of this gene (8). The 3′ untranslated region of the RML mRNA synthesized in *A. oryzae* is therefore composed of 90 bp from the native RML mRNA and 160 bp from the 3′ end of the glucoamylase mRNA.

This expression vector was cotransformed into *A. oryzae* wild type strain A1560 with the *AmdS* plasmid p3SR2 (9), as described (4). Transformants were selected by their ability to use acetamide as sole nitrogen source. This ability is encoded on p3RS2.

Stable transformants were picked from selective plates and maintained on Czapek-dox (10) agar slants. After 5–10 days of growth at 30°C spores were harvested from the agar slants and inoculated in 500 ml culture flasks containing 100 ml of SSP-medium (3% soybean meal, 1.5% potato starch and 0.5% Bacto peptone). The cultures were grown for 4 days at 30°C, and the lipolytic activity was assayed in the culture supernatants as the ability to cleave tributyrin (2).

Purification and characterization of rRML. An *A. oryzae* transformant that secreted lipolytic activity to the growth medium was selected for production of rRML, and was subsequentially grown in a small fermentor (4). Before the preparation of a crude rRML powder (2), the culture broth was adjusted to alkaline pH (approximately pH 10) in order to loosen the lipase protein from the mycelium. The crude rRML was chromatographed on DEAE Sepharose (2) to generate a partially purified rRML preparation, which was further purified by gel filtration chromatography (flow rate 5 ml/min) on a TSK G3000 SWG column (21.5 × 600 mm) mounted with a precolumn TSK SWPG (21.5 × 75 mm) (3). Procedures for the determination of the amino acid composition, carbohydrate content and isoelectric point of the purified rRML as well as protocols for the SDS-polyacrylamide gel-electrophoresis and tandem-crossed immunoelectrophoresis have previously been described (2,3). The N-terminal amino acid sequence of the purified rRML was determined by automated Edman degradation using an Applied Biosystems Model 470A gas phase sequencer (11). Approximately 4 nmol of purified rRML was applied to the filter of the sequencer, and 24 Edman degradation cycles were carried out.

RESULTS

The cDNA encoding the precursor (3) of the triglyceride lipase from *Rhizomucor miehei* was inserted in an *Aspergillus oryzae* expression vector (4) under control of the *A. oryzae* α-amylase gene promoter and the *A. niger* glucoamylase gene terminator to generate the plasmid pRML-787 (Fig. 1). We have recently (4) demonstrated that this type of expression plasmid directed efficient production of the aspartic proteinase from *Rhizomucor miehei* when the cDNA (5) for this protein was inserted into a similar vector. This construct was cotransformed into *A. oryzae* with the *amdS* gene (cloned on the plasmid p3SR2 [9]) from *A. nidulans* as selective marker. The *amdS* gene encodes an acetamidase enabling *A. oryzae* to grow on acetamide as sole nitrogen source. In the present study, approximately 80% of the transformants selected on acetamide expressed lipolytic activity in the supernatant. Untransformed *A. oryzae* did not produce any tributyrine hydrolyzing activity.

One such transformant was grown in a small fermentor in order to obtain enough rRML for purification and characterization. Purification was performed by consecutive steps of chromatography on DEAE Sepharose and TSK G3000 SWG columns. The 280 nm absorbance profile from the gel filtration step is shown on Figure 2. The two main peaks of eluted material represent an α-amylase secreted from *A. oryzae* and the rRML, respectively. On the SDS-polyacrylamide gel shown in Figure 3,

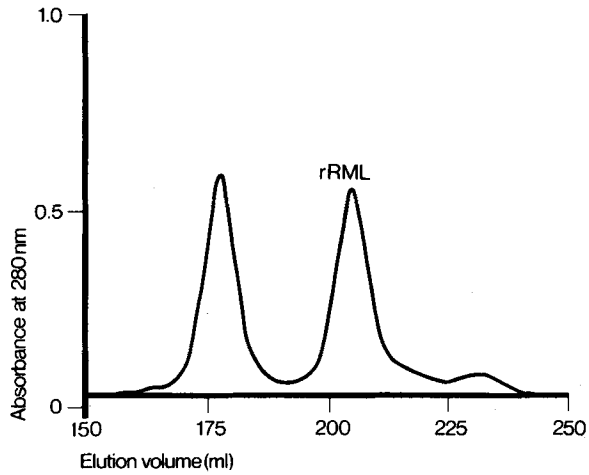

FIG. 2. Gel filtration chromatography of recombinant lipase on a TSK G3000 SWG column. The partially purified preparation of the rRML from the DEAE Sepharose chromatography was further purified on the TSK G3000 SW column (3). The peak to the left in the chromatogram represents an α-amylase endogenous to *A. oryzae*. Tributyrine hydrolyzing activity was only found in the pool represented by the peak designated rRML.

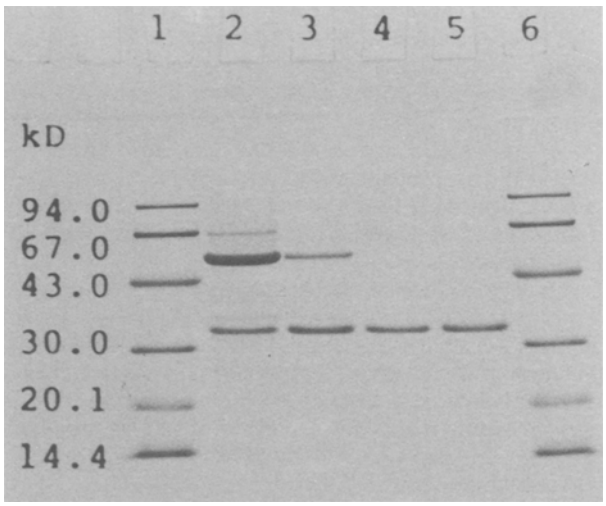

FIG. 3. SDS-Polyacrylamide gel electrophoresis of rRML. Lanes 1 and 6: molecular weight standards; Lane 2: Proteins in the crude powder from the ultra filtration step (17 μg protein applied). Lane 3: Partially purified rRML from the DEAE Sepharose chromatography (6 μg protein applied). Lane 4: Purified rRML from the TSK G3000 SWG column (3 μg protein applied). Lane 5: Purified RML-B from *Rhizomucor miehei* (3 μg protein applied). The gel was stained with Coomassie Brilliant Blue.

the results from the different purification steps were analyzed. Lanes 2–5 show the composition of the crude powder, the partially purified material from the DEAE Sepharose chromatography, the finally purified rRML from the gel filtration chromatography and the purified native RML-B (3) for comparison, respectively. The purified rRML as well as the purified native RML-B have apparent molecular weights of 32 kD. The specific activity of the purified rRML was 8810 LU/mg protein as compared to the 7500 LU/mg protein of the previously described (3) native RML-B. These values are based on protein determinations according to the Lowry method (12). The corresponding values based on protein determinations by amino acid compositional analysis were 12150 LU/mg protein of the rRML and 11200 LU/mg protein of the native RML-B. We have consistently observed a difference in the estimated amount of protein in the purified lipase as determined by the two methods.

The amino acid composition of the purified rRML is compared to the amino acid composition of RML as deduced from the cDNA sequence (3) in Table 1. Only minor deviations between the two compositions are noticed. As a further characterization of the recombinant lipase product determination of the isoelectric point was performed (Fig. 4). In this experiment the pI of rRML is compared to the pI of both native RML-A and RML-B (2). The pI of rRML is 4.3 as compared to 3.9 for RML-A and 4.3 for RML-B. We have previously shown that alkaline conditions (as employed in the purification of rRML in this study) or acidic conditions result in a partial deglycosylation of the native RML, and we have designated this partially deglycosylated variant the B form (2). The isoelectric point of rRML purified as described in this report indicates that this protein is on a form similar to the B type. The carbohydrate content of the purified rRML was approximately 1.2% as compared to 4% (w/w) for the purified native RML-B (3).

The antigenic identity between the rRML and the native RML was investigated using tandem-crossed immunoelectrophoresis. It is evident from the results shown in Figure 5 that the two lipases share a very high degree of antigenic identity.

As a final characterization we performed N-terminal amino acid sequence analysis on the purified recombinant lipase. The result of the analysis is shown in Table 2. Based on the relative yields of the amino acid derivatives in the first three cycles of the Edman degradation, we concluded the following: among the lipase molecules secreted and purified from *A. oryzae*, approximately 70% had the

TABLE 1

Amino Acid Composition of RML and rRML

Amino acid	RML[a]	rRML[b]
Asp/Asn	26	29
Thr[c]	27	25
Ser[c]	25	24
Glu/Gln	21	25
Pro	13	14
Gly	20	19
Ala	18	18
Cys-SH[d]	7	7
Val[c]	21	21
Met	1	1
Ile[c]	17	16
Leu	22	22
Tyr	15	14
Phe	10	10
Lys	7	8
His	6	6
Trp	3	4
Arg	10	10
Totals	269	273

[a] Deduced from cDNA sequence (3).

[b] rRML is the recombinant lipase secreted by *A. oryzae*.

[c] Determined as extrapolated values to 0 or infinite hydrolysis time.

[d] Determined as S-β-(4-pyridylethyl)-cysteine.

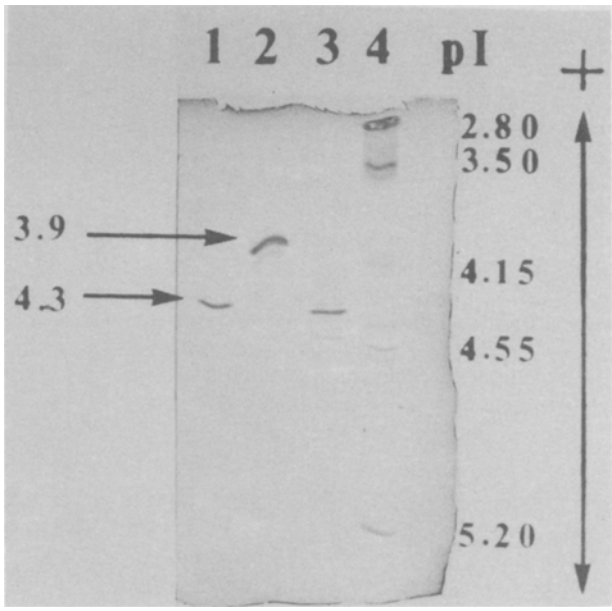

FIG. 4. Isoelectric focusing of purified rRML in comparison with the purified native RML-A and RML-B. Lane 1: rRML. Lane 2: RML-A. Lane 3: RML-B. Lane 4: Reference proteins.

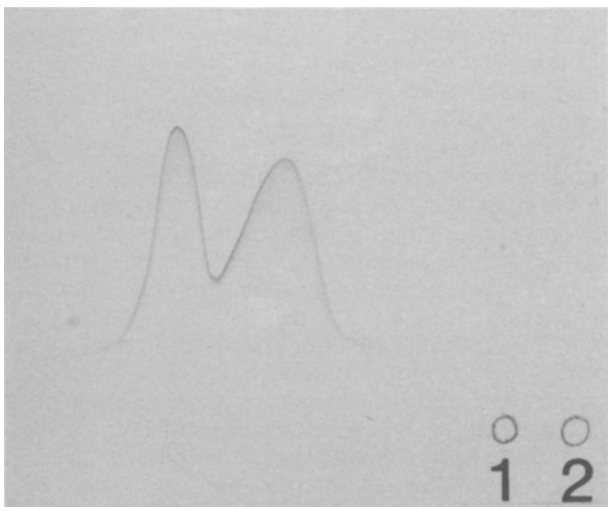

FIG. 5. Tandem-crossed immunoelectrophoresis stained with Coomassie Brilliant Blue. Well 1: the purified rRML. Well 2: a crude powder of the native RML. The rabbit antibodies applied in the electrophoresis were raised against purified native RML-B.

TABLE 2

Automated Edman Degradation of Purified rRML[a]

Cycle no.	PTH-a.a.[b]	Yield (nmol)	PTH-a.a.[b]	Yiled (nmol)
1	Ser	1.0	Ile	0.9
2	Ile	2.1	Asp	0.8
3	Asp	1.5	Gly	0.9
4	Gly	(1.6)[c]	Gly	(1.6)[c]
5	Gly	1.3	Ile	0.7
6	Ile	1.7	Arg	0.3
7	Arg	1.2	Ala	1.2
8	Ala	(2.5)[c]	Ala	(2.5)[c]
9	Ala	1.9	Thr	0.4
10	Thr	0.7	Ser	0.5
11	Ser	0.7	Gln	0.4
12	Gln	0.9	Glu	0.8
13	Glu	1.3	Ile	0.6
14	Ile	1.1	Asn	0.5
15	Asn	1.0	Glu	0.6
16	Glu	1.1	Leu	0.6
17	Leu	1.1	Thr	0.3
18	Thr	0.5	Tyr	0.7
19	Tyr	(1.2)[c]	Tyr	(1.2)[c]
20	Tyr	1.0	Thr	0.3
21	Thr	(0.4)[c]	Thr	(0.4)[c]
22	Thr	0.3	Leu	0.6
23	Leu	0.8	Ser	0.4
24	Ser	0.6	Ala	0.5

[a]The sequence determination was stopped after 24 cyles. The average repetitive yield during operation of the sequencer was 90.5%.

[b]PTH-a.a. is phenylthiohydantoin amino acid.

[c]The same residue is present in both sequences.

same N-terminal amino acid sequence as the native RML (Ser-Ile-Asp-Gly-Gly-, etc.) (3); the remaining 30% of the molecules had lost the N-terminal serine residue, and therefore had isoleucine at the N-terminal.

DISCUSSION

Recently, we demonstrated the versatility of *A. oryzae* as a potential host organism for the production of recombinant proteins (4). We demonstrated that the *Rhizomucor miehei* derived aspartic proteinase was secreted in high quantities (more than 3 grams/liter) to the growth medium. Furthermore, the specificity of the N-terminal processing of the propeptide from the zymogen of this enzyme was identical to that reported for the processing of the native enzyme precursor (13). Processing of the zymogens to the aspartic proteinases takes place through an autocatalytic mechanism, and therefore, correct processing of this type of zymogen in *A. oryzae* is not totally unexpected.

There is a rapidly growing interest in lipases from different sources. Many lipases have been purified and characterized, and an increasing number of lipase cDNAs have been cloned (14). We have continued our studies on the RML (2,3) by investigating the possibility of providing a recombinant system for production of the enzyme.

The precursor of RML has 24 amino acid residues in the signalpeptide, 70 amino acid residues in an N-terminal propeptide and 269 residues in the mature enzyme (3). The maturation of the enzyme thus involves a proteolytic cleavage between the C-terminal amino acid residue (Met) of the propeptide and the N-terminal residue (Ser) of the mature enzyme. We have no knowledge of the enzyme(s) involved in this processing in *R. miehei.* The versatility of *A. oryzae* as a host organism for the expression of recombinant triglyceride lipases will depend on the ability of this fungus to secrete and correctly process the lipase precursors. To answer that question we have purified and characterized the recombinant RML enzyme produced in *A. oryzae.*

Purified rRML showed the same mobility as purified native RML-B in a reducing SDS-polyacrylamide gel. The molecular weight of 32 kD for both enzymes compares well with 29,472 dalton, which is the calculated value for the protein backbone of the mature enzyme.

Within experimental error the amino acid composition of purified rRML was identical to the values for the RML cDNA sequence (3). The recombinant produced protein has the same pI as native RML-B, and has a high degree of antigenic identity with this molecule as determined through the tandem-crossed immunoelectrophoresis. About one third of the molecules in the secreted and purified rRML population had lost their N-terminal serine. This heterogeneous N-terminal processing of the triglyceride lipase precursor in *A. oryzae* does not influence the specific activity of the secreted recombinant molecule as compared to the native enzyme.

We anticipate that the primary translation product synthesized from the transfected construct in *A. oryzae* is the preprolipase, as it is specified by the cloned cDNA sequence (3). Thus, the simplest interpretation of our data is that *A. oryzae* and *R. miehei* share the enzymatic capacity to process both the signalpeptide of this precursor and the 70 amino acid propeptide present in the proRML molecule. The heterogeneity at the N-terminus of rRML would then be a result of degradation of the released protein. Other alternatives, however, such as formation of mature-sized rRML as a result of aminopeptidase activity in the fermentation broth of *A. oryzae,* have yet to be rigorously excluded.

In all the investigated characteristics of the recombinant-derived lipase molecule, we have noticed a high degree of resemblance to the native RML. We have thus demonstrated the ability of *A. oryzae* to synthesize, process and secrete the triglyceride lipase from *R. miehei.* The ability of *A. oryzae* to process and secrete an active heterologous triglyceride lipase significantly broadens the industrial potential of this filamentous fungus as a host organism for expression of recombinant proteins.

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Synthesis of Trideuterated *O*-Alkyl Platelet Activating Factor and Lyso Derivatives

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Racemic heavy isotope analogs of 1-*O*-alkyl-*sn*-glycero-3-phosphocholine (lysoPAF) and 1-*O*-alkyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine (PAF) were prepared for use as internal standards to facilitate quantitative studies based on mass spectrometry. Starting from pentadecane-1,15-diol and *rac*-glycerol-1,2-acetonide, a convergent synthesis of 1-*O*-[16′-$^2H_3$]hexadecyl and 1-*O*-[18′-$^2H_3$]octadecyl *rac*-glycero-3-phosphocholine and their acetyl derivatives is described. Three deuterium atoms were introduced at the terminal position of the 1-*O*-alkyl group by displacement of the *p*-toluensulfonyl group from 1-*O*-alkyl-15′-*p*-toluensulfonate and 1-*O*-alkyl-17′-*p*-toluensulfonate with [$^2H_3$]-methylmagnesium iodide. The 1-*O*-alkyl-17′-*p*-toluensulfonate was obtained by reaction of the 1-*O*-alkyl-15′-*p*-toluensulfonate with allylmagnesium bromide, followed by reductive ozonolysis and treatment with *p*-toluenesulfonyl chloride. The hydroxyl group at C-2 was protected by a benzyl group and removed at a late stage in the synthesis. This provided the corresponding lyso-derivatives or allowed preparation of racemic PAF by subsequent acetylation of the free hydroxy group. The phosphocholine moiety was introduced at glycerol C-3 by reaction with bromoethyldichlorophosphate and trimethylamine. The synthetic compounds were analyzed by FAB/MS and GC/NICIMS. They were shown to contain less than 0.6% protium impurity.

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Since the discovery of PAF (1,2) there has been an enormous interest in the pathophysiological role of this naturally occurring phospholipid in human disease. PAF has been characterized as an AGEPC (3-6). It is also a major constituent of antihypertensive polar renomedullary lipid (7). Two of the biologically important forms of PAF contain saturated C_{16} and C_{18} alkyl moieties attached at C-1 of the glycerol backbone. PAF exerts diverse biological actions such as platelet aggregation, hypotension, bronchoconstriction and increased vascular permeability (8-10). It undergoes ready enzymic hydrolysis *in vivo* to the corresponding lyso derivative (10). Thus, estimates of PAF excretion often require quantitative analyses of both PAF and lysoPAF (11). These compounds are normally present in very low concentrations in biological fluids so that highly sensitive and specific methods are required for their analysis.

Quantitative analysis of PAF has been carried out by FAB/MS (12) and GC/EIMS (13). More recently, GC/NICIMS (14-16) has been employed for high sensitivity quantitative analysis. All of these techniques require an appropriate isotopically labelled internal standard. 1-*O*-Alkyl-2-*O*-[$^2H_3$]acetylglycero-3-phosphocholine, prepared by acetylation of lysoAGEPC, has been used extensively (12-16). This standard is not suitable for the quantification of lysoPAF, as it has quite different chromatographic properties. Haroldsen *et al.* (11) have reported a stable isotope dilution technique coupled with GC/EIMS for the quantitative analysis of lysoPAF. However, the standards that were employed contained considerable protium impurity (5%) as a consequence of the method of preparation. This limited the sensitivity and specificity of the assay.

With these thoughts in mind, a route was devised for the synthesis of racemic [$^2H_3$]PAF and lyso analogues having three deuterium atoms on the terminal position of the *O*-alkyl chain. A number of syntheses of PAF and their analogues have been previously reported (17; and references cited therein), although none of them describe methodology that would allow the preparation of stable isotope labelled material. We report a convergent synthesis of racemic [$^2H_3$]C_{16}-lysoPAF **1a** and racemic [$^2H_3$]C_{18}-lysoPAF **2a** and their corresponding acetyl derivatives **1b** and **2b**.

RESULTS AND DISCUSSION

Our synthetic approach was based on the use of racemic isopropylidene-glycerol and pentadecane-1,15-diol **3** as the initial building blocks (Scheme 1). The [$^2H_3$]methyl group at the terminal position of the alkyl chain was introduced by *p*-toluenesulfonyl activation of the hydroxyl group followed by [$^2H_3$]methylmagnesium iodide-displacement of the leaving roup (18). The phosphodiester moiety of C-3 was introduced by sequential reaction with 2-bromoethyldichlorophosphate and trimethylamine (19). The hydroxyl group at C-2 was protected as a benzyl ether and was removed late in the synthetic sequence. Tosylation of 1,15-pentadecanediol **3** in pyridine with *p*-toluenesulfonyl chloride gave *bis*-tosyl derivative **4**. Condensation of **4** with the anion from 2,3-isopropylidene-*rac*-glycerol, prepared by reaction of NaH (1 equiv.) in dimethylformamide, afforded the intermediate 1-*O*-15′-*O*-*p*-toluenesulfonylpentadecyl-*rac*-glycerol-2,3-acetonide **5**. Displacement of the *p*-toluensulfonate with 2.5 equiv. of [$^2H_3$]methylmagnesium iodide in the presence of lithium tetrachlorocuprate (Li_2CuCl_4) (20) gave the 1-*O*-[$^2H_3$]alkyl derivative **6**. Deprotection of the diol function using cation exchange resin Dowex AG 50W-X8 at room temperature in methanol for 6 hr, followed by silylation of diol **7** with *t*-butyldimethylchlorosilane/dimethylaminopyridine gave monosilyl ether **8** in 78% yield. The hydroxyl group at C-2 was protected as a benzyl ether by treating **8** with benzyl bromide in the presence of NaH. The resulting benzyl ether **9** was desilylated using Bu_4NF in THF to yield alcohol **10** in 87% yield. The

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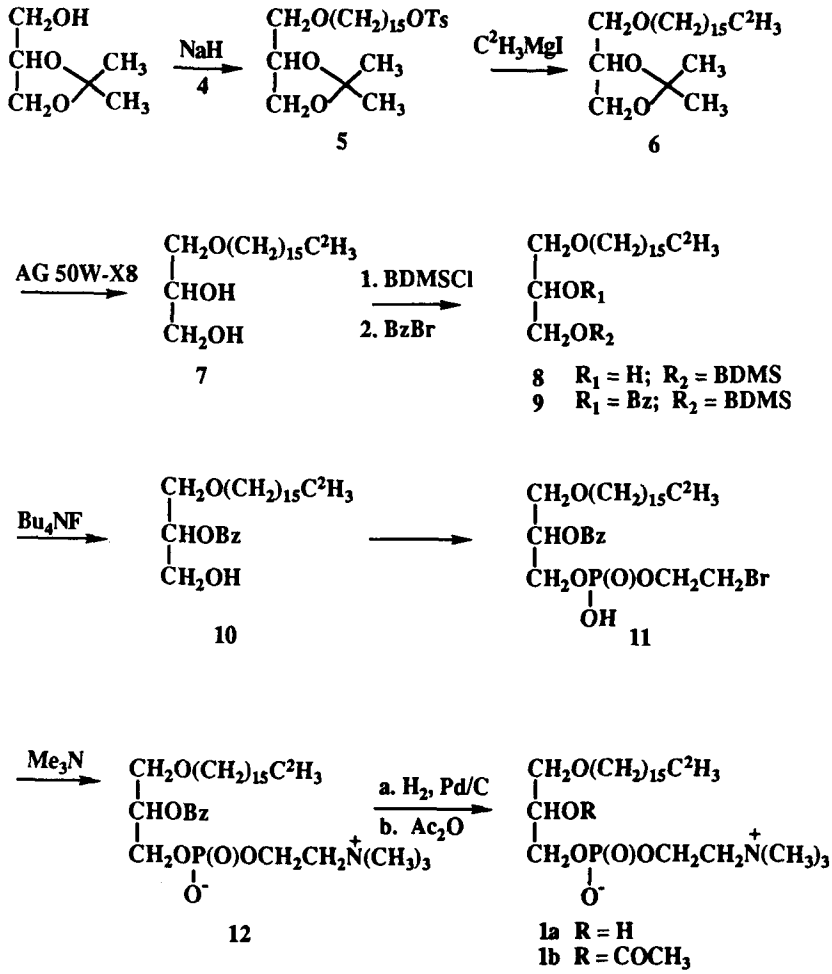
Abbreviations used: PAF, platelet activating factor, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; lysoPAF, 1-*O*-alkyl-*sn*-glycero-3-phosphocholine; AGEPC, acetylglyceryl ether phosphocholine; MS, mass spectrometry; FAB, fast atom bombardment; GC, gas-liquid chromatography; EI, electron impact; NICI, electron capture negative chemical ionization; TLC, thin-layer chromatography; PLC, phospholipase C; Bu_4NF, tetrabutylammonium fluoride.



SCHEME 1. Synthesis of $[^2H_3]C_{16}$-lysoPAF 1a and $[^2H_3]C_{16}$-PAF 1b.

alcohol **10** was dried *in vacuo* over P_2O_5 and then phosphorylated with 2-bromoethyldichlorophosphate (19) in dry CCl_4 to give bromide **13**. Displacement of the bromide with anhydrous trimethylamine in CCl_4 at 60°C overnight gave crude phosphocholine **12** which was purified by silica column chromatography. Removal of the benzyl group was carried out by hydrogenation with Pd/C for 24 hr to afford the $[^2H_3]C_{16}$-lysoPAF **1a**. The positive FAB spectrum of the trideuterated lysoPAF **1a** showed a molecular ion at *m/z* 485 together with the expected fragment ions at *m/z* 184, 166, 86 and 58 (Fig. 1a).

Acetylation of **1a** using acetic anhydride/perchloric acid as previously described (14) failed to go to completion. However, a quantitative yield of $[^2H_3]$PAF **1b** was obtained when acetylation was carried out with acetic anhydride/pyridine in CH_2Cl_2. The positive FAB spectrum of $[^2H_3]C_{16}$-PAF **1b** showed a molecular ion at *m/z* 527 three daltons higher than native C_{16}-PAF (Fig. 1b). The expected, structurally significant fragment ions were observed at *m/z* 184, 166, 86 and 58 (Fig. 1b). The ion reported to arise from fragmentation (with charge retention) of the O-alkyl side-chain at *m/z* 224 for C_{16}-PAF (12) was unexpectedly also observed at *m/z* 224 in the trideuterated compound. This revealed that the ion at *m/z* 224 in fact arose as a consequence of the loss of the alkyl and acyl side-chains (Fig. 1b), and not by charge retention of an O-alkyl chain fragment. The fragmentation pathway was similar to that observed previously for acyl phospholipids (21).

$[^2H_3]C_{18}$-lysoPAF **2a** and $[^2H_3]C_{18}$-PAF **2b** were synthesized using a similar strategy (Scheme 2). Since 1,17-hepatadecanediol was not commercially available, the intermediate tosylate **15** required for introduction of the $[^2H_3]$methyl group was synthesized from the acetonide **5**. Condensation of **5** with allylmagnesium chloride in THF gave the allyl derivative **13**. Ozonolysis, followed by reduction with sodium borohydride gave the alcohol **14**, which was converted to tosylate **15**. Treatment of **15** with $[^2H_3]$methylmagnesium iodide in the presence of Li_2CuCl_4 gave the trideuterated alkyl derivative **16**, which was then treated with Dowex AG 50W-X8 in methanol to afford the diol **17**. Silylation of diol **17** with *t*-butyldimethylchlorosilane (1.1 equiv.), followed by benzylation of resulting mono-silyl ether **18** yielded the benzyl ether **19**. After removal the silyl group with Bu_4NF, the alcohol **20** was phosphorylated to give the bromide **21**, which on reaction with trimethylamine, gave phosphocholine derivative **22**. The benzyl group was removed with Pd/C to yield $[^2H_3]C_{18}$-lysoPAF **2a**. Acetylation of **2a**

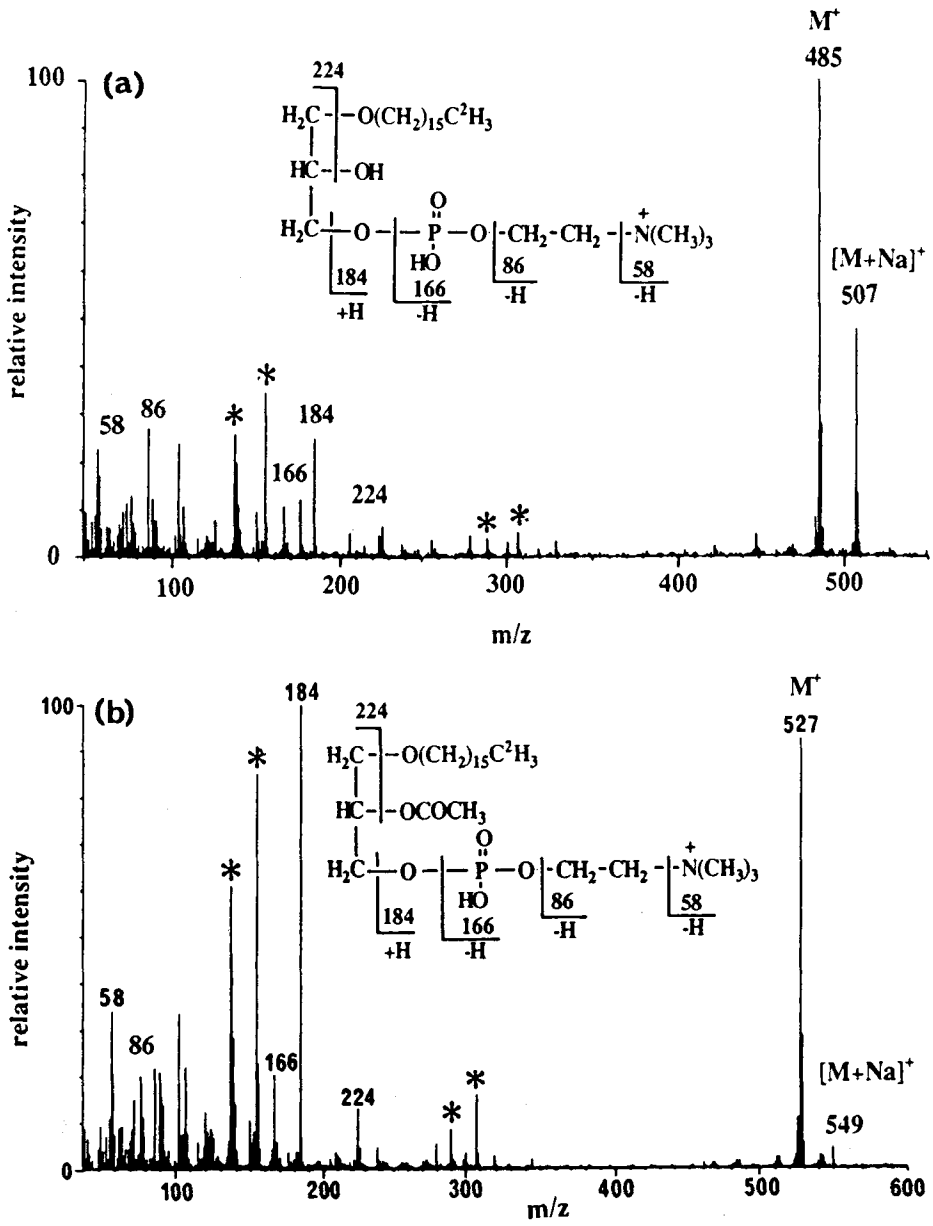
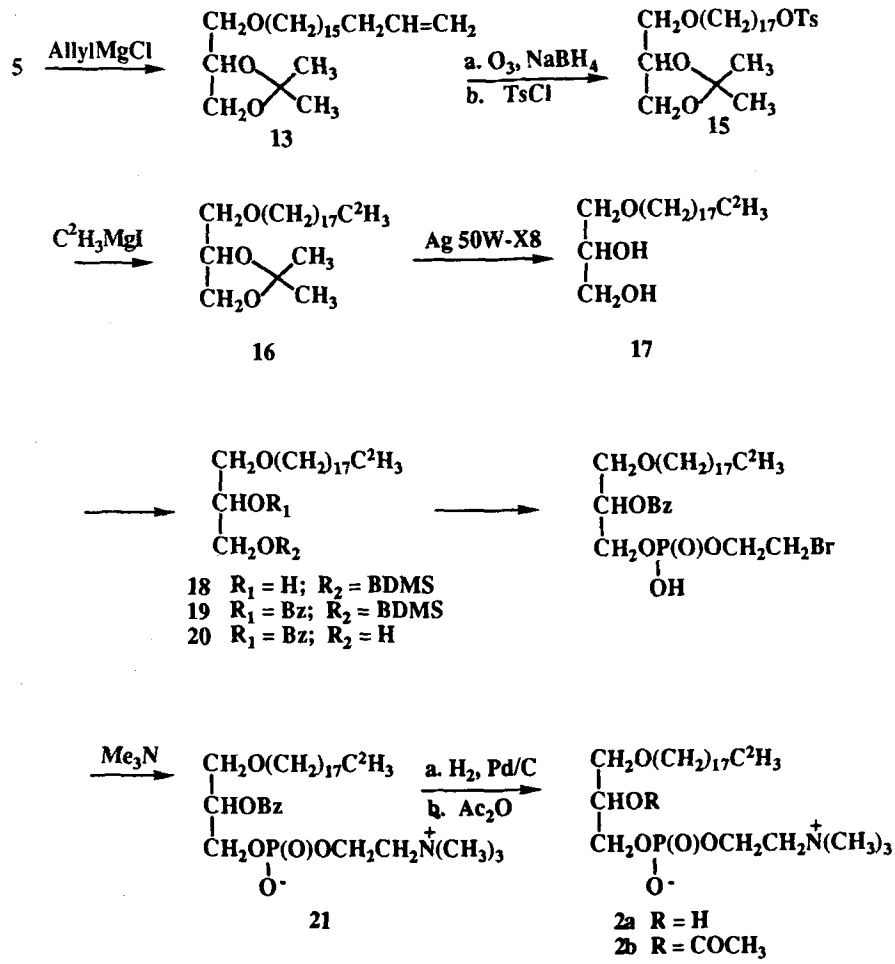


FIG. 1. FAB mass spectra in 3-nitrobenzyl alcohol of (a) $[^2H_3]C_{16}$-lysoPAF 1a and (b) $[^2H_3]C_{16}$-PAF 1b.

afforded $[^2H_3]C_{18}$-PAF **2b**. The positive FAB mass spectrum of **2a** and **2b** showed protonated molecular ions at *m/z* 513 and *m/z* 555, respectively, together with the expected structurally relevant fragment ions.

The trideuterated standards were analyzed as their corresponding mono- and diglycerides by capillary column GC/NICIMS in order to confirm the deuterium distribution. Chemical noise in the FAB mass spectra obscured the exact isotope incorporation. Synthetic $[^2H_3]C_{16}$-lysoPAF **1a** and $[^2H_3]C_{18}$-lysoPAF **2a** and their respective acetyl derivatives (**1b,2b**) were hydrolyzed with PLC, and then converted to their pentafluorobenzoyl derivatives (pentafluorobenzoyl chloride/toluene/pyridine) (16). During TLC purification, acetyl groups at the C-2 position migrated to C-3 as described previously (16). Each of the derivatives eluted as a single homogeneous peak on GC analysis. Their NICI mass spectra showed intense $M^{-\bullet}$ ions at *m/z* 707, 735, 555 and 583 for **1a**, **2a**, **1b** and **2b**, respectively. The content of non-deuterated material in each of the synthetic standards was <0.6%. A typical NICI mass spectrum obtained for the pentafluorobenzoyl derivative derived from $[^2H_3]C_{16}$-PAF **1b** is shown in Figure 2.

In summary, a convergent route for the synthesis of racemic $[^2H_3]C_{16}$-PAF, $[^2H_3]C_{18}$-PAF, and their lyso derivatives, has been developed. Optically active deuterated PAF analogs for biochemical studies can be synthesized by the route described in this paper by starting with the relevant optically active isopropylideneglycerol. The racemic deuterated compounds will be used as internal



SCHEME 2. Synthesis of $[^2H_3]C_{18}$-lysoPAF 2a and $[^2H_3]_{18}$-PAF 2b.

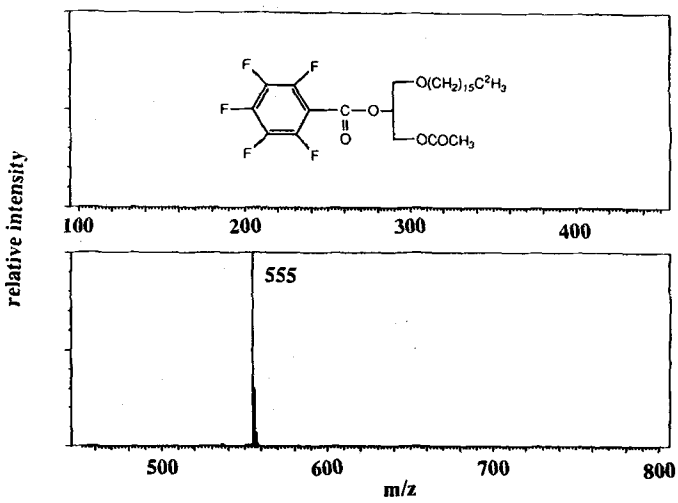


FIG. 2. NICI mass spectrum of 1-*O*-16′-$[^2H_3]$hexadecyl-2-pentafluorobenzoyl-3-acetyl-glycerol derived from $[^2H_3]C_{16}$-PAF 1b.

standards for the quantification of PAF and lysoPAF in biological fluids using either FAB/MS or combined capillary column GC/NICIMS. The results of these observations will be published separately. The GC/MS procedure involves PLC cleavage of the phospholipid (16). It is possible that the unnatural antipode derived from the internal standard will not undergo complete hydrolysis. However, this would only affect the slope of the standard curve. The precision and accuracy of analysis would not be affected.

EXPERIMENTAL

Racemic glycerol-1,2-acetonide, p-toluenesulfonyl chloride, benzyl bromide, phosphorus oxychloride, bromoethanol, triethylamine, trimethylamine, sodium hydride, acetic anhydride, allylmagnesium chloride, pyridine, $[^2H_3]$methylmagnesium iodide, $NaBH_4$, Dowex AG 50W-X8 and 4-dimethylaminopyridine were obtained from Aldrich Chemical Co. (Milwaukee, WI). Pentadecane-1,15-diol was purchased from Columbia Organic Chemical Co. (Camden, SC). 2-Bromoethyldichlorophosphate and Li_2CuCl_4 were prepared as previously described (19,20). THF was distilled from sodium benzophenone ketyl immediately prior to use. DMF was vacuum distilled from calcium hydride and stored over 3 Å molecular sieves. Dry CH_2Cl_2 was obtained by distillation from P_2O_5. All other solvents were of reagent grade and were used directly.

Reactions were carried out under a dry nitrogen atmosphere.

Procedures. Flash chromatography was carried out either on S/P silica gel 60 Å or florisil (Fisher Scientific, Atlanta, GA). TLC was performed on Analtech silica gel GF uniplates. Organic extracts were dried with $MgSO_4$. The standard workup procedure involved dilution of the reaction mixture with saturated aqueous sodium chloride and extraction with ethyl acetate. The organic extract was washed with water and dried. After concentration *in vacuo,* the residue was purified on a silica gel column.

Instruments. Melting points were determined on a capillary Hoover apparatus and are uncorrected. Elemental analyses were carried out by Galbraith Laboratories (Knoxville, TN). The NMR Spectra were recorded in $CDCl_3$ on a Bruker AM 400 or an IBM NR 300. Chemical shifts (δ ppm) are reported relative to Me_4Si as an internal standard. FAB mass spectra were obtained on a VG 70/250 double focusing magnetic sector instrument at a resolving power of 2000. GC/MS was carried out on a Nermag R1010C quadrupole instrument interfaced to a Varian Vista gas chromatograph. Injections were made in the splitless mode on a 10 m SPB 5 fused silica capillary column (0.32 mm internal diameter, 0.25 μm coating thickness, Supelco, Bellefonte, PA). Under standard GC conditions, the column was temperature programmed from 100°C to 320°C at 15°C/min with a helium as carrier gas at a flow rate of 1 ml/min. Methane was used as the reagent gas for NICIMS at an analyzer pressure of 6.4×10^{-6} Torr.

*1,15-*Bis-(p-*toluenesulfonyloxy*)-*pentadecane* (**4**). A solution of pentadecane-1,15-diol **3** (5.0 g, 20.50 mmol) in pyridine (50 ml) was treated with *p*-toluenesulfonyl chloride (11.72 g, 61.5 mmol) in portions at 0°C, and the reaction mixture was stirred overnight in a cold room at 4°C. Ethyl acetate (200 ml) and water (20 ml) were added and the organic layer was separated, washed sequentially with HCl (2 N, 20 ml), $NaHCO_3$ (5%, 20 ml), water (20 ml), and then dried ($MgSO_4$). The solvent was removed *in vacuo* and the crude *bis*-tosylate **4** was purified on silica gel column using ethyl acetate in hexane (1:19, v/v) to give (8.8 g, 77.8%) as a white powder, m.p. 79–81°C. ^1H NMR 7.77 (*d,* 4H, J = 8.3 Hz, aromatic), 7.32 (*d,* 4H, J = 8.1 Hz, aromatic), 4.05 (*t,* 4H, J = 6.5 Hz, 2 × $\underline{CH_2}OTS$), 2.43 (*s,* 6H, 2 × CH_3 of tosyl), 1.59 (*m,* 4H) and 1.20 (*m,* 22H). FAB/MS (*m/z*) 553 (MH^+), 537 (M-15), 460 [M−(C_6H_5)CH_3+H]. Anal. Calcd for $C_{29}H_{44}O_6S_2$: C, 63.01; H, 8.02; S, 11.60. Found: C, 63.05; H, 8.08; S, 11.74.

*1-*O-*15′*-O-p-*Toluenesulfonylpentadecyl-*rac-*glycerol-2,3-acetonide* (**5**). A solution of glycerol-2,3-acetonide (1.0 g, 7.56 mmol) in DMF (5 ml) was added dropwise, over a 30 min period, to a stirred suspension of prewashed (hexane) NaH (362 mg, 7.56 mmol, 50% mineral oil dispersion) in DMF (20 ml). After being stirred for 15 min it was cooled in an ice bath and a solution of above *bis*-tosyl derivative **4** (5.02 g, 9.08 mmol) in 5 ml DMF was added all in one portion. The mixture was stirred overnight at room temperature, quenched with water and extracted with ether (3 × 50 ml). The combined ether solutions were washed with a saturated solution of NaCl (20 ml), water (20 ml) and dried. Concentration of the solvent followed by purification of the residue on silica gel column using ethyl acetate in hexane (1:5, v/v) yielded pure compound **5** (2.38 g, 62%), m.p., 57–59°C. NMR 7.78 (*d,* 2H, J = 8.2 Hz, aromatic), 7.31 (*d,* 2H, J = 8.1 Hz, aromatic), 4.26 (*m,* 1H, H-2), 4.04 (*m,* 3H, $\underline{CH_2}OTS$ and H-3a), 3.71 (*dd,* 1H, H-3b), 3.46 (*m,* 4H, H-1 and H-1′), 2.46 (*s,* 3H, CH_3 of tosyl), 1.58 (*m,* 4H), 1.38 (*s,* 3H, CH_3 of isopropylidene), 1.32 (*s,* 3H, CH_3 of isopropylidene) and 1.22 (*m,* 22H). FAB/MS (*m/z*) 513 (MH^+), 497 (M − CH_3), 455 [M − CH_3 − $C(CH_3)_2$]. Anal. Calcd for $C_{28}H_{48}O_6S$: C, 65.62: H, 9.37. Found: C, 65.65; H, 9.38.

*1-*O-*16′*-[$^2H_3$]*Hexadecyl-*rac-*glycerol-2,3-acetonide* (**6**). A solution of [$^2H_3$]methylmagnesium iodide (5.0 ml, 5.0 mmol, 1 M) at 0°C was added dropwise to a stirred solution of tosyl derivative **5** (1.024 g, 2.0 mmol) in THF (5.0 ml) and Li_2CuCl_4 (100 μl, 0.01 mmol). The reaction mixture was allowed to warm to room temperature during 1 hr and stirring was continued for a further 12 hr. The reaction was quenched with a saturated solution of NH_4Cl (5.0 ml) and extracted with ether (3 × 20 ml). The combined ether solutions were dried ($MgSO_4$) and evaporated *in vacuo.* Purification of the residue on silica gel column using hexane gave the pure compound **6** as a white powder (624 mg, 87%), m.p. 44–46°C. NMR 4.24 (*m,* 1H, H-2), 4.06 (*dd,* 1H, J = 6.5 Hz, H-3a), 3.68 (*dd,* 1H, J = 6.5 Hz, H-3b), 3.46 (*m,* 4H, H-1 and H-1′), 1.58 (*m,* 2H), 1.38 (*s,* 3H, CH_3 of isopropylidene), 1.33 (*s,* 3H, propylidene) and 1.23 (*m,* 26H). FAB/MS (*m/z*) 360 (MH^+), 344 (M − CH_3), 302 [MH − $OC(CH_3)_2$], 115 and 101. Anal. Calcd for $C_{22}H_{41}{}^2H_3O_3$: C, 73.54; H, 13.09. Found: C, 73.29; H, 12.72.

*1-*O-*16′*-[$^2H_3$]*Hexadecyl-*rac-*glycerol* (**7**). The acetonide **6** (500 mg, 1.4 mmol) in methanol (2 ml) was stirred with Dowex resin AG 50W-X8 (2.5 g) for 6 hr. The resin was removed by filtration and washed with more methanol. The combined methanol solution was concentrated and the residue was crystallized from acetone as white prismatic needles (410 mg, 92%), m.p. 65–66°C (lit $^1H_3$, 65.5°C, ref. 22). NMR 3.79 (*m,* 1H, H-2), 3.64 (*m,* 2H, H-3), 3.42 (*m,* 4H, H-1 and H-1′), 1.56 (*m,* 2H) and 1.22 (*m,* 26H). FAB/MS (*m/z*) 320 (MH^+), 302 (MH − H_2O), 289 (M − HCHO). Anal. Calcd for $C_{19}H_{37}{}^2H_3O_3$: C, 71.47; H, 13.48. Found: C, 71.12; H, 13.59.

*1-*O-*16′*-[$^2H_3$]Hexadecyl-3-O-t-*butyldimethylsilyl-*rac-*glycerol* (**8**). 4-Dimethylaminopyridine (146 mg, 1.2 mmol) and *t*-butyldimethylsilyl chloride (180 mg, 1.2 mmol) at 0°C was added to a stirred solution of alcohol **7** (320 mg, 1.0 mmol) in CH_2Cl_2 (3 ml). After stirring for 4 hr, the reaction mixture was diluted with water (10 ml) and CH_2Cl_2 (20 ml). The organic layer was separated, washed with brine, and dried ($MgSO_4$). Removal of the solvent and purification of the residue on silica gel column using ethyl acetate in hexane (1:9, v/v) provided the pure silyl ether **8** as a colorless oil (340 mg, 78%). NMR 3.78 (*m,* 1H, H-2), 3.62 (*dd,* 2H, J = 5.4 Hz, $\underline{CH_2}OBDMS$), 3.42 (*m,* 4H, H-1 and H-1′), 1.53 (*m,* 2H), 1.24 (*m,* 26H), 0.87 (*s,* 9H, butyl) and 0.05 (*s,* 6H, 2 × CH_3). FAB/MS (*m/z*) 434 (MH^+), 376 (M − Bu), 358 (M − Bu − H_2O) and 302 (MH − BDMSOH).

*1-*O-*16′*-[$^2H_3$]*Hexadecyl-2-*O-*benzyl-3-*O-t-*butyldimethylsilyl-*rac-*glycerol* (**9**). Sodium hydride (38 mg, 0.76 mmol, 50% mineral oil dispersion) was washed with petroleum ether under nitrogen and suspended in THF (1 ml). It was cooled at 0°C (ice bath) and a solution of alcohol **8** (280 mg, 0.65 mmol) in THF (2.0 ml) was added dropwise. After being stirred for 1 hr, benzyl bromide (132 mg,

0.78 mmol) in THF (0.5 ml) was added. The mixture was stirred for 30 min at −5°C, and then for 3 hr at room temperature. It was then diluted with water (10 ml) and extracted with ether (2 × 50 ml). The combined ether extracts were washed with a saturated solution of NaCl (10 ml) and dried ($MgSO_4$). Evaporation of solvent and purification of the residue on silica gel column using ethyl acetate:hexane (1:19, v/v) afforded pure **9** (250 mg, 68%) and with (1:4) ethyl acetate hexane unreacted **8** (50 mg). Benzyl ether **9** was obtained as a colorless syrup. NMR 7.29 (*m*, 5H, aromatic), 4.72 (*s*, 2H, $\underline{CH_2}C_6H_5$), 3.42–3.76 (*m*, 7H), 1.60 (*m*, 2H), 1.38 (*m*, 26H), 0.94 (*s*, 9H, butyl), and 0.05 (*s*, 6H, 2 × CH_3). FAB/MS (*m/z*) 524 (MH^+), 508 (M − CH_3), 466 (M − Bu), 432 (M − $CH_2C_6H_5$) and 392 (MH − BDMSOH).

1-O-16′-[$^2H_3$]Hexadecyl-2-O-benzyl-rac-*glycerol* (**10**). Tetrabutylammonium fluoride (0.05 ml, 0.05 mmol, 1 M) was added to a magnetically stirred solution of **9** (210 mg, 0.4 mmol) in THF (2 ml). The reaction mixture was stirred for 2 hr and diluted with ether (50 ml). The ether layer was washed with saturated solution of NaCl (10 ml) and dried. Concentration and purification on silica gel column using ethyl acetate hexane (1:4, v/v) gave pure **10** (140 mg, 87%) as an oil (lit $^1H_3$, oil, ref. 23). NMR 7.29 (*m*, 5H, aromatic), 4.65 (*s*, 2H, $\underline{CH_2}C_6H_5$), 3.40–3.75 (*m*, 7H), 2.06 (*bm*, 1H, OH), 1.54 (*t*, 2H) and 1.24 (*m*, 26H). FAB/MS (*m/z*) 410 (MH^+), 318 (M − $CH_2C_6H_5$) and 302 (M − $OCH_2C_6H_5$).

1-O-16′-[$^2H_3$]Hexadecyl-2-O-benzyl-rac-*glycero-3-phospho-2′-bromoethanol* (**11**). A solution of 2-bromoethyldichlorophosphate (95 mg, 0.39 mmol) in CCl_4 (1.5 ml) was added dropwise with stirring to a solution of alcohol **10** (100 mg, 0.246 mmol) and triethylamine (50 mg, 0.39 mmol) in CCl_4 (3 ml). The reaction mixture was stirred for 2 hr at room temperature and then diluted with toluene (10 ml). The mixture was filtered and the solvent was removed. The residue was stirred in a mixture of THF and 0.5 M sodium acetate (20 ml, 1:1) for 2 hr. After removal of the solvent, the residue was acidified with dilute HCl and the mixture was extracted with ether (3 × 50 ml). The combined extracts were washed with a saturated solution of NaCl (10 ml) and dried. The solvent was evaporated *in vacuo* and the residue was chromatographed on Florisil using sequentially $CHCl_3$ followed by $CHCl_3$-CH_3OH (4:1, v/v) to give the pure product **11** as a viscous oil (90 mg, 62%). NMR 7.28 (*bm*, 5H, aromatic), 4.67 (*s*, 2H, $CH_2C_6H_5$), 3.82–4.22 (*m*, 5H), 3.20–3.75 (*m*, 6H), 1.53 (*m*, 2H) and 1.22 (*m*, 26H).

1-O-16′-[$^2H_3$]Hexadecyl-2-O-benzyl-rac-*glycero-3-phosphocholine* (**12**). The bromide **11** (60 mg, 0.10 mmol) was dissolved in a mixture of $CHCl_3$/CH_3CN (2 ml, 1:1, v/v) and cooled at 0°C (ice bath). To this cold stirred solution, an excess of trimethylamine (1 ml) was added and the reaction mixture was stirred at room temperature for 30 min. It was then heated (50°C) in an oil bath until the starting compound had disappeared as indicated by TLC (18 hr). After cooling to room temperature, the solvent was removed and the residue was stirred with methanol (2 ml) containing Ag_2CO_3 (30 mg, 0.11 mmol) for 3 hr. The grey solution was filtered and the residue was washed with methanol. The combined methanol solution was evaporated *in vacuo* and the crude product was purified on a silica gel column using sequentially $CHCl_3$/MeOH (70:30) and methanol to afford the pure **12** (52 mg, 82%). NMR 7.28 (*bm*, 5H, aromatic), 4.72 (*s*, 2H, $\underline{CH_2}C_6H_5$), 4.29 (*m*, 3H), 4.06 (*m*, 2H), 3.66 (*m*, 2H), 3.54 (*m*, 2H), 3.48 (*m*, 2H), 3.22 (*s*, 9H, $N[CH_3]_3$), 1.58 (*m*, 2H) and 1.28 (*m*, 26H). FAB/MS (*m/z*), 575 (M^+).

1-O-16′-[$^2H_3$]Hexadecyl-rac-*glycero-3-phosphocholine* (*[$^2H_3$]C_{16}-lyso PAF*) (**1a**). A mixture of **12** (50 mg) and Pd/C (50 mg, 10%) in absolute ethanol (10 ml) was hydrogenated at atmospheric pressure till the starting compound had disappeared (TLC) (24 hr). The catalyst was removed by filtration and the residue was washed with more ethanol. Evaporation of the combined solvent gave the lysolipid **1a** (38 mg, 95%). FAB/MS (*m/z*) 485 (MH^+). The NICI mass spectrum of the *bis*-pentafluorobenzoyl derivative (16) obtained by GC/MS, showed ions at (*m/z*) [$^2H_3$]; 707 ($M^{-\cdot}$, 100%), [$^2H_2$]; 706 (1.50%), [$^2H_1$]; 705 (0.59%), $^2H_0$; 704 (0.53%).

1-O-16′-[$^2H_3$]Hexadecyl-2-O-acetyl-rac-*glycero-3-phosphocholine* (*[$^2H_3$]C_{16}-PAF*) (**1b**). [$^2H_3$]C_{16}-lysoPAF **1a** (38 mg, 0.08 mmol) in CH_2Cl_2 (0.5 ml) was treated with acetic anhydride (25 μl, 0.25 mmol) and pyridine (25 μl, 0.3 mmol) and the reaction mixture was heated at 60°C overnight. After cooling, saturated aqueous brine (1 ml) was added and the reaction mixture was extracted with CH_2Cl_2 (2 × 10 ml). The combined organic solvents were evaporated and passed through a small column of silica gel. Elution with $CHCl_3$ followed by $CHCl_3$/methanol (4:1, v/v) gave the pure compound **1b** (35 mg, 92%). NMR (CD_3OD), 5.16 (*m*, 1H, CHOAc), 4.30 (*m*, 2H), 4.02 (*m*, 2H), 3.67 (*m*, 2H), 3.59 (*d*, 2H), 3.47 (*m*, 2H), 3.25 (*s*, 9H, $N(CH_3)_3$), 2.08 (*s*, 3H, $COCH_3$), 1.55 (*m*, 2H) and 1.26 (*m*, 26H). FAB/MS (*m/z*) 527 (M^+). The NICI mass spectrum of the pentafluorobenzoyl derivative (16) obtained by GC/MS, showed ions at (*m/z*) [$^2H_3$]; 555 ($M^{-\cdot}$, 100%), [$^2H_2$]; 554 (0.78%), [$^2H_1$]; 553 (0.96%) and [$^2H_0$]; 552 (0.49%).

1-O-Octadec-17′-enyl-rac-*glycerol-2,3-acetonide* (**13**). A solution of allylmagnesium chloride (1.35 ml, 2.70 mmol, 2 M) at 0°C was added dropwise to a solution of tosyl compound **5** (1.0 g, 1.8 mmol) in THF (10 ml). The reaction mixture was warmed to room temperature during 1 hr, and it was then refluxed for 1 hr at 60°C. The mixture was diluted with saturated solution of NH_4Cl (10 ml) and extracted with ether (3 × 50 ml). The combined solutions were washed with water and dried. After evaporation of the solvent *in vacuo*, the residue was purified on silica gel column using ethyl acetate: hexane (1:19, v/v) to give the pure compound **13** as an oil (660 mg, 96%). NMR, 5.76 (*m*, 1H, H-17′, olefin), 4.98 (*m*, 2H, H-18′, olefins), 4.24 (*m*, 1H, H-2), 4.04 (*dd*, 1H, J = 6.5 Hz, H-3a), 3.70 (*dd*, 1H, J = 6.4 Hz H-3b), 3.44 (*m*, 4H, H-1 and H-1′), 2.04 (*m*, 2H), 1.58 (*m*, 4H), 1.40 (*s*, 3H, CH_3 of acetonide), 1.34 (*s*, 3H, CH_3 of acetonide) and 1.24 (*m*, 24H). FAB/MS (*m/z*) 383 (MH^+), 381, 367 (M − CH_3) and 325 (MH − $OC(CH_3)_2$) and 307 (MH − 2H − $O_2C(CH_3)_2$).

1-O-Heptadecan-17′-ol-rac-*glycerol-2,3-acetonide* (**14**). To a cooled solution of olefin **13** (600 mg, 1.57 mmol) in methanol (3 ml), ozone gas was bubbled for 15 min. $NaBH_4$ (60 mg, 1.57 mmol) in methanol (3 ml) was then added at 0°C and the reaction mixture was stirred for 1 hr at 0°C. The reaction mixture was quenched by the addition of saturated solution of NaCl and then extracted with ethyl acetate (2 × 20 ml). The combined organic solutions were washed with water and dried ($MgSO_4$).

Evaporation of the solvent and purification of the residue on silica gel column using ethyl acetate in hexane (1:9, v/v) to provide the pure alcohol **14** as an oil (550 mg, 91%). NMR 4.24 (*m*, 1H, H-2), 4.03 (*dd*, 1H, J = 6.4 Hz, H-3a), 3.68 (*dd*, 1H, J = 6.4 Hz, H-3b), 3.61 (*t*, 2H, CH_2OH), 3.43 (*m*, 4H, H-1 and H-1'), 1.54 (*m*, 4H), 1.40 (*s*, 3H, CH_3 of acetonide), 1.33 (*s*, 3H, CH_3 of acetonide) and 1.23 (*m*, 26H). FAB/MS (*m/z*) 387 (MH^+), 371 ($M - CH_3$), and 329 ($MH - OC(CH_3)_2$), 311 ($MH - 2H - O_2C(CH_3)_2$).

1-O-17'-O-p-Toluenesulfonylheptadecyl-rac-glycerol-2,3-acetonide (**15**). The alcohol **14** (460 mg, 1.19 mmol) in CH_2Cl_2 (1.2 ml) was stirred at 0°C with pyridine (184 μl, 2.38 mmol). *p*-Toluenesulfonyl chloride (330 mg, 1.8 mmol) was then added in three portions, and the reaction mixture was stirred overnight at 0°C. After addition of water (5 ml) and CH_2Cl_2 (20 ml), the organic layer was separated and the aqueous solution was extracted with more CH_2Cl_2 (10 ml). The combined organic solutions were washed with dilute HCl (5 ml, 1 N), water (10 ml) and dried. Evaporation of the solvent followed by purification of the crude product on silica gel column gave the pure tosyl compound **15** as a white powder (508 mg, 79%), m.p. 67-68°C. NMR 7.78 (*d*, 2H, J = 8.2 Hz, aromatic), 7.30 (*d*, 2H, J = 8.2 Hz, aromatic), 4.25 (*m*, 1H, H-2), 4.04 (*m*, 3H, CH_2OTS and H-3a), 3.72 (*dd*, 1H, J = 6.4 Hz, H-3b), 3.46 (*m*, H-1 and H-1'), 2.44 (*s*, 3H, CH_3 of tosyl), 1.57 (*m*, 4H), 1.38 (*s*, 3H, CH_3 of isopropylidene), 1.32 (*s*, 3H, CH_3 of isopropylidene) and 1.22 (*m*, 26H). FAB/MS (*m/z*) 541 (MH^+), 525 ($M - CH_3$), 483 ($MH - OC(CH_3)_2$). Anal. Calcd for $C_{30}H_{52}O_6S$; C, 66.62; H, 9.69; S, 5.92. Found: C, 65.65; H, 9.95; S, 5.83.

1-O-18'-[$^2H_3$]Octadecyl-rac-glycerol-2,3-acetonide **16**. Displacement of tosyl group of **15** with [$^2H_3$]methylmagnesium iodide gave **16** as white powder, m.p., 59-61°C. NMR 4.23 (*m*, 1H, H-2), 4.06 (*dd*, 1H, J = 6.5 Hz, H-3a), 3.68 (*dd*, 1H, J = 6.5 Hz, H-3b), 3.45 (*m*, 4H, H-1 and H-1'), 1.58 (*m*, 2H), 1.36 (*s*, 3H, CH_3 of isopropylidene), 1.33 (*s*, 3H, CH_3 of isopropylidene) and 1.24 (*m*, 30H). FAB/MS (*m/z*) 388 (MH^+), 386 ($M - H$), 372 ($M - CH_3$), 330 ($MH - OC(CH_3)_2$).

1-O-18'-[$^2H_3$]Octadecyl-rac-glycerol (**17**). Removal of isopropylidene group of **16** using Dowex resin AG 50W-X8 afforded **17**, m.p., 73-75°C (lit $^1H_3$, 71-71.5°C; ref. 22). NMR 3.78 (*m*, 1H, H-2), 3.63 (*m*, 2H, H-3), 3.42 (*m*, 4H, H-1 and H-1'), 1.55 (*m*, 2H) and 1.22 (*m*, 30H). FAB/MS (*m/z*), 348 (MH^+), 330 ($MH - H_2O$).

1-O-18'-[$^2H_3$]Octadecyl-3-O-t-butyldimethylsilyl-rac-glycerol (**18**). Silylation of **17** as described for **8** gave **18** as an oil. NMR 3.76 (*m*, 1H, H-2), 3.62 (*dd*, 2H, J = 5.4 Hz, CH_2OBDMS), 3.42 (*m*, 4H, H-1 and H-1'), 1.53 (*m*, 2H), 1.25 (*m*, 30H), 0.88 (*s*, 9H, butyl) and 0.06 (*s*, 6H, 2 × CH_3). FAB/MS (*m/z*) 462 (MH^+), 404 (M − Bu), 386 (M − Bu − H_2O) and 330 (MH − BDMSOH).

1-O-18'-[$^2H_3$]Octadecyl-2-O-benzyl-3-O-t-butyldimethylsilyl-rac-glycerol (**19**). Benzylation of **18** with benzyl bromide as in **9** yielded benzyl ether **19** as a viscous oil. NMR 7.28 (*m*, 5H, aromatic), 4.70 (*s*, 2H, $CH_2C_6H_5$), 3.42-3.74 (*m*, 7H), 1.58 (*m*, 2H), 1.38 (*m*, 30H), 0.94 (*s*, 9H, butyl) and 0.06 (*s*, 6H, 2 × CH_3). FAB/MS (*m/z*) 552 (MH^+), 536 ($M - CH_3$), 494 (M − Bu), 460 ($M - CH_2C_6H_5$) and 420 (MH − BDMSOH).

1-O-18'-[$^2H_3$]Octadecyl-2-O-benzyl-rac-glycerol (**20**). Removal of silyl group of **19** with Bu_4NF gave **20** as an oil (lit $^1H_3$, oil; ref. 23). NMR 7.28 (*m*, 5H, aromatic), 4.64 (*s*, 2H, $CH_2C_6H_5$), 3.42-3.76 (*m*, 7H), 2.06 (*bm*, 1H, OH), 1.54 (*t*, 2H) and 1.32 (*m*, 30H). FAB/MS (*m/z*), 438 (MH^+), 346 ($M - CH_2C_6H_5$) and 330 ($M - OCH_2C_6H_5$).

1-O-18'-[$^2H_3$]Octadecyl-2-O-benzyl-rac-glycero-3-phosphocholine (**21**). Treatment of **20** with 2-bromoethyldichlorophosphate and trimethylamine as described for **12** afforded pure **21** as a viscous oil. NMR 7.28 (*bm*, 5H, aromatic), 4.71 (*s*, 2H, $CH_2C_6H_5$), 4.28 (*m*, 2H), 4.06 (*m*, 2H), 3.65 (*m*, 2H), 3.55 (*m*, 2H), 3.48 (*m*, 2H), 3.21 (*s*, 9H, $N(CH_3)_3$), 1.58 (*m*, 2H) and 1.28 (*m*, 30H). FAB/MS (*m/z*) 603 (M^+).

1-O-18'-[$^2H_3$]Octadecyl-rac-glycero-3-phosphocholine ([$^2H_3$]C$_{18}$-lysoPAF) (**2a**). Hydrogenation of **21** with Pd/C gave [$^2H_3$]*C$_{18}$-lysoPAF* **2a**. FAB/MS *m/z* 513 (MH^+). The NICI mass spectrum of the *bis*-pentafluorobenzoyl derivative (16) obtained by GC/MS, showed ions at (*m/z*) [$^2H_3$]; 735 (M^-•, 100%), [$^2H_2$]; 734 (0.88%), [$^2H_1$]; 732 (0.99%) and [$^2H_0$]; 732 (0.52%).

1-O-18'-[$^2H_3$]Octadecyl)-2-O-acetyl-rac-glycero-3-phosphocholine ([$^2H_3$]C$_{18}$-PAF) (**2b**). Acetylation of **2a** with acetic anhydride/pyridine yielded **2b**. FAB/MS (*m/z*) 555 (M^+). The NICI mass spectrum of the pentafluorobenzoyl derivative (16) obtained by GC/MS, showed intense ions at (*m/z*) [$^2H_3$]; 583 (M^-•, 100%), [$^2H_2$]; 582 (0.91%), $^2H_1$; 581 (0.92%) and [$^2H_0$]; 580 (0.47%).

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Serum Fatty Acid Profiles After Intravenous Medium Chain Triglyceride Administration

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The serum fatty acid profiles of patients receiving either intravenous medium or long chain triglycerides were studied. Seventeen hospitalized patients, dependent on total parenteral nutrition, were randomly enrolled into a prospective study. The total parenteral nutrition (TPN) delivered amino acids and glucose and either a 75% medium chain triglyceride and 25% long chain triglyceride (MCT group) physical mixture or all long chain triglyceride (LCT group), as the respective fat sources. The amino acids and glucose were given continuously, and the lipid was given for 10 hours each day over five days. Fatty acid profiles on serum triglycerides and free fatty acids were done in the morning before any lipid was given and also later in the afternoon, near the end of the lipid administration, on days 1, 3 and 5.

Medium chain fatty acids rose quickly in the triglyceride fraction in patients given MCT. Rapid MCT hydrolysis occurred as evidenced by the appearance of medium chain fatty acids in the free fatty acid fraction in the afternoon sampling. Clearance of the hydrolyzed medium chain free fatty acids (MCFFA) occurred so that little, if any, were present in the morning sampling one day later. Long chain fatty acids, as either triglycerides or free fatty acids, showed expected increases during the daily infusion, but not of such relative magnitude as the medium chain fatty acids. Medium chain fatty acid incorporation into the phospholipid or cholesterol ester fractions by the end of the five-day feeding period was present but minimal. As opposed to conventional long chain triglycerides, intravenously administered medium chain triglycerides are hydrolyzed and cleared rapidly and do not accumulate in other lipid fractions, and are therefore a more readily available lipid fuel.

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As a parenteral nutrient, intravenous fat currently in use is soybean or safflower oil. Problems with this type of fat include slow hydrolysis and clearance from the blood and subsequent slow oxidation (1). Medium chain triglyceride (MCT), long given enterally, has been shown to be an excellent lipid fuel (2). Besides being hydrolyzed and absorbed rapidly from the intestines, it is poorly stored and quickly oxidized (3,4).

The intravenous use of MCT has been under investigation for several years (5). These triglycerides have been shown to be readily cleared from the blood and oxidized to form ketone bodies (6). We report the fatty acid profile changes in serum from patients administered either long chain (LCT) or medium chain triglycerides over five days.

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Abbreviations used: TPN, total parenteral nutrition; MCT, medium chain triglyceride; LCT, long chain triglyceride; MCFFA, medium chain free fatty acid.

METHODS

Seventeen patients were studied. All were dependent on total parenteral nutrition (TPN) for their nutrition during the course of the study. Oral intake, if any, was less than 500 kcal on any study day. Any oral calories were mostly from carbohydrates, and some from protein. Ages ranged from 29-75; the mean age for the MCT group was 59.5 years, that for the LCT group was 55.3. Eight patients received MCT, 9 patients LCT. There were 7 women and 10 men; 4 each in the MCT group and 3 women and 6 men in the LCT group. All patients gave written informed consent. This study was approved by the Institutional Review Board on Human Studies at the New England Deaconess Hospital (Boston, MA). The respective diagnoses of the patients are given in Table 1.

Protocol. Patients dependent on total parenteral nutrition were eligible for inclusion into the study. After written informed consent was obtained, the patients were randomized into either the MCT or LCT group. They were fed for 24 hours before the start of the study with amino acids and glucose only. For 10 hours of every day of the 5-day study, the respective lipid emulsion was given in addition to amino acids and glucose. On the mornings of days 1, 3 and 5, serum samples were drawn before the lipid emulsion was infused. The respective lipid emulsion was then infused over 10 hours and, near the end of the lipid infusion, serum samples were drawn again for fatty acid analyses.

Total parenteral nutrition. The parenteral nutrition solution was individualized for each patient. Total water

TABLE 1

Patient Characteristics

	Age	Sex	Diagnosis
MCT group	52	M	Ulcerative colitis
	35	F	Chronic pancreatitis
	72	M	Colon cancer, colectomy
	75	F	Adenocarcinoma of stomach, esophagogastrectomy
	57	M	Lymphoma
	59	F	Orthotopic liver transplant
	64	M	Crohn's disease
	62	F	Abdominal pain, ? etiology
LCT group	70	M	Prostatic carcinoma, radical prostatectomy
	62	F	Acute pancreatitis
	65	M	Abdominal lymphoma
	29	M	Subtotal gastrectomy
	63	M	Lymphoma
	61	F	Cirrhosis, portacaval shunt
	72	M	Colon cancer, colectomy
	35	F	Chronic pancreatitis
	41	M	Sclerosing cholangitis, lymphoma

and electrolytes were adjusted daily per patient as needed. The day before the study began and for each study day, the amount of amino acids and glucose was fixed per patient. Patients received 1.5 gm of amino acids per kilogram of body weight and 4.4 gm of glucose each day, given over 24 hours. The lipid was dosed at 1.3 gm of total fat per kgBW/day. The TPN, therefore, gave 32.7 kcal/kgBW/day. This gave 18% of calories as protein, 46% as carbohydrate and 36% as fat.

As opposed to the continuous administration of amino acids and glucose, the intravenous fat was given over 10 hours at a rate of 130 mg of lipid per kgBW/hr. This allowed a caloric delivery of 0.88 kcal/kgBW/hr during the 14 hours when the patient was not receiving any lipid (or a rate of 21 kcal/kgBW/day), and 2.04 kcal/kgBW/hr during the 10 hours that the lipid was infusing along with the amino acids and glucose (or at a rate of 49 kcal/kgBW/day). The overall rate of caloric delivery per 24-hour period was 32.7 kcal/kgBW/day.

The LCT group received Travamulsion®, a 20% soybean oil emulsion as the control lipid. The MCT group received the same amount of lipid, but as 75% MCT and 25% LCT. The LCT was Travamulsion® as above. The MCT was medium chain triglycerides fractionated from coconut oil. Its fatty acid composition was 72% octanoic acid and 27% decanoic acid. Table 2 shows the fatty acid profile of the MCT and LCT emulsion mixture which the MCT group received. Except for the MCT oil, it was made identically to the LCT emulsion by the same company (Clintec Nutrition Company, Deerfield, IL) using the same emulsifier.

Fatty acid analyses. The lipids were extracted from the serum using chloroform and methanol (2:1) (7). Internal standards in the form of free heptadecanoic acid and phospholipid, triglyceride and cholesterol ester esterified with heptadanoic acid were added to each sample. The various classes of lipids in the serum were then separated by thin-layer chromatography using LK5D silica gel plates with 0.25 mm layer thickness (Whatman Chemical Separation, Inc., Clifton, NJ). The solvent system used was petroleum ether/diethyl ether/glacial acetic acid (80:20:1) (v/v/v). The bands were visualized using 0.1% 2,7-dichlorofluoroscein and scraped into appropriate vials. The fatty acids were then converted to fatty acid methyl esters using 14% boron trifluoride in methanol as the methylating reagent. The methyl esters were then extracted using petroleum ether. The samples were stored under a nitrogen blanket at −70°C until ready for gas chromatographic analysis.

The gas chromatograph used was a Perkin Elmer Sigma 2000 (Perkin Elmer, Norwalk, CT) with a 50 meter fused silica capillary column (Qudrex Corporation, New Haven, CT). The column was coated with cyanopropyl silicone with a two-way thickness of 0.25 μ. The internal diameter of the column was 0.32 mm. The split ratio was 100:1. The program used an initial oven temperature of 70°C, lasting 3 minutes. An increase in temperature to 162°C occurred at a rate of 30°C/min. After reaching 162°C, a subsequent rise in temperature to 165°C occurred at 0.5°C/min. The column temperature was then raised to 190°C at 1°C/min and then held for 6 more minutes, giving a total run time of 43 min. The fatty acid methyl esters were detected by use of a flame ionization detector. The chromatograms, retention times and peak areas were printed by a Perkin Elmer LCI-100 Computing Integrator. The fatty acids were identified using standards from Nu Chek Prep (Elysian, MN). The micromolar concentrations of each fatty acid were computed using internal standards, and statistical analyses were done on these values. Values of less than 0.1 μmol/100 ml serum were not detected and are noted as n.d.

Statistics. The data were analyzed using a three-way general linear model procedure with type of lipid feeding (MCT or LCT), day (1, 3 or 5) and hour (0 or 10) as the three independent variables. Outlier tests to detect extreme observations were done prior to the general linear model procedure. The software program (SAS, SAS Institute, Cary, NC) was used on an IBM 370. It allowed independent effects from variables to be discerned, as well as two- or three-way interactions. If an interaction effect was significant, least square means was performed to discern the specific difference. The level of statistical significance was set at $\alpha = 0.05$.

TABLE 2

Fatty Acid Profiles of Lipid Emulsions[a]

Fatty acid	Travamulsion®	MCT/LCT
6:0 caproic		tr
8:0 octanoic		54
10:0 decanoic		20
12:0 lauric		tr
16:0 palmitic	11	3
18:1 oleic	23	6
18:2 linoleic	56	14
18:3 α-linolenic	6	1.5

[a]Expressed as relative percent.

RESULTS

Total caloric, total fat, carbohydrate or protein intakes did not differ between the two groups (data not shown). All the fatty acid data are expressed as μmol/100 ml serum with the total number of samples in parentheses. The medium chain fatty acid profile of the serum triglyceride fraction is shown in Table 3. The octanoic acid concentration in the triglyceride fraction was higher at hour 10, at the end of the infusion than at hour 0, $p < 0.0008$. On days 1, 3 and 5, respectively, the concentrations rose from less than 1 μmol/100 ml to 13, 8 and 37 μmol/100 ml. Decanoic acid concentrations changed over the course of the daily infusions in the MCT group, $p < 0.0001$. For days 1, 3 and 5, the concentrations rose from 2 or less μmol/100 ml to 11, 11 and 21 μmol/100 ml, respectively. Dodecanoic acid concentrations also rose during the course of the infusion, although only in the MCT group, $p < 0.0001$. Hour 0 concentrations were less than 1 μmol/100 ml and rose to 2, 1 and 1.5 μmol/100 ml, respectively, on days 1, 3 and 5.

The long chain fatty acid profile of the serum triglyceride fraction is shown in Table 4. Palmitic acid concentrations in the triglyceride fraction were highest for the average of day 1, as compared to days 3 or 5, in the LCT group, $p < .05$. For days 1, 3 and 5, respectively, the serum concentrations from hour 0 to hour 10 were 51–83,

TABLE 3

Serum Triglyceride Medium Chain Fatty Acid Concentrations[a]

Fatty acid	Lipid group	Day 1 Hour 0	(n)	Day 1 Hour 10	(n)	Day 3 Hour 0	(n)	Day 3 Hour 10	(n)	Day 5 Hour 0	(n)	Day 5 Hour 10	(n)	(p)
8:0	MCT	n.d.	(5)	13 ± 5	(6)	.4 ± .2	(4)	8 ± 3	(6)	.4 ± .1	(3)	37 ± 21	(5)	.0008[b]
	LCT	n.d.	(6)	n.d.	(8)	n.d.	(8)	n.d.	(6)	n.d.	(7)	n.d.	(6)	
10:0	MCT	n.d.	(7)	11 ± 3	(6)	2 ± 1	(6)	11 ± 3	(7)	.6 ± .2	(3)	21 ± 9	(5)	.0001[b]
	LCT	n.d.	(6)	n.d.	(7)	n.d.	(7)	n.d.	(7)	n.d.	(8)	n.d.	(7)	
12:0	MCT	.2 ± .1	(7)	2 ± .5	(7)	.4 ± .2	(6)	1 ± .1	(7)	.4 ± .1	(3)	1.5 ± .4	(5)	.0001[b]
	LCT	.3 ± .1	(6)	.6 ± .3	(8)	.3 ± .06	(8)	.8 ± .3	(9)	.3 ± .1	(8)	.2 ± .1	(6)	

[a]Expressed in μmol/100 ml serum as $\bar{X}$ ± SEM with number of samples in parentheses.
[b]Hour 0 average less than hour 10 average.

TABLE 4

Serum Triglyceride Long Chain Fatty Acid Concentrations[a]

Fatty acid	Lipid group	Day 1 Hour 0	(n)	Day 1 Hour 10	(n)	Day 3 Hour 0	(n)	Day 3 Hour 10	(n)	Day 5 Hour 0	(n)	Day 5 Hour 10	(n)	(p)
16:0	MCT	36 ± 9	(7)	45 ± 4	(7)	43 ± 11	(6)	48 ± 3	(7)	48 ± 24	(3)	50.5 ± 5	(5)	
	LCT	51 ± 9	(6)	83 ± 18	(8)	32 ± 6.5	(7)	51 ± 4	(8)	37 ± 5	(8)	47 ± 9	(6)	.05[b]
16:1ω7	MCT	8 ± 3	(7)	5 ± 1	(6)	7.5 ± 3	(6)	5 ± .8	(6)	6 ± 3	(3)	8.4 ± 3	(5)	
	LCT	11 ± 2	(7)	8 ± 2	(8)	4 ± 1	(8)	4.4 ± .7	(8)	4 ± 1	(8)	5 ± 1.3	(7)	.05[b]
18:0	MCT	3 ± 1	(7)	5 ± .5	(7)	4.4 ± 1	(6)	5.6 ± .6	(6)	7 ± 4	(3)	8 ± 1.6	(5)	
	LCT	104 ± .7	(6)	9 ± 2	(8)	4.7 ± 1.3	(8)	9 ± 2	(9)	4 ± .6	(8)	7 ± 1.5	(6)	
18:1ω9	MCT	45 ± 14	(7)	38 ± 5	(7)	29 ± 5	(5)	40.5 ± 4	(6)	19 ± 2	(2)	35 ± 3	(4)	
	LCT	63 ± 12	(6)	104 ± 22	(8)	48 ± 13	(8)	63 ± 7	(8)	40 ± 6	(8)	82 ± 24	(7)	.0009[c]
18:2ω6	MCT	20 ± 5	(7)	35 ± 5	(7)	28 ± 4	(6)	41 ± 8	(7)	23 ± 5	(3)	49 ± 12	(5)	.05[d]
	LCT	20 ± 5	(6)	102 ± 18	(8)	20 ± 2	(6)	67 ± 12	(8)	30.6 ± 3	(8)	77 ± 14	(6)	.0001[d]
18:3ω3	MCT	.6 ± .2	(7)	2 ± .5	(7)	1 ± .2	(5)	3 ± 1	(7)	.8 ± .3	(3)	3 ± 1	(5)	
	LCT	.5 ± .1	(6)	7.6 ± 1.5	(8)	.8 ± .1	(6)	6 ± 1.8	(9)	1 ± .1	(7)	11 ± 4	(7)	.0001[d]
20:4ω6	MCT	2.6 ± .7	(7)	2 ± .1	(6)	3 ± .7	(6)	2.4 ± .5	(6)	4 ± 1	(3)	3 ± .8	(5)	
	LCT	1.8 ± .4	(7)	2.8 ± .5	(8)	1.8 ± .4	(8)	3 ± .7	(9)	2 ± .2	(8)	2 ± .3	(6)	.05[d]

[a]Expressed in μmol/100 ml serum as $\bar{X}$ ± SEM with numbers in parentheses.
[b]Day 10 average greater than day 3 or 5.
[c]LCT group average greater than that for MCT.
[d]Hour 0 average less than hour 10 average.

32–51 and 37–47 μmol/100 ml. The same pattern was seen for palmitoleic acid concentrations, with the highest daily average seen on day 1, $p < 0.05$. Stearic acid concentrations in serum triglyceride did not show any changes. This is not unexpected, given its absence from the emulsions. Oleic acid concentrations in serum triglycerides over all time points were higher in the LCT group than in the MCT group, $p < 0.0009$. Linoleic acid concentrations in the serum triglyceride fraction rose during the infusion. This was true for each lipid group (MCT, $p < 0.05$ and LCT, $p < 0.0001$). α-Linolenic acid concentrations rose significantly in the LCT group ($p = 0.0001$). On days 1, 3 and 5, respectively, in the LCT group, α-linolenic acid levels rose from 0.5–7.6, 0.8–6 and from 1–11. There were increases in the MCT group, but these did not reach statistical significance. Arachidonic acid concentrations increased each day with the infusions in the LCT group only ($p < 0.05$). The changes on days 1, 3 and 5 were, respectively, 1.8–2.8, 2–3 and 2–2. In the MCT group, the changes were not significant.

The medium chain fatty acid profile of the serum free fatty acid fraction is shown in Table 5. Free octanoic acid concentrations rose during the MCT infusions ($p < 0.0005$), in contrast with the LCT group. The daily changes were 0.1–82, 5–12 and 0.8–22, respectively. Not surprisingly, there were no changes in the LCT group. Decanoic acid concentrations in the free fatty acid fraction also rose during the infusion ($p < 0.0005$). For days 1, 3 and 5 the changes were from not detectable to 22, 4–9 and 0.4–11, respectively. In the LCT group, decanoic

TABLE 5

Medium Chain Free Fatty Acid Concentrations in Serum[a]

Fatty acid	Lipid group	Day 1 Hour 0	(n)	Day 1 Hour 10	(n)	Day 3 Hour 0	(n)	Day 3 Hour 10	(n)	Day 5 Hour 0	(n)	Day 5 Hour 10	(n)	(p)
8:0	MCT	.1 ± .05	(6)	82 ± 41	(6)	5 ± 2	(5)	12 ± 5	(6)	.8 ± .3	(4)	22 ± 6	(5)	.0005[b]
	LCT	n.d.	(6)	.06 ± .04	(8)	.04 ± .03	(8)	n.d.	(6)	n.d.	(7)	n.d.	(6)	
10:0	MCT	n.d.	(5)	22 ± 13	(5)	4 ± 1	(5)	9 ± 4	(6)	.4 ± .1	(5)	11 ± 2	(4)	.0005[b]
	LCT	n.d.	(6)	n.d.	(7)	.03 ± .02	(7)	n.d.	(7)	n.d.	(8)	n.d.	(7)	
12:0	MCT	8 ± 4	(8)	4 ± 2	(6)	6 ± 4	(5)	1 ± .3	(5)	.4 ± .08	(4)	1.5 ± .4	(5)	
	LCT	.3 ± .1	(6)	.6 ± .3	(8)	.3 ± .05	(8)	.8 ± .3	(9)	.3 ± .08	(8)	.2 ± .1	(6)	

[a]Expressed in μmol/100 ml serum as $\bar{X}$ ± SEM with number of samples in parentheses.
[b]Hour 0 average less than hour 10 average.

TABLE 6

Long Chain Free Fatty Acid Concentrations in Serum[a]

Fatty acid	Lipid group	Day 1 Hour 0	(n)	Day 1 Hour 10	(n)	Day 3 Hour 0	(n)	Day 3 Hour 10	(n)	Day 5 Hour 0	(n)	Day 5 Hour 10	(n)	(p)
16:0	MCT	57 ± 30	(8)	37 ± 11	(5)	98 ± 52	(5)	22 ± 6	(6)	24 ± 4	(5)	26 ± 3	(4)	
	LCT	16 ± 5	(5)	399 ± 187	(8)	24 ± 6.6	(8)	34 ± 9	(9)	15 ± 4	(5)	37 ± 5	(7)	
16:1ω7	MCT	4 ± 1.5	(7)	14 ± 7	(7)	14 ± 8	(5)	2.6 ± .9	(6)	4 ± 1.3	(5)	3.5 ± 1.5	(5)	
	LCT	1.8 ± .3	(5)	49 ± 26	(8)	2 ± .7	(8)	2 ± .5	(9)	2 ± .6	(6)	2.3 ± .4	(7)	
18:0	MCT	11 ± 5	(8)	9.6 ± 3	(5)	16 ± 8	(5)	6 ± 1	(6)	4 ± .7	(5)	7 ± 1.4	(5)	
	LCT	6 ± 2	(6)	28 ± 10.6	(7)	5 ± 1.2	(8)	5 ± .8	(7)	3 ± 1	(5)	11 ± 1.5	(7)	.05[b]
18:1ω9	MCT	56 ± 28	(8)	38 ± 14	(5)	78 ± 41	(5)	23 ± 6	(6)	20.7 ± 5.5	(5)	29 ± 8	(5)	
	LCT	22 ± 7	(5)	455 ± 207	(8)	26 ± 7	(8)	23 ± 3.3	(7)	16 ± 5	(5)	50.5 ± 6	(7)	.05[c]
18:2ω6	MCT	20 ± 9	(8)	29 ± 8	(5)	129 ± 71	(6)	11 ± .6	(4)	15 ± 5	(5)	23 ± 5	(4)	
	LCT	17 ± 7	(6)	161 ± 63	(7)	18 ± 5	(8)	27 ± 4	(7)	77 ± 44	(7)	76 ± 13	(7)	.05[d]
18:3ω3	MCT	1.4 ± .6	(8)	11 ± 6	(7)	3.4 ± 1.8	(5)	1.7 ± .4	(6)	.7 ± .1	(5)	3.7 ± 1.3	(5)	
	LCT	.15 ± .1	(6)	6 ± 2	(7)	1 ± .4	(8)	2.5 ± .5	(7)	.5 ± .2	(5)	8 ± 1	(7)	
20:4ω6	MCT	4.5 ± 1.7	(8)	4 ± 1.2	(6)	12.5 ± 5	(6)	1.6 ± .3	(6)	1.7 ± .3	(5)	1.5 ± .4	(5)	
	LCT	1.4 ± .5	(5)	18 ± 8	(8)	1 ± .2	(7)	1.4 ± .4	(9)	1.2 ± .4	(6)	1.6 ± .5	(6)	.03[c,e]

[a]Expressed in μmol/100 ml serum as $\bar{X}$ ± SEM with number of samples in parentheses.
[b]Hour 0 average lower than hour 10 average.
[c]Day 1 average higher than day 3 or 5.
[d]Hour 10, MCT average less than hour 10, LCT average.
[e]Hour 0 average lower than hour 10 average.

acid was not detectable. Dodecanoic acid levels in the free fatty acid fraction, although present, did not show any statistically significant changes with the daily infusions or differences between the LCT and MCT groups. The levels in the MCT group seem higher than those in the LCT group, suggesting elongation from the infused decanoic acid, but baseline levels were higher as well on day 1, hour 0.

The long chain fatty acid profile of the serum free fatty acid fraction is shown in Table 6. Palmitic acid concentrations did not vary because of the daily infusions or because of the type of lipid infused. Likewise, free palmitoleic acid concentrations did not show any effects between the lipid groups or because of the infusions. Stearic acid concentrations in the free fatty acid fraction rose in the LCT group during the infusion ($p < 0.05$), but did not vary in the MCT group. The daily changes within the LCT group were, respectively, on days 1, 3 and 5; 6-28, 5-5 and 3-11. Since stearic acid was not infused, it may have increased because of elongation of infused palmitic acid. Oleic acid concentrations in the free fatty acid fraction tended to fall over the five days, but only in the LCT group ($p < 0.05$). In the MCT group, for days 1, 3 and 5, the hour 0 to hour 10 values were, respectively, 56-38, 78-23 and 20.7-29. However, in the LCT group the concentrations rose from 22 to 455 on day 1, but fell from 26 to 23 on day 3 and rose from 16 to 50 on day 5. Free linoleic acid concentrations were higher at hour 10 in the

LCT group than in the MCT group ($p < 0.05$). The values did not differ at hour 0. The hour 10 values for the MCT group were, for days 1, 3 and 5, respectively, 29, 11, 23; whereas for the LCT group, they were 161, 27 and 76. This is reflective of the amount infused since linoleic acid is an essential fatty acid. Free α-linolenic acid concentrations did not differ between the two lipid groups. Presumably a small enough quantity was delivered to either group so that it was able to be cleared. Arachidonic acid concentrations in the free fatty acid fraction rose in the LCT group during the daily infusions but not for the MCT group ($p = 0.03$). In the LCT group, for days 1, 3 and 5, respectively, the hour 0 to hour 10 values were 1.4-18, 1-1.4 and 1.2-1.6, whereas in the MCT group, the respective changes were 4.5-4, 12.5-1.6, and 1.7-1.5. Free arachidonic acid also tended to fall within the LCT group over the course of the five-day study ($p < 0.03$). The same amount of lipid delivered did not result in the same daily elevations of arachidonic acid. The values in the MCT group did not show as much variability.

Table 7 shows the fatty acid composition in the serum cholesterol ester fraction at the end of the study, on day 5 at hour 10. Very little (less than 1/5 of 1% of all fatty acids) medium chain fatty acids were seen in the MCT group as compared to the long chain fatty acids normally seen.

Table 8 shows the fatty acid composition in the serum phospholipids on day 5 at hour 10. As in the cholesterol ester fraction, medium chain fatty acids accounted for a small proportion (less than 1%) of the total fatty acids.

TABLE 7

Serum Cholesterol Ester Fatty Acid Concentrations on Day 5 Hour 10[a]

Fatty acid	MCT (n)	LCT (n)
8:0	n.d. (3)	n.d. (5)
10:0	n.d. (3)	n.d. (5)
12:0	n.d. (2)	n.d. (5)
16:0	14.1 ± 2.2 (3)	21.5 ± 4.8 (5)
16:1ω7	6.1 ± 1.9 (3)	6.4 ± 1.4 (5)
18:0	1.2 ± 0.2 (3)	1.8 ± 0.4 (5)
18:1ω9	27.1 ± 4.4 (3)	37.8 ± 7.9 (5)
18:2ω6	43.4 ± 6.8 (3)	62.3 ± 16.5 (5)
18:3ω3	0.8 ± 0.4 (3)	n.d. (3)
20:4ω6	8.6 ± 3.4 (3)	4.2 ± 1.5 (5)

[a]Expressed in μmol/100 ml serum as $\bar{X}$ ± SEM with numbers in parentheses.

TABLE 8

Serum Phospholipid Fatty Acid Concentrations on Day 5 Hour 10[a]

Fatty acid	MCT (n)	LCT (n)
8:0	1.6 ± 0.1 (2)	n.d. (5)
10:0	1.9 ± 0.9 (3)	n.d. (5)
12:0	0.7 ± 0.3 (3)	n.d. (5)
16:0	147 ± 10.1 (3)	130 ± 15.6 (5)
16:1ω7	5.5 ± 0.9 (3)	10.9 ± 2.5 (5)
18:0	48.8 ± 6.1 (3)	54.7 ± 3.1 (5)
18:1ω9	59.5 ± 10.0 (3)	84.8 ± 10.3 (5)
18:2ω6	74.0 ± 5.0 (3)	82.2 ± 7.4 (5)
18:3ω3	2.3 ± .8)4)	0.7 ± 0.5 (5)
20:4ω6	47.5 ± 20.4 (4)	21.8 ± 1.7 (4)

[a]Expressed in μmol/100 ml serum as $\bar{X}$ ± SEM with numbers in parentheses.

DISCUSSION

These data collectively point to many properties of intravenously administered medium chain fatty acids.

Among triglycerides, the appearance of medium chain fatty acids was not surprising, due to the infusion. Rapid hydrolysis of these fatty acids was evidenced by their levels reaching baseline values on the subsequent mornings. This is consistent with the rapid clearance reported by Sailer and Muller (6) and others (8-11). Interestingly, dodecanoic acid rose substantially during the infusion, yet accounted for only trace amounts in the emulsion, leading one to suspect either endogenous elongation, or relatively much slower hydrolysis as compared to octanoic and decanoic acids.

Likewise, in the free fatty acid fraction, octanoic and decanoic acids rose quickly during the infusion and were back down to baseline levels on the subsequent mornings, supporting rapid clearance of the free medium chain fatty acids.

These data show that intravenously administered medium chain triglycerides, when accounting for approximately 24% of the total caloric delivery in hospitalized patients on TPN, are rapidly hydrolyzed as evidenced by appearance of medium chain fatty acids in the free fatty acid fraction by the end of the infusion, and subsequently cleared from the blood as evidenced by baseline levels for medium chain fatty acids in the triglyceride and free fatty acid fractions 14 hours later. Coupled with the fact that these fatty acids are rapidly oxidized intracellularly (4), this advances the use of some MCT as a better metabolic lipid fuel than solely LCT for the hospitalized patient.

Long chain fatty acids showed predictable increases in both the triglyceride and free fatty acid fractions during the infusion, most consistently in the LCT treatment group. Of note was an effect during the five-day study where the subsequent daily levels of some of the long chain fatty acids in the triglyceride fraction decreased. For example, in the LCT group, linoleic acid had its highest hour 10 value on day 1 (1.02 vs 0.67, 0.77 on days 3 and 5, respectively). This enhanced clearance of intravenous fat with continued administration has been observed before (12).

The changes in arachidonic acid are interesting. Since it was not infused, its rise during the infusion in the LCT group, in either the triglyceride or free fatty acid fraction, may have been caused indirectly by the infused linoleic acid. Elongation and desaturation is one possible route.

The increase in arachidonic acid may not be beneficial. For example, in endotoxic shock we have shown improved survival from feeding marine oils to guinea pigs, either enterally or parenterally, as opposed to a safflower oil-based diet (13,14). Marine oils contain eicosapentaenoic acid (EPA), an antagonist to arachidonic acid. Not much change occurred in the cholesterol ester or phospholipid fractions with MCT infusion, so arachidonate changes in the triglyceride or free fatty acid fractions may not translate into effects on eicosanoid metabolism.

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Occurrence of Geometrical Isomers of Eicosapentaenoic and Docosahexaenoic Acids in Liver Lipids of Rats Fed Heated Linseed Oil

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Mono *trans* geometrical isomers of 20:5 n-3 and 22:6 n-3 were detected in liver lipids of rats fed heated linseed oil. The isomers were identified as being 20:5 Δ5*c*,8*c*,11*c*,14*c*,17*t* and 22:6 Δ4*c*,7*c*,10*c*,13*c*,16*c*,19*t*. These fatty acids were isolated as methyl esters by preparative high-performance liquid chromatography (HPLC) on reversed phase columns followed by silver nitrate thin layer chromatography ($AgNO_3$-TLC). The structures were identified using partial hydrazine reduction, $AgNO_3$-TLC of the resulting monoenes, oxidative ozonolysis of each monoene band, and gas-liquid chromatography (GLC) of the resulting dimethyl esters and monomethyl esters. Fourier-transform-infrared spectrometry confirmed the *trans* geometry in isolated 20:5 and 22:6 isomers. The isomers of eicosapentaenoic and docosahexaenoic acids in liver lipids probably resulted from desaturation and elongation of 18:3 Δ9*c*,12*c*,15*t*, a geometrical isomer of linolenic acid present in the heated dietary oil.
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Heat treatment of vegetable oils results in geometrical isomerization of linolenic and linoleic acids (1,2). The quantity of *trans* polyunsaturated fatty acids formed depends on the temperature and on the duration of the heat treatment (3). No positional fatty acid isomers were observed except for some conjugated fatty acids (2).

Among the questions raised by the detection of such compounds in heated edible oils, one attracted our attention. Could these geometrical isomers of essential fatty acids be desaturated and elongated *in vivo*? A previous study by our group indicated the presence of three unknown peaks in the fatty acid composition of testicular and adrenal lipids of rats fed with a heated linseed oil (4). In gas-liquid chromatographic (GLC) analyses, these compounds (previously named X, Y and Z) had equivalent chain length (ECL) values close to those of 20:5 n-3, 22:5 n-3 and 22:6 n-3 respectively. Their hydrogenation gave 20:0 (X) and 22:0 (Y, Z). The same compounds were also observed in liver lipids of rats fed with heated soybean or rapeseed oils, but not in rats receiving heated sunflower oil (5).

We assumed that these compounds were geometrical isomers of 20:5 n-3, 22:5 n-3 and 22:6 n-3, formed by desaturation and elongation of one or more geometrical isomers of linolenic acid. It was recently confirmed (6) that the compound X was, in fact, a geometrical isomer of 20:5 n-3. A sufficient quantity of this compound was isolated from the liver lipids of rats fed with heated linseed oil to allow its identification after hydrazine reduction, separation of the *cis* and *trans* monoenes, and their oxidative ozonolysis. We thus identified the compound X as being 20:5 Δ5*c*,8*c*11*c*,14*c*,17*t* (6).

The present work was aimed at identifying compound Z and confirming the geometry of the ethylenic bonds of components X and Z using gas-liquid chromatography coupled with Fourier-transform-infrared spectrometry (GLC-FTIR).

MATERALS AND METHODS

Animals and dietary fat. Eighteen specific pathogen free (SPF) Wistar rats from the animal breeding of the "Station de Recherches sur la Qualité des Aliments de l'Homme" were used. They were nine weeks old at the beginning of the experiment. They were fed for three weeks with a purified diet (7) containing 10% in weight of linseed oil heated at 275°C for 12 hr under nitrogen.

The composition of octadecatrienoic acid isomers present in this oil was obtained through the following procedures. The non-polar fraction (as methyl esters) was obtained as described elsewhere (8). The trienes were obtained from this fraction by semi-preparative high-performance liquid chromatography (HPLC) as described in the next paragraph. These were then fractionated by silver nitrate-thin layer chromatography ($AgNO_3$-TLC) (9) and quantified by gas-liquid chromatography (GLC) with methyl heptadecanoate as the internal standard. Their chemical structures were determined using the TLC and GLC data previously obtained (1,10).

Isolation of unknown compound Z. Rats were killed by decapitation and their livers were rapidly removed and placed in a vessel containing a mixture of chloroform/methanol (2:1, v/v). Total liver lipids were extracted using the method of Folch *et al.* (11), and the fatty acids were converted to methyl esters according to Morrison and Smith (12).

The liver fatty acid methyl esters were fractionated by preparative HPLC, using a Waters Prep LC 500 A with a Prep-Pak 500 C18 column (30 cm × 5.7 cm i.d.). About 2.5 g of fatty acid methyl esters dissolved in acetone were injected and pure acetonitrile

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Abbreviations: $AgNO_3$-TLC, silver nitrate thin layer chromatography; DMC, dimethyl esters; ECL, equivalent chain length; FAME, fatty acid methyl esters; GLC, gas-liquid chromatography; GLC-FTIR, gas-liquid chromatography-Fournier-transform-infrared; GLC-MS, mass spectrometry gas-liquid chromatography; HPLC, high performance liquid chromatography; IR, infrared; MCT, mercury-cadmium telluride; MMC, monomethyl esters; SPF, specific pathogen free.

at 200 ml/min was used as the eluent solvent. The eluting components were detected by refractometry, fractions collected, and the solvent evaporated.

The composition of the fractions was obtained by GLC. The fractions containing the more unsaturated fatty acids were then submitted to a semi-preparative HPLC fractionation, using a F 6000 A Waters pump, a Hibar LiChrosorb RP-18 (7 μm) Merck column (25 cm × 10 mm i.d.), a Waters differential refractometer R 401, and methanol as solvent at 3 ml/min.

After evaporation of the solvent, the fraction containing a mixture of 22:6 n-3 and Z was submitted to $AgNO_3$-TLC. Silila gel plates (Merck, 0.25 mm thickness, 20 cm × 20 cm) were impregnated by dipping for 30 minutes in a 10% solution of silver nitrate in acetonitrile. The developing solvent was a mixture of diethyl ether/methanol (9:1, v/v), as described by Morris (13). The bands were detected under ultraviolet light at 254 nm, after spraying with a 0.2% solution of 2′,7′-dichlorofluorescein in ethanol, and scraped off into glass centrifuge tubes. A 1% solution of sodium chloride in 90% methanol was added until the red color of the silver-dichlorofluorescein complex disappeared, as described by Hill *et al.* (14). The methyl esters were extracted with hexane after addition of water.

Structural determination of the unknown compound. The 22:6 n-3 and the fraction containing the unknown compound Z were submitted to hydrazine reduction, according to Ratnayake (15) and Conway *et al.* (16). After seven hr of reaction, water was added and the methyl esters extracted with hexane. Each reduced fraction was separated by $AgNO_3$-TLC, as described above, except that developing solvent was a mixture of hexane/benzene (1:1, v/v). TLC standards 22:0, 22:1 Δ13*c*, 22:1 Δ13*t* (Nu-Check Prep, Elysian, MN) were used. Methyl esters were extracted from the silica gel by a mixture of chloroform/hexane (1:1, v/v). The resulting monoene fractions were submitted to oxidative ozonolysis in boron trifluoride/methanol, using a technique recently described (1) and adapted from the method by Ackman and Sebedio (17, 18). A Supelco micro-ozonizer was used to produce ozone. The ozonolysis products (mono- and dimethyl esters) were extracted into chloroform, analyzed by GLC, and identified by comparison with authentic standards (Sigma, St. Louis, MO).

Gas-liquid chromatography (GLC). Each step of preparation or identification of the unknown compound was monitored by GLC of the methyl esters. Girdel 3000, Becker-Packard 417 or 420 chromatographs fitted with flame ionization detectors and solid injectors (19) were used. Three capillary columns were employed: a home-made glass column (35 m × 0.35 mm i.d.) coated with Carbowax 20M, a glass column (50 m × 0.25 mm i.d.) coated with Silar-10C (Alltech) and a fused silica column (50 m × 0.32 mm i.d.) coated with CP-Sil-88 (Chrompack). Helium was used as carrier gas. The analyses were performed at 175°C or at 180°C. The temperature of the injectors and detectors were 240°C. A Vista CDS 401 (Varian) or an Autolab System 4 (Spectraphysics) integrator were used for quantitative analyses. The equivalent chain lengths (ECL) of the unsaturated fatty acids were calculated according to the method described by Ackman (20).

Gas-liquid chromatography coupled with Fourier-transform infrared spectroscopy (GLC-FTIR). The gas-phase infrared spectra were obtained with a Bruker IFS 85 Fourier-transform-infrared spectrometer. This was connected to a Carlo-Erba 5160 gas chromatograph equipped with an on-column injector and a flame ionization detector maintained at 300°C.

The interface consisted of a gold-coated light-pipe (20 cm × 0.8 mm i.d.) maintained at 250°C. A narrow band (4800–600 cm^{-1}) liquid nitrogen-cooled mercury-cadmium telluride (MCT) detector was used.

A DB-5 (J and W Scientific) fused silica capillary column (30 m × 0.32 mm i.d.) with a film thickness of 1.0 μm was used; oven temperatures were programmed from 60 to 250°C at 10°C/min, and then held isothermally for completion of the analyses. The spectral resolution was fixed at 8 cm^{-1} and 12 interferograms were collected per second.

Gas-liquid chromatography coupled with mass spectrometry (GLC-MS). A Hewlett-Packard 5890 gas chromatograph coupled with a 5970 Mass Selective Detector (Hewlett-Packard) was used to monitor the TLC separation of the monoenes and dienes, after partial hydrazine reduction. The column used was a fused silica column (J and W Scientific, DB-Wax, 30 m × 0.25 mm i.d., film thickness 0.5 μm). The temperature was programmed from 50 to 200°C at 20°C/min, held at 200°C for 25 minutes, then programmed from 200°C to 220°C and held at 220°C until completion of the analyses. Splitless injection was used with the injection port maintained at 250°C.

RESULTS AND DISCUSSION

With the procedure used to quantify the octadecatrienoic fatty acid isomers in the heated oil, these compounds were obtained free from polymers and other polar compounds (8), and from cyclic monomers (21). The linseed oil heated at 275°C for 12 hr under nitrogen contained no more than 1.7% of linolenic acid, compared to 54% before heating. Consequently, all the geometrical isomers of linolenic acid were detected in the oil. Their relative quantities differed from those observed in oils heated at 240°C for 10 hr (1): the three di-*cis*, mono *trans* compounds were detected in similar proportions, but 18:3 Δ9*t*,12*c*,15*t* was the major trienoic isomer (Table 1).

TABLE 1

Geometrical Isomers of 18:3 in the Linseed Oil Heated at 275°C for 12 Hours Under Nitrogen (Percent of Chromatographed Nonpolar Methyl Esters)

18:3 Isomer		wt%
• 18:3 Δ 9,12,15		
	t, t, t	0.3
	c, t, t	2.2
	t, c, t	12.1
	t, t, c	1.5
	c, c, t	4.6
	c, t, c	3.8
	t, c, c	4.1
	c, c, c	1.7
• Unknowns		0.4

As already observed (4-6), the rats that received heated linseed oil in their diet showed (in their tissues lipids) three unknown compounds (marked X, Y and Z) with GLC retention times close to those of 20:5 n-3, 22:5 n-3 and 22:6 n-3 (Table 2). The quantities of these compounds were substantial, especially in the case of compound X. This latter compound was recently identified (6) as being 20:5 Δ5*c*,8*c*,11*c*,14*c*,17*t*. The GLC-FTIR spectrum obtained (Fig. 1) confirmed this structure. An absorption band, characteristic of a *trans* ethylenic bond, was observed at 966 cm^{-1} (δCH), along with the bands characteristic of *cis* ethylenic bonds:3022 cm^{-1} (γCH) and 702 cm^{-1} (δCH).

A previous study (22) showed that the IR spectra were slightly different in the vapor phase compared to those obtained in condensed phase: for example, methyl oleate showed an absorption band at 704 cm^{-1} for the δCH out-of-plane deformation in vapor phase instead of near 690 cm^{-1} in the condensed phase; likewise in the CH stretching region, the absorption band was at 3013 cm^{-1} in vapor phase compared to 3040-3010 cm^{-1} in the condensed phase.

The isolation and identification of compound Z was made easier through the experience gained during the isolation and identification of compound X. The isolation of Z was effected using preparative reverse phase HPLC followed by semi-preparative HPLC, and then by $AgNO_3$-TLC. The identification of Z was carried out in three steps. First the fraction enriched in compound Z was partially reduced using hydrazine. The *cis* and *trans* monoenes were then separately obtained by $AgNO_3$-TLC and the positions of the ethylenic bonds determined by oxidative ozonolysis and GLC of the resulting dimethyl esters (DMC) and monomethyl esters (MMC). This methodology (Fig. 2, right panel) was slightly different from that used for the isolation and identification of compound X (Fig. 2, left panel). They differed in three points: (i) A supplementary step of semi-preparative HPLC was added after the preparative HPLC, in order to make the $AgNO_3$-TLC step easier, (ii) the reaction time of the hydrazine reduction was extended to seven hours in order to obtain a larger proportion of monoenes at the expense of the polyenes, (iii) this allowed us to omit the step of methoxybromercuric derivative formation and separation, which is difficult when highly unsaturated esters are present.

Seven fractions (A to G) were obtained using the preparative HPLC fractionation (Fig. 3). Fractions B (28.6 mg) and C (120.6 mg) were enriched in 22:6 n-3 and compound Z [48.0 and 21.0 percent of the total fatty acid methyl esters (FAME), respectively]. These fractions were then submitted to semi-preparative reverse phase HPLC (Fig. 4). Three peaks of FAME were collected. Peak 1 contained eicosapentaenoic acids and peak 3 the 20:4 n-6. Peak 2 was essentially (87.8%) composed of the two compounds of interest, 22:6 n-3 and Z; only 0.6% of 20:4 n-6 and 6.5% of eicosapentaenoic acids remained in this fraction. This fraction was then separated by $AgNO_3$-TLC into two bands. The first band (R_f 0.22) contained 22:6 n-3 (95%) and the second (R_f 0.33) 73.5% of Z and 11.8% of 22:6 n-3. These two fractions were considered sufficiently pure for subsequent analysis and identification, and were separately submitted to hydrazine reduction $AgNO_3$-TLC and oxidative ozonolysis (Table 3).

The hydrazine reduction of a hexaenoic fatty acid gives a mixture of pentaenes, tetraenes, trienes, dienes, monoenes and saturated compounds. However, only 22:0, monoenes and some dienes were obtained under the experimental conditions described above. This facilitated the subsequent isolation of monoenes which are each representative of the geometry and the position of the ethylenic bonds in the parent molecule (15, 23). Overlaps between the different fatty acids were observed in GLC. Thus, it was necessary to frac-

TABLE 2

Hepatic Content (wt% of Total Chromatographed Fatty Acids as Methyl Esters) of Selected Fatty Acids

Fatty acid	ECL[a]	Percentage w/w
20:5 Δ5*c*,8*c*,11*c*,14*c*,17*t* (X)	21.64	1.33
20:5 n-3	21.70	0.48
unknown compound Y	23.62	0.06
22:5 n-3	23.68	0.40
unknown compound Z	23.87	0.15
22:6 n-3	23.93	5.06

[a]Equivalent chain length on a Carbowax-20M column at 175°C.

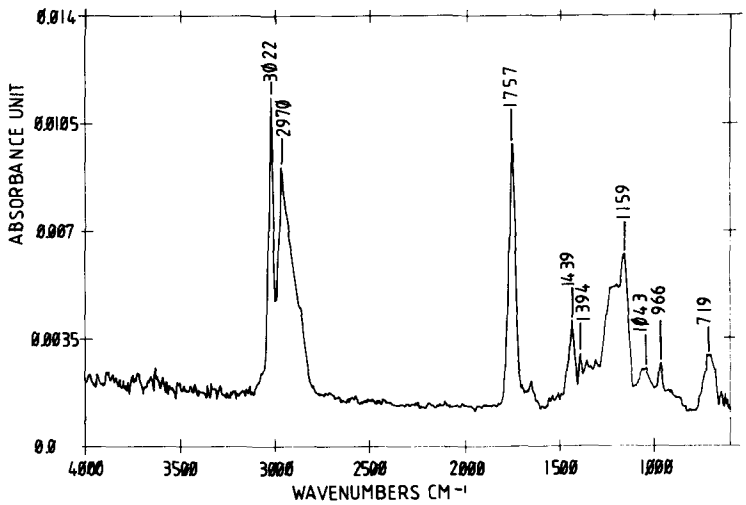

FIG. 1. GLC-FTIR spectrum of compound X (20:5 Δ5*c*,8*c*,11*c*,14*c*,17*t*).

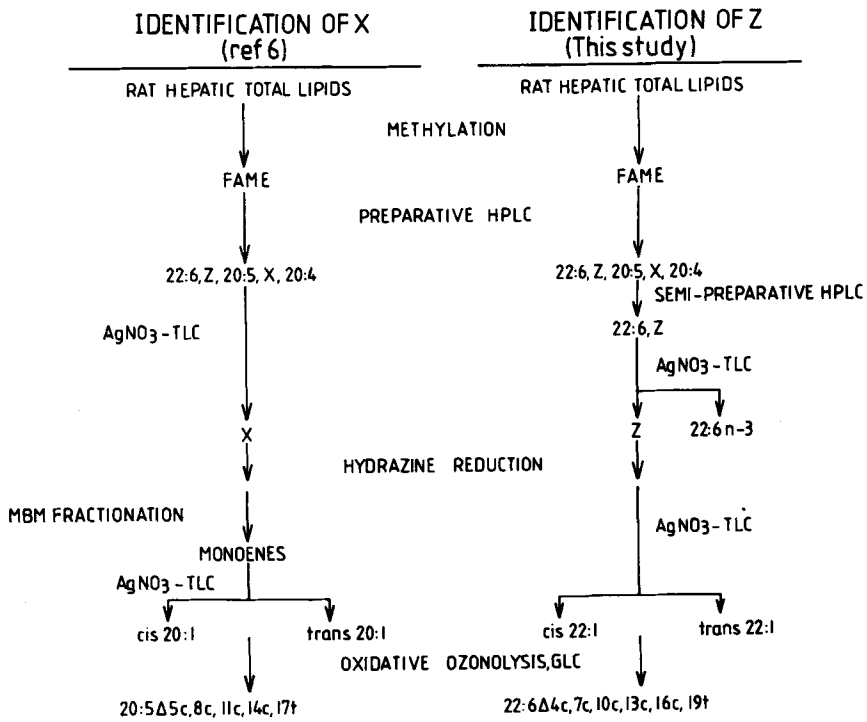

FIG. 2. Methods used for the identification of compounds X (20:5 Δ5*c*,8*c*,11*c*,14*c*,17*t*) and Z.

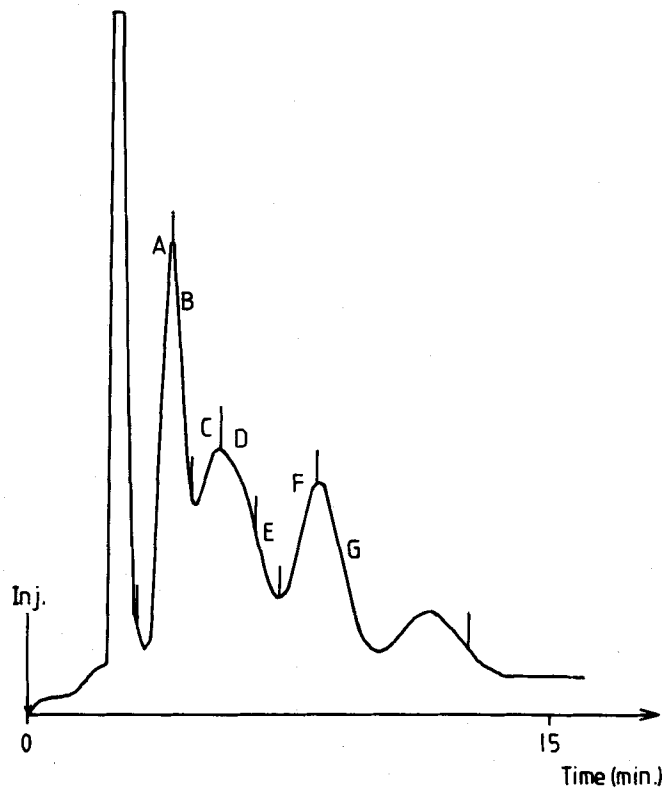

FIG. 3. Preparative HPLC fractionation of FAME derived from hepatic total lipids of rats fed with heated linseed oil (C18 reverse phase column, 30 cm long and 5.7 cm i.d., acetonitrile at 200 ml/min).

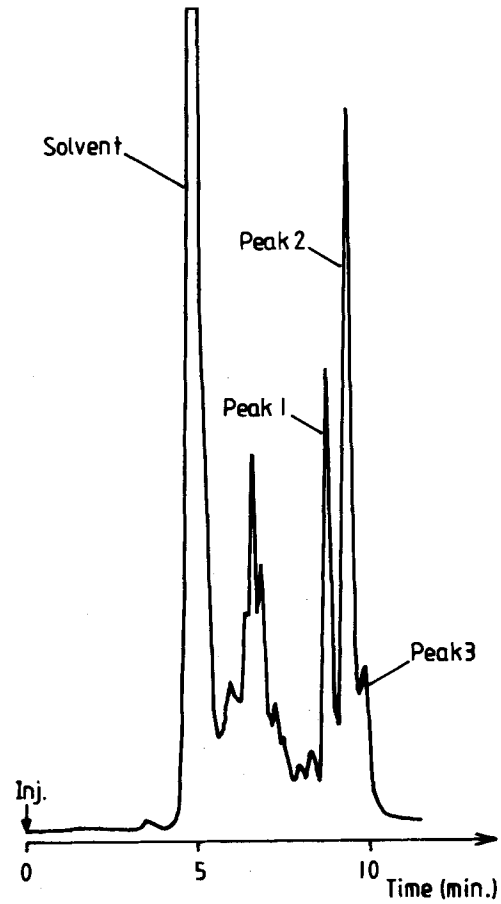

FIG. 4. Semi-preparative HPLC fractionation of a mixture enriched in 22:6 n-3 and compound Z (C18 reverse phase column, 25 cm long and 10 mm i.d., methanol at 3 ml/min).

TABLE 3

Results Obtained by Silver Nitrate-TLC, GLC and Ozonolysis of the Monoene Fractions from the Partial Hydrazine Reduced Unknown Compound Z and 22:6 n-3

Silver nitrate-TLC		GLC				
Band	R_f	Number of peaks	ECL on Carbowax -20M	ECL on CP-Sil-88	Ozonolysis products	Position of the ethylenic bonds (Δ)
Hydrazine reduced unknown compound Z fraction						
A	0.19	1	22.9	22.34	DMC7 + MMC15	7
B	0.24	2	22.00 22.21	22.34 22.34	DMClO + MMC12 + MMC18	4, 10
C	0.29	3	22.14 22.21 22.36	22.49 22.34 22.76	DMClO + DMC13 + DMC16 + MMC9 + MMC12	10, 13, 16
D	0.39	1	22.54	22.67	DMC19	19
Hydrazine reduced 22:6 n-3						
A′	0.16	1	22.09	22.34	DMC7 + MMC15	7
B′	0.22	3	22.00 22.21 22.64	22.34 22.34 23.04	DMClO + DMC19 + MMC12 + MMC18	4, 10, 19
C′	0.28	3	22.13 22.34 22.64	22.49 22.76 23.04	DMC13 + DMC 16 + DMC 19 + MMC9	13, 16, 19

tionate the mixture. This was accomplished by $AgNO_3$-TLC. The 22:0 (R_f 0.56) was easily located by comparison with the 22:0 standard (R_f 0.58). The 22:1 Δ13*t* and 22:1 Δ13*c* standards had R_f values of 0.42 and 0.29, respectively. Four poorly separated bands A, B, C, D, having R_f values ranging from 0.19 to 0.39 were observed for the TLC fractionation of the partially reduced Z mixture. Three bands, A′, B′, C′ (R_f 0.16 to 0.28), were obtained by fractionation of 22:6 n-3 itself (Table 3). These bands (A, B, C, D, A′, B′ and C′) were assumed to contain the monoenes, while the dienes remained near the origin (R_f < 0.11). These assumptions were verified using GLC-MS. All the compounds of fractions A, B, C, D, A′, B′ and C′ presented a molecular ion at 352 with two intense fragments, 320 and 321, corresponding respectively to M−32 and M−31. These spectra were similar to those of the 22:1 *cis* and *trans* standards. The dienes (*m/z* 350) were effectively located in the bottom band (R_f 0.0-0.11), and clearly separated from the monoenes.

Only one peak was observed by GLC on the two phases (Carbowax-20M and CP-Sil-88) for the TLC band A′ (R_f 0.16). By oxidative ozonolysis of this band, two compounds were observed; these compounds were identified as DMC7 and MMC15 by GLC and comparison with standards. The peak for this band was thus identified as the 22:1 Δ7. With the same method, three compounds were observed (Table 3) in the B′ band (R_f 0.22). The DMC10 and MMC12 indicated the presence of the 22:1 Δ10. The DMC19 came from the 22:1 Δ19, and in this case the MMC3 was not observed under our GLC conditions because of its greater volatility. The MMC18 observed probably originated from a 22:1 Δ4, and the DMC4 was also too volatile to be seen under our GLC conditions. It must be pointed out that the 22:1 Δ4, 22:1 Δ7 and 22:1 Δ10 were not separated by GLC on the more polar column CP-Sil-88 (ECL 22.34), but were well separated on the Carbowax-20 M column (respective ECL, 22.00, 22.09 and 22.21). The band C′ (R_f 0.28) also contained three compounds (Table 3). One of these compounds was the 22:1 Δ19 (DMC19). The two other peaks were the 22:1 Δ13 (DMC13 + MMC9) and the 22:1 Δ16 (DMC16). In this last case, the MMC6 was also not seen under our GLC conditions. All these results confirmed that the original compound was the 22:6 Δ4,7,10,13,16,19 (22:6 n-3).

The same methodology was used for the unknown compound Z fraction (Table 3). The ethylenic bonds were detected in positions 4, 7, 10, 13, 16 and 19, but the 22:1 Δ19 obtained using hydrazine reduction was different in this case: (i) its R_f was 0.39 by $AgNO_3$-TLC instead of a R_f close to 0.22-0.28, and (ii) its GLC retention time was different on the two columns. On carbowax 20 M phase, the ECL was 22.54 instead of 22.64 for the 22:1 Δ19*c*. On the cyanosilicone CP-Sil-88 the ECL was 22.67 instead of 23.04.

These chromatographic properties are characteristic of a *trans* fatty acid. The comparison (Table 3) between the products of 22:6 (n-3) and of the unknown compound Z fraction lead us to think that the Z compound is a 22:6 isomer with a *trans* ethylenic bond in position Δ19. This compound is thus the 22:6 Δ4*c*,7*c*,10*c*,13*c*16*c*,19*t*. Unfortunately, we did not obtain a sufficient quantity of 22:1 Δ19*t* to confirm this structure by GLC coupled with FTIR. But the compound Z itself was examined by GLC-FTIR under these conditions and gave an IR spectrum of a compound containing a *trans* ethylenic bond (964 cm^{-1} for δCH).

Compound Z would thus be formed from the compound X already identified as 20:5 Δ5*c*,8*c*,11*c*,14*c*,17*t*, by subsequent elongation and Δ4 desaturation (Fig. 5). The unknown compound Y could by analogy be the

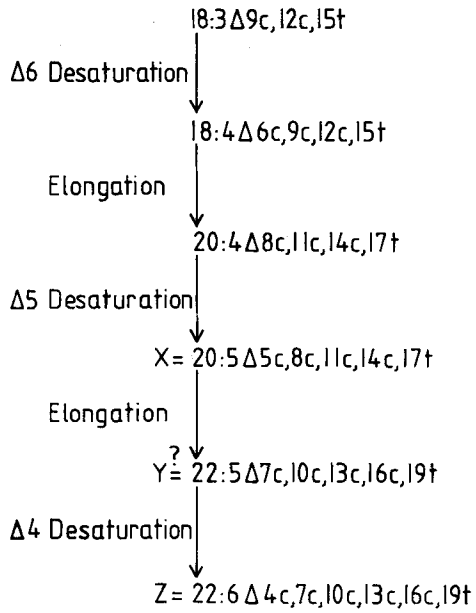

FIG. 5. Proposed scheme for the formation *in vivo* of the compounds X (20:5 Δ5*c*,8*c*,11*c*,14*c*,17*t*) and Z (22:6 Δ4*c*,7*c*,10*c*,13*c*,16*c*,19*t*).

22:5 Δ7*c*,10*c*,13*c*,16*c*,19*t*. In our previous work (4), we observed that 22:0 was obtained by hydrogenation of Y. Furthermore, its ECL on Carbowax-20 M (Table 2) differed from the ECL of 22:5 n-3 by 0.06, in parallel with the respective ECL values of X and Z, which, respectively differed from those of 20:5 n-3 and 22:6 n-3 by 0.06. The 22:5 Δ7*c*,10*c*,13*c*,16*c*,19*t* is likely the intermediary compound in the formation of 22:6 Δ4*c*,7*c*,10*c*,13*c* ,16*c*,19*t* from 20:5 Δ5*c*,8*c*,11*c*,14*c*,17*t*. Confirmation of this proposed structure for Y compound will be attempted later.

The most probable pathway of formation for compounds X, Y and Z is represented in Figure 5. All of these compounds originated from the 18:3 Δ9*c*,12*c*,15*t*, which is present in the oil included in the diet of the rats. This linolenic acid isomer is formed during heat treatment of the oil (1). Such a compound was also observed in deodorized oils by Ackman *et al.* (24) and in frying oils collected from restaurants and market vendors in France (25). Among the geometrical isomers present in the diet (Table 1), only 18:3 Δ9*c*,12*c*,15*t* appears to undergo desaturation and elongation in noticeable quantity. Other unknown compounds were also observed along with the 20:5 n-3 and the 20:5 Δ5*c*,8*c*,11*c*,14*c*,17*t*, but in very low quantities; these compounds may be other geometrical isomers of 20:5 n-3, but their chemical structure is still under investigation.

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Plasma Lipoproteins in Dairy Cows with Naturally Occurring Severe Fatty Liver: Evidence of Alteration in the Distribution of Apo A-I-containing Lipoproteins

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The relationships between fatty liver in dairy cows and reduced levels of plasma lipoproteins, and particularly of low density lipoproteins (LDL), has been previously described. Since electrophoretic heterogeneity of ultracentrifugally isolated LDL (d, 1.006–1.063 g/ml) has been found, the exact nature of this reduction in cows with fatty liver was investigated. Lipoproteins from control and severely afflicted animals were isolated by ultracentrifugation and affinity chromatography on heparin-Sepharose CL 6 B. Gradient gel electrophoresis of lipoproteins on 4–30% gels and an immunolocalization study of apoprotein A-I (apo A-I) showed that control animals have two subpopulations of apo A-I-containing particles with a mean radius of 6.52 and 5.05 nm. In the fatty liver cows, the former was clearly shifted toward smaller particles. We concluded that the depressed level and compositional modifications of LDL in severe fatty liver cows result from a decrease in the oversized apo A-I-containing lipoproteins which can be isolated in the LDL density range. This could stem from the decreased supply of triglyceride-rich lipoprotein surface components for the production of these lipoproteins. The modifications can be plausibly explained by a reduced synthesis or secretion of very low density lipoproteins (VLDL) by the liver.
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In early lactation many high-yielding dairy cows undergo a period of energy deficit. In order to make the necessary energetic adjustment they mobilize body reserves (1). Increased fat mobilization from adipose tissue results in a rise in the plasma levels of free fatty acids (FFA). One of the consequences of the increase in plasma concentration of FFA is their enhanced uptake by the liver and, thereafter, their reesterification, principally to triglycerides (1,2). The liver's capacity for secreting triglycerides is lower than its ability for synthesis, therefore, triglycerides accumulate in the liver. Normally, maximal lipid infiltration takes place in the first month of lactation and then disappears progressively (2,3). Nevertheless, fatty liver develops in most dairy cows and has significant effects on hepatic structure and function. This fatty liver occurs frequently in a subclinical form and has been associated with an increased incidence of metabolic, infectious, and reproductive disorders (3,4).

In previous work, we have demonstrated that the increase in the liver triglyceride content at the beginning of lactation is associated with a low concentration of plasma lipids and lipoproteins. The level of LDL (d, 1.006–1.063 g/ml), in particular, drops in this period (5). Cows with severe fatty liver often present abnormally low concentrations of this fraction (6). Ultracentrifugally isolated LDL in bovines is heterogenous and consists of two distinct electrophoretic groups: α- and β-lipoproteins which contain apo A-I and apo B, respectively, as the major apoproteins (7–12). In fact, bovine animals have unusually large α-lipoproteins in this density range, in varying quantities depending on their physiological stage. Lactating cows, in particular, have increased proportions of large α-lipoproteins associated with hyperalphalipoproteinemia. A possible mechanism involved in the formation of large α-lipoproteins in bovine animals has recently been proposed by Puppione (11). The metabolic relationship of triglyceride-rich lipoproteins to large α-lipoproteins appears as evidence of this phenomenon.

In view of the origin and importance of the apo A-I-containing particles in bovine animals, we have undertaken to determine whether these particles undergo a redistribution in size and composition when liver lipoprotein secretion is limited.

MATERIALS AND METHODS

Animals. Holstein × Friesian multiparous cows with an average milk yield of 6.400 kg from the Institute's dairy herd were used in this study. The cows were housed and individually fed a complete diet based on maize silage supplemented with concentrate. Samples of blood and liver were collected from cows 12–21 days after calving, 2–3 hr after milking and feeding. Blood samples were taken from the jugular vein and collected on 0.01% Na_2-EDTA, and plasma was prepared by low speed centrifugation. Liver samples were taken by percutaneous needle biopsy under local anaesthesia and assayed for triglyceride content as previously described (5). The cows in this study were divided into experimental groups according to the extent of the fatty liver development. Cows with less than 50 mg of triglycerides per gram of wet liver weight were considered normal (control animals) and those with more than 100 mg were considered severely fatty (3,6). Six apparently healthy cows, with no depressed milk yield, were retained from each group.

Lipoprotein separation. Plasma lipoproteins were separated into various density classes by sequential preparative ultracentrifugation (13) as previously described (5,6,12). Ultracentrifugation was performed at 20°C in a Beckman L-5 model ultracentrifuge (Beck-

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Abbreviations: apo, apoprotein; d, density; EDTA, ethylene diamine tetraacetic acid; FFA, free fatty acids; HDL, high density lipoproteins (d, 1.063–1.21 g/ml); LCAT, lecithin:cholesterol acyltransferase; LDL, low density lipoproteins (d, 1.006–1.063 g/ml); LPL, lipoprotein lipase; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; TBS, Tris buffered saline; VLDL, very low density lipoproteins (d, <1.006 g/ml).

man Instruments, Palo Alto, CA) with a 50 titanium rotor. Before the lipoproteins were separated, NaN_3 (0.02%), merthiolate (0.005%) and Na_2-EDTA (0.04%) were added. Lipoprotein fractions were isolated by sequentially raising the density by adding cristalline KBr. The LDL, 1.006–1.063 g/ml and HDL, 1.063–1.21 g/ml density fractions were obtained after centrifugation at 100.00 × *g* for 20 and 24 hr, respectively.

For lipid and apoprotein analysis, fractions were then washed for a further period of ultracentrifugation at the same density. All isolated lipoprotein fractions were dialyzed against 0.02 M phosphate buffer (pH 7.4) containing 0.15 M NaCl, 0.01% Na_2-EDTA, and 0.02% NaN_3.

Chemical analysis. FFA (Wako Chemicals, Biolyon, France), triglycerides (Triglyenzyme Color, Biotrol, France), total and free cholesterol (Cholestérol enzymatique PAP, BioMérieux, France) and phospholipids (Phospholipides enzymatiques PAP, BioMérieux, France) in plasma and lipoprotein fractions were determined by sensitive enzymatic procedures. Cholesterol ester content was calculated by free cholesterol subtracted from total cholesterol and the difference multiplied by 1.67 to account for weight contribution of fatty acids. The protein concentration of all isolated lipoproteins was determined by a modified Lowry method (14). Bovine serum albumin (Fraction V, Sigma) was used as a standard.

Liver triglycerides were determined as previously described (5). Liver samples were homogenized and the lipids were extracted (15). Extracts were evaporated to dryness under nitrogen. Triglycerides in the lipid residue were saponified by KOH-ethanol (0.25 ml) at 70°C for 30 min. Then 0.15 M $MgSO_4$ (0.5 ml) was added to neutralize the mixture. After centrifugation at 2.000 × *g* for five minutes, the supernatant was assayed for glycerol determination.

Apo A-I quantification. A single radial immunodiffusion assay was developed to determine the apo A-I plasma concentration (16). The assay was performed using a 1.25% agarose gel mixed with anti-bovine apo A-I rabbit antiserum. Standard (bovine HDL) or diluted plasma (10 times with 0.15 M NaCl) were applied in 3 μl amounts to each well in the gel. Incubation was conducted for 48 hr at 25°C and the ring precipitates were measured, after staining with Amido Black 10 B, by using a projector for immunoanalysis.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Apoprotein composition was determined by SDS-PAGE, in 4–20% polyacrylamide gradient gel, carried out on samples solubilized by 1% SDS according to Weber and Osborn (17). The gels after electrophoresis were stained with 0.1% Coomassie Brillant Blue R 250 in methanol/acetic acid/H_2O (25:10:65) and destained with methanol/acetic acid/H_2O (10:7.5:82.5). Bands were identified by their apparent molecular weights using protein standards (Pharmacia Fine Chemicals, Uppsala, Sweden) run simultaneously.

Affinity chromatography on heparin-Sepharose. The ability of heparin-Sepharose to bind β-lipoproteins at low salt concentrations has recently been exploited to separate α-and β-lipoproteins in bovine plasma (18,19). In our study, lipoproteins of the d, 1.006–1.21 g/ml from some control and severe fatty liver cows, were fractionated by affinity chromatography on heparin-Sepharose CL 6-B (Pharmacia Fine Chemicals, Uppsala, Sweden) as described by Weisgraber and Mahley (20).

Nondenaturing gradient polyacrylamide gel electrophoresis. The molecular size distribution of lipoprotein particles was estimated by nondenaturing gradient polyacrylamide gel electrophoresis (21) on Pharmacia precast PAA 4–30 gels (Pharmacia Fine Chemicals, Uppsala, Sweden). Aliquots of ultracentrifugation or affinity chromatography-isolated lipoprotein samples were subjected to electrophoresis at 125 V for 17 hr in a Tris-borate buffer (pH 8.35). Particle size of lipoproteins was calculated by reference to coelectrophoresed standards of thyroglobulin (radius 8.5 nm), ferritin (radius 6.1 nm), lactate dehydrogenase (radius 4.08 nm), and bovine serum albumin (radius 3.55 nm) from a high molecular weight electrophoresis calibration kit (Pharmacia Fine Chemicals, Uppsala, Sweden). Gels were fixed in 10% sulfosalicylic acid for one hour, stained for four hours in 0.04% Coomassie Brillant Blue R 250 in perchloric acid (3.5%), and destained in acetic acid (5%). The gels were then scanned in a Vernon densitometer.

For immunodetection of apo A-I distribution, the lipoproteins were electrophoretically transferred (22) to nitrocellulose sheets (Biorad, France). Transfer was carried out in Tris-glycine buffer, pH 8.4, at 250–500 mA for 36 hr. After blocking unreacted sites with a mixture containing 0.05% Tween 20 and 3% gelatin in Tris buffered saline (TBS) (30 min at 37°C and 30 min at room temperature) and subsequent washing with the TBS (3×), the sheets were reacted with anti-bovine apo A-I rabbit antiserum for three hours. After washing again, the sheets were reacted for one hour with ^{125}I-labeled conjugate protein A (IM. 144; Amersham France SA). The transfers were autoradiographed by overnight exposure to X-ray film (Kodak XAR-5; Sigma) at −70°C. A nonimmune rabbit serum was used for the control of nonspecific binding.

Statistics. Individual mean differences were determined by Student's unpaired t-test (23).

RESULTS

Plasma lipids and apo A-I levels. The general lipid and apo A-I profiles of the cows in this study, divided into experimental groups according to the extent of the fatty liver as determined by the hepatic triglyceride level, are shown in Table 1. The levels of plasma cholesterol, triglycerides and apo A-I were significantly lower and the level of FFA was significantly higher in cows with severe fatty liver than in control cows.

Plasma lipoprotein levels and composition. Plasma levels and the chemical composition of LDL and HDL ultracentrifugally isolated fractions are shown in Table 2. The levels of these fractions were significantly lower in cows with severe fatty liver than in control cows. The composition of both fractions was altered in the fatty liver group. In this group, an elevation in the percentage of triglycerides was found together with a decrease in the percentage of protein and phospholipids in LDL fraction. This group was also characterized by an elevation in the percentage of protein and a

TABLE 1

Lipid and apo A-I Levels in Plasma from Control and Fatty Liver Cows

Cows	Control	Severe fatty liver
Liver triglycerides[a] (mg/g wet weight)	29.0 ± 7.7[b]	164.3 ± 20.1
Total cholesterol (mg/dl)	99.0 ± 5.9	73.3 ± 7.6[c]
Free cholesterol (mg/dl)	24.4 ± 2.1	17.7 ± 2.1[c]
Triglycerides (mg/dl)	16.1 ± 1.4	11.3 ± 1.3[c]
Free fatty acids (mM)	0.44 ± 0.08	0.96 ± 0.18[c]
Apo A-I (mg/dl)	126.7 ± 4.9	112.0 ± 2.5[c]

[a]The basis of classification.
[b]Means ± standard error of six samples per group.
[c]Significant difference from control cows, P<0.05.

decrease in the percentage of phospholipids in the HDL fraction.

When the apoproteins of the LDL were electrophoresed on 4-20% gradient SDS-PAGE (Fig. 1), we observed less apo A-I and more apo B_h in severe fatty liver than in control cows. Apo B_l was present as a small protein band without appreciable differences between both groups. The electrophoretic pattern of HDL apoproteins (not shown) demonstrated that apo A-I was the major apoprotein in all animals and the presence of other protein bands was very weak.

Nondenaturing gradient polyacrylamide gel electrophoresis analysis. For particle size analysis, isolated lipoproteins were separated by nondenaturing gradient polyacrylamide gel electrophoresis and stained for protein. Densitometric scans depicting the gels of LDL and HDL ultracentrifugally-isolated fractions are shown in Figures 2a and 2b, respectively. Analysis of the LDL in the control cows provides evidence for the presence of different lipoprotein subpopulations with the mean radius of 9.92, 6.91 and 5.00 nm. In contrast, in the fatty liver cows only trace amounts of the last two lipoprotein components were observable in this density range; however, the very large particles, with the mean radius of 9.92 nm, became more prominent. HDL from control cows comprises two subpopulations of mean particle radius 6.49 and 5.03 nm, respectively. The first subpopulation is the major component of bovine HDL. In fatty liver cows this subpopulation has a smaller size with a mean particle radius of 5.88 nm.

Next, the lipoproteins of the d, 1.006-1.21 g/ml, without subfractionation at d, 1.063 g/ml, were analyzed to demonstrate the modifications in total lipoprotein distribution (Fig. 2c). In the control cows, isolated lipoproteins mostly comprised three discrete size subpopulations with a mean radius of 9.92, 6.52 and 5.05 nm. When the results of the control and severe fatty liver groups were compared, one of the subpopulations (with a mean radius of 6.52 nm in control cows) was clearly shifted toward smaller particles.

To separate apo A-I-containing lipoproteins from apo B-containing lipoproteins the fraction of the d, 1.006-1.21 g/ml from control and severe fatty liver cows were subfractionated by affinity chromatography on heparin-Sepharose. The pattern of size distribution of this last population (Fig. 2d) was similar to those of d, 1.006-1.21 g/ml fraction (Fig. 2c) in the range of small particles, in a given group of cows. The large particles, corresponding to apo B-containing lipoproteins, were not present in this population. The differences observed between the control and the severe fatty liver group were present in both preparations. Apo B-containing lipoproteins, eluted from the heparin-Sepharose, contained, in both animal groups, only one population of large particles (not shown). Particles of the smallest size were either not detected or were found only in trace quantities in this fraction.

The immunolocalization study of apo A-I-containing particles of lipoproteins in the d, 1.006-1.21 g/ml is shown in Figure 3. The immunoblot results are in good agreement with the gradient gel analysis. The modification of lipoprotein distribution in fatty liver

TABLE 2

Plasma Levels and Percent Content of Components in the LDL (d, 1.006-1.063 g/ml) and HDL (d, 1.063-1.21 g/ml) Ultracentrifugal Fractions Isolated from Control and Severe Fatty Liver Cows

	LDL		HDL	
Fraction	Control	Severe fatty liver	Control	Severe fatty liver
		mg/dl		
Fraction plasma level[a]	47.3 ± 2.9[b]	25.9 ± 1.6[c]	292.1 ± 14.1	233.8 ± 12.4[c]
		%		
Triglycerides	11.4 ± 0.2[d]	17.2 ± 0.3[c]	0.4 ± 0.1	0.5 ± 0.1
Esterified cholesterol	30.0 ± 0.6	29.7 ± 0.4	32.4 ± 0.6	31.0 ± 0.4
Free cholesterol	6.2 ± 0.2	7.0 ± 0.2[c]	5.8 ± 0.2	5.8 ± 0.2
Phospholipid	20.1 ± 0.3	17.2 ± 0.3[c]	25.7 ± 0.4	24.1 ± 0.5[c]
Protein	32.3 ± 0.3	28.9 ± 0.3[c]	35.7 ± 0.4	38.6 ± 0.7[c]

[a]Expressed as sum of components: cholesterol, phospholipids, triglycerides and protein.
[b]Means ± standard error of six samples per group.
[c]Significant difference from control cows, P<0.05.
[d]Four samples per group.

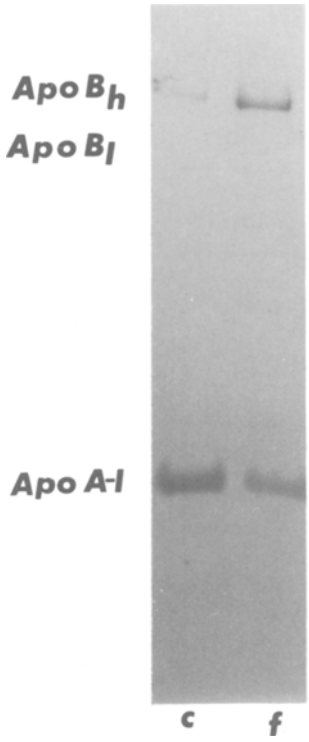

FIG. 1. SDS-polyacrylamide 4–20% gradient gel electrophoresis of the apoproteins of ultracentrifugally isolated LDL, d, 1.006–1.063 g/ml fraction (20 μg of protein) from representative individual control (c) and severe fatty liver cows (f).

cows was consistent with that of major subpopulation of apo A-I-containing lipoproteins.

DISCUSSION

We have recently described a previously undocumented association between severe fatty liver in dairy cows in early lactation and a reduction in plasma LDL, d, 1.006–1.063 g/ml fraction (6). In the present study, the depressed level of the LDL in severe fatty liver cows was associated with a decrease in plasma concentration and the size of apo A-I-containing lipoproteins. We conclude that the decrease in the LDL in severe fatty liver cows results, at least in part, from a decrease in apo A-I-containing particles, which can be isolated in the LDL density range. This modification is due to a change in distribution of apo A-I-containing lipoprotein subpopulations between LDL and HDL.

Puppione *et al.* (24) have shown that a considerable physical and chemical heterogeneity exists among bovine α-lipoproteins. The majority of bovine α-lipoproteins can be isolated in the ultracentrifugal density fraction referred to as the HDL (d, 1.063–1.21 g/ml) which contains apo A-I as the major apoprotein. In comparison with human plasma, the size and density of the major component of bovine HDL has been reported to be similar to HDL_2 (11, 25). Using gradient gel electrophoresis, human HDL_2 is separable into at least two subpopulations, HDL_{2a} and HDL_{2b}, with a mean particle radius of 4.6 and 5.3 nm, respectively. In general, in humans, particles of sizes 4–5.4 nm constitute the bulk (over 95%) of plasma apo A-I containing particles (26). Comparison of the size distribution of bovine and human HDL by gel filtration chromatography has demonstrated that approximately half of bovine HDL is larger than human HDL (27). Our results confirm the previous observations and show that the lactating cow has at least two subpopulations of HDL. One of these subpopulations, of mean radius of 6.49 nm, is larger than human HDL and constitutes the major component of bovine HDL.

In fact, analysis of total lipoproteins, without subseparation of LDL and HDL, provides evidence for the presence of two different subpopulations of α, apo A-I-containing lipoproteins in lactating cows, with a mean radius of 6.52 and 5.05 nm, respectively. The subpopulation with mean radius of 6.52 nm appears as the major component of α-lipoproteins and part of them can be isolated in the LDL density range. According to previous observations (11), the lactating cow has a relatively high concentration of this unusually large, α-lipoprotein.

The larger size of bovine α-lipoproteins may be the result of the particularities of lipoprotein metabolism in these animals. The action of LCAT and the lack of hepatic lipase in this species, are the major factors involved in the increased size of these lipoproteins (11). The relatively high concentration of oversized α-lipoproteins in lactating cows appears to be the consequence of extensive lipolysis of triglyceride-rich lipoproteins. The transfer of lipids and apoproteins between lipoproteins is known to be accompanied by the intravascular lipolysis of triglyceride-rich lipoproteins by LPL. Their surface lipids and soluble apoproteins move to the HDL, transforming them into larger particles (28).

In severe fatty liver cows the subpopulation of larger apo A-I-containing lipoproteins was clearly displaced to smaller particles which, in turn, leads to a decrease in the level of the LDL fraction. This appears to result from the decreased supply of triglyceride-rich lipoprotein surface components for the production of large α particles as a consequence of the reduced synthesis or secretion of VLDL by the liver in these animals. The VLDL fraction was not studied in the present work. Determination of this fraction provides little information on lipid metabolism in dairy cows since VLDL's have a much greater turnover rate than other lipoprotein fractions and their plasma level is very low (27,29). On the other hand, reduction and changes in distribution of apo B-containing lipoproteins are also possible and await further investigations. Apo B-containing particles in bovines can be isolated in VLDL, IDL and LDL fractions, but their level is relatively low, which corresponds to the low level of plasma triglycerides. Additionally, their distribution among the various density ranges depends on nutritional factors and on the conditions of their isolation (11). It has recently been demonstrated (30,31) that a part of VLDL could change density range when the lipoproteins are isolated at a temperature lower than 37°C. The relatively high percentage of triglycerides observed in the LDL fraction isolated in our study may be explained by this phenomenon. Recent studies conducted in our laboratory using a direct immunological estimation of apo B support the hypothesis of a decrease in apo B levels in

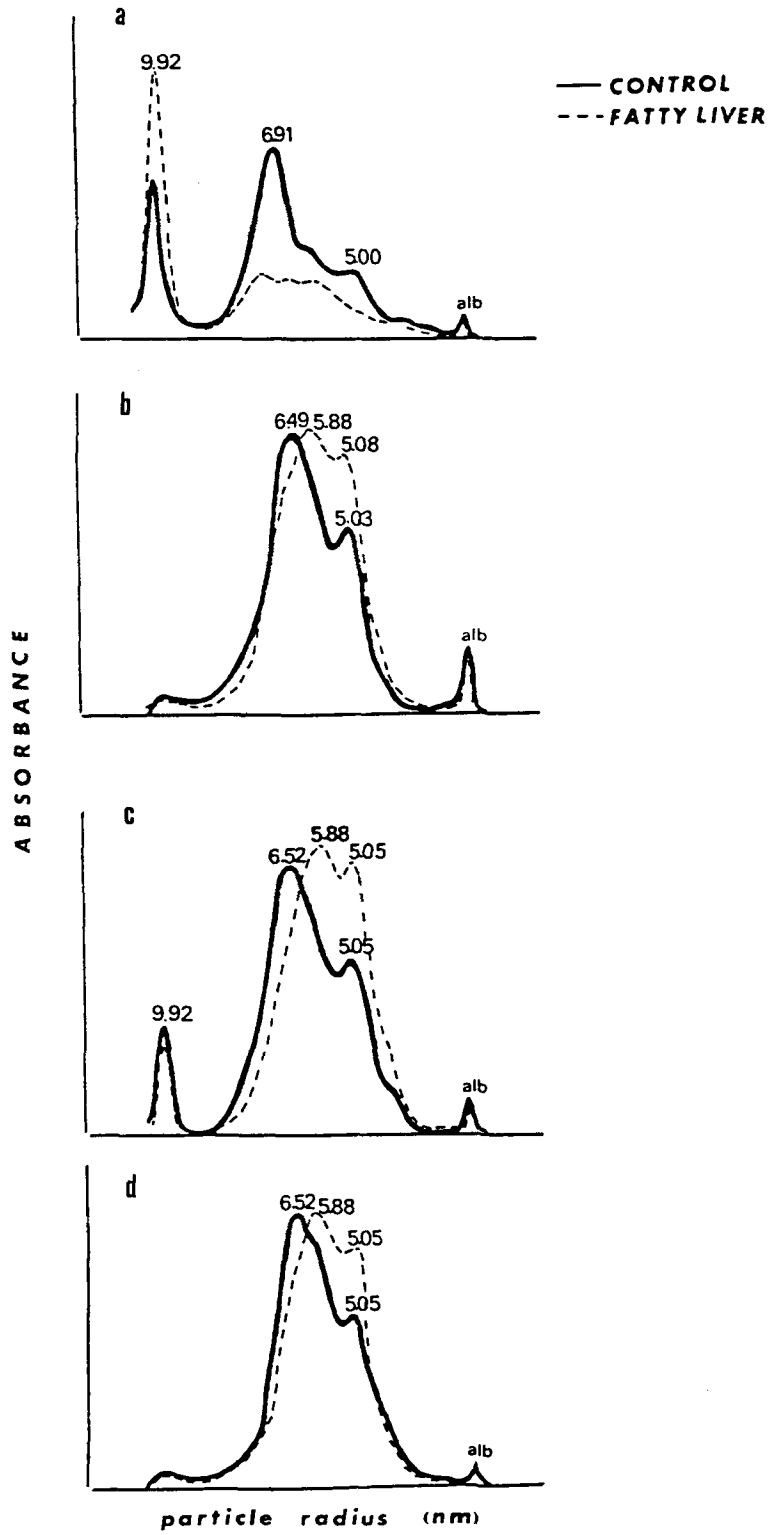

FIG. 2. Densitometric scans of 4-30% nondenaturing gradient gel electrophoresis of lipoprotein fractions from control and severe fatty liver cows. a: LDL (d, 1,006-1,063 g/ml); b: HDL (d, 1,063-1,21 g/ml); c: Lipoproteins of d, 1,006-1,21 g/ml; d: α-lipoproteins isolated from lipoproteins of d, 1,006-1,21 g/ml by heparin-sepharose chromatography.

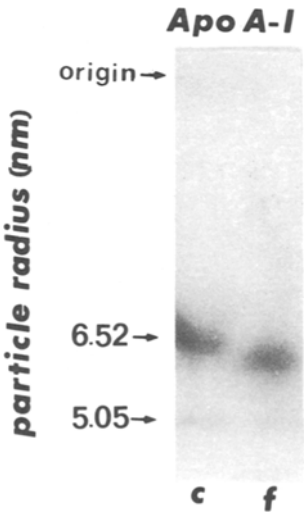

FIG. 3. Immunolocalization of apo A-I. Autoradiograph of immunoblot of lipoproteins (d, 1.006-1.21 g/ml) from control (c) and severe fatty liver cows (f) electrophoresed on 4-30% polyacrylamide gradient gel. Molecular weights were estimated from calibration of the gel with high molecular weight protein standards.

fatty liver cows. The study of large populations of dairy cows in early lactation indicates that severe fatty liver is associated with a decreased level of plasma apo B (about 30%) (unpublished data).

If the observed modifications in lipoproteins result from a decreased supply of triglyceride-rich lipoproteins the question arises: why do cows which develop fatty liver often have no depressed milk yield and milkfat level? One possible explanation of stable milk production is that these animals use the FFA for lipid synthesis in the mammary gland in greater proportion than the control cows. The origins of milk fat in cows are varied (32). Approximately 50% of fatty acids are synthesized *de novo* in the mammary gland (from acetate and butyrate) and blood fat usually contributes about 50% of the fatty acids in milk. Fatty acids released from adipose tissue can be directly taken up by the mammary gland or can be reformed into lipoprotein triglyceride. The long-chain fatty acids are obtained from triglycerides by the action of LPL on triglyceride-rich lipoproteins. Triglycerides are the only plasma lipid class that can be taken up by the mammary glands of ruminant animals on an adequate plane of nutrition and the metabolic importance of VLDL as a primary source of lipids, particularly in lactating animals, has already been established (32). In an abnormal nutritional state, such as fasting, the gland could enhance FFA uptake from the blood (32). In early lactation and especially in fatty liver cows, a marked increase in the plasma concentration of FFA has been reported (3,5,33), and was also observed in our study.

Finally, the present work demonstrates a considerable reduction in the level and the size of apo A-I-containing lipoproteins in severe fatty liver cows. This modification appears to be a consequence of the reduced availability of triglyceride-rich lipoproteins. HDL subpopulations in humans and rats have been shown to have differing metabolic roles (34-36) although this remains to be elucidated in bovines. Further studies are required to determine the origin and fate of apo A-I-containing lipoprotein subpopulations in the dairy cow.

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Platelet-Activating Factor Regulates Phospholipid Metabolism in Human Neutrophils

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This study extended the earlier finding that platelet-activating factor (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) promotes arachidonic acid incorporation into neutrophil phosphatidylinositol (PI) and phosphatidylcholine (PC). In the present study the effect of PAF on fatty acid uptake by human neutrophils and the incorporation of extracellular linoleic acid and palmitic acid into phospholipids were investigated. Incubation of 10^{-7} M PAF with neutrophils and radiolabeled arachidonic acid or linoleic acid or palmitic acid for 1–10 min resulted in an increased rate of loss of label from the incubation medium. PAF stimulated the incorporation of linoleic acid and palmitic acid most significantly into PI and PC. The magnitude of stimulation was greater in PI than in PC for the incorporation of linoleic acid, and vice versa for the incorporation of palmitic acid. The positional distribution of linoleic acid and palmitic acid in PI and PC and the mass of these phospholipids were not altered in PAF-stimulated neutrophils. An increased incorporation of all three fatty acids into both diacyl and alkylacyl species of PC was demonstrated after a two minute incubation of cells with PAF. While more radioactivity was recovered in the diacyl species, the magnitude of increase of radioactivity in the alkylacyl species was more pronounced than that in the diacyl species of PC. These results suggest that both increased fatty acid uptake and increased available lysophospholipids may be contributory to the increased phospholipid acylation induced by PAF.
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Platelet-activating factor (PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is synthesized in various tissues and stimulated blood cells including neutrophils (1). It has been shown that the major portion of PAF synthesized by stimulated neutrophils is either released into the extracellular medium (2–5) or remains membrane bound (6,7). High affinity binding of PAF receptors by exogenous PAF having been identified on neutrophil cell membranes (8,9), PAF release into the extracellular medium could occur *in vivo*.

Investigations on the biological effects of PAF on neutrophil metabolism were performed largely in the absence of extracellular fatty acids. However, the concentrations of free fatty acids in the plasma have been estimated to be about 0.5 μeq/ml in the basal state (10), and neutrophils have been shown to be capable of incorporating free fatty acids bound to albumin into complex lipids (11). For this reason, it appears to be important to examine the effect of PAF on the uptake and incorporation of extracellular fatty acids into phospholipids by neutrophils. It was demonstrated earlier (12) that PAF promotes the incorporation of arachidonic acid into PI and PC by human neutrophils. However, it has not been established whether the increased phospholipid acylation by exogenous fatty acids is secondary to increased fatty acid uptake by PAF-stimulated neutrophils. The present study demonstrated that PAF stimulates the uptake of not only arachidonic acid but also of linoleic and palmitic acid by human neutrophils. It also demonstrated that PAF stimulates the incorporation of all three fatty acids into phosphatidylinositol (PI) and both diacyl and alkylacyl phosphatidylcholine (PC).

MATERIALS AND METHODS

Preparation of human neutrophils. Human blood was obtained from healthy donors who had received no medication in the previous two weeks. Each 33 ml portion of venous blood was mixed with 333 units of sodium heparin (Sigma Chemical Co., St. Louis, MO) in 1 ml of 0.9% NaCl and 5 ml of 5% dextran T500 (Pharmacia LKB, Piscataway, NJ) in 0.9% NaCl. The mixture was allowed to sediment at room temperature for 30 min. A leukocyte-rich plasma was removed and centrifuged at 250 × g for 10 min at 20°C. The cell pellet obtained from each 100 ml blood was washed once with 20 ml of 0.9% NaCl and resuspended in 10 ml of the same solution. Contaminated erythrocytes were lysed by adding 30 ml of ice-cold water to each 10 ml of cell suspension. After inversion of the tube for 15 sec, the isotonicity was restored by adding 10 ml of ice-cold 3.4% NaCl to the tube. After centrifuging the mixture at 250 × g for five minutes at 20°C, the cell pellet from 100 ml blood was resuspended in 12 ml Dulbecco's phosphate buffered saline (PBS) (without Ca^{2+} and Mg^{2+}, GIBCO, Grand Island, NY). Each 6 ml cell suspensions were layered on 5 ml Lymphocyte Separation Medium (Litton Bionetics, Charleston, SC) in a 15 ml Falcon tube and centrifuged at 400 × g for 20 min at 20°C. The resulting neutrophil pellet was washed twice each with 20 ml PBS and once with 20 ml Dulbecco's balanced salt solution (DBSS) (13) containing 5 mM glucose. It was finally resuspended in DBSS containing 5 mM glucose at a concentration of 20×10^6 cells/ml. Cell counts were made in a hemocytometer, and cell viability was measured by trypan blue exclusion. Cell preparations contained more than 95% neutrophils.

Incubation of cells. Each radiolabeled fatty acid (New England Nuclear Corp., Boston, MA) was suspended in 0.9% NaCl containing fatty acid-free bovine serum albumin (4 mg/ml) (Sigma) at a concentration of 38–43 μM. PAF (Avanti Polar Lipids, Birmingham, AL) was suspended in fatty acid-free bovine serum

Abbreviations: DBSS, Dulbecco's balanced salt solution; 5-HETE, 5-hydroxyeicosatetraenoic; LTB_4, leukotriene B_4; MOPS, morpholinopropanesulfonic acid; PAF, platelet-activating factor (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine); PBS, phosphate buffered saline; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; TLC, thin-layer chromatography.

albumin (2.5 mg/ml 0.9% NaCl) at a concentration of 20 μM. Each incubation tube in a final volume of 2 ml contained 0.97 μM (0.1 μCi) [1-^{14}C]arachidonic acid (52.0 Ci/mol) or 1.14 μM (0.125 μCi) [1-^{14}C]linoleic acid (59.0 Ci/mol) or 0.95 μM (0.1 μCi) [1-^{14}C]palmitic acid (56.0 Ci/mol), 10^{-7} M PAF and 20×10^6 cells. An equivalent volume of 0.9% NaCl containing bovine serum albumin (2.5 mg/ml) was included in control tubes. Incubations were started by adding 20×10^6 cells to each tube and were performed at 37°C.

Measurement of fatty acid uptake. Incubations were terminated by adding 5 ml ice-cold PBS containing fatty acid-free serum albumin (4 mg/ml) and 0.2 mM phloretin (Sigma) (stop solution) to each tube. The tubes were immediately centrifuged at 400 × g for three minutes at 4°C. An aliquot of the supernatant solution was removed for counting radioactivity by liquid scintillation spectrometry. In zero time incubations, cell suspension was added after stop solution had been delivered to the tube. Fatty acid uptake is expressed as a percent of total radioactivity remaining in the incubation medium after correction for zero time incubation.

Phospholipid extraction and analysis. Incubations were terminated by adding 5 ml methanol to each tube. Total lipids were extracted according to the method of Bligh and Dyer (14) and dissolved in chloroform/methanol (2:1, v/v) containing 0.01% butylated hydroxytoluene. Individual phospholipids were resolved by two-dimensional thin-layer chromatography (TLC) and analyzed as previously described (15). Since the basal levels of phosphatidic acid from as many as 20×10^6 neutrophils could not be accurately measured by phosphate assay, the radioactivity of individual phospholipids is expressed as dpm/6×10^6 cells.

Hydrolysis of phospholipids by phospholipase A_2. Phosphatidic acid (PA), PI and PC were each extracted from the thin-layer chromatography plate as described previously (15). For the identification of PA on the TLC plates, standard PA from egg yolk lecithin (Sigma) was applied together with neutrophil lipid extract to each TLC plate. Phospholipase A_2 hydrolysis of phospholipids was performed according to the method of Brockerhoff (16), with modification. Each phospholipid (0.5 to 1.5 μg phosphorus) was dissolved in 1.0 ml diethyl ether and was incubated with 10 μl (20 μg protein) porcine pancreatic phospholipase A_2 (Sigma) in the presence of 0.1 ml of 0.1 M MOPS (morpholinopropanesulfonic acid) buffer containing 1 mM $CaCl_2$, pH 7.2. Hydrolysis of phospholipids was carried out for 18 hr at room temperature. It was terminated by adding 0.5 ml of 2% acetic acid in ethanol. The precipitated salt was removed by brief centrifugation and was washed once more with the same solution. The combined ethanol solution was evaporated to dryness, and the lipid residue was redissolved in chloroform-methanol (2:1, v/v) containing 0.01% butylated hydroxytoluene. Fatty acids and lysophospholipids were separated by thin-layer chromatography in a solvent system consisting of chloroform/methanol/28% ammonia (25:10:2, v/v/v). Radioactivity in the fatty acid and lysophospholipid bands was measured by scraping the gel into vials and counting by liquid scintillation spectrometry.

Mild alkali hydrolysis of [^{14}C]palmitic acid-labeled PC. Each [^{14}C]palmitic acid-labeled PC fraction (1.5 μg phosphorus) from resting and PAF-stimulated neutrophils was dissolved in 0.5 ml of chloroform and mixed with 0.5 ml of 0.2 N NaOH in methanol. The hydrolysis was conducted at room temperature for 1 hr as described by Rider *et al.* (17). The samples were then neutralized with 1 N hydrochloric acid and extracted with the solvent system of Bligh and Dyer (15). The resulting fatty acids and ether-linked lysoPC fractions were resolved by TLC, and the radioactivity in each fraction was measured by liquid scintillation spectrometry.

The distribution of labeled fatty acid in the diacyl and alkylacyl PC was analyzed as previously described (15).

RESULTS

Effect of PAF on fatty acid uptake. Previous studies (12) demonstrated a maximum stimulatory effect by PAF at 10^{-7} M on phospholipid acylation by arachidonic acid, hence in the present study, 10^{-7} M PAF was used in all experiments. Table 1 demonstrates that the percent of total radiolabeled fatty acid remaining in the incubation medium was decreased in the presence of PAF, indicating an increased uptake of fatty acids by neutrophils. All three fatty acids tested became rapidly cell associated with a corresponding loss of label from the incubation medium. A wide variation was found among different donors in the rate of loss of label from the incubation medium for all three fatty acids. This was also reported for arachidonic acid uptake by resting rabbit neutrophils (18). However, PAF consistently increased the loss of label from the incubation medium at all time intervals (1–10 min).

Time course of PAF effect on the incorporation of [1-^{14}C]linoleic acid and [1-^{14}C]palmitic acid into phospholipids. Figure 1 demonstrates the time course of the effect of PAF on the incorporation of linoleic acid into phospholipids. PAF inhibited the incorporation of [1-^{14}C]linoleic acid into PA, but it enhanced the incorporation of this fatty acid into PI and PC. In two separate experiments, the average radioactivities of PI and PC were increased to 1980 and 600% of control after 1 and 10 min incubations, respectively. The magnitude of stimulation (percentage of control) on the formation of labeled PI and PC varied with incubation time. The labeling of phosphatidylethanolamine (PE) and phosphatidylserine (PS) by [1-^{14}C]linoleic acid was not influenced by the presence of PAF even after a 10-min incubation. Analysis of the phosphorus content of the major phospholipid classes including PC, PE, PI, PS and sphingomyelin revealed no measurable changes during neutrophil-PAF interaction.

Figure 2 shows the time course of the effect of PAF on the incorporation of [1-^{14}C]palmitic acid into phospholipids. PAF promoted the incorporation of [1-^{14}C]palmitic acid most significantly into PC. In two separate experiments, the average radioactivity of PC was increased to 825 and 134% of control after 1 and 10 min incubations, respectively. An increased incorporation of palmitic acid into PI in the presence of PAF was measurable after a 5-min incubation. The stimulation by PAF of palmitic acid

TABLE 1

Time Course of the Effect of PAF on the Uptake of Fatty Acids by Human Neutrophils

Incubation minutes	Percent of total radiolabeled fatty acid remaining in the incubation medium[a]					
	Arachidonate		Linoleate		Palmitate	
	Control	PAF	Control	PAF	Control	PAF
1	91 ± 3.2	85 ± 3.8	89 ± 3.7	83 ± 4.4	95 ± 3.2	86 ± 4.0
2	78 ± 4.0	65 ± 5.6	75 ± 5.7	64 ± 5.1	85 ± 5.3	72 ± 4.4
5	43 ± 3.5	35 ± 3.2	38 ± 5.5	28 ± 3.0	67 ± 4.7	48 ± 3.9
10	24 ± 2.0	19 ± 1.7	15 ± 1.8	12 ± 1.5	29 ± 4.0	20 ± 3.7

[a]Neutrophils (20×10^6) were incubated at the indicated period of time with 0.1 μCi [1-^{14}C]arachidonic acid (0.97 μM), or 0.125 μCi [1-^{14}kC]linoleic acid (1.14 μM), or 0.1 μCi palmitic acid (0.95 μM) in the absence or presence of 10^{-7} M PAF. Incubation conditions and measurement of fatty acid uptake were described in Materials and Methods. The data are means ± SD from four separate experiments.

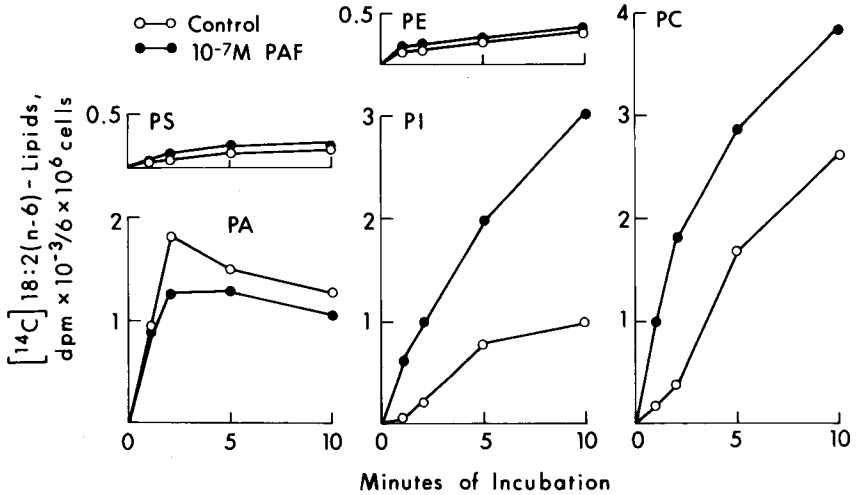

FIG. 1. Time course of the effect of PAF on the incorporation of [1-^{14}C]linoleic acid (18:2 [n-6]) into phospholipids by human neutrophils. Neutrophils (20×10^6) were incubated at the indicated period of time (1-10 min) with 0.125 μCi [1-^{14}C]linoleic acid in the absence (○) or presence (●) of 10^{-7} M PAF. Each point represents the average value of duplicate incubations from two separate neutrophil preparations. PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine.

incorporation into PA was less pronounced than that into PC. After a 1-min incubation the radioactivity of PA was increased to 153% of control, but it returned to basal level after a 10-min incubation.

Positional distribution of radiolabeled linoleic acid and palmitic acid in phospholipids. Extracellular linoleic acid and palmitic acid have been found to be incorporated by neutrophils into both *sn*-1 and *sn*-2 positions of PC (19,20). If the increased incorporation of these fatty acids into PI and PC in the presence of PAF is caused only by increased phospholipase A_2 activity, then the percentage of labeled fatty acids in the *sn*-2 position of phospholipids from PAF-stimulated cells would be greater than that from control cells. The results show that the positional distribution of neither labeled fatty acid in PA, PI and PC was affected by the presence of PAF. After treatment of each phospholipid with pancreatic phospholipase A_2, 79, 92 and 74% of radiolabeled linoleic acid was released from PA, PI and PC, respectively. 2-Min and 10-min incubations exhibited identical positional distribution of radiolabeled linoleic acid for each phospholipid.

The positional distribution of radiolabeled palmitic acid in phospholipids was found to vary with incubation time. After a 2-min incubation, 72, 85 and 78% of radiolabeled palmitic acid in PA, PI and PC, respectively, were found in the *sn*-2 position, whereas after a 10-min incubation the radiolabeled palmitic acid in the *sn*-2 position became 60, 67 and 53% in PA, PI and PC, respectively. Phospholipids isolated from PAF-stimulated neutrophils demonstrated a similar positional distribution for both fatty acids.

Effect of PAF on the distribution of labeled fatty acids in diacyl and alkylacyl PC. Neutrophil PC has a high content of alkylacyl species (21,22), and the *sn*-2 position of endogenous alkylacyl PC contains linoleic acid and palmitic acid besides arachidonic acid (21). Thus it is important to examine whether PAF alters the distribution of labeled fatty acids in the diacyl and alkylacyl PC. The results showed that PAF stimulated the incorporation of extracellular fatty acids into both diacyl and alkylacyl PC. While more radioactivity was

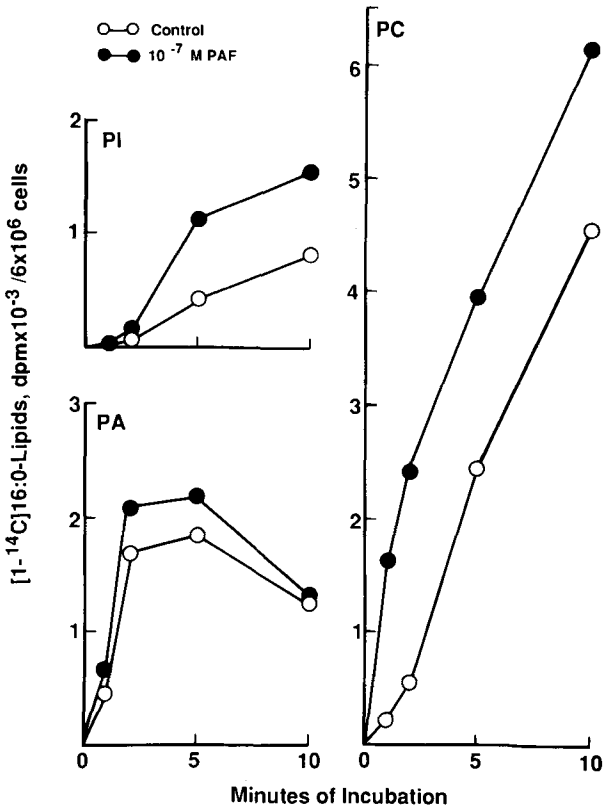

FIG. 2. Time course of the effect of PAF on the incorporation of [1-^{14}C]palmitic acid (16:0) into phospholipids by human neutrophils. Neutrophils (20 × 10^6) were incubated at the indicated period of time (1-10 min) with 0.1 [1-^{14}C]plamitic acid in the absence (○) or presence (●) of 10^{-7} M PAF. Each incubation represents the average value of duplicate incubations from two separate neutrophil preparations. PA, phosphatidic acid; PC, phosphatidylcholine; PI, phosphatidylinositol.

recovered in the diacyl species, the magnitude of increase of radioactivity in the alkylacyl species was more pronounced than that in the diacyl species.

Earlier studies (12) only demonstrated an increased incorporation of labeled arachidonic acid into the diacyl species of PC after a 1-min incubation of neutrophils with PAF. The present study (Table 2) demonstrated that after a longer period of incubation, PAF stimulated the incorporation of arachidonic acid into both diacyl and alkylacyl PC. Analysis of the formation of arachidonic acid-labeled diacyl and alkylacyl species at 2 and 10 min indicated that the increment of arachidonic acid labeled diacyl species induced by PAF was more evident after two min than after 10 min of incubation. The formation of labeled alkylacyl species was not further increased at 10 min in the presence of PAF.

As shown in Table 3, in resting neutrophils exogenous linoleic acid was mainly incorporated into diacyl PC and only about 3% of the total radioactivity in PC was recovered in the alkylacyl species after a 10 min incubation. In PAF-stimulated neutrophils there were about 39% and 23% of the total radioactivity in PC being recovered in the alkylacyl species after 2 and 10 min incubations, respectively. The increment in the formation of linoleic acid-labeled alkylacyl PC induced by PAF appeared to have reached a maximum before 10 min, as alkylacyl PC exhibited similar radioactivity after incubation of cells with PAF for 2 and 10 min. In the presence of PAF, the radioactivity of linoleic acid-labeled diacyl PC became 400% of control at two minutes, but it remained the same as that in control cells at 10 min.

The effect of PAF on the distribution of labeled palmitic acid in the diacyl and alkylacyl PC is shown in Table 4. In resting neutrophils 20 and 12% of total PC radioactivity were recovered in the alkylacyl spe-

TABLE 2

Effect of PAF on the Distribution of [1-^{14}C]Arachidonic Acid in Diacyl and Alkylacyl Phosphatidylcholine (PC)

Incubation minutes	Radioactivity (dpm × 10^3)[a]			
	Diacyl PC		Alkylacyl PC	
	Control	PAF	Control	PAF
2	0.81 ± 0.2	2.12 ± 0.3	0.11 ± 0.02	0.55 ± 0.1
10	2.52 ± 0.4	3.0 ± 0.3	0.34 ± 0.1	0.75 ± 0.1

[a]Neutrophils (20 × 10^6) were incubated with 0.1 μCi [1-^{14}C]arachidonic acid (0.97 μM) for 2 and 10 min in the absence and presence of 10^{-7} M PAF. Each PC fraction isolated from 60 × 10^6 cells was resolved into diacyl and alkylacyl species as described in Materials and Methods. The radioactivity in each species was derived from 1.5 μg phosphorus of PC, and represents the mean ± SD from three experiments.

TABLE 3

Effect of PAF on the Distribution of [1-^{14}C]Linoleic Acid in Diacyl and Alkylacyl Phosphatidylcholine (PC)

Incubation minutes	Radioactivity (dpm × 10^3)[a]			
	Diacyl PC		Alkylacyl PC	
	Control	PAF	Control	PAF
2	0.78 ± 0.2	3.0 ± 0.4	0.02 ± 0.01	1.8 ± 0.3
10	5.2 ± 0.3	5.4 ± 0.4	0.19 ± 0.02	2.0 ± 0.2

[a]Neutrophils (20 × 10^6) were incubated with 0.125 μCi [1-^{14}C]arachidonic acid (1.14 μM) for 2 and 10 min in the absence and presence of 10^{-7} M PAF. Each PC fraction isolated from 60 × 10^6 cells was resolved into diacyl and alkylacyl species as described in Materials and Methods. The radioactivity in each species was derived from 1.5 μg phosphorus of PC, and represents the mean ± SD from three experiments.

TABLE 4

Effect of PAF on the Distribution of [1-^{14}C]Palmitic Acid in Diacyl and Alkylacyl Phosphatidylcholine (PC)

Incubation minutes	Radioactivity (dpm × 10^3)[a]			
	Diacyl PC		Alkylacyl PC	
	Control	PAF	Control	PAF
2	1.0 ± 0.2	4.2 ± 0.6	0.28 ± 0.02	2.5 ± 0.4
10	9.2 ± 1.2	11 ± 1.5	1.28 ± 0.4	3.4 ± 0.5

[a]Neutrophils (20 × 10^6) were incubated with 0.1 μCi [1-^{14}C]arachidonic acid (0.97 μM) for 2 and 10 min in the absence and presence of 10^{-7} M PAF. Each PC fraction isolated from 60 × 10^6 cells was resolved into diacyl and alkylacyl species as described in Materials and Methods. The radioactivity in each species was derived from 1.5 μg phosphorus of PC, and represents the mean ± SD from three experiments.

cies after 2 and 10 min incubation, respectively. In PAF-stimulated cells, a 2-min incubation with [1-^{14}C]palmitic acid resulted in an increase of labeled alkylacyl PC to 772% of control and of labeled diacyl species to 352% of control. After 10 min incubation of cells with PAF and [1-^{14}C]palmitic acid, the radioactivity of alkylacyl PC was increased to 264% of control, and that of diacyl species became 122% of control.

Mild alkaline hydrolysis of palmitic acid-labeled PC. This experiment was carried out to examine if the radioactivity in palmitic acid-labeled alkylacyl PC was solely derived from the acyl moiety and not from the alkyl moiety. After deacylation of PC with methanolic NaOH, the resulting fatty acids and 1-alkyl-*sn*-glycero-3-phosphocholine (lysoPAF) were separated by TLC. It was found that the radioactivity was completely recovered in the fatty acid fraction and no radioactivity was detected in ether-linked lysophosphatidylcholine.

DISCUSSION

The present study extended the earlier studies (12), and demonstrated that PAF stimulates the uptake of fatty acids from extracellular medium. It also demonstrated that PAF increases the incorporation of linoleic acid and palmitic acid into PI and PC. In addition, the present study illustrated that PAF enhances the incorporation of extracellular fatty acids into both diacyl and alkylacyl PC.

In the absence of competing labeled fatty acids, human neutrophils did not appear to exhibit a preferential uptake of arachidonic acid. Linoleic acid and palmitic acid also rapidly became cell associated. The mechanism by which fatty acid enters into neutrophils has not yet been defined. It may be a transport process or a simple diffusion. Due to the rapid incorporation of fatty acids into phospholipids, it is difficult to separate the uptake from activation and esterification of fatty acids under the experimental conditions. However, the finding that PAF promotes the loss of labeled fatty acids from the incubation medium suggests an increased fatty acid uptake during PAF-neutrophil interaction. It is not known whether the increased fatty acid uptake is specific for PAF among the agonists for neutrophil activation. There is evidence for increased PAF formation in neutrophils in response to formyl-methionyl-leucyl-phenylalanine (1,7), leukotriene B_4 (23), ionophore A23187 (24), and phorbol esters (25). Interpretation of fatty acid uptake by neutrophils in the presence of any one of these agonists would be complicated by increased PAF formation under these conditions. Parallel studies on fatty acid uptake and PAF formation would be required to ascertain the specific effects of these agonists on fatty acid uptake.

The magnitude of stimulation by PAF on the formation of labeled PI and PC appears to reflect the specificity of the acyltransferases catalyzing the acylation of the respective lysophospholipids. It was greater in PI than in PC for the incorporation of linoleic acid and vice versa for the incorporation of palmitic acid. Increased phospholipid acylation could be brought about by increased: (a) fatty acid uptake; (b) phospholipase A_2 activity; (c) fatty acyl-CoA synthetase activity; and (d) acyltransferase activity. If the increased phospholipid acylation induced by PAF is solely secondary to increased phospholipase A_2 activity, the percentage of radioactivity in the *sn*-2 position of PI and PC from PAF-stimulated cells would be greater than that from control cells. However, PAF was not found to alter the positional distribution of labeled fatty acids in PI and PC after incubation of neutrophils for either 2 or 10 min. Since there were no detectable changes in the mass of PI and PC during PAF-neutrophil interaction, both increased fatty acid uptake and increased available lysophospholipids may be contributory to the increased phospholipid acylation by exogenous fatty acids. Indeed, an activation of phospholipase A_2 by exogenous PAF was demonstrated in cytocholasin B-primed rabbit neutrophils (26). The incorporation of exogenous palmitic acid into the *sn*-2 position of PC was also reported in human neutrophils (20). The present study demonstrated a variation of the positional distribution of palmitic acid in phospholipids with incubation time. More labeled palmitic acid being recovered in the *sn*-2-position after a 2-min incubation may indicate a higher basal level of 1-acyl-2-lysophospholipids than that of 1-lyso-2-acyl-phospholipids in neutrophils, and more labeled palmitic acid being recovered in the *sn*-1 position after a longer period of incubation may indicate a shift to a *de novo* pathway.

Although neutrophil alkylacyl PC has a high content of linoleic and palmitic acid (21), rabbit neutrophils incorporated these two fatty acids only into the diacyl species (20). The present study also demonstrated that resting human neutrophils incorporated linoleic acid mainly into the diacyl species of PC. Although the present study demonstrated that resting human neutrophils incorporated a significant amount of exogenous palmitic acid into alkylacyl PC, the incorporation of this fatty acid into alkylacyl PC is expected to be attenuated by the presence of arachidonic acid in the incubation medium. The increased incorporation of fatty acids into alkylacyl PC in PAF-stimulated neutrophils could be due to increased lysoPAF. LysoPAF can be derived from endogenous alkylacyl PC after an activation of phospholipase A_2 during PAF-neutrophil interaction; it can also be derived from PAF after deacetylation (27).

An increased incorporation of exogenous fatty acids into PI and PC by neutrophils in response to PAF may serve to replenish these phospholipids following deacylation. This may also divert exogenous arachidonic acid from the 5-lipoxygenase pathway, thereby attenuating the formation of leukotriene B_4 (LTB_4) and 5-hydroxyeicosatetraenoic acid (5-HETE), both of which are mediators of inflammation (28,29). Furthermore, increased acylation of lysoPAF induced by PAF would allow a rapid termination of further synthesis of PAF, as it has been shown that PAF stimulates its own synthesis in human neutrophils (23,30). It remains to be tested if exogenous palmitic acid and linoleic acid are more effective than arachidonic acid in attenuating PAF synthesis by PAF-stimulated neutrophils, since activated neutrophils may metabolize part of the exogenous arachidonic acid to LTB_4 and 5-HETE, both of which have been shown to increase PAF synthesis (23).

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Polyphosphoinositide Formation in Isolated Cardiac Plasma Membranes

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Phosphatidylinositol (PtdIns) and phosphatidylinositol 4-phosphate (PtdIns4P) kinase activities in plasma membranes isolated from canine left ventricle were partially characterized, and their sensitivity to a number of intracellular variables was established. PtdIns and PtdIns4P kinase activities were estimated by the formation of [^{32}P]PtdIns4P and [^{32}P]phosphatidylinositol 4,5-*bis*phosphate ([^{32}P]PtdIns(4,5)P_2), respectively, when membranes were incubated with [γ-^{32}P]ATP and 0.1% Triton X-100. Unlike [^{32}P]-PtdIns4P formation, [^{32}P]PtdIns(4,5)P_2 formation required exogenous (PtdIns4P) substrate. [^{32}P]-PtdIns4P and [^{32}P]PtdIns(4,5)P_2 formation were insensitive to Ca^{2+} at concentrations ranging from 0.1-30 μM. The hydrolysis of [^{32}P]PtdIns4P was less than 15% under standard assay conditions for measuring its formation, and was unaffected by any of the variables tested. The apparent Km of the PtdIns kinase for ATP was 53 $\pm$ 13 (S.E.M.) μM (N=3). ADP inhibited [^{32}P]PtdIns4P formation competitively with respect to ATP, the Ki being 0.4 mM. The data indicate that ADP is a poor competitive inhibitor of PtdIns kinase at the concentrations which are believed to be present intracellularly normally or which may be attained during mild hypoxia provided ATP levels are maintained in the millimolar range. Hence, any response of the myocardium to α-adrenergic hormones during mild hypoxia would be largely unimpaired by effects of Ca^{2+} on PtdIns and PtdIns(4,5)P_2, or of ADP on PtdIns kinase activity.
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α-adrenergic hormones, which produce a positive inotropic action in the intact heart (1), increase Ins(1,4,5)P_3 formation in isolated cardiomyocytes (2,3). Little information exists on the cardiac enzymes involved in phosphoinositide turn-over other than the known presence of PtdIns and PtdIns4P kinases in the tissue (4,5). We have previously characterized cardiac PI synthase activity, which is present on the SR membranes, and found the synthase to be inhibited by Ca^{2+}, half-maximal inhibition occurring at about 10 μM (6). A recent report indicates that Ca^{2+} activates sarcolemmal membrane phospholipase C (7). Some reports have implicated Ca^{2+} in the regulation of PtdIns kinase activity in a number of tissues (8-11).

In the present communication, we partially characterize the PtdIns4P and PtdIns(4,5)P_2 kinase and phosphoesterase activities present in a highly enriched cardiac plasma membrane preparation (12). We show that these enzyme activities are insensitive to μM Ca^{2+} when measured under conditions where saturating ATP levels are maintained. Our data, furthermore, suggest that ADP, a competitive inhibitor of [^{32}P]PtdIns4P formation, is not likely to be an important regulator of PI kinase when present at intracellular levels estimated to occur normally or during mild hypoxia when ATP levels remain in the mM range.

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Abbreviations: PtdIns, phosphatidylinositol; PtdIns4P, phosphatidylinositol 4-phosphate; PtdIns(4,5)P_2, phosphatidylinositol (4,5)-*bis*phosphate; Ins(1,4,5)P_3, inositol (1,4,5)-trisphosphate; PS, phosphatidylserine; PC, phosphatidylcholine; SR, sarcoplasmic reticulum; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

EXPERIMENTAL PROCEDURES

Materials. Enzyme grade sucrose was obtained from Schwarz-Mann Biotech (Cambridge, MA). Disodium ATP, alamethicin, phosphoinositide mixture (sodium salt) and all other phospholipids were obtained from Sigma Chemical Corp. (St. Louis, MO). [γ-^{32}P]ATP triethylammonium salt (13-40 Ci/mmol) was obtained from Amersham Corp. (Arlington Heights, IL). Polyethylenimine-coated plastic sheets (Polygram CEL PEI) were obtained from Brinkman Instruments, Inc. (Westbury, NY). All organic solvents used in this study were of high performance liquid chromatography grade and were obtained from Fisher Scientific (Springfield, NJ).

Preparation of cardiac plasma membranes and assay of marker enzyme activities. Purified cardiac plasma membranes were prepared from canine left ventricle and characterized with respect to marker enzyme activities as described by Jones and Besch (13). This membrane preparation will be referred to as plasma membranes instead of sarcolemmal membranes in view of the report by Tomlins *et al.* (14), which indicates the presence of some plasma membranes derived form endothelial cells in a preparation of sarcolemmal membranes obtained from rat heart. Protein was determined by the biuret method using bovine serum albumin as a standard. UDP-galactose:*N*-acetylglucosamine galactosyl transferase activity, used as a marker for Golgi membranes, was measured by the method of Fleischer and Smigel (15).

Assay of [^{32}P]PtdIns4P and [^{32}P]PtdIns4P$_2$ formation. To assay [^{32}P]PtdIns4P formation, membranes (0.2 mg/ml) were incubated in polypropylene tubes at 25°C in 50 μl of a standard assay medium consisting of 40 mM histidine-HCl, pH 6.8, 5 mM $MgCl_2$, 2 mM [γ-^{32}P]ATP at a specific radioactivity of 0.16 Ci/μmol, 1.0 mM EGTA, and 0.1% Triton X-100. Reactions were started by the addition of membranes to the temperature equilibrated medium. The reactions were stopped at the times indicated in the text by adding 300 μl of ice-cold chloroform:methanol (1:1), containing 90 mM HCl (acidified chloroform-methanol) and vortexing the tubes for 30 sec. Lipids were extracted and separated by thin layer chromatography and counted, as described below. When alamethicin was used to unmask latent enzyme activity, the membranes were preincubated with alamethicin at room temperature (16). [^{32}P]PtdIns4P formation was measured in the presence of exogenous PtdIns4P in the standard assay medium for [^{32}P]PtdIns4P formation. In some experiments, exogenous phospholipids were included in the assay mixture. The chloroform in which they were dis-

solved was evaporated and the dried phospholipids were suspended in 280 μl of 69 mM histidine-HCl, pH 6.8, by sonication (6) prior to their addition to the reaction medium. To monitor the extent of ATP breakdown, approximately 0.5 μl of reaction mixture was spotted on polyethyleneimine-coated plastic thin layer chromatography sheets 4.5 min after starting the reaction. Adenine nucleotides were separated using 0.75 M KH_2PO_4 as the solvent and identified by autoradiography and UV illumination.

To determine the effect of Ca^{2+} on polyphosphoinositide formation and hydrolysis, membranes were incubated in the standard assay media containing 0.1 to 30 μM (free) Ca^{2+}, which was maintained by $CaCl_2$-EGTA buffers (17). These were prepared by fixing the final $CaCl_2$ concentration at 0.5 mM, and varying the EGTA concentration to give the desired Ca^{2+} concentration. A value of 10^6 M^{-1} was used for the apparent binding constant of Ca^{2+} and EGTA. The binding constant used for Mg^{2+} and ATP^{4-} was 88,000 M^{-1} (18).

To obtain kinetic constants, plasma membranes were incubated as described above, except that the ATP concentration was varied between 0.01 μM and 5 mM. Reaction tubes contained 3.6 x 10^7 cpm [γ-^{32}P]ATP. The counting error for each sample was 2% or less. Kinetic constants were determined from double reciprocal plots of initial rates of [^{32}P]PtdIns4P formation vs ATP concentration. Ki was derived from a secondary plot of the slopes (apparent Km/Vmax) in the presence of inhibitor (ADP) vs inhibitor concentration (19).

Assay of the hydrolysis of endogenous [^{32}P]PtdIns4P. Membrane phospholipids were labeled with ^{32}P in the presence of [γ-^{32}P]ATP, as described in the previous section, except that 0.1% Triton X-100 was omitted. The reaction was stopped after a 20-min incubation by addition of 20 ml of an ice-cold solution containing 10 mM Hepes-HCl, pH 6.8, 0.25 M sucrose, and 1 mM dithiothreitol. Membranes were centrifuged at 170,000 × g for 30 min in a Beckman 50.2Ti rotor. The pellet was suspended in the same solution as before at a protein concentration of 2 mg per ml. [^{32}P]PtdIns4P hydrolysis by plasma membranes was measured using a standard assay medium containing 40 mM histidine-HCl, pH 6.8, 5 mM $MgCl_2$, 1 mM EGTA, and 0.1% Triton X-100. The reactions, carried out at 25°C, were started by the addition of [^{32}P]labeled membranes to a final concentration of 0.2 mg/ml, and stopped by addition of 300 μl of ice-cold acidified chloroform-methanol. The [^{32}P]labeled phospholipids were extracted and separated by thin-layer chromatography (6). For 0-time controls, 300 μl of acidified chloroform-methanol was added to the reaction medium before the addition of [^{32}P]labeled plasma membranes. [^{32}P]labeled lipids were detected on the chromatograms by exposing the plates to iodine vapor and by autoradiography on Kodak X-Omat AR X-ray film for 16 hr at -70°C with the aid of a Dupont Cronex® Lightning Plus intensifying screen. The spots of interest were scraped off the plates and transferred to counting vials. Recovery of [^{32}P]labeled phospholipids from membranes was >99% as determined by addition of [^{32}P]labeled phospholipids to unlabeled membranes in acidified chloroform-methanol, re-extraction, and thin-layer chromatography. Radiolabeled phospholipids were identified by comparison of their R_f values with those of phospholipid standards. The R_f values for PtdIns, PtdIns4P, and PtdIns4P$_2$ were 0.76, 0.54, and 0.32, respectively. The extent of hydrolysis of [^{32}P]PtdIns4P was expressed as the percent of the radioactivity that had disappeared from the [^{32}P]PtdIns4P after incubation for various periods.

RESULTS

Marker enzyme activities of cardiac plasma membrane preparations. Marker enzyme activities in the cardiac plasma membrane preparation used in this study are shown in Table 1. (Na^+ + K^+)-activated ATPase activity, a marker for plasma membranes, was high relative to (K^+ + Ca^{2+})-activated ATPase and azide sensitive ATPase activities, markers for SR and mitochondrial membranes, respectively. These enzyme activities are consistent with those reported by Jones and Besch (13). The contamination of plasma membranes by Golgi membranes was estimated to be 1.8%, taking as 100% the activity reported for rat liver Golgi membranes (300 nmol.mg^{-1}.h^{-1}) (20), and using the same assay conditions (15,20).

TABLE 1

Marker Enzyme Activities in Cardiac Plasma Membrane Preparations[a]

Enzyme	Activity (μmol.mg^{-1}.h^{-1})	% Contamination of plasma membranes
(Na^+-K^+)-ATPase (μmol.mg^{-1}.h^{-1})	106.6 ± 7.8	—
(K^+-Ca^{2+})ATPase (μmol.mg^{-1}.h^{-1})	7.3 ± 2.6	9
Azide-sensitive ATPase (μmol.mg^{-1}.h^{-1})	6.0 ± 1.1	6

[a]Assays were carried out as described by Jones and Besch (13). The percent contamination of plasma membranes was calculated on the basis of 79 μmol Pi.mg^{-1}.h^{-1} and 100 μmol.mg^{-1}.h^{-1} (6) as 100% (K^+-Ca^{2+})-ATPase and azide-sensitive ATPase activities in SR and mitochondrial membrane preparations, respectively.

Establishment of assay conditions for PtdIns and PtdIns4P kinase activities. When the cardiac plasma membranes were incubated with [γ-^{32}P]ATP in a standard reaction medium, ^{32}P was incorporated into [^{32}P]PtdIns4P by PtdIns kinase(s), which utilized endogenous PtdIns as the phospholipid substrate (Fig. 1). A two-fold increase in [^{32}P]PtdIns4P formation was observed upon addition of 20 μM exogenous PtdIns; higher concentrations were inhibitory. [^{32}P]PtdIns4P formation was linear with membrane protein up to a concentration of 0.4 mg/ml for at least two min of incubation (Fig. 1). The inhibition by the higher concentrations of PI was non-specific for PI since all other phospholipids tested produced inhibition to various extents (Table 2). Maximum [^{32}P]PtdIns4P formation occurred at 5-30 mM $MgCl_2$, and varied by less than 15% over this range. No [^{32}P]PtdIns4P formation was detected in the absence of $MgCl_2$.

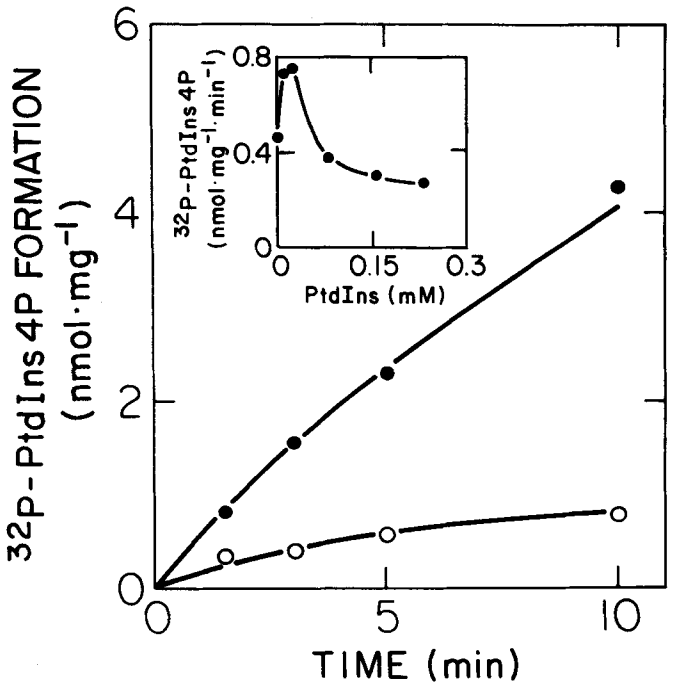


FIG. 1. Time course of [^{32}P]PtdIns4P formation in cardiac plasma membranes. Plasma membranes (0.2 mg/ml) were incubated in the presence (•) and absence (○) of 0.1% Triton X-100 in the standard assay medium including [γ-^{32}P]ATP. Reactions were stopped at the indicated times and [^{32}P]PtdIns4P was extracted and quantitated as described under "Experimental Procedures."

Inset: Concentration dependency of [^{32}P]PtdIns4P formation on exogenous PtdIns.

TABLE 2

Effects of Exogenous Phospholipids on [^{32}P]PtdIns4P Formation in Cardiac Plasma Membranes[a]

Phospholipids	% Inhibition 100 μM	300 μM
PtdIns	31	40
LysoPtdIns	20	62
PS	58	74
LysoPS	24	52
PC	65	66
Sphingomyelin	39	72
PtdIns4P	7	46
PtdIns(4,5)P_2	8	19

[a][^{32}P]PtdIns4P formation was measured in a standard assay medium containing, in addition, the indicated phospholipids at concentrations of 100 and 300 μM. The percentages shown represent the inhibition observed as compared to control incubates which were run with the addition to the reaction mixture of sonicated vehicle alone. Reactions were stopped after 10 min of incubation.

Omission of 0.1% Triton X-100 from the standard reaction medium decreased [^{32}P]PtdIns4P formation to approximately 1/5 of the value obtained in its presence. A concentration of 0.1% Triton X-100 was found to be the lowest concentration necessary to obtain maximal [^{32}P]PtdIns4P formation. Of all of the membrane permeabilizing agents tested, Triton X-100 produced the highest [^{32}P]PtdIns4P formation and was the most effective in preventing hydrolysis of [γ-^{32}P]ATP. At an initial concentration of 2 mM ATP, 2% and 10% of the ATP was hydrolyzed after five minutes of incubation in the presence and absence, respectively, of 0.1% Triton X-100; at 0.2 mM ATP, the corresponding amounts of ATP hydrolyzed were 20% and 45%. Alamethicin pretreatment of membranes or 0.05% saponin was considerably less effective in preserving the ATP concentration. No detectable [^{32}P]PtdIns4P was formed when 0.05% deoxycholate (mg membrane protein:mg deoxycholate, 1:1) or 0.003% sodium dodecyl sulphate was used.

Only negligible [^{32}P]PtdIns(4,5)P_2 formation was detected when the plasma membranes were incubated in the standard assay medium for assaying [^{32}P]PtdIns4P formation (Fig. 2). The addition of exogenous PtdIns did not increase [^{32}P]PtdIns(4,5)P_2 formation. However, when 0.25 mM exogenous PtdIns4P was included in the reaction medium, [^{32}P]PtdIns(4,5)P_2 formation increased to 0.37 ± 0.02 nmol.mg.$^{-1}$.5 min^{-1} (mean of three membrane preparations ± S.E.M.). [^{32}P]PtdIns(4,5)P_2 formation was maximum at about 0.25 mM exogenous PtdIns4P, and decreased slightly at the higher concentrations tested. No [^{32}P]labeling of polyphosphoinositides was observed in heat-denatured membranes. Approximately 90% of the apparent PtdIns and PtdIns4P kinase activities was retained upon storage of membranes in liquid nitrogen for up to six months.

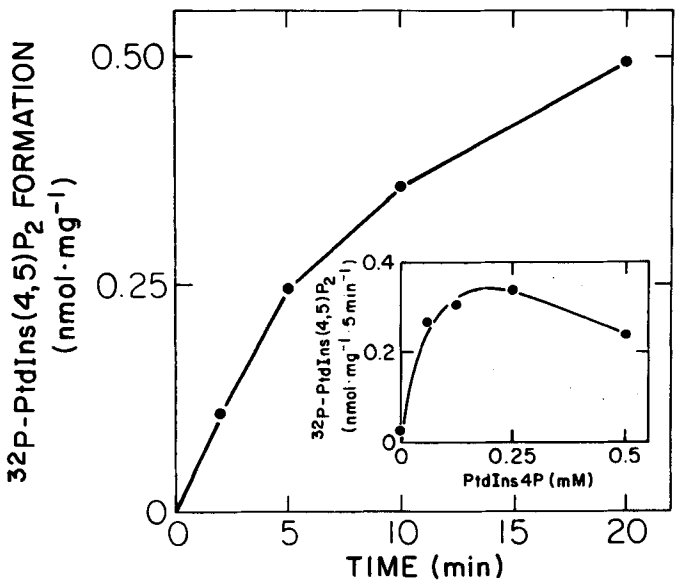


FIG. 2. Time course of [^{32}P]PtdIns(4,5)P_2 formation. Plasma membranes were incubated in the standard assay medium, except that 0.25 mM PtdIns4P was included. Inset: PtdIns4P concentration dependency of [^{32}P]PtdIns4P_2 formation. Reactions were stopped after 5 min.

Effect of Ca^{2+} on the formation of [^{32}P]PtdIns4P and [^{32}P]PtdIns(4,5)P_2 and on the hydrolysis of [^{32}P]PtdIns4P. No change in membrane [^{32}P]PtdIns4P or [^{32}P]PtdIns(4,5)P_2 formation was observed when assays were carried out under standard assay conditions at Ca^{2+}

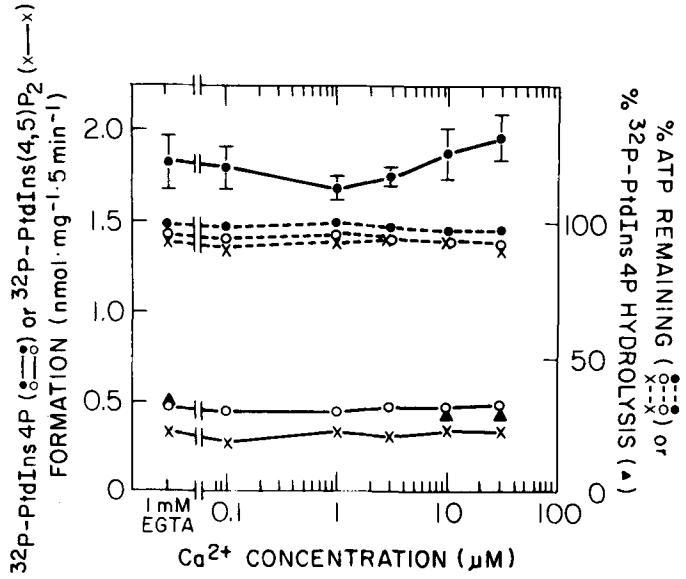


FIG. 3. Effect of Ca^{2+} on [^{32}P]PtdIns4P and [^{32}P]PtdIns(4,5)P_2 formation and hydrolysis of [^{32}P]PtdIns4P. [^{32}P]PtdIns4P and [^{32}P]PtdIns(4,5)P_2 formation and the amount of ATP remaining near the end of the reaction were measured at the indicated Ca^{2+} concentrations in the presence (•, X) and absence (○) of 0.1% Triton X-100. [^{32}P]PtdIns4P_2 formation was measured in the presence of 0.25 mM PtdIns4P (and 0.1% Triton X-100). Reactions were stopped after 5 min for measurement of [^{32}P]PtdIns4P and [^{32}P]PtdIns(4,5)P_2 formation, after 4.5 min for determining ATP hydrolysis, and after 5 min for determining [^{32}P]PtdIns4P hydrolysis in [^{32}P]labeled plasma membrane. Values are the means ± S.E.M. obtained with three membrane preparations; where no error bar is shown, the error is within the size of the symbol.

concentrations ranging from 0.1-30 μM or in the presence of 1 mM EGTA (Fig. 3). Similar results were obtained in the absence of 0.1% Triton X-100. [^{32}P]PtdIns4P hydrolysis, also, was unaffected by Ca^{2+} within the concentration range tested.

Effects of ADP and related compounds on the formation and hydrolysis of [^{32}P]PtdIns4P. [^{32}P]PtdIns4P formation was decreased to different extents by ADP and related compounds when assayed at 5 mM ATP (Table 3). When microsomes which had been incubated under standard conditions for measuring [^{32}P]PtdIns4P formation were centrifuged and resuspended in assay medium for measuring [^{32}P]PtdIns4P hydrolysis, 65% of the initial amount of [^{32}P]PtdIns4P was hydrolyzed after a 10 min incubation. The hydrolysis of [^{32}P]PtdIns4P was not significantly changed by 10 mM ADP during the 10-min incubation period. No detectable hydrolysis of endogenous [^{32}P]PtdIns4P occurred (<5% in 15 min) when Triton X-100 was omitted from the assay medium. Compounds related to ADP also had no effect on the hydrolysis of [^{32}P]PtdIns4P when tested during a 5-min incubation period (Table 3).

To determine the nature of the inhibition by ADP, [^{32}P]PtdIns4P formation was measured in the presence of 0, 0.4, 1 and 3 mM ADP at different ATP concentrations (Fig. 4A). Reactions were linear during the two min incubation at each ATP concentration tested and [^{32}P]PtdIns4P hydrolysis was <15% during this time. The

TABLE 3

Effects of ADP and Related Compounds on [^{32}P]PtdIns4P Formation and the Hydrolysis of Endogenously Produced [^{32}P]PtdIns4P in Cardiac Plasma Membrane Preparations

Additions	[^{32}P]PtdIns4P formation[a] %	[^{32}P]PtdIns4P hydrolysis[b] %
None	100	33 ± 6
ADP	49 ± 5	31 ± 5
GDP	85 ± 2	35 ± 10
UDP	82 ± 7	32 ± 4
Adenine	56 ± 5	29 ± 5
Adenosine	24 ± 5	33 ± 8
5'-AMP	63 ± 4	31 ± 4

[a][^{32}P]PtdIns4P formation was measured in the presence and absence of the indicated additions to the standard assay medium, except that 5 mM [γ-^{32}P]ATP was used. All additions were present at a concentration of 5 mM. 100% [^{32}P]PtdIns4P formation was 2.2 ± 0.2 (mean ± S.E.M.) nmol [^{32}P]PtdIns4P.mg^{-1}.5 min^{-1}.

[b]The hydrolysis of endogenously produced [^{32}P]PtdIns4P was assayed by incubating the [^{32}P]labeled membranes in the presence of the indicated additions. The 0-time control (0-hydrolysis) was 887 ± 38 cpm (mean ± S.E.M.), which were attributable to [^{32}P]PtdIns4P. Reactions were stopped after 5 min, and the remaining [^{32}P]PtdIns4P was extracted and quantitated. The values shown in the table are the means ± S.E.M. of three experiments with different membrane preparations.

presence of ADP increased the apparent Km of the enzyme for ATP but did not change Vmax, indicating that the inhibition was competitive (Fig. 4B). To assess the extent of possible interference by esterase activity, additional reactions were carried out in the absence of Triton

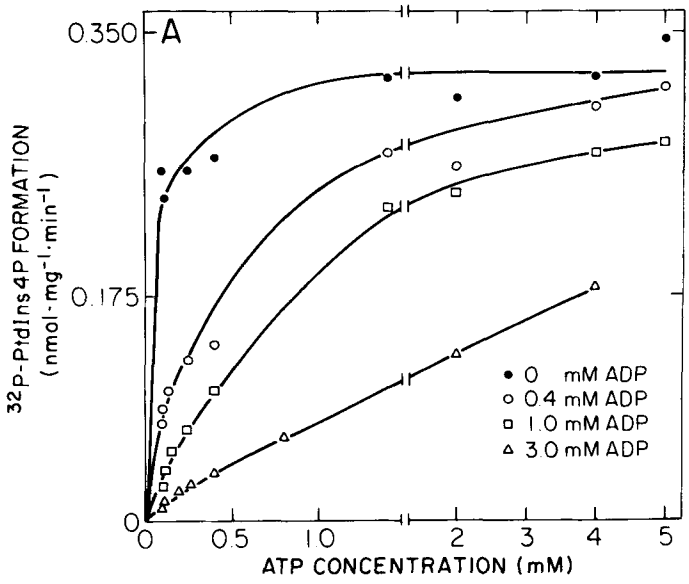
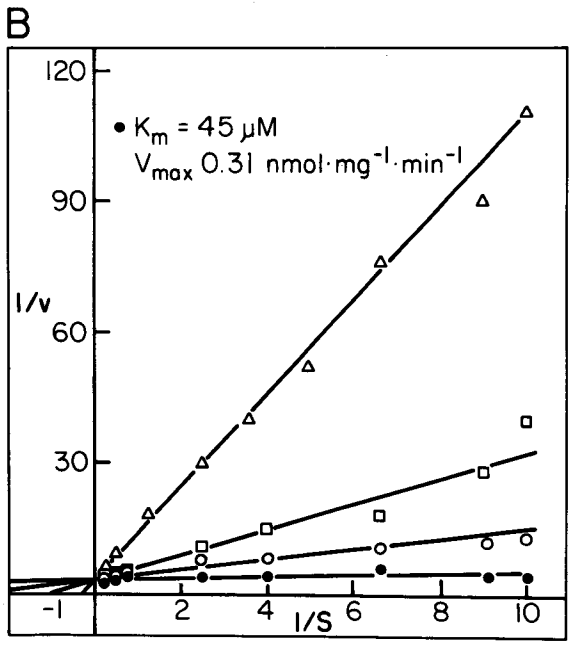


FIG. 4. Effect of ADP on [^{32}P]PtdIns4P was extracted and quantitated. The rate of [^{32}P]PtdIns4P formation at different ATP concentrations. A) Plasma membranes were incubated at the indicated ATP and ADP concentrations in the presence of 20 mM $MgCl_2$. Under these conditions, >99% of the ATP was present as $MgATP^{2-}$. Reactions were stopped after 1 and 2 min, and [^{32}P]PtdIns4P formation at each ATP concentration was linear for at least 2 min. B) Double reciprocal plot of the data shown in Figure 4A.



X-100 because esterase activity was undetectable when endogenously produced [^{32}P]PtdIns4P was used as substrate. Almost identical Km and apparent Km values were obtained as before in the absence and presence of ADP, respectively. The Vmax values obtained in the absence and presence of ADP also were similar to each other but were 1/5 of those observed in the presence of Triton X-100 (data not shown). The apparent Km of the PtdIns kinase was 53 ± 13 μM (mean of three membrane preparations ± S.E.M.) when assayed in different membrane preparations at ATP concentrations ranging from 0.01 to 5 mM. The Ki of ADP for the PtdIns kinase was 0.4 mM. The inhibition of [^{32}P]PtdIns formation at 5 mM ATP by 0.4 mM and 1.0 mM ADP was approximately 13% and 21%, respectively, only the latter being statistically significant ($p < 0.02$) when tested by Student's t-test.

DISCUSSION

Our findings demonstrate that [^{32}P]PtdIns4P and [^{32}P]PtdIns(4,5)P_2 formation by the cardiac plasma membranes are unaffected by up to 30 μM Ca^{2+}. Since in previous studies where a Ca^{2+} dependent decrease in PtdIns kinase activity was reported (8-11), ATP levels had not been monitored during the course of the reaction, depletion of the substrate cannot be eliminated as an explanation for the apparent decrease in enzyme activity. Such an effect would be particularly likely to occur in isolated SR membranes (10) or membrane preparations containing SR membranes as a contaminant (8,10) in view of the high Ca^{2+}-ATPase activity of these membranes. An artifactual stimulation of PtdIns4P formation by protein kinase A has previously been shown to be the result of an inhibition of ATPase activity in isolated rat lymphocyte membranes (21). This finding was confirmed in our own studies with cardiac membranes when subsaturating concentrations of ATP were used (Kasinathan *et al.*, unpublished observations). The lack of effect of Ca^{2+} at the level of the kinases, as demonstrated in our study, is consistent with a possible regulation by Ca^{2+} at the level of phospholipase C (7).

PtdIns kinase activity in cardiac plasma membranes was inhibited by ADP and related compounds when tested at mM concentrations, adenosine being most inhibitory (Table 3). Estimates of adenosine concentrations in normal and anoxic tissues range from 10 μM to 100 μM, respectively (22). Intracellular levels of ADP have been estimated to increase to 0.05 or 0.7 mM in tissue exposed to two minutes of anoxia during which time ATP decreased from 6.2 to 4.7 mM (23). In our study, 0.4 and 1 mM ADP produced only a small decrease in [^{32}P]PtdIns formation at 5 mM ATP, the effect at 1 mM ADP being statistically significant. The high Ki of the enzyme for ADP relative to its Km for ATP is consistent with the low likelihood of ADP being a significant regulator of PtdIns kinase activity in the intact cell when ATP levels are maintained in the millimolar range. A competitive inhibition by adenosine or adenine nucleotides has been previously reported for brain PtdIns kinase (24,25). The inhibitory effects of adenosine (22,24) and ADP (24,25) on PtdIns kinases in other tissues were observed at 0.5 mM or less ATP.

Many of the properties of the cardiac PtdIns kinase resemble those described for PtdIns kinases in other systems. Like brain type 2 PtdIns kinase activity (24), the cardiac PtdIns kinase activity was inhibited nonspecifically by higher than optimum concentrations of PtdIns substrate. A possible explanation for the findings in both studies may be a requirement for a higher Triton X-100 concentration when exogenous phospholipids are added to the reaction mixture. This interpretation is consistent with the lower inhibition observed in our study with the charged phospholipids compared to the uncharged ones.

A lack of PtdIns(4,5)P_2 formation in our cardiac membrane preparation from either exogenous or endogenous PtdIns (in the absence of added PtdIns4P) cannot readily be explained on the basis of different pools of phosphoinositides, based on a lack of evidence for the existence of multiple pools in other tissues (26). A failure to detect PtdIns(4,5)P_2 formation in the absence of added PtdIns4P has also been reported for other tissues (4,21).

The apparent Km of the cardiac PtdIns kinase activity for ATP of 53 ± 13 μM (S.E.M.) is similar to that of type 2 PtdIns kinase in brain, which is 54 μM (24), and to PtdIns kinase purified from porcine liver microsomes, which is 60 μM (27). The Ki of the cardiac enzyme for ADP of 0.4 mM is higher than that reported for the brain type 2 PtdIns kinase, namely 27 μM (24).

Like the PtdIns kinase in rat liver nuclear envelopes (28) and murine T lymphocytes (29), the cardiac PtdIns kinase is dependent upon added $MgCl_2$. The requirement for $MgCl_2$ by the cardiac PtdIns kinase can be attributed primarily to the formation of $MgATP^{2-}$. At 2 mM ATP and 5 mM $MgCl_2$, more than 97% of the ATP is present as $MgATP^{2-}$. The presence of Mg^{2+} in excess of $MgATP^{2-}$ produced little or no change in maximum [^{32}P]PtdIns4P formation. A requirement for additional Mg^{2+} cannot be eliminated, however, in view of the endogenous magnesium present in the membranes.

In summary, our data show that the PtdIns and PtdIns4P kinases in cardiac tissue are not regulated by

μM Ca^{2+}. Our data suggest that any response of the myocardium to α-adrenergic hormones under normal conditions or during mild hypoxia when millimolar concentrations of ATP are maintained (23,30,31) would be largely unimpaired by effects of ADP on plasma membrane PtdIns kinase activity.

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COMMUNICATIONS

Temperature Lability and cAMP-Dependent Protein Kinase Activation of Cholesteryl Ester Hydrolase as a Function of Age in Developing Rat Testis

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Cholesteryl ester hydrolase (CEH) was measured at 32°C and 37°C, and with and without cofactors for stimulation of cyclic AMP-dependent protein kinase, in 104,000 × *g* supernatants from rats aged 14–365 days. Activity at the two temperatures was also partially resolved by cation exchange FPLC. Total specific activity of CEH was relatively constant, with or without addition of cofactors, from 14 to 47 days, during which time temperature labile CEH was a very small fraction of total CEH activity. At later times, 51–150 days, activity was increased as much as two-fold, both with and without cofactors, with most of the increase occurring in the temperature labile fraction. Activation of temperature stable and temperature labile activities, where present, by protein kinase cofactors could be demonstrated in all age groups, but was highly variable as a function of age and protein concentration used in the assay. Apparent induction of temperature labile activity over the interval 47–51 days coincides with reported increases in testosterone synthesis and first appearance of spermatozoa in the testis. This and other lines of evidence suggest unique roles for these enzymes in regulation of availability of free cholesterol for testosterone and membrane synthesis, respectively.
Lipids 24, 824–828 (1989).

Durham and Grogan have reported both temperature stable and temperature labile cytosolic cholesteryl ester hydrolases (CEH) which account for most CEH activity in rat testis (1,2). Temperature stable CEH (TSCEH), with M_r = 28 kDa, exhibited similar activities at 32° and 37°C, and was found in both Sertoli cells, which maintain the blood-testis barrier and support spermatogenesis and in steroidogenic Leydig cells. TSCEH was greatly reduced by hypophysectomy and induced by luteinizing hormone (LH) and follicle stimulating hormone (FSH), both of which are essential for spermatogenesis, and whose target cells are Leydig and Sertoli cells, respectively.

In contrast, temperature labile CEH (TLCEH), with M_r = 72 kDa and 420 kDa (multimer), was inactivated by increasing incubation temperature from 32°C, the approximate scrotal temperature, to 37°C, near the abdominal temperature of the rat. Loss of activity was reversible following brief exposure to 37°C, but irreversible following a 24 hr surgical implantation of testis in the abdomen. TLCEH was found only in Sertoli cells, was undetectable in hypophysectomized rats, and was stimulated by FSH but not LH. Germinal cells had no measurable CEH activity at either temperature.

Bailey and Grogan (3) later showed that both enzymes were activated by cAMP-dependent protein kinase, the probable mediator of induction by FSH and LH, and inhibited by phosphatase. In view of the obligate role of these hormones in initiation and maintenance of spermatogenesis (4), the well-known temperature lability of spermatogenesis (5), and obvious parallels with CEH activity, we have proposed that both enzymes play important roles in spermatogenesis. In the current study we have measured TSCEH and TLCEH activities in rats at various ages in order to correlate changes in CEH activity with anatomical, morphological, physiological and biochemical changes which take place in testis during postpartum development and maturation. We also discuss implications of observed correlations for possible roles of CEH in initiation and maintenance of spermatogenesis.

METHODS AND MATERIALS

Chemicals and supplies. [4-^{14}C]cholesteryl oleate (59.4 mCi/mmol) was purchased from New England Nuclear (Boston, MA). All solvents were purchased from Fisher Scientific (Columbia, MD). Adenosine-5′-triphosphate (ATP), $MgCl_2$, ethylenediaminetetraacetic acid (EDTA), adenosine-3′,5′-cyclic monophosphate, cholic acid, digitonin and thioglycolate were purchased from Sigma Chemical Co. (St. Louis, MO). Sprague-Dawley rats with certified dates of birth were purchased from Flow Laboratories (Dublin, VA), and fed standard laboratory chow *ad libitum* until use. Rats were maintained on a uniform 12 hr light/12 hr dark cycle at all times.

Preparation of rat testes for CEH assay. Rats were sacrificed by decapitation and testes were removed, detunicated on ice, minced with a scalpel blade and homogenized in a glass test tube with a loose fitting teflon pestle in ice-cold 200 mM Tris-HCl buffer (pH 7.4, 1.5 ml/g tissue). The buffer also contained 0.05% cholic acid, 0.1 mM EDTA and 0.1 mM thioglycolate. A 104,000 × *g* supernatant (S104) was prepared from homogenate by differential centrifugation at 2,000 × *g* for 30 min, 10,000 × *g* for 30 min and 104,000 × *g* for 90 min, discarding the pellet at each step. Protein was assayed in S104 by the Pierce Bicinochoninic Acid method (6) using bovine serum albumin as the standard.

Cholesteryl ester hydrolase assay. CEH was measured in aliquots of S104 containing 15–20 μg protein diluted

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Abbreviations: cAMP, adenosine 3′,5′-cyclic-monophosphate; ATP, adenosine-5′-triphosphate; CEH, cholesteryl ester hydrolase; EDTA, ethylenediaminetetraacetic acid; LH, luteinizing hormone; FSH, follicle stimulating hormone; GPHPLC, gel permeation high performance liquid chromatography; S104, 104,000 × *g* supernatant; TLCEH, temperature labile CEH; TSCEH, temperature stable CEH.

to 0.5 ml by addition of homogenizing buffer. Twelve and one-half μl acetone containing 100 nmols cholesteryl oleate and 30-50 × 10^3 dpm [4-^{14}C]cholesteryl oleate was added to assay. The reaction mixture was preincubated for 5 min at 32°C prior to addition of substrate and for 1 hr at the indicated temperature, following addition of substrate. Hydrolysis of cholesteryl ester was determined by measurement of radioactivity in digitonin precipitated free cholesterol essentially according to the method of Chen and Morin (7). The reaction was terminated by addition of 5 ml acetone/ethanol (1:1, v/v). The resulting mixture was centrifuged for 15 min at 1,000 × *g* and 5 ml of supernatant was decanted into a 15 ml test tube to which was added 100 μg unlabeled cholesterol carrier. Free cholesterol was precipitated by addition of 2 ml 0.5% digitonin in 50% ethanol. This mixture was allowed to sit overnight and was centrifuged for 15 min at 1,000 × *g* and the supernatant discarded. In order to remove residual cholesteryl ester, precipitate was resuspended twice in 2 ml acetone/diethyl ether and 2 ml diethyl ether, respectively, followed each time by centrifugation. The final pellet was dissolved in 500 μl methanol and its radioactivity measured by liquid scintillation counting.

Activation by cAMP-dependent protein kinase. Conditions for activation of CEH by endogenous cAMP-dependent protein kinase were those established as optimal by Bailey and Grogan (3). Activation was carried out for 5 min at 32°C in an aliquot of S104 containing 15-20 μg protein (specific activity was constant up to 25 μg), 0.5 mM $MgCl_2$, 0.1 mM ATP, and 1.0 μM cAMP brought to a volume of 0.5 ml with assay buffer. Some studies were also done with 50 μg protein (beyond the optimal range). Following activation, CEH was assayed as described above.

$(NH_4)_2SO_4$ precipitation. S104 was adjusted to 40% $(NH_4)_2SO_4$ and centrifuged at 10,000 × *g* for 30 min. Supernatant was assayed for CEH activity and the pellet was stored at −20°C until needed.

Cation exchange chromatography. The 40% $(NH_4)_2SO_4$ pellet was dissolved in 500 μl Buffer A and desalted on a Sephadex G-25 column. The desalted fraction was centrifuged at 10,000 × *g* for 20 min. Two ml of supernatant containing 8-10 mg protein was loaded on a Mono S HR 10/10 FPLC column (Pharmacia, Uppsala, Sweden), which was then eluted with a buffer gradient. Buffer A consisted of 20 mM Tris, 0.05% cholic acid, 20 mM KCl, 0.1 mM EDTA and 0.1 mM thioglycolate. Buffer B differed only in Tris (1.0 M). The elution program was as follows: At a flowrate of 4 ml/min; 40 ml Buffer A; 24 ml linear gradient, 0-35% Buffer B; 56 ml 35% Buffer B; 24 ml linear gradient, 35-55% Buffer B; 16 ml 55% Buffer B; 40 ml linear gradient, 55-100% Buffer B. Eight-ml fractions were collected and aliquots were adjusted to assay conditions and assayed for CEH.

Gel permeation HPLC. Fractions 7-9 and 18-20 from cation exchange FPLC were concentrated using a Centriprep 10 apparatus (Amicon), according to the instructions provided. Two hundred μl of concentrate was fractionated on a Waters Protein Pak 300 SW HPLC column eluted at 0.5 ml/min with 200 mM phosphate buffer, pH 7.4, containing 0.05% cholic acid, 20 mM KCl, 0.1 mM EDTA and 0.1 mM thioglycolate. One-ml fractions were collected and 200 μl aliquots were assayed for CEH at 32°C and 37°C.

RESULTS

As shown in Figure 1, CEH activity remained relatively constant at time points from 14-47 days when assayed in presence and absence of protein kinase cofactors, at both 32°C and 37°C. Between 47 and 51 days, a two-fold increase in CEH activity occurred both in presence and absence of cofactors when measured at 32°C. This elevated level was maintained up to 365 days.

There was no difference in CEH activity at 32° and 37°C prior to 51 days, in presence or absence of cofactors, suggesting absence or very low levels of TLCEH in both active, i.e., phosphorylated, or less active, dephosphorylated forms. At 51 days, a substantial fraction of activity became temperature labile as evidenced by a 37% decrease at 37°C. Lability persisted through 150 days without cofactors, although the decrease was not statistically significant at 64 or 150 days in presence of cofactors, apparently due to selective stimulation of TSCEH at these time points, which obscured the temperature lability somewhat.

Inasmuch as previous studies of testicular CEH were confined to adult rats aged 50-70 days (1-3), we partially purified CEH activities of both immature (20 day old) and adult (70 day old) rats by $(NH_4)_2SO_4$ precipitation and cation exchange FPLC in order to examine the contributions of isoenzymes at two representative developmental ages (Fig. 2). Overall recovery of activity was 90-100%. At both ages, CEH activity eluted from the cation exchange column in two well-separated ranges (fractions 7-11, 17-21, respectively) of buffer concentration (Fig. 2).

With immature rats, each elution range from the cation exchange column showed two peaks of activity, one reduced at 37°C. However, TLCEH comprised only a small fraction of total CEH, consistent with the results of studies with S104 (Fig. 1). With adult rats, on the other hand, over 50% of CEH activity from the cation exchange column was labile to 37°C, both major elution ranges contained TLCEH, and there was no clear separation between TLCEH and TSCEH (Fig. 2). The elution profile for activity measured at 37°C (i.e., TSCEH) was very similar to that of immature rats, although total activity was lower in proportion to testis weight.

Cation exchange fractions exhibiting TLCEH activity were further fractionated by GPHPLC (data not shown). The two elution ranges (fractions 7-11 and 17-21 in Fig. 2) apparently represent previously reported multimeric and monomeric forms (2) characteristic of this class of enzymes (8,9) as the bulk of CEH activity in fractions 7-9, exhibited GPHPLC elution volumes corresponding to M_r < 100 kDa, whereas the bulk of activity from fractions 18-20, eluted with M_r > 400 kDa (data not shown). Elution profiles were consistent with those which we have published for GPHPLC of S104 (2), showing peaks of temperature labile activity suggestive of monomeric or multimeric forms of the enzyme, although low recoveries at this step (~5%) precluded estimation of relative contributions from various isoenzymes. Thus, temperature lability is found primarily in adult rats and persists through three purification steps, strongly supporting the view that this property is characteristic of a specific inducible enzyme.

At protein concentrations used in these assays (30-40 μg/ml), activity in S104 was not significantly affected by

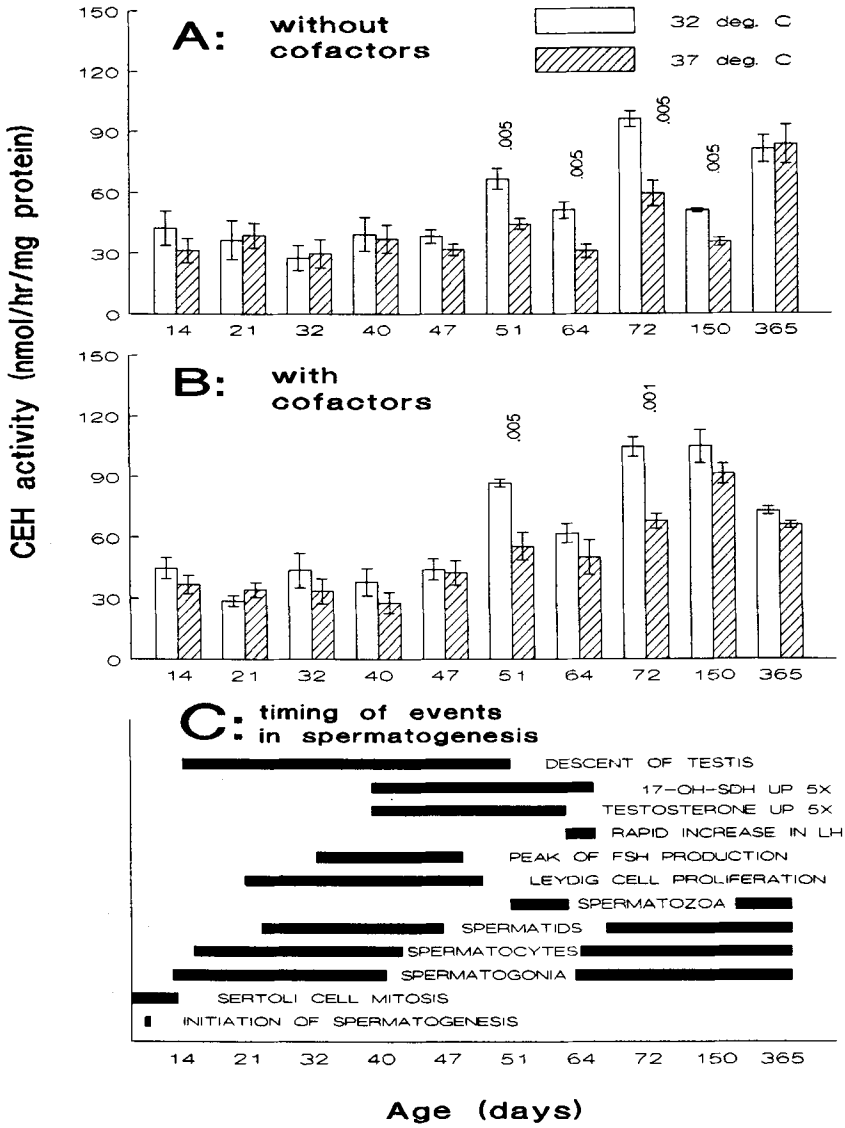

FIG. 1. Variation with age of testicular CEH activity, temperature lability and activation by cAMP-dependent protein kinase and correlation with reported anatomical, morphological, physiological and biochemical changes during postpartum development in the rat. Cholesteryl ester hydrolase was assayed at 32° and 37°C as described in Methods and Materials, in 104,000 × *g* supernatant prepared from testes of rats of various ages, postpartum. For rats 14–40 days old, testes from two rats were pooled and assayed in triplicate under each assay condition with five replicate experiments. For older rats, testes from each of two rats were assayed individually in triplicate with five replicates (three replicates for 150 days; two replicates for 365 days). Error bars represent standard errors of the mean. Numbers written above bars are p-values for differences between activities at 32° and 37°C. (A) Supernatant was assayed without addition of cAMP-dependent protein kinase cofactors. (B) Cyclic AMP, ATP and Mg^{2+} were added to the incubation as described in Methods and Materials. (C) Timing of various important events in testicular development. Time scale varies according to intervals between sampling times. Presence of germinal cell types is indicated from time of first appearance. Time of peak production or sudden increase are indicated for other components. Data are summarized from Ghanadian *et al.* (testicular steroid production [18]); Lee *et al.* (Leydig cell proliferation, FSH and LH [17]); Clermont (stages of spermatogenesis [12]); Van der Molen *et al.* (testicular enzyme levels [19]); Baker (descent of testis [11]). 17-OH-SDH = 17-hydroxysteroid dehydrogenase.

presence of cofactors at either temperature prior to 51 days (Fig. 1), although increases in protein concentration beyond the range of constant specific activity (100 μg/ml) resulted in two- to six-fold activation by cofactors at each time point shown in Figure 1 (data not shown). None of this induced activity was temperature labile prior to 51 days.

At 51 and subsequent days, activation by cofactors was significant but highly variable with age, reaching a peak, two-fold activation at 150 days at both temperatures.

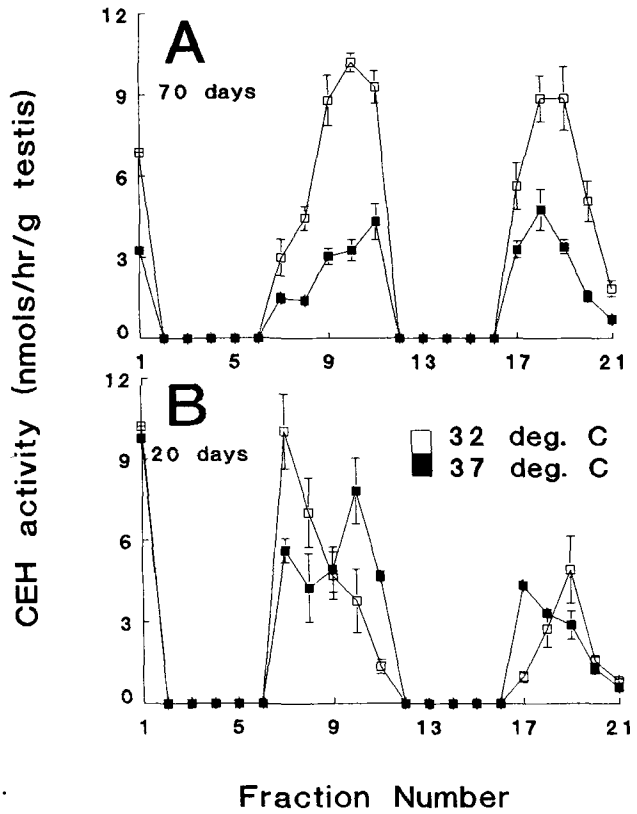

FIG. 2. Elution of temperature stable and temperature labile CEH activity from cation exchange FPLC. For each experiment, S104 was prepared from pooled rat testes (40-120 immature; 4-6 adult), as described in Methods and Materials. Protein was precipitated from 40% $(NH_4)_2SO_4$ with a two-fold increase in specific activity and recovery of 90-100% of total activity, and separated on a preparative cation exchange FPLC column as described in Methods and Materials, with overall recovery of 90-100% of S104 activity. Eluent was collected in 8 ml fractions and aliquots were assayed for CEH activity under optimal conditions at the indicated temperatures. Values are means of three experiments, each assayed in triplicate. Error bars represent standard errors of the mean. Where no error bar is visible, SEM is obscured by the symbol at that point.

Means of all adult values with cofactors at 32° and 37°C (86 ± 4 and 64 ± 4 nmol/hr/mg protein, respectively) were significantly higher ($p < .02$, $p < .005$) than corresponding values without cofactors (70 ± 5 and 43 ± 4 nmols/hr/mg protein). In no case did cofactors have a significant effect on temperature lability.

DISCUSSION

As studies from this laboratory have previously shown, adult rat testis contains two major CEH activities—TSCEH, which retains activity at both scrotal (~32°C) and abdominal (~37°C) temperatures, and TLCEH, which, like spermatogenesis, is inactivated at 37°C (1,2). Both enzymes are activated by cAMP-dependent protein kinase and reversibly inhibited by phosphatase (3).

Current studies suggest that very little TLCEH is present in testis prior to day 51, in that CEH activities are not different at 32° and 37°C (Fig. 1). That decreases in activity between 32°C and 37°C are indicative of a discrete TLCEH isoenzyme is supported by assays of partially purified enzymes, which show very little temperature lability in 20 day old, immature rats, in contrast to marked temperature lability in 70 day old adult rats (Fig. 2). Overall recovery of 90-100% of total activity, rules out selective loss of isoenzymes during purification. Persistence of temperature lability through $(NH_4)_2SO_4$ precipitation, cation exchange FPLC (Fig. 2) and GPHPLC (data not shown) provides strong evidence that this property is characteristic of a specific isoenzyme rather than artifactual or due to some other cytosolic factor (Fig. 2).

Comparison of the developmental pattern of CEH enzymes with anatomical, morphological, physiological and biochemical events may yield insight into functional roles of the enzymes. Landmarks of testicular development are shown in Figure 1C. Sertoli cells, in which TLCEH is found, are present at birth and cease mitosis at 14-15 days postpartum (10). Whereas TLCEH may not be active until descent of testis from abdomen to scrotum is complete at ~51 days (11), it is unclear when TLCEH protein synthesis begins. Present results show a small amount of TLCEH present as early as 20 days postpartum (Fig. 2B). Whereas temperature sensitive spermatocytes appear in testis at about 25 days (12,13), testicular temperature must be regulated at this age, though not necessarily low enough to prevent inactivation of TLCEH. On the other hand, the sudden increase in TLCEH at ~51 days suggests that this enzyme is induced in coordination with appearance of spermatozoa and puberty which occur at 50 days in the rat.

Whereas effects of protein kinase cofactors were highly variable and dependent on protein concentration and age, participation of cAMP-dependent protein kinase in induction at 51 days cannot be ruled out. Other researchers have described wide swings in levels of protein kinase A, protein kinase inhibitor, adenyl cyclase and cAMP during development (14-16). However, none of these events correlates well with induction of TLCEH. Moreover, neither FSH nor LH, whose actions are mediated by cAMP-dependent protein kinase, shows a marked increase which might account for the sudden increase in CEH activity at 51 days (17).

The increase in CEH activity does correlate well with a reported five-fold increase in testosterone synthesis beginning at about day 40 and leveling off at 60-70 days (18). Other enzymes of steroid metabolism are induced in testis with time courses similar to those of induction of testosterone synthesis and increased CEH activity (19). Whereas the product of TSCEH in Leydig cells is the substrate for testosterone synthesis and TSCEH is modulated by LH *in vivo* (2), TSCEH may play a role in regulation of testosterone synthesis. A similar role has been suggested for CEH in adrenocorticoid hormone (20) and bile acid synthesis (21). Although there is no similar rationale for regulation of TLCEH in Sertoli cells, temporal relationships are consistent with induction of this CEH by testosterone in the Sertoli cell, which is its target cell in the testis.

TLCEH is present in Sertoli cells but not in cells of Leydig, and thus cannot play a significant role in androgen synthesis (2). This is consistent with the observation that testosterone synthesis is not impaired by temperature elevation. Nevertheless, it has long been

known that elevated testicular temperature leads to impaired spermatogenesis and accumulation of cholesteryl esters in testis (22). Moreover, we have reported selective loss of TLCEH with surgical implantation of testes in the abdomen, which also disrupts spermatogenesis (1). These observations support physiological relevance for temperature lability of this CEH, which may prevent expression of TLCEH at temperatures which are inappropriate for spermatogenesis. TLCEH may play a role in regulating intracellular free cholesterol required for membrane synthesis. This could be especially critical during the burst of germinal cell proliferation and marked changes in morphology following initiation of spermatogenesis. In the first 60 days, postnatal, testicular mass increases 500-fold, with more than half of the increase taking place in the last 26 days (23). In the current study, the magnitude of increase in TLCEH from 47–51 days, coinciding precisely with appearance of spermatozoa, also supports importance of TLCEH to spermatogenesis. It is not clear whether inhibition of TLCEH alone is sufficient to disrupt spermatogenesis. However, phenylmethylsulfonyl fluoride, a relatively specific esterase inhibitor at appropriate concentrations, inhibits spermatogenesis as well as CEH activity (24). TLCEH comprises a significant fraction of total CEH from 51–150 days, falling off only in 365 day old retired breeders, suggesting a continuing role in maintenance of spermatogenesis.

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Epidermal Growth Factor Modulates Release of Arachidonic Acid from Embryonic Cells

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The calcium ionophore A23187 stimulates release of free [^{3}H]arachidonic acids from radiolabeled cultures of MEPM cells which are growing, but not from those which are confluent. However, when confluent MEPM cells are pretreated with EGF or PMA, release of [^{3}H]arachidonic acids does occur in response to A23187. Since neither EGF nor PMA themselves stimulate release of [^{3}H]arachidonic acids from these cells, but do activate protein kinase C, these data support the hypothesis that protein kinase C modulates the activities of phospholipid hydrolases in MEPM cells.
Lipids 24, **829–832 (1989).**

Administration of glucocorticoids to some strains of pregnant mice results in a high incidence of cleft palate in the neonate (1). Since (a) the antiinflammatory properties of glucocorticoids seem to be due to their inhibitory effects on expression of activities of those phospholipases necessary for mobilization and metabolism of arachidonic acid (2), and (b) activities of phospholipases expressed by embryonic palatal cells from the glucocorticoid-sensitive A/J strain are more readily inhibited by dexamethasone than activities expressed by cells from the less sensitive C57BL/6J strain (3), it is reasonable to hypothesize that regulation of mobilization and metabolism of arachidonic acid is critical to normal morphogenesis of the palate (1,4). However, mechanisms underlying regulation of activities of phospholipases in (embryonic) cells are not known.

There is some evidence that mobilization and/or metabolism of arachidonic acid may be regulated as a function of growth in various cells. In some cases such regulation reflects (a) loss of arachidonic acid from cellular phospholipids as a function of time spent in culture (5), or (b) release from confluent cells of some uncharacterized substance (6) which inhibits conversion of arachidonic acid to prostaglandins. Recent reports indicate MEPM cells grown to confluency release less prostaglandin E_2 (7,8) and $F_{2\alpha}$ (8) than do cells in the log phase of growth in response to agents known to activate phospholipase A_2. Therefore, experiments were initiated to explore relations between growth and mobilization of arachidonic acid by MEPM cells in an effort to establish a model with which to study the biochemical basis for regulation of phospholipases in these embryonic cells.

MATERIALS AND METHODS

Preparation of cultures. Pregnant C57BL/6J mice were killed on the fourteenth day of gestation, their embryos collected, and primary cultures of MEPM cells prepared according to Chabot and Chepenik (7). Cells were incubated at 37°C in a humidified incubator with an atmosphere of air:CO_2 (95:5); medium was changed every other day. Under these conditions the cells enter the log phase of growth on the second day after plating, are subconfluent on the fourth day, and are confluent and epitheleoid on the seventh day after plating (9).

Radiolabeling of cellular lipids. Cells in the log or confluent phase of growth were incubated for 24 hr in 1 ml complete medium which contained 0.5 μCi [^{3}H]arachidonic acid. Greater than 90% of the radiolabel incorporated into cellular lipids is found in the *sn*-2 position of endogenous phospholipids as a result of this protocol (10). In some cases, cells were also incubated for 30 min in 50 μCi [^{32}P]O_4^{3-}/ml buffered saline (0.9 mM $CaCl_2$, 0.49 mM $MgSO_4$, 0.373 mM KCl, 0.13 M NaCl, 0.1% dextrose, 22 mM *N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid) to radiolabel endogenous pools of phosphate prior to treatment with various agents.

Extraction and chromatography of cellular lipids. The distribution of radiolabel into cellular lipids was determined by extracting the cells (and medium, where appropriate) according to Bligh and Dyer (11), except that the methanol contained 2% acetic acid. Lipids recovered in the organic phase were chromatographed on silica gel H thin layer plates (Whatman LK6) using $CHCl_3/CH_3OH/CH_3COOH/H_2O$ (50:25:6:1, by vol; system I) to separate phospholipids, or $C_6H_{14}/(C_2H_5)_2O/CH_3COOH$ (50:50:1, by vol; system II) to separate neutral lipids. Since system I does not reliably separate phosphatidylserine from phosphatidylinositol, the lipids extracted into chloroform were sometimes chromatographed in system III according to Esko and Raetz (12), in which the solvent for the first dimension was $CHCl_3/CH_3OH/CH_3COOH$ (65:25:10, v/v/v) and the solvent for the second dimension was $CHCl_3/CH_3OH/$88%HCOOH (65:25:10, v/v/v). The *O*-alk-1-enyl linkages present in ethanolamine plasmalogens were broken by exposing the plates to HCl fumes (13) after the first dimension of system III and the resultant lysophosphatidylethanolamine isolated in the second dimension. Lipids were visualized under UV light after spraying the chromatograms with primulin (14), and then the silica gel containing each lipid was either scraped into mini-scintillation vials containing scintillation fluid or "spiked" with 0.4 μg heptadecanoic acid, which was methylated according to Rogozinski (15), and the various fatty acid methyl esters separated and quantitated by capillary-column gas-liquid chromatography, as detailed previously (16).

Materials. [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid (83.8 Ci/mmol) was from NEN Products/Dupont (Wilmington, DE); [^{32}P]O_4^{3-}(carrier free) was from ICN Biomedicals (Irvine, CA). Culture medium and antibiotics were from Grand Island Biological Co. (GIBCO,

Abbreviations: DBI, double bond index; DMSO, dimethylsulfoxide; EGF, epidermal growth factor; MEPM, mouse embryo palate mesenchyme; PC,PE,1,2-diradyl-*sn*-glycero-3-phosphocholine, phosphoethanolammine; PS/PI, combined inositol and serine phospholipids.

Grand Island, NY), EGF was from Collaborative Research Inc. (Lexington, MA), and sera were from Hazelton Research Products Inc(Lenexa, KS). A23187 from CALBIOCHEM(San Diego, CA) was dissolved in DMSO to yield 19 mM and then frozen under N_2. PMA from LC Services Corp. (Woburn, MA) was dissolved in DMSO to yield 5 mg/ml and frozen under N_2. Commerically prepared thin-layer plates were from Bodman Chemicals(Media, PA), HPLC-grade solvents were from Fisher Scientific(King of Prussia, PA), authentic phospholipid and neutral lipid standards were from Serdary Research Laboratories(Ontario, Canada). All other chemicals were analytical reagent grade from either Fisher Scientific or A.H. Thomas(Swedesboro, NJ).

RESULTS AND DISCUSSION

There was significant release of free [^{3}H]fatty acid from phospholipids and concomitant incorporation of some [^{32}P]O_4^{3-} into phosphatidic acid and phosphatidylinositol (Fig. 1) when log phase cultures of MEPM cells were stimulated with 10 μM A23187. In contrast, treatment of similarly radiolabeled confluent(stationary) cells with A23187 resulted in a significant increase in incorporation of [^{32}P]O_4^{3-} into phosphatidylinositol and little release of [^{3}H]free-fatty acids (Fig. 1). These data are interpreted to mean (a) phospholipases in growing cells may be activated readily by the calcium ionophore A23187, but not those in confluent cells, and (b) such failure of activation does not reflect unresponsiveness of the confluent cells to the ionophore per se, since A23187 did stimulate enhanced uptake of [^{32}P]O_4^{3-}into phosphatidylinositol of confluent cells.

The differential responsiveness of growing and confluent cells to stimulation of release of esterified stores of arachidonic acid could not be attributed to (a) a failure of incorporation of [^{3}H]arachidonic acid into major classes of phospholipids of confluent cells, as significant amounts of radiolabel could be found in all major classes of cellular phospholipids regardless of the state of growth (Fig. 2), or (b) depletion of arachidonic acid from cellular phospholipids, since there was an almost singular increase in their relative amounts of arachidonic acid during the period of culture (Table 1). This increase in content of polyunsaturated fatty acid was reflected by an increase in the double-bond index calculated for the major cellular phospholipids extracted from confluent cells compared to growing cells (Table 1). Absence of release of [^{3}H]fatty acid from confluent cultures treated with the ionophore did not reflect release of uncharacterized phospholipase inhibitory factors from confluent cultures since medium conditioned for three days by confluent cultures had no effect on release of [^{3}H]fatty acid from growing cultures treated with the ionophore(data not shown), nor did it reflect alterations in the Ca^{2+} requirements of the phospholipid hydrolases in confluent MEPM cells as they were identical (data not shown) to the Ca^{2+} requirements

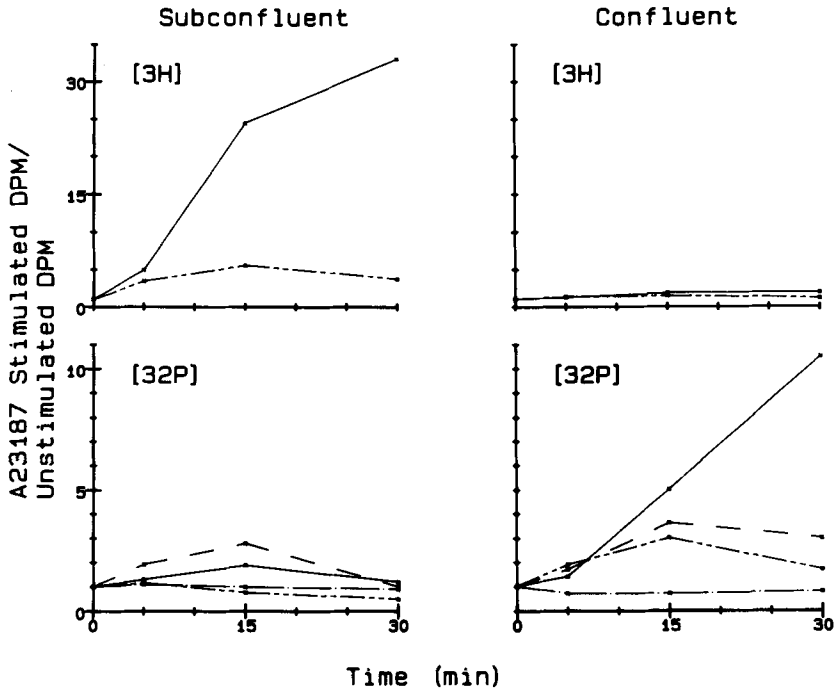

FIG. 1. Time course of release of free [^{3}H]fatty acid and uptake of [^{32}P]O_4^{3-}into phospholipids by subconfluent and confluent cultures of MEPM cells stimulated with 10 μM A23187. Cells were labeled with [^{3}H]arachidonic acid and [^{32}P]O_4^{3-} prior to challenge with the ionophore or vehicle, as described in Materials and Methods. Data are expressed as the amount of radiolabel released or incorporated in response to the ionophore relative to the amount of radiolabel released or incorporated in response to the vehicle, and are the means of three separate determinations. [^{32}P]:1,2-diradyl-*sn*-glycero-3-phosphocholine, —·— ; phosphoethanolamine, ——— ; phosphoinositol, —— ; phosphate, — —. [^{3}H]: free fatty acid, ——; diacylglycerol, ——— .

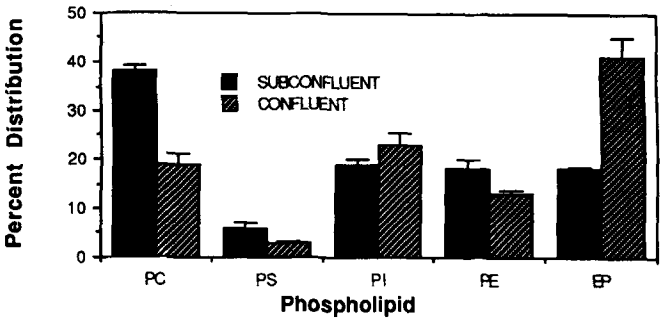

FIG. 2. Distribution of radiolabeled arachidonic acid amongst phospholipids of growing and confluent MEPM cells. Subconfluent or confluent cultures of MEPM cells were radiolabeled 24 hr with [^{3}H]arachidonic acid. Lipids were extracted, separated by two-dimensional thin-layer chromatography in system III, and scraped into scintillation vials for quantitation of radioactivity by scintillation counting, as described in Materials and Methods. Data are the means ± SEM(n=3). PC, PS, PI, PE: 1,2-diacyl-*sn*-glycero-3-phosphocholine, phosphoserine, phosphoinositol, phosphoethanolamine; EP: 1-*O*-alk-1′-enyl-2-acyl-*sn*-glycero-3-phosphoethanolamine. An average of 180,300 DPM were incorporated into the phospholipids of growing cells and 429,954 DPM were incorporated into the phospholipids of confluent cells.

reported for growing MEPM cells (10). However, 15 min pretreatment of confluent cultures of MEPM cells radiolabeled with [^{3}H]arachidonic acid with either EGF or PMA enhanced release of [^{3}H]arachidonic acid in response to A23187 (Fig. 3).

This latter finding is particularly interesting as we observed recently that such treatment of confluent MEPM cells with EGF or PMA will activate protein kinase C (17). Others have shown that treatment with EGF for 20 min is sufficient to stimulate phosphorylation of endogenous substrates by protein kinase C in 3T3-L1 cells (18) or potentiate release of prostaglandins from MEPM cells stimulated with A23187 (19).

The finding that A23187 did not stimulate release of arachidonic acid from confluent cultures of MEPM cells, while affecting synthesis of PI, permits the conclusion that elevation of intracellular levels of Ca^{2+} alone is not sufficient to activate phospholipid hydrolases. This conclusion is similar to that reached by Billah *et al.* (20), wherein A23187 stimulated phospholipid hydrolases in differentiated, but not undifferentiated, HL60 cells. Furthermore, it would seem that activation of protein kinase C enhances activities of phospholipase A in MEPM cells since PMA or EGF promoted release of arachidonic acid in response to A23187. Neither of these agents alone stimulated release of arachidonic acid while each one has been shown to activate protein kinase C in these cells (17). Regulation of phospholipid hydrolases by protein kinase C may be a biochemical mechanism common to a number of types of cells since agents (PMA, oleoyl acetyl glycerol) which activate protein kinase C also have been found to enhance ionophore-stimulated release of arachidonic acid from human platelets (21), rabbit neu-

TABLE 1

Acyl Composition (wt%)[a] of Phospholipids Isolated from MEPM[b] Cells in Log (Grw) or Confluent (Confl) Phase of Growth

	PC[d]		PS/PI[d]		PE[d]	
Aliphatic moiety[c]	Grw	Confl	Grw	Confl	Grw	Confl
14:0	3.6	2.4	3.0	0.5	2.0	1.8
16:0	33.9	33.7	19.4	13.3	14.3	13.3
16:1	7.4	5.8	5.2	2.2	5.7	2.9
18:0	9.4	9.0	27.9	26.5	12.3	14.3
18:1	31.5	33.2	24.3	24.2	17.2	17.5
18:2	2.0	2.5	1.4	1.9	1.7	1.5
18:3	<1	<1	1.4	<1	1.0	<1
20:1	1.0	<1	<1	<1	2.3	<1
20:2	1.8	1.6	1.5	<1	3.5	2.1
20:3	<1	<1	<1	1.4	<1	1.2
20:4	2.9	5.1	10.7	15.3	14.6	23.7
22:4	<1	<1	1.3	2.3	3.9	6.0
22:5	<1	<1	1.9	1.6	4.4	4.8
22:6	<1	1.4	1.3	1.9	5.5	7.1
24:0	<1	<1	1.6	<1	4.0	<1
DBI[e]	61	86	107	131	169	225

[a]Weight (wt%) = (μg of each individual aliphatic moiety − sum of the μg amounts of total aliphatic moities found in PC, PS/PI, or PE) × 100. Each value is the mean of either three(Grw) or four(Confl) separate determinations. SEM (<5%) omitted for clarity of table.

[b]Phospholipids were extracted into chloroform, isolated by chromatography in system I, and methyl esters of fatty acids prepared as described in Materials and Methods. Fatty acid methyl esters were identified by comparison of retention times obtained on gas-liquid chromatography to those obtained with authentic

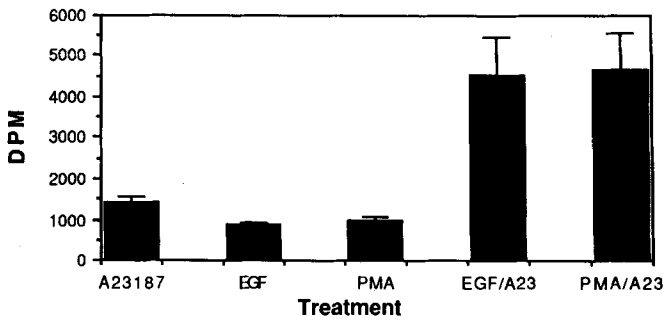

FIG. 3. Effects of PMA or EGF on release of free [^{3}H]fatty acids ([^{3}H]FFA) from confluent cultures of MEPM cells treated with A23187. Confluent MEPM cells radiolabeled with [^{3}H]arachidonic acid were washed, then incubated 15 min with vehicle, 50 ng EGF/ml, or 100 ng phorbol PMA/ml, prior to stimulation with 10 μM A23187 for 15 min. Radiolabeled free fatty acids were extracted into organic solvent, separated by thin-layer chromatography in system II, and their radioactivities were quantitated as described in Materials and Methods. Data are the mean ± SEM(n=3).

trophils (22), and human polymorphonuclear leukocytes (23).

ACKNOWLEDGMENTS

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Ingestion of High Doses of Fish Oil Increases the Susceptibility of Cellular Membranes to the Induction of Oxidative Stress

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Feeding rats with 4 g/kg body weight of sardine oil during 7 or 14 days increases the content of eicosapentaenoic acid and docosahexanoic acid in the erythrocyte and hepatic microsomal membranes by 2 to 6%. These membranes show increased susceptibility to the induction of oxidative stress, expressed as lipid peroxidation, when they are exposed to Fe^{2+}-ascorbate and to NADPH-Fe^{3+}-ADP, respectively. The results indicate that in order to prevent the increased susceptibility to lipid peroxidation, supplementation with larger amounts of antioxidants may be needed than those required to stabilize the oil.
Lipids 24, 833-835 (1989).

Fish oil concentrates containing a high proportion of n-3 polyunsaturated fatty acids have been reported to have beneficial effects on cardiovascular and cerebrovascular disease (1), peripheral vascular disease (2), and kidney disease (3). Among the n-3 unsaturated fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have received greatest attention as these acids are found in considerable amounts in fish oil. Feeding a diet rich in polyunsaturated fatty acids increases the content of these acids in the phospholipids of cellular membranes (4). Moreover, a high degree of unsaturation in membrane phospholipids may increase membrane susceptibility to the induction of oxidative stress, expressed as lipid peroxidation. The present study was undertaken to determine whether erythrocyte membranes and hepatic microsomes obtained from rats fed high doses of fish oil are more susceptible to the induction of *in vitro* lipid peroxidation by prooxidants such as Fe^{2+}-ascorbate or the NADPH-Fe^{3+}-ADP system (5).

MATERIALS AND METHODS

Male Wistar rats 180-200 g body weight (n=20), fed a standard diet and water *ad libitum*, received 4 g of sardine oil/kg body weight daily (9 a.m.) during 7 (n=10) or 14 days (n=10). Control animals (n=5) received an equivalent amount of mineral oil. Sardine oil (34% EPA plus DHA) obtained from a local fish meal factory (Corpesca, S.A.) was deodorized by molecular distillation and maintained under a N_2 atmosphere until use. The peroxide value of the oil was less than 2 meq/kg. At the end of the experimental period, animals were exsanguined under light anesthesia by cardiac puncture. Blood was centrifuged at 2300 × g for 10 min at 4°C, and after removal of the buffy coat, erythrocytes were washed three times with PBS buffer (150 mM NaCl and 5 mM sodium phosphate, pH 8). Membrane ghosts were obtained according to Burton *et al* (6). Livers were extracted after perfusion with saline, and homogenized in 0.25 M mannitol containing 0.025 M morpholinpropanesulfonic acid buffer pH 7.4 and 0.2 M triethylenetetramine hydrochloride. Microsomes were prepared according to Albro *et al.* (7).

Erythrocyte membrane lipid peroxidation was induced by Fe^{2+}-ascorbate (50 μM Fe^{2+}, 400 μM ascorbate). Microsomal lipid peroxidation was induced by a NADPH-Fe^{3+}-ADP system (400 μM NADPH, 50 μM Fe^{3+}, 4 mM ADP) according to Devasagayam (5). Lipid peroxidation was assessed as thiobarbituric acid reactive products (TBAR) as described by Fee and Teitelbaum (8). Proteins were measured by a modification of the method of Lowry *et al.*, as described by Albro (9). Fatty acid methyl esters were prepared by transesterification with boron trifluoride-methanol (10) of lipid extracts of erythrocyte membranes or hepatic microsomes. Gas chromatography was done on a Perkin-Elmer Sigma 300 gas chromatograph using a GP 10% SP 2330 column and a Sigma 15 data station for peak area measurements. All chemicals were reagent grade obtained from Sigma Chemical Co. (St. Louis, MO). Results are expressed as means ± S.D., and the significance of the differences between mean values was assessed by Student's t-test for unpaired results.

RESULTS AND DISCUSSION

Feeding of rats with sardine oil during either 7 or 14 days produced an increase in some polyunsaturated fatty acids in erythrocyte membranes and hepatic microsomes, as is shown in Figure 1. The most significant change was the increase in EPA and DHA of these membranes in the range from 1.5-6.5%, depending on when the animals received the oil. Linoleic acid content was decreased in both cellular fractions, arachidonic acid being variable depending on the origen of the fraction and on the time of oil consumption.

Although basal TBAR levels of erythrocyte membranes and hepatic microsomes are similar to those obtained from controls (time=0, Fig. 2A and B), which can be interpreted as an absence of oxidative stress in these fractions, their response to the induction of lipid peroxidation by the prooxidant Fe^{2+}-ascorbate or by the NADPH-Fe^{3+}-ADP system is very different from that of the controls. Figure 2A shows the peroxidative effect on erythrocyte membranes after the addition of Fe^{2+}-ascorbate. The response is related to the duration of the oil ingestion period. Figure 2B shows a similar effect on hepatic microsomes after the addition of NADPH-Fe^{3+}-ADP. These results demonstrate that, under our experimental conditions, both erythrocyte membranes and hepatic microsomes from rats fed high doses of sardine oil appear to be more susceptible to the induction of lipid peroxidation. Low basal TBAR

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DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; MDA, malondiadehyde; PBS, poly (butene-1-sulfone); TBAR, thiobarbituric acid reactive products.

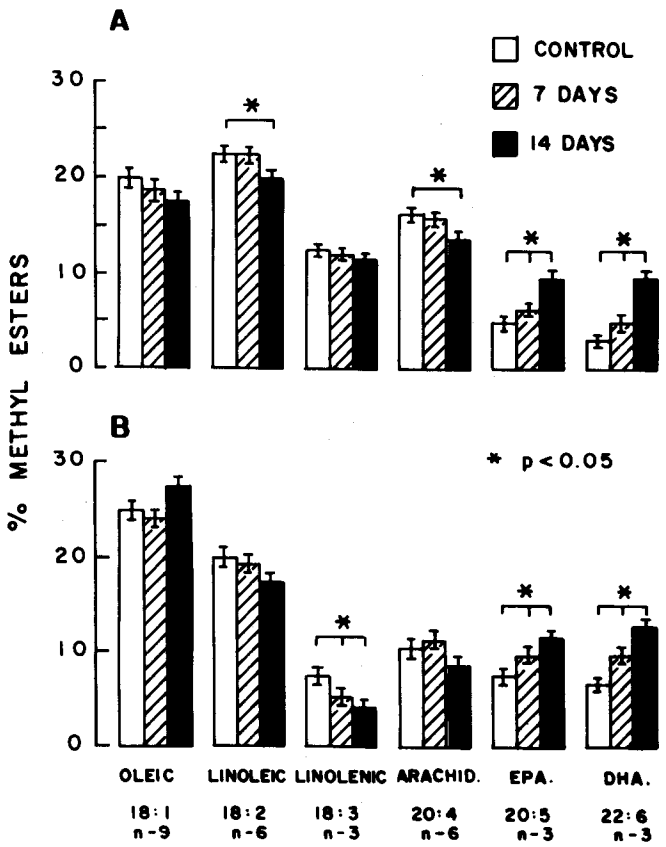

FIG. 1. Changes (%) in the composition of some unsaturated fatty acids from rat erythrocyte membranes (A) and rat hepatic microsomes (B) after feeding rats sardine oil for 7 or 14 days. Results are the mean of five assays ± S.D.

levels observed in control and experimental membranes do not indicate the absence of *in vivo* oxidative stress. Malondialdehyde (MDA), which is the main component of the TBAR reactive products of lipid peroxidation, is water-soluble and may be metabolized and/or excreted from cells and tissues into the bloodstream and finally into the urine. Piché *et al.* (11) have observed high concentrations of urinary MDA from rats fed marine oils. Although these authors interpreted the observation to be due to lipid peroxides and other oxidation products present in marine oils, the possibility that the effect could, in part be the consequence of induction of tissue oxidative stress should not be discarded.

Our results, in addition to those of Piché *et al.* (11), raise the question at which level cellular defenses against oxidative stress may counteract the oxidative pressure from the increase in polyunsaturation in membrane structures. Long-term feeding with marine oils may produce a depletion of some cellular molecular antioxidants such as glutathione, ascorbic acid or tocopherols. Although the sardine oil used in our study does not contain any added antioxidant, the marine oils which are widely sold on the market contain α-tocopherol in the range of 0.8—1.0 g/kg oil, which is enough to stabilize the oil against oxidation, but may not be sufficient to compensate for the increased susceptibility of highly unsaturated cellular structures against oxidative stress. Although, to our knowledge, the activity of protective enzymes such as superoxide dismutase, glutathione peroxidase or catalase has not been tested in experimental models to which high doses of marine oils had been administered, the possibility of adding higher concentrations of α-tocopherols or other antioxidants to the oil, in order to compensate

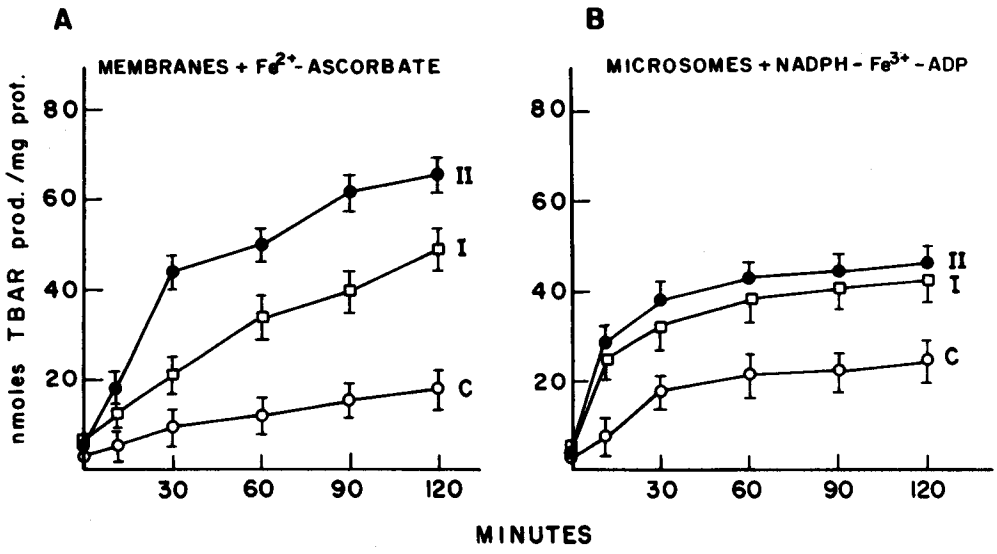

FIG. 2. Effect of feeding sardine oil to rats on the susceptibility of erythrocyte membranes (A) and hepatic microsomes (B) to induction of lipid peroxidation. I: 7 days of ingestion. II: 14 days of ingestion. C: controls. Results are the mean of six experiments ± S.D.

for the cellular antioxidant capacity, may be considered. Experiments to study this possibility are currently being worked out in our laboratory.

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Metabolism of Peripheral Nerve Monogalactosylceramides[1]

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The metabolism of hydroxy galactocerebrosides (GalCe-OH) and nonhydroxy galactocerebrosides (GalCe) was investigated during nerve development, degeneration and regeneration by endoneurial injection of [^{14}C]acetate and by *in vitro* incubation of rat sciatic endoneurium with [^{14}C]acetate, [^{3}H]galactose or [^{3}H]glucose. After endoneurial microinjection, [^{14}C]acetate was found to be incorporated first into GalCe-OH and later, and to a much lesser degree, into GalCe. The ratio of ^{14}C-labeled GalCe-OH to GalCe decreased with time and remained fairly constant after 4 hr. On the other hand, *in vitro* incorporation of [^{14}C]acetate resulted in higher ^{14}C-labeling in GalCe and monogalactosyl diacylglycerol (MGDG) and lower ^{14}C-labeling in GalCe-OH, diminishing with incubation time. After 24 hr, only GalCe and MGDG were labeled. When [^{3}H]galactose or [^{3}H]glucose, instead of [^{14}C]acetate, were used as precursor *in vitro*, a similar preference for labeling of GalCe-OH was demonstrated in regenerating nerve. These data suggest that hydroxy fatty acids and hydroxy ceramides are the preferred substrates in peripheral nervous system for the sphingosine acyltransferase and the UDP-galactose:ceramide galactosyltransferase reactions, respectively. The α-hydroxylation system did not appear to be fully functional under *in vitro* conditions. The biosynthesis of GalCe-OH was greatly enhanced during nerve fiber regeneration and decreased rapidly with increasing age. This suggests that a close interrelation exists between α-hydroxylation and peripheral nerve myelination.
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Galactocerebrosides and galactosulfatides are the major glycolipids of peripheral nerve myelin (1), which is an extension of the plasma membrane of Schwann cells (2). Previous studies on peripheral nerve glycolipid composition (3) have shown that hydroxycerebrosides and hydroxysulfatides are the main constituents of glycolipids of immature rat sciatic endoneurium. The ratio of hydroxy to nonhydroxy cerebrosides decreases rapidly as myelination proceeds, and then remains fairly constant throughout adulthood. More than 50% of the adult content of endoneurial galactolipids is reached before 21 days of age. In the absence of myelin assembly, e.g., in Wallerian degeneration, the maximum decrease of endoneurial galactocerebrosides coincides with axonal degeneration and demyelination (3). These results suggest that the biosynthesis of endoneurial galactolipids take place preferentially at the time when the peripheral nerve is undergoing active myelination. The main biosynthetic pathway of peripheral nerve galactocerebrosides appears to involve the transfer of galactose to ceramide by the UDP-galactose:ceramide galactosyltransferase reaction (4).

On the other hand, glucocerebrosides, which are the major monohexosylceramides of extraneural tissues, are not considered constituents of the myelin membrane. Recently, we have shown (5) that the normally inactive pathway for the biosynthesis of glucocerebroside and oligohexosylceramides is activated in adult rat sciatic endoneurium following permanent nerve transection. Furthermore, only glucocerebroside homologues, not galactocerebroside, are synthesized in purified Schwann cell culture (Yao and Yoshino, unpublished data). Thus, it is likely that the cells responsible for glucocerebroside biosynthesis are Schwann cells as these comprise 90% of the total endoneurial cell area in the distal nerve segment at 35 days after transection (6). However, this does not exclude the possibility that other cell types, such as macrophages, may contribute to glucocerebroside biosynthesis during nerve fiber degeneration. To further evaluate the metabolic role of galactosylceramides in peripheral nerve myelination, the metabolism of galactolipids was investigated by *in vivo* and *in vitro* incorporation of [^{14}C]acetate, [^{3}H]galactose or [^{3}H]glucose into rat sciatic endoneurium during nerve development, degeneration and regeneration.

MATERIALS AND METHODS

Materials. [^{14}C]Acetate, [^{3}H]galactose and [^{3}H]glucose were purchased from Amersham (Arlington Heights, IL). Unlabeled galactose was obtained from Sigma Chemical Co. (St. Louis, MO). High-performance thin-layer chromatography (HPTLC) plates (LHP-K with preadsorbent area) were from Whatman Chemical Separation (Clifton, NJ). Sep-Pak silica gel cartridges and cartridge rack were from Waters Associates (Milford, MA). EN^{3}HANCE was from New England Nuclear (Boston, MA). Lipid standards were from Supelco (Bellefonte, PA). All solvents (HPLC grade) were from Burdick and Jackson Laboratories (Muskegon, MI).

Endoneurial injection of [^{14}C]acetate. Using a nanoliter pump (model 1400 EC, W-P Instruments, Inc., New Haven, CT), the procedure used for endoneurial microinjection was essentially the same as that described previously (4,7). [1-^{14}C]Acetate (54-60 mCi/mmol) was diluted in Evans blue solution (0.5 mg/ml) to yield a concentration of 1.1×10^{7} dpm/μl. Under the dissecting microscope, the micropipette tip was placed inside the sciatic endoneurium and 0.4 μl of [^{14}C]acetate solution was injected at a rate of 0.1 μl/min. Unless otherwise indicated, one-month-old male Sprague-Dawley rats were used throughout this study.

[1] A preliminary report of this work was presented at the 19th Annual Meeting of the American Society for Neurochemistry, New Orleans, LA, March 7, 1988. Part of this work was done while the author was associated with the Mayo Clinic, Rochester, MN.

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Abbreviations: HPTLC, high-performance thin-layer chromatography; GalCe, galactocerebroside; GalCe-OH, hydroxy galactocerebroside; MGDG, monogalactosyl diacylglycerol; Su, sulfatide; Su-OH, hydroxy sulfatide; C, cholesterol; NL, non-polar lipids; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; Sp, sphingomyelin; TG, triacylglycerol.

Tissue preparation. Approximately 1 cm of sciatic nerve, including the injection site, was removed at various times after injection. Each group consisted of four or more nerve samples. Immediately after removal, the sciatic nerve was desheathed on a cold plate under a dissecting microscope. The desheathed portion was blotted dry with filter paper before the wet weight was taken. The endoneurium was then lyophilized overnight to obtain the dry weight.

Surgical procedure. Wallerian degeneration and regeneration were produced in rat sciatic nerve by either crush injury or transection according to the method described previously (8). Reattachment of transected nerve was according to the procedure of nerve xenograft described by Dyck *et al.* (9).

In vitro radiolabeled precursor incorporation. The method used for the *in vitro* incubation of sciatic endoneurium with [1-^{14}C]acetate, [^{3}H]galactose or [^{3}H]-glucose was essentially the same as described by Yao and Poduslo (5). Following dissection and weighing, the desheathed nerve was immediately placed in Lab-Tek tissue culture chamber/slides (Miles Scientific, Naperville, IL) which contained 0.5 ml of modified Krebs glucose solution (10). Incubation medium was mixed with 2.5 μCi of the sodium salt of [1-^{14}C]acetic acid (~60 mCi/mmol), 62 μCi of [1-^{3}H]glucose (19.4 mCi/mg) or 58 μCi of [^{3}H]galactose (58 mCi/mg) plus 71 μg of unlabeled galactose just before addition of nerve sample. Each nerve was incubated in a metabolic incubator at 37°C under a mixture of 95% O_2 and 5% CO_2. After various incubation times, the nerve segments were removed and washed five times with ice-cold unlabeled medium to ensure complete removal of free labeled precursor. The labeled nerve segment was then lyophilized to obtain the dry weight.

Lipid extraction and separation. The lipids were extracted from lyophilized tissues according to the procedure described previously (8,11). An aliquot was counted with a Beckman liquid scintillation counting system (model LS 5801) to obtain the total incorporation of [^{14}C]acetate, [^{3}H]galactose or [^{3}H]glucose into endoneurial lipids. Radiolabeled lipid profiles of endoneurium were analyzed by HPTLC using the procedure described by Yao and Rastetter (12). Separation of GalCe and glucocerebroside was achieved on borate-impregnated plates (13) using chloroform/methanol/water (60:17:2, v/v/v) as development solvent (14). Fluorography was used to identify the radiolabeled lipids following HPTLC (4), thus ensuring the accuracy and resolution of radioactivity distribution. Quantification of fluorograms was done using a LKB Ultrascan Laser Densitometer and Apple IIe computer. Isolation of neutral glycosphingolipids from total lipid extracts was achieved by column separation on a Sep-Pak silica cartridge (12).

Chemical reactions. To remove any phospholipids or glycolipids containing ester-linked acyl moiety, the isolated glycolipids were subjected to mild alkaline methanolysis according to the procedure described by Vance and Sweeley (15). To determine the radioactivity distribution among different moieties, the labeled glycosphingolipids were reacted with 0.5 N methanolic HCl at 100°C for 3 hr (16). The procedure used to extract fatty acid methyl esters, methyl glycosides, and sphingosine bases was essentially the same as described by Desnick *et al.* (17).

RESULTS

Endoneurial microinjection and in vitro *incubation of [^{14}C]acetate.* When [^{14}C]acetate was injected into the endoneurial portion of one-month-old rat sciatic nerve, approximately 9% of labeled lipids were found to be glycolipids (4). Following separation of ^{14}C-labeled glycolipids from nonpolar lipids and phospholipids on HPTLC and as revealed by fluorography (Fig. 1), [^{14}C]acetate was found to be incorporated first into GalCe-OH and later, although to a much lesser degree, into GalCe. Six hours after injection, only minute amounts of ^{14}C-labeled sulfatides were detected on the fluorogram when the HPTLC plate was exposed to Kodak X-Omat S film for 14 days. On the other hand, *in vitro* incorporation of [^{14}C]acetate (Fig. 2) resulted in higher ^{14}C-labeling in GalCe and MGDG, and lower ^{14}C-labeling in GalCe-OH, diminishing with incubation time. After 24 hr, only GalCe and MGDG were found to be labeled from [^{14}C]acetate. No radioactivity was found in the region of glucocerebroside following borate-impregnated HPTLC.

FIG. 1. Fluorograms of HPTLC of total ^{14}C-labeled lipids following endoneurial microinjection of [^{14}C]acetate into rat sciatic nerve after various periods of time (hr). Each sample represents total ^{14}C-labeled lipids extracted from 0.05 mg dry wt of endoneurium, except 0.15 hr (sample was extracted from 0.15 mg). After TLC development, the plate was exposed to Kodak X-Omat S film at −70°C for four days. O = origin.

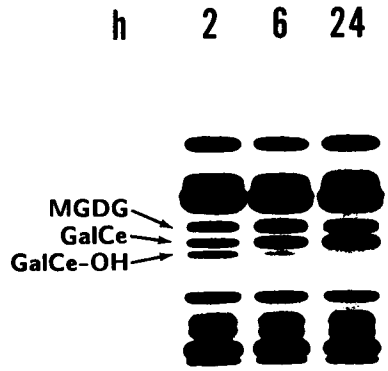

FIG. 2. Effect of incubation time on peripheral nerve galactolipid biosynthesis. Following *in vitro* incorporation of [^{14}C]acetate into rat sciatic endoenurium after various time periods, ca. 10,000 cpm/sample were applied to a thin-layer plate which was exposed to Kodak X-Omat S film at −70°C for five days. h = incubation time.

In vitro incubation of [^{3}H]galactose. The incorporation of [^{3}H]galactose into endoneurial lipids was substrate-dependent with an optimal concentration of 0.2 μmol/mg dry weight of endoneurium. There was a linear relationship between incorporation and incubation time up to 24 hr. [^{3}H]Galactose was rapidly incorporated into endoneurial GalCe-OH, and later into GalCe and MGDG (Fig. 3). Approx. 80% of radioactivity incorporated into endoneurial glycolipids were found in the carbohydrate moiety. In addition, ^{3}H-labeled nonpolar lipids and phospholipids were also identified by fluorography (Fig. 3). The ratio of ^{3}H-labeled GalCe-OH to GalCe decreased with incubation time and remained fairly constant after 6 hr (Fig. 4). A similar change in the ratio of ^{14}C-labeled GalCe-OH to GalCe with time was also demonstrated after endoneurial microinjection of [^{14}C]acetate into rat sciatic nerve (Fig. 4), except that GalCe-OH was predominantly labeled early in the experiment.

Effect of age on galactolipid biosynthesis. In vitro incorporation of [^{3}H]galactose into endoneurial lipids of rat sciatic nerve decreased rapidly with increasing age (Fig. 5). Approximately 70% of the labeled lipids were found to be galactolipids in rats four days of age as compared with <45% in rats five months of age or older. In addition to GalCe-OH and GalCe, [^{3}H]galactose was also selectively incorporated into MGDG, galactosulfatides (Su) and oligohexosylceramides (GL-4). The proportions of MGDG, Su and GL-4 to total labeled lipids all decreased substantially with increasing age (Fig. 6). When glycolipids were labeled by endoneurial injection of [^{14}C]acetate into rat sciatic nerve, a similar decrease with age was demonstrated (Fig. 7).

Biosynthesis of galactolipids during peripheral nerve degeneration and regeneration. To further demonstrate the preference of GalCe-OH biosynthesis during nerve regeneration, [^{14}C]acetate was incorporated *in vitro* into

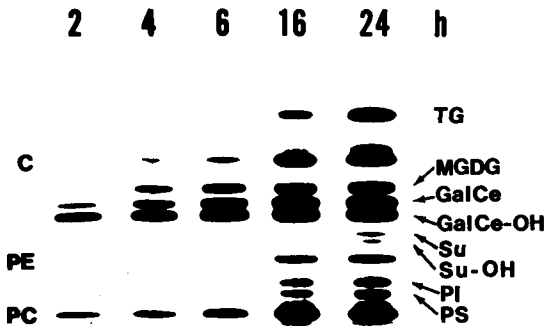

FIG. 3. *In vitro* incorporation of [^{3}H]galactose into endoneurial lipids of rat sciatic nerve after various incubation times. Each sample represents ^{3}H-labeled lipid extracts from 0.02 mg dry wt of endoneurium. After development, the thin-layer plate was exposed to Kodak X-Omat S film at −70°C for four days.

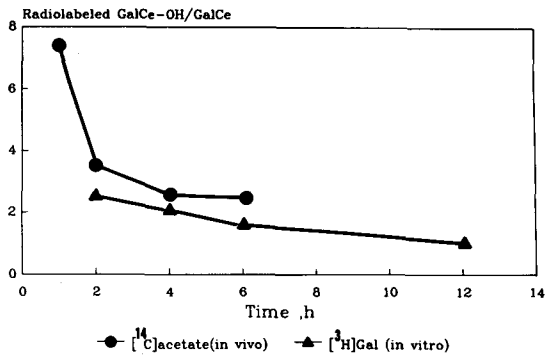

FIG. 4. Effect of incorporation time on the biosynthesis of GalCe-OH and GalCe following endoneurial microinjection of [^{14}C]acetate or *in vitro* incubation of [^{3}H]galactose with rat sciatic endoneurium.

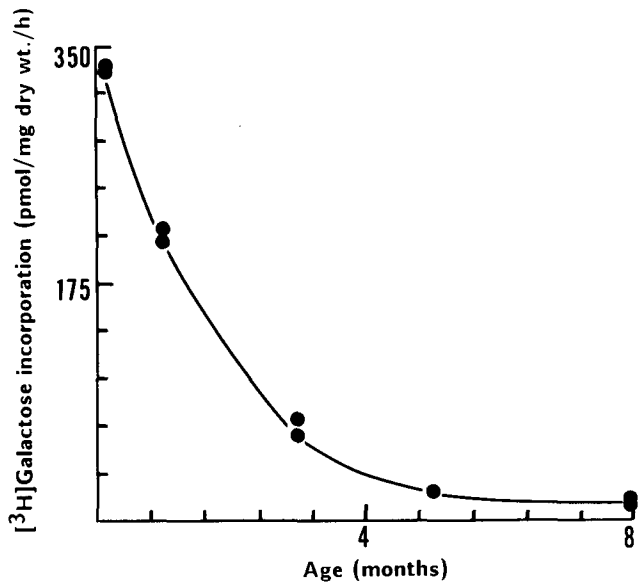

FIG. 5. Effect of age on peripheral nerve lipid biosynthesis after 24 hr *in vitro* incorporation of [^{3}H]galactose into endoneurial lipids of rat sciatic nerve.

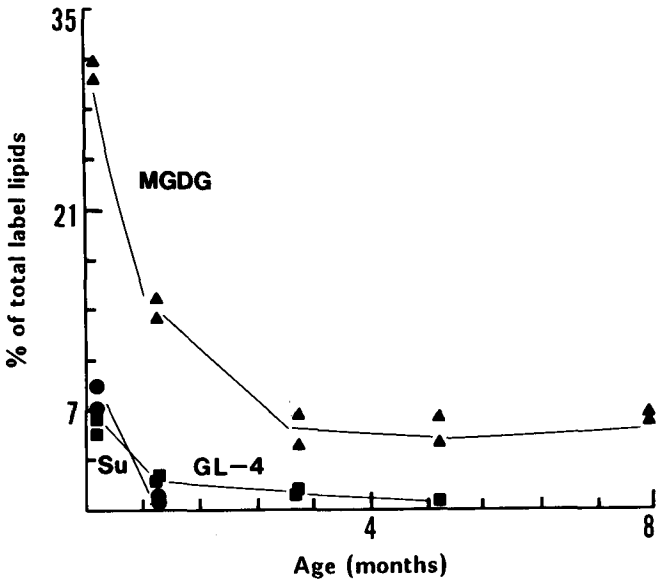

FIG. 6. Effect of age on the biosynthesis of MGDG, Su and tetrahexosylceramide (GL-4) after 24 hr *in vitro* incorporation of [^{3}H]galactose into rat sciatic endoneurium.

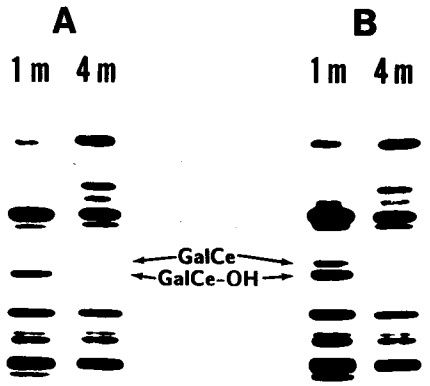

FIG. 7. Effect of age on biosynthesis of peripheral nerve galactocerebroside. Two hours after endoneurial microinjection of [^{14}C]-acetate into rat sciatic nerve, labeled lipid extracts from 5,000 cpm/sample (A) and from 0.25 mg wet wt of endoneurium (B) were applied to a thin-layer plate. After development, the plate was exposed to Kodak X-Omat S film at −70°C for three days.

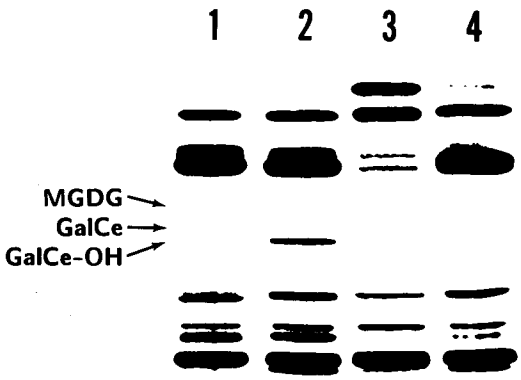

FIG. 8. Fluorograms of HPTLC of ^{14}C-labeled lipids following 2 hr *in vitro* incorporation of [^{14}C]acetate into endoneurial lipids of control nerve-contralateral uncrushed nerve (1), regenerated nerve-30 days after crush injury (2), degenerated nerve-60 days after transection (3), and regenerated nerve-30 days after reattachment of the transected nerve (4). Ca. 10,000 cpm/sample were applied to a thin-layer palte which was exposed to Kodak X-Omat S film at −70°C for four days. The unmarked bands are non-polar lipids (above MGDG) and phospholipids (below GalCe-OH).

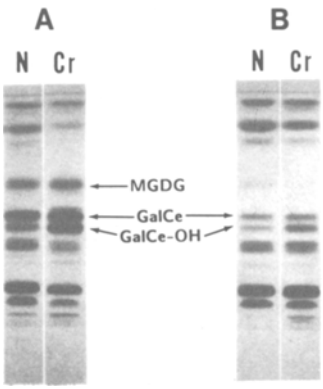

FIG. 9. Separation of ^{3}H-labeled galactolipids by borate-impregnated HPTLC. Following incorporation of [^{3}H]galactose (A) or [^{3}H]glucose (B) into normal nerve (N) and regenerated nerve 30 days after crush injury (Cr), ca. 10,000 cpm/sample were applied to a thin-layer plate which was exposed to Kodak X-Omat S film at −70°C for 13 days.

either crush-injured nerve or transected nerve after reattachment. As expected, there was a very low incorporation of [^{14}C]acetate into glycolipids of two-month-old nerve (Fig. 8, lane 1, contralateral uncrushed nerve). On the other hand, ^{14}C-labeling of glycolipids, particularly GalCe-OH, was substantially increased in regenerated nerve (Fig. 8, lane 2, crush-injured nerve 30 days after surgery). Glycolipid biosynthesis from [^{14}C]acetate was not observed (Fig. 8, lane 3, degenerated nerve 60 days after transection) in the absence of myelin assembly. Enhanced ^{14}C-labeling in GalCe-OH was demonstrated in regenerated nerve 30 days after reattachment of transected nerve (Fig. 8, lane 4). When [^{3}H]galactose or [^{3}H]glucose was substituted for [^{14}C]acetate as precursor in the *in vitro* incubation, increased ^{3}H-labeling in GalCe-OH was observed in regenerating nerves (Fig. 9).

DISCUSSION

The biosynthetic pathways of peripheral nerve glycosphingolipids are still not fully understood. Our previous data (4) had suggested that GalCe in rat sciatic endoneurium may be derived *in vivo* from ceramide via acylation of sphingosine. In newborn rats, the predominant glycolipid in sciatic endoneurium is GalCe-OH (3). The present study demonstrates that preferential labeling of GalCe-OH over GalCe occurs following either *in vivo* incorporation of [^{14}C]acetate or *in vitro* incorporation of [^{3}H]galactose into rat sciatic endoneurium. This suggests that hydroxy fatty acids and hydroxy ceramides in rat sciatic nerve may be the preferred substrates for the sphingosine acyltransferase and the UDP-galactose:ceramide galactosyltransferase reactions, respectively. It is not clear, however, whether hydroxy fatty acids must be activated to hydroxy fatty acyl-CoA prior to sphingosine acyltransfer.

A selectively increased proportion of nonhydroxy GalCe in adult rat sciatic nerve (3) may reflect the decreased availability of hydroxy fatty acids, because a substantial reduction of α-hydroxylation activity has, for example, been shown to occur in mature rat brain (18,19). Lack of ^{14}C-labeling in GalCe-OH after microinjection of [^{14}C]acetate into four-month-old rat sciatic endoneurium (Fig. 7) also implies that α-hydroxylation activity was diminished in mature adult rat sciatic nerve. Furthermore, the fatty acid α-hydroxylation activity, which regulates the rate of GalCe-OH biosynthesis, appears to be closely related to the process of rat brain myelination (18,19). In the present study, GalCe-OH biosynthesis was markedly increased in regenerating nerve, suggesting that the ratio of GalCe-OH to GalCe could be used as a biochemical index to measure peripheral nerve myelination.

Although the ratio of [^{3}H]GalCe-OH to [^{3}H]GalCe decreased with increased incubation time (Fig. 4) when [^{3}H]galactose was used as a precursor, the percentage of ^{3}H-labeling in total galactosylceramides (GalCe and GalCe-OH) decreased slightly from 60% at 2 hr, to 50% at 24 hr. On the other hand, there was virtually no ^{14}C-labeling in GalCe-OH after 24 hr when [^{14}C]acetate was used as precursor (Fig. 2). Thus, lack of *in vitro* incorporation of [^{14}C]acetate into GalCe-OH of rat sciatic endoneurium may indicate that certain components required for α-hydroxylation to occur may be lacking or destroyed under extended *in vitro* incubation conditions. Changes

due to age or incubation conditions should be taken into consideration when galactolipid biosynthesis is investigated in biopsied human nerve *in vitro*.

In normal adult rat sciatic nerve, where the rate of myelin assembly is substantially reduced and Schwann cells are mainly involved in maintaining the existing myelin membrane, [^{3}H]galactose was primarily incorporated into MGDG, GalCe and GalCe-OH (Fig. 3). In contrast, at 35 days after permanent nerve transection, in the absence of axonal regeneration or myelin assembly, incorporation of [^{3}H]galactose into galactolipids was hardly detectable. The ^{3}H-labeled glycolipids in transected nerve were identified as the glucocerebrosides and oligohexosylceramides (5). A similar increase in ^{3}H-labeling of oligohexosylceramides was demonstrated in explant cultures from neonatal nerves (20), in neonatal nerves (Fig. 6), and in bopsied sural nerves from patients with various types of peripheral neuropathy (21). Recently, we have also demonstrated (Yao and Yoshino, unpublished data) that the biosynthesis of glucocerebroside homologues, but not of galactocerebrosides, takes place in purified Schwann cell cultures. Thus, the shift in biosynthesis from GalCe to glucocerebroside homologues would support the idea of a possibly direct role of axons in specifying Schwann cell biosynthesis of galactolipids. The absence of such Schwann cell-axon signalling would result in the phenotypic expression of glucocerebroside homologues by the Schwann cell.

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Lipids

Notices
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10th Anniversary Meeting of the European Association for Cancer Research (University College, Galway, Ireland, September 11–13, 1989). For further information, contact Dr. S. M. Lavelle, Experimental Medicine, University College, Galway, Ireland; or in North America, contact Dr. J. H. Weisburger, American Health Foundation, Valhalla, New York, NY 10595-1599. The program involves plenary lectures, workshops, symposia and poster sessions.

Symposium on Lipid Lowering Drugs: Actions and Mechanisms, Ulm, Federal Republic of Germany, September 21–22, 1989. Plenary lectures will focus on current hypolipidemic drugs including fibrates, nicotinic acid and its derivatives, bile acid sequestrants and cholesterol absorption blockers, HMG-CoA-reductase inhibitors, and on newly developed agents. Mechanisms of action and clinical applications will be discussed. For further information contact: Eduard F. Stange, M.D., Department of Internal Medicine II, University of Ulm, Robert Koch Str. 8, D-7900 Ulm, Federal Republic of Germany. Telephone: 49-731-1776/3982; Fax: 49-731-1762038.

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Effect of Aging and Dietary Restriction on Bile Acid Metabolism in Rats

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The aim of the present study was to determine whether increased output of phospholipid in bile during aging may be due to alteration of bile acid composition and stimulated hydrophobic bile acid formation. In female Sprague-Dawley rats we examined the influence of aging and life long dietary restriction (60% of the *ad libitum* intake) on bile flow, total bile acid secretion, bile acid composition and conjugation pattern, as well as phospholipid output. Rats were cannulated at 3.5, 8-12 and 24-27 months of age and bile collected for analysis. With age, there was a significant reduction in bile flow and total bile acid secretion, however, phospholipid output increased. Restriction of dietary intake exerted a beneficial effect on the age-related decline in bile formation. Studies of bile composition indicated that 12α-hydroxylated bile acids (cholic acid and deoxycholic acid) secretion decreased in aged rats compared to 3.5-month-old rats. This was associated with a corresponding increase in secretion of chenodeoxycholic acid and hyodeoxycholic-ursodeoxycholic acid. However, the magnitude of the change in secretion of these bile acids could not account for the increased output of phospholipid in bile.
Lipids 24, 842-848 (1989).

The formation of bile is an important and specific function of the liver. Biliary bile acids are major determinants of bile flow. However, it has become evident that a substantial fraction of bile flow is not directly related to the transport of bile acids, but rather to the active transport of other substances (e.g., electrolytes) (1-3). Thus, canalicular bile consists of two fractions, a bile acid dependent fraction (BADF) and a bile acid independent fraction (BAIF). Studies from several laboratories, including our own (4-7), have shown that bile formation, like other liver functions, is altered with age. Both BADF and BAIF are reduced in aged rats. Moreover, dietary restriction, which is an effective intervention to increase mean and maximal survival of rodents, exerts a beneficial effect on the age-related decline in bile formation (7).

Bile contains a mixture of primary, secondary, and tertiary bile acids with different physiochemical and biological properties (3). Although infusion of all bile acids promotes the secretion of phospholipids, their effects differ significantly. Deoxycholic acid and chenodeoxycholic acid induce the secretion of significantly more phospholipid per mol of bile acid than cholic acid (8,9).

It is noteworthy that phospholipid secretion rates increase with age, but do not correlate with bile acid secretion (6,7). The uncoupling between bile acid and lipid component is unexpected and could be due to age-related alterations in bile acid metabolism, which promote higher secretion of phospholipid. Thus, in the present study, we examine biliary bile acid distribution and conjugation in rats during aging, and life-long dietary restriction.

EXPERIMENTAL

Sprague-Dawley strain female rats (Charles River, St. Constant, Quebec, Canada) were used. Animals were obtained at weaning age and housed individually in standard cages in an environment in which the temperature was maintained at a constant 22°C, with a 12 hr light/dark cycle (7 a.m.-7 p.m.). Rats were fed *ad libitum* until four weeks of age, with a diet prepared in pellet form by Teklad (Madison, WI), which consisted of 26% casein, 43% saccharose, 15% cornstarch, 4% corn oil, 0.1% cholesterol, 7.5% non-nutritive fiber (cellulose), 3.5% mineral mix (William-Briggs modified), and 1% vitamin mix (Teklad). Detailed diet composition has been published previously in the literature (10). At four weeks of age, rats were divided into groups of 15-20, one group of each time interval under study (i.e., 3.5, 8-12, and 24-27 months of age). Each of these groups was divided into two subgroups of 5-10 rats each.

The subgroups consisted of a control group which was fed *ad libitum,* and the experimental group, which was fed 60% (by weight) of the mean amount of food consumed by the control population. Food intake of the control group was recorded daily, and, the next day, 60% of this amount was fed to the experimental group. This procedure was followed until six months of age, when the food intake was monitored bi-weekly. The diet fed to the experimental groups contained an increased amount of minerals and vitamins, in order to provide the same intake as controls. All rats had unlimited quantity of drinking water. At each time interval under study, rats were anesthesized with pentobarbital (48 mg/kg) before the introduction of a PE 10 catheter into the bile duct. Body temperature was monitored and maintained throughout at 37 ± 1°C, with a rectal probe and a thermostatically controlled infrared heat lamp. Rats of both groups were not fed for 17-18 hr before they were cannulated. Bile was collected for 60 min, in 10 min aliquots, and bile flow, total bile acid output and bile acid distribution, as well as biliary lipid composition, was determined.

Following the last bile collection, all the animals were killed by an anesthetic overdose, and their livers excised and weighed. Liver specimens were taken, immediately fixed and processed as previously described for light and electron microscopy (11). Bile samples were collected in pre-weighed tubes and stored at −20°C until required for analysis. Bile flow was determined gravimetrically, assuming a density of 1 g/ml. Total biliary bile acids were determined by using 3-hydroxy steroid dehydrogenase as described by Nicolas *et al.* (12).

The individual biliary bile acids were analyzed by gas liquid chromatography. Details of procedure, characteristics of the column, and details of temperatures used have been given previously in the literature (13). With this method, hyodeoxycholic and ursodeoxycholic acids are not separated. Thus, the data obtained will be reported

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Abbreivations: ANOVA, analysis of variance; BADF, bile acid dependent fraction; BAIF, bile acid independent fraction.

as hyo-ursodeoxycholic acid. Bile acid conjugates were identified by thin-layer chromatography in butanol/acetic acid/water (10:1:1 by vol). The specific spots (identified with the aid of bile acid conjugates) were then scraped, and bile acid quantitated by an enzymatic method, using the Sterognost-3α P^{h}o kit supplied by Nyegaard (Oslo, Norway). Total biliary phospholipid was analyzed according to the Bartlett method (14), after digestion of all biliary components in perchloric acid, as described by Galliard *et al.* (15). To ascertain that changes occurred in phospholipid and not in phosphorus secretion, selected samples from the various groups were analyzed by an enzymatic method (16) employing a kit provided by Wako Chemicals (Dallas, Texas). This procedure has been proven to be adequate for measuring 90–100% of organic phosphorus (choline-containing phospholipids) in rat bile (17). Results indicated that changes in phosphorus were due to altered phosphatidylcholine secretion. Cholesterol was measured with a kit supplied by Boehringer-Mannheim (Montreal, Quebec, Canada) based on cholesterol oxidase. Cholesterol was extracted from bile by the method of Bligh and Dyer (18) prior to analysis because some biliary components interfered with this assay.

The values were assessed for significance with two-way analysis of variance (ANOVA) and Newman-Keul's test. A value of $P < 0.05$ was considered to be significant.

RESULTS

Characteristics of the rat population. The percent survival in groups fed *ad libitum* and 40% restriction is shown in Figure 1. As expected, the percentage of survival decreases markedly with age after 12 months in the *ad libitum* fed group. At 27 months of age, 53% of rats were alive in the *ad libitum* fed group compared to 74% for the group fed restricted diet.

The records of food intake (g/day) (Fig. 2) indicate that the group fed *ad libitum* consumed significantly more food in the period between 1 and 3 months and 15 and 21 months of age. Besides these periods, food intake was quite stable. As expected, the food intake of the diet restricted group paralleled that of the control group.

Details of the disease processes occurring in our colony have been described elsewhere in the literature (19). It is sufficient here to indicate that nephropathy was found in all rats fed *ad libitum* at the time of spontaneous death. Age-associated progression of renal disease was evident in the *ad libitum* fed group and significantly prevented by diet restriction, as judged by study of tissue taken at various ages. The development of spontaneous neoplastic disease was another process markedly affected by diet restriction. In rats fed *ad libitum*, mammary gland adenomas were the most frequent tumors occurring with increasing age. Thus, at 27 months of age, 41% of rats fed *ad libitum* had tumors, as opposed to 10% in diet restricted group.

Studies of liver morphology at the light microscopy level indicated that the only conspicuous change observed in old rats (24–27 months of age) was lipid droplet accumulation. This was seen in rats fed *ad libitum* and the restricted diet, but the lipid accumulation was more marked in the *ad libitum* group. By electron microscopy, qualitative analysis of hepatocytes of midzonal areas corroborated lipid droplet accumulation. In addition, lysosomes, autophagic vacuoles and dense bodies were more numerous in older than in younger animals. These findings agree with those described earlier by our laboratory (20) for male Sprague-Dawley rats. With regard to the bile secretory apparatus of the hepatocytes, the appearance of the bile canaliculi at all ages was virtually similar. Canaliculi showed abundant microvilli and the Golgi apparatus was well developed.

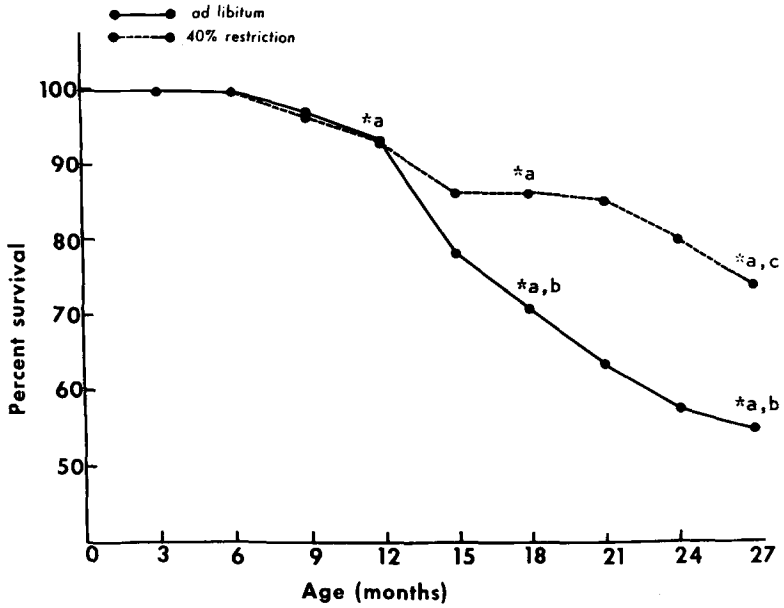

FIG. 1. Percent survival in groups of rats fed *ad libitum* and restricted diet. n = 35. *a, $p < 0.05$ when compared to rats of 3.5 months fed the same diet; b, $p < 0.05$ when compared to rats of 8–12 months fed the same diet; c, $p < 0.05$ when compared to rats of same age fed *ad libitum*.

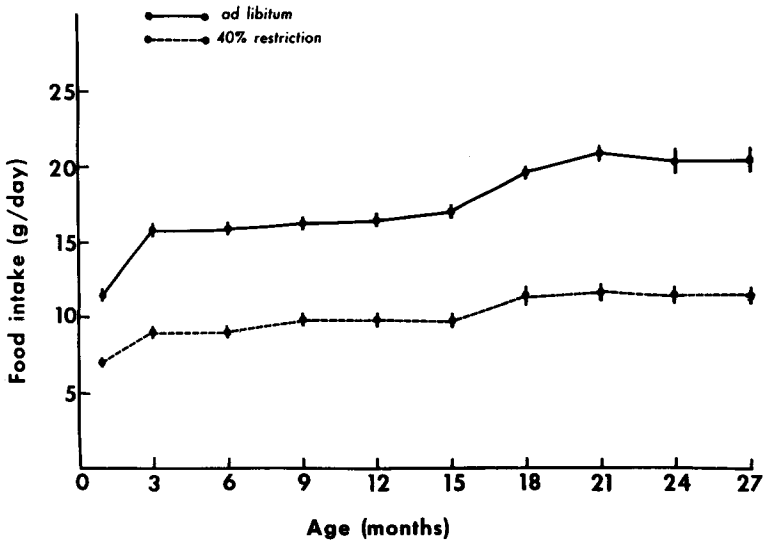

FIG. 2. Food intake in rats fed *ad libitum* and restricted diet.

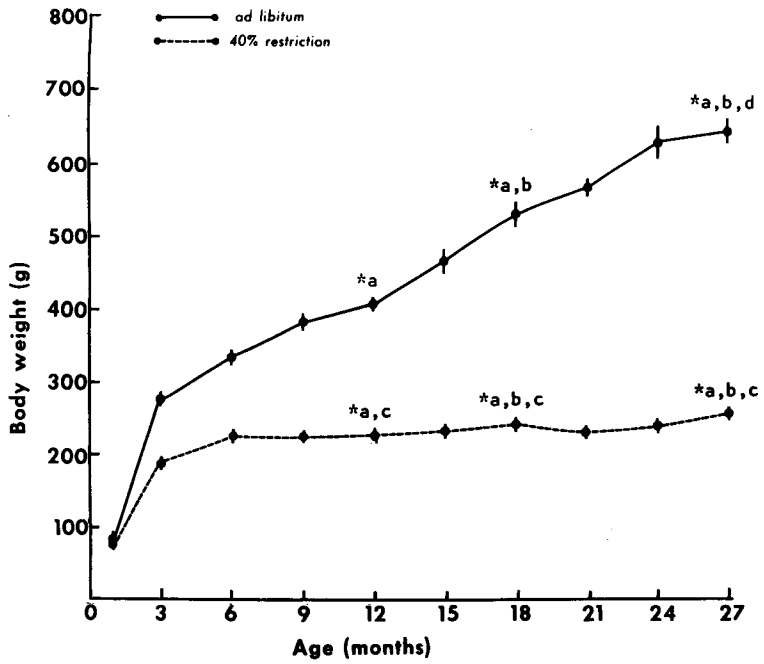

FIG. 3. Body weight changes with aging in rats fed *ad libitum* and restricted diet. *a, $p < 0.05$ when compared to rats of 3.5 months fed the same diet; b, $p < 0.05$ when compared to rats of 8–12 months fed the same diet; c, $p < 0.05$ when compared to rats of same age fed *ad libitum*; d, $p < 0.05$ when compared to rats of 18 months fed the same diet.

Body and liver weights. Figure 3 shows that the body weight of rats fed *ad libitum* increased with age, and that in the group fed restricted diets, body weight increased up to 12 months, remaining stable thereafter. At each time period, body weights of rats fed restricted diet were significantly lower than rats fed *ad libitum.* The liver weights (Table 1) also increased with age, and this may be accounted for in part by the lipid droplet accumulation as shown by morphologic analysis.

Bile flow and biliary secretion of bile acids, phospholipid, and cholesterol. Changes in bile formation observed in the present study corroborate those recently published by this laboratory (7). Briefly, bile flow (expressed as μl/min/g) decreased significantly ($p < 0.05$) in aged rats fed *ad libitum* (0.87 μl at 24–27 months of age vs 1.17 μl at 3.5 months of age). By contrast, bile flow of rats fed the restricted regimen did not decrease with age, being (at 8–12 and 24–27 months, respectively) 34% and 43%

higher than that of corresponding controls. Total bile acid secretory rate also decreased significantly ($p < 0.05$) in the old *ad libitum* fed rats (25.38 nmol/min/g liver at 24–27 months, vs 31.05 nmol/min/g liver at 3.5 months of age). Bile acid output of diet restricted rats was significantly higher at 24–27 months of age (40–50%, $p < 0.05$) when compared to age-matched controls. The secretion of phospholipid in rats fed *ad libitum* increased with age (2.97 vs 1.93 nmol/min/g liver at 24–27 and 3.5 months of age, respectively), while that of cholesterol

TABLE 1

Body and Liver Weights of Aging Rats Fed *Ad Libitum* (A) or Restricted Diets (R)

Age	Diet	Body weight (g)	Liver weight (g)	Liver weight (g/100 g body weight)
3.5 months	A	276 ± 29	7.39 ± 0.79	2.54 ± 0.27
	R	187 ± 15	5.08 ± 0.20	2.63 ± 0.24
8–12 months	A	410 ± 81[a]	9.17 ± 0.68	2.28 ± 0.12
	R	226 ± 23[c]	6.70 ± 0.14[a]	2.92 ± 0.07
24–27 months	A	637 ± 120[a,b]	18.24 ± 0.96[a,b]	3.20 ± 0.23[b]
	R	239 ± 20[c]	7.75 ± 0.39[c]	3.31 ± 0.12

Values are means ±SEM.

[a] $p < 0.05$ when compared to rats at 3.5 months, fed the same diet.

[b] $p < 0.05$ when compared to rats at 8–12 months fed the same diet.

[c] $p < 0.05$ when compared to rats of the same age, fed *ad libitum*.

TABLE 2

Percent of Biliary Bile Acids: Effect of Age and Diet

	3.5 months		8–12 months		24–27 months	
Bile acids (%)	A	R	A	R	A	R
Cholic acid	59.87 ± 2.88	70.34 ± 3.66	72.95 ± 3.52[a]	57.76 ± 2.87	48.00 ± 3.33[a,b]	44.95 ± 2.55[a,b]
Deoxycholic acid	5.21 ± 1.06	3.94 ± 1.04	4.36 ± 0.39	4.65 ± 0.39	6.02 ± 0.82	6.50 ± 0.60
Chenodeoxycholic acid	7.15 ± 0.77	5.70 ± 1.51	5.92 ± 1.58	8.66 ± 1.41	13.94 ± 2.23[a,b]	10.02 ± 1.63[a]
Hyo-ursodeoxycholic acid	7.89 ± 2.45	5.21 ± 1.27	Non-detectable	6.64 ± 1.81	13.77 ± 1.51[a]	14.39 ± 2.29[a,b]
Muricholic acids	11.44 ± 0.47	10.05 ± 0.94	11.53 ± 0.81	10.22 ± 1.01	9.96 ± 1.23	9.04 ± 0.70
Ketolithocholic acid	6.81 ± 2.03	4.29 ± 0.48	1.90 ± 0.40	11.44 ± 1.06[c]	3.43 ± 1.00	8.30 ± 1.36[c]
Other bile acids	2.02 ± 0.80	1.77 ± 0.52	1.85 ± 0.27	2.29 ± 0.50	5.62 ± 1.53[b]	6.80 ± 1.70[a,b]

Values are means ± SEM. A, *ad libitum;* R, restricted diet.

[a] $p < 0.05$ when compared to rats at 3.5 months, fed the same diet.

[b] $p < 0.05$ when compared to rats at 8–12 months, fed the same diet.

[c] $p < 0.05$ when compared to rats of the same age, fed *ad libitum*.

TABLE 3

Actual Rate of Bile Acids Secreted (nmol/min/g liver): Effect of Age and Diet

	3.5 months		8–12 months		24–27 months	
Bile acids	A	R	A	R	A	R
Cholic acid	18.24 ± 1.85	37.90 ± 7.62[c]	18.64 ± 1.91	20.42 ± 3.22[a]	11.45 ± 2.08[a]	27.20 ± 3.77[a,c]
Deoxycholic acid	1.64 ± 0.45	1.93 ± 0.45	1.10 ± 0.11	1.59 ± 0.16	1.31 ± 0.24	2.21 ± 0.30
Chenodeoxycholic acid	1.13 ± 0.12	2.73 ± 0.39	1.43 ± 0.30	2.98 ± 0.49	3.26 ± 0.82[a,b]	3.91 ± 0.93
Hyo-ursodeoxycholic acid	2.47 ± 0.75	2.48 ± 0.64	Non-detectable	2.16 ± 0.23	3.00 ± 0.41	5.07 ± 1.03[a]
Muricholic acids	3.45 ± 0.18	5.25 ± 0.90	2.94 ± 0.32	3.54 ± 0.44	2.18 ± 0.33	3.67 ± 0.76
Ketolithocholic acid	1.95 ± 0.55	2.22 ± 0.39	0.45 ± 0.06[a]	3.87 ± 0.17[a,c]	0.85 ± 0.34	3.13 ± 0.55[c]
Other bile acids	0.66 ± 0.31	0.88 ± 0.23	0.48 ± 0.09	0.78 ± 0.18	1.19 ± 0.30	2.16 ± 0.46[a,b]

Values are means ± SEM. A, *ad libitum;* R, restricted diet.

[a] $p < 0.05$ when compared to rats at 3.5 months, fed the same diet.

[b] $p < 0.05$ when compared to rats at 8–12 months, fed the same diet.

[c] $p < 0.05$ when compared to rats of the same age, fed *ad libitum*.

decreased (0.32 vs 0.43 nmol/min/g liver at 24-27 vs 3.5 months of age, respectively). Similarly to what was observed for bile acid, the phospholipid and cholesterol secretory rates of diet restricted rats were higher (28-48%) than those observed in age-matched controls.

Biliary bile acid composition. Table 2 shows that the percentage distribution of bile acids is altered with age. The contribution of cholic acid decreased significantly between 3.5 and 24-27 months of age, while that of chenodeoxycholic acid, hyo-ursodeoxycholic acid, and that of bile acids listed as other bile acids increased. There was a tendency towards a decrease in keto-lithocholic acid, but the differences did not prove to be statistically significant. The bile acid distribution was not markedly altered by diet restriction, the only exception being keto-lithocholic acid levels, which increased with age.

The actual rate of bile acids secreted is shown in Table 3. The pattern of change with age is similar to that obtained when results were expressed as percentage bile acid distributions. Since the rate of total bile acid secreted was higher in diet-restricted rats, the secretion rate of all bile acids increased. However, the differences were statistically significant only for cholic and keto-lithocholic acid.

The results of bile acid secretion rate given, grouped according to their biosynthetic pathway, generally tended to corroborate the results presented in Tables 2 and 3. As seen in Figure 4, there is a significant decrease ($p < 0.05$) in the aged rats of bile acids containing the 12-hydroxyl group. No statistically significant changes were observed for the bile acids containing the 6 β-hydroxyl group, or the other bile acids.

The percentage of tauro and glyco conjugated bile acids are given in Figure 5. In rats fed *ad libitum*, the T/G (tauro/glyco) ratio remained constant, regardless of age. In diet-restricted rats, no change in conjugation pattern was observed at 3.5 and 12 months of age. However, at 24-27 months, a significant decrease in tauro conjugates and a proportional increase of glyco conjugates were observed. In the restricted group, the higher bile acid output rate was mainly contributed by the tauro conjugated bile acids.

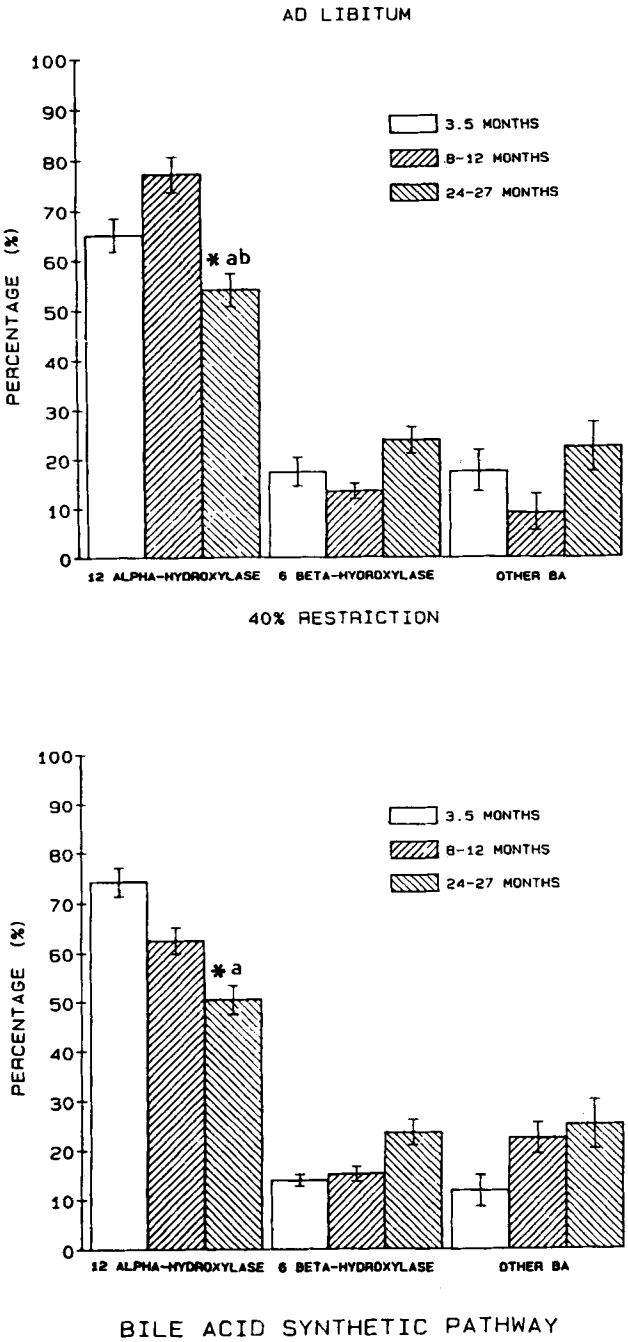

FIG. 4. Percent of biliary bile acids secreted according to their synthetic pathway: Effect of age and diet. 12α-hydroxylase indicates bile acids with 12-hydroxyl groups. 6β-hydroxylase indicates bile acid with 6-hydroxyl groups. *a, $p < 0.05$ when compared to rats of 3.5 months fed the same diet; c, $p < 0.05$ when compared to rats of the same age fed *ad libitum*.

DISCUSSION

The present results corroborate previous data that bile formation decreases with age and that decreased bile acid output rate per unit liver weight is associated with higher phospholipid output (6,7).

It is important to note that although all studies which examined bile formation with aging in rats reported decreased bile flow (5-7,21,22), not all observed a decreased bile acid secretion rate (22). This is difficult to explain as being associated with sex or strain of rat examined since male (5,6) and female (7) Sprague-Dawley rats, as well as Fisher male rats, showed decreased bile flow and bile acid secretion rate. Perhaps liver pathology affecting the hepatobiliary system varies between the various rat populations, and this influences bile formation. Thus, the findings on bile acid secretion with aging are not universal in rats and future studies assessing hepatobiliary function should take into account age related liver pathology. Another factor that may influence bile formation is the diet composition, which will also alter bile acid metabolism. It was suggested that changes in bile acid composition could account for the increase in phospholipid secretion rate with aging. However, the data obtained in the present study indicates that although the contribution of chenodeoxycholic acid increased, the change was too small to account for the phospholipid secreted. In studies of biliary lipid secretion during bile

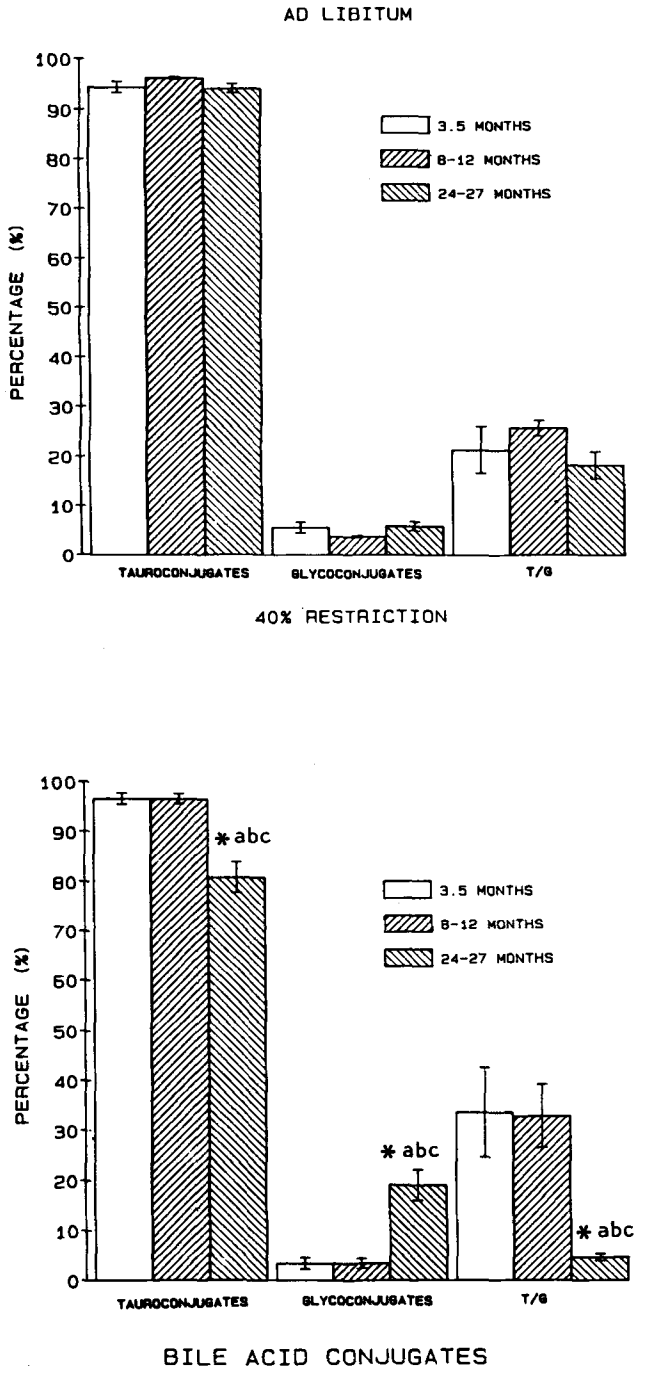

FIG. 5. Percentage of tauro- and glyco-conjugated bile acids: Effect of age and diet. *a, $p < 0.05$ when compared to rats of 3.5 months fed the same diet; b, $p < 0.05$ when compared to rats of 8–12 months fed the same diet; c, $p < 0.05$ when compared to rats of same age fed *ad libitum*.

acids infusions in adult rats, we showed that 1 nmol of chenodeoxycholic acid secreted per min and per g liver promoted the secretion of 0.15 nmol of phospholipid (9). The increase of chenodeoxycholic acid with age secreted was of 1.13 nmol which would promote 0.16 nmol of phospholipid. The actual phospholipid output increased by 1.04 nmol per min, a value too high to be accounted for by the change in the secretion of chenodeoxycholic acid. One possibility is that the bile canalicular membrane of the aged rats may be more susceptible to the detergent properties of the bile acids. There is support to this view since, in recent preliminary studies of bile canalicular membrane composition, it was shown that membranes from 12-month-old rats exhibited lower enzyme activity (e.g., Na+, K+, ATPase) and phospholipid content than 4.5 months old rats (23). It has been proposed that the secretion of phospholipid and plasma membrane enzymes in bile is linked to the secretion of bile acids (7). Moreover, these materials are solubilized from the canalicular membranes and there is continuous replacement by repair components which will prevent extensive damage to the membrane by the bile acids (24). Thus, the lower phospholipid content of the membrane could also result from inadequate replacement by repair components because of limited supply or altered transport from the site of synthesis to the canalicular membrane. Alternatively, it is also possible to explain the higher phospholipid output with age from misdirected secretion from the sinusoidal to the canalicular pole of the cell. Such phenomenon does occur when microtubule network function is disrupted by colchicine.

As the contribution of chenodeoxycholic acid increased with age, that of cholic acid decreased, the differences having been statistically significant when results were expressed as percentage of biliary bile acid composition. However, such changes in bile acid distribution were not found by other researchers (6). In comparing the data, it may be that factors such as animal's sex (25) and diet may be responsible for the difference since earlier work examined only male rats and did not use defined diets. The bile acid distribution was not markedly altered by the dietary restriction, except for keto-lithocholic acid, which had a tendency to increase in the diet restricted group when compared with the corresponding controls. This may be of interest as it has been shown that keto-lithocholic acid (the precursor in the synthesis ursodeoxycholic acid from chenodeoxzycholic acid) possesses choleretic properties greater than that of other bile acids (26), and could contribute to the higher bile acid dependent and independent bile flow observed in the food restricted rats (7) and the increase in hyo-ursodeoxycholic acid. Since hyo-ursodeoxycholic acid in rats may derive from the keto intermediate formed mainly in the intestine, it is possible that aging influences the intestinal bacteria involved in the bile acid oxidation.

Analysis of the pattern of conjugation of the bile acids indicates that the tauroconjugates predominate in the rats of all ages. The ratio of taurine to glycine conjugates was not altered during aging in rats fed *ad libitum*, but it decreased in the older age group fed the restricted diet. Kroker *et al.* (5) also examined the pattern of bile acid conjugation with age in rats fed *ad libitum*, but in rats of the Wistar strain.

They noted a lower ratio of tauro to glyco conjugates in the older age group examined, similar to what we observed in the diet restricted aged rat. It may be that the diet intake of the Wistar rats was low compared to that of the Sprague-Dawley, rendering the Wistar group fed *ad libitum* comparable to the diet restricted group. The shift in conjugation with age may be explained by a reduction of the hepatic taurine available for bile acid conjugation. Hardison (27) has demonstrated that the

proportion of taurine conjugates is directly related to the hepatic taurine concentration. Decreased activity of the enzyme cysteine sulfinate decarboxylase (the limiting factor in taurine synthesis) may occur with aging. It is known that the biosynthesis of taurine is reduced in early development and this was attributed to decreased activity of the cysteine sulfinate decarboxylase (28). A similar situation may occur in the aged rat when the dietary supply of precursor aminoacids is limited. It is known that vitamin B6, in the form of pyridoxal 5-phosphate, is a necessary co-enzyme in the synthesis of hypotaurine and that vitamin B6 deficiency reduces taurine synthesis (29). This factor could be excluded in our experiments because diets fed to restricted groups provided similar amounts of vitamins and minerals to that fed to *ad libitum* groups.

In summary, aging alters bile acid metabolism in female Sprague-Dawley rats, but the changes are not sufficient to account for the greater output of phospholipid observed in the aged rat. Dietary restriction does not have a significant effect on bile acid distribution but it decreased the proportion of tauroconjugates in the older age group. Further work is needed to elucidate the relationship between diet, taurine metabolism and aging.

ACKNOWLEDGMENTS

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Characterization of Acylmono-, Mono-, Di-, Tri- and Tetraglycosylsterol and Saponin in Adzuki Bean (*Vigna angularis*) Seeds

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Five sterylglycosides (acylmono-, mono-, di, tri- and tetraglycosylsterol) and a saponin were isolated from Adzuki beans and characterized. In the glycosylsterols, the principal component sterols were sitosterol and stigmasterol; the major sugar component was glucose. The glucose units were shown to be linked by β1,6-bonds. The three oligoglycosylsterols were shown to be gentiobiosylsterol, gentiotriosylsterol and gentiotetraosylsterol; the latter two are novel sterylglycosides. The saponin was identified as glucopyranosyl-(β1→2)-glucopyranurosyl-(β1→3′)-soyasapogenol B (Azukisaponin I) which had previously been found in Adzuki beans.
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Sterylglycosides are typical glycolipids which are widely distributed in the plant kingdom (1,2). As the monoglycosyl type has recently been reported to be present in animal tissues (3,4) and in some microorganisms (5,6), sterylglycosides may be wide-spread in nature. In earlier studies, in addition to monoglycosylsterol (G_1ST) and acylmonoglycosylsterol (AG_1ST), which are the well-known classes, small amounts of di-, tri-, tetra- and pentaglycosylsterol ($G_{2-5}ST$) were isolated and their basic structures were identified in rice bran and corn seeds (7-9). Oligoglycosylsterols have also been isolated from leaves such as rice (10), green tea (11), *Lindenbergia indica* (12), and *Musa paradisiaca* (13,14), and were shown to differ in their glycosidic structures among plant species. On the other hand, saponins, which are closely related structurally to sterylglycoside, are known to be present in higher plants. Although the lipid analogues exhibit various pharmacological effects (13,15), little information is available concerning the biological activity of sterylglycoside, except for their anti-ulcerogenic activity (13,15) and their growth-promoting effects (16). Structural and metabolical studies on plant sterol lipids have been done, but the physiological role of these sterol lipids remains to be elucidated.

Previously, we reported on the chemical composition of non-polar sterol lipids including triterpene compounds in Adzuki bean *(Vigna angularis)* seeds (17). Further analysis of the sterol lipids revealed the existence of several glycolipids, which showed a positive test for sterol-specific reagents. AG_1ST, G_1ST, G_2ST, G_3ST and G_4ST, as well as saponin with a disaccharide moiety were included. In the present paper, the structural characterization of the polar steryl glycolipids found in Adzuki beans is described.

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Abbreviations: AG_1ST, acylmonoglycosylsterol; FD-MS, field desorption-mass spectrometry; G_2SA, diglycosyl type of Adzuki bean saponins; G_1ST, monoglycosylsterol; $G_{2-5}ST$, di-, tri-, tetra-, pentaglycosylsterol; GC-MS, gas chromatography-mass spectrometry; GLC, gas-liquid chromatography; IR, infrared; PMR, proton magnetic resonance; TLC, thin-layer chromatography; TMS, trimethylsilyl.

EXPERIMENTAL METHODS

Isolation of glycosylsterols and saponins. Adzuki beans (Erimo variety, harvested in 1983) were milled and immediately steamed to inactivate the enzymes. Total lipids were extracted with chloroform/methanol (2:1, v/v) and water-saturated butanol, as reported previously (7,17). Part of the lipids were subjected to silicic acid column chromatography and eluted successively with chloroform, acetone and methanol in order to obtain neutral, glycolipid and phospholipid fractions (18). The commonly present AG_1ST and G_1ST were isolated and purified from the glycolipid fraction by a combination of silicic acid column chromatography and thin-layer chromatography (TLC) on silica gel G (19).

To separate the oligoglycosylsterols and saponins, the residual total lipids were subjected to silicic acid column chromatography. Polar lipids were eluted with methanol and chloroform/methanol/water (16:16:5, v/v/v) after removal of neutral lipids by eluting with adequate amounts of chloroform, followed by methanolic 0.4N KOH treatment at 37°C for two hours to remove the glycerolipids. The alkali-stable polar lipids thus obtained were subjected to silicic acid column chromatography using the stepwise chloroform/methanol elution (7). G_2ST, G_3ST, and G_4ST, including saponins, were eluted with chloroform/methanol in the ratios of 80:20-70:30, 65:35 and 40:60-0:100, respectively. Each sterylglycoside was isolated by preparative TLC on silica gel G with chloroform/methanol/water (65:25:4, v/v/v) as developing solvent, acetylated, and purified further by TLC on silica gel G using chloroform/benzene/acetone (80:20:20 or 80:20:40, v/v/v) (7). The obtained lipids were deacetylated with 0.4N KOH, as described above, to obtain pure G_2ST, G_3ST and G_4ST. On the other hand, saponins eluted together with G_4ST from the silicic acid column were fractionated into individual compounds by preparative TLC on silica gel G with chloroform/methanol/water (60:35:8, v/v/v). Only one pure form of saponin was isolated from the crude saponin fraction.

Analyses of components. Each glycosylsterol was methanolyzed in order to obtain component sterols, fatty acid methylesters and methylglycosides, and were analyzed by gas-liquid chromatography (GLC) (7). However, the configuration at C-24 of the component sterols in each of the glycosylsterols were not identified.

The isolated saponin was heated under reflux with 5% HCl in methanol for two hours. After cooling, the reaction mixture was neutralized with Ag_2CO_3, filtered to remove salts, and then evaporated to dryness. The methanolysis products, which contained methylglycosides and sapogenins, were converted to trimethylsilyl (TMS) ether derivatives for GLC analysis. Because some saponins are known to contain uronic acid as the component sugars, the saponin was converted to acetyl derivatives followed by reduction with $LiAlH_4$ (20). The reduced saponin was methanolyzed as described above to obtain methylglycosides.

Analyses of glycosidic chains. To examine the binding positions in the saccharide moieties, each glycosylsterol and saponin were permethylated according to Kuhn (21) and Hakomori (22). Methylated products recovered from the chloroform phase by partitioning the reaction mixture with chloroform and water were purified by TLC on silica gel G with chloroform/benzene/acetone (80:20:10 or 80:20:30, v/v/v) (7) as solvent. A portion of the permethylated sterol glycolipids were hydrolyzed with 2N HCl for two hours at 100°C to yiled partially methylated sugars, which were later reduced with $NaBH_4$ and acetylated to get partially methylated alditol acetates (7), while the other portion was methanolyzed to give methylated methylglycosides (7).

In order to determine the anomeric configuration in the sugar chains, glycosylsterols were acetylated along with inositol as an internal standard, followed by oxidation in acetic acid with CrO_3 (23). The oxidized product was recovered from the reaction mixture with chloroform and was methanolyzed as described above. The surviving methylglycosides were determined by GLC (7).

Instrumental analyses. Infrared (IR) spectra were taken on a JASCO infrared spectrophotometer (Model A-3, Nippon Bunko Kogyo Co., Tokyo) using KBr pellets. GLC analyses were performed with the Hitachi Gas Chromatograph (Model 163, Hitachi Seisakusho Co., Tokyo) equipped with a hydrogen ionization detector. Sterols, methylglycosides and fatty acid methylesters were analyzed under the same conditions as described in the previous report (17). Partially methylated alditol acetates and methylated methylglycosides were analyzed on 3% ECNSS-M at 150°C, and on 3% NPGS at 175°C, respectively. Gas chromatography-mass spectrometry (GC-MS) and field desorption-mass spectrometry (FD-MS) were carried out under the same conditions as described previously (7,24). Proton magnetic resonance (PMR) spectra were measured using a JEOL JNM-FX-200 (200 MHz) instrument (24). Glycosylsterols were assayed in dimethylsulfoxide. The chemical shifts were recorded in parts/million by using tetramethylsilane as an internal standard.

RESULTS

Species of polar steryllipids. TLC of the polar lipid fraction with chloroform/methanol/water (65:25:4, v/v/v) produced four fractions (Rf 0.84, 0.66, 0.16 and 0.08) which gave positive color reactions with anthrone and 50% sulfuric acid. The observed Rf values of 0.84 and 0.66 and the IR spectra were identical to those of authentic AG_1ST and G_1ST prepared from rice bran (19). Based on the Rf values reported in the literature (7,20), the other two spots were presumed to be saponins. The IR spectrum of the purified saponin with Rf 0.16 exhibited an absorption peak at 1720 cm^{-1} due to the carboxyl group, in addition to notable peaks due to methyl, methylene and alcoholic hydroxy groups usually found in glycosylsterols (19). When the alkali-stable polar lipids were subjected to TLC under the same conditions as described above, three minor spots (Rf 0.43, 0.28 and 0.17) containing sterol and sugar were found. The Rf values and the IR spectra were almost identical to those of G_2ST, G_3ST and G_4ST isolated from rice bran (7,8). Ten, 6 and 4 mg of G_2ST, G_3ST and G_4ST, respectively, were isolated from 10 kg of Adzuki beans. The relative proportions of AG_1ST, G_1ST and Rf 0.16 saponin in the polar lipid fraction were roughly 3.5, 1.5 and 0.7%, respectively.

Sterol composition of glycosylsterols. TLC of the lipophilic fraction in the methanolysates of AG_1ST and G_1ST using hexane/diethyl ether/acetic acid (70:30:1, v/v/v) revealed only 4-desmethylsterol, whereas the three oligoglycosylsterols contained 4-desmethylsterol and small amounts of methylsterol (19). 4-Desmethylsterol composition of glycosylsterols from Adzuki beans is shown in Table 1. At least seven sterols were found in the form of free sterol and acylsterol (17). The predominant components were sitosterol and stigmasterol. It could be assumed that the 24-methylcholesterol may probably be an epimeric mixture of 24α- and 24β-epimers (campesterol and dihydrobrasicasterol), and the 24-ethylcholesterol may have only the α-configuration (25,26). However, nuclear magnetic resonance (NMR) analyses were not carried out to establish the configurations.

Sapogenin composition of saponins. TLC of the component sapogenins in the methanolysates of the crude saponin fractions with hexane/diethyl ether/acetic acid (70:30:1, v/v/v) revealed several spots with Rf values lower than those of 4-desmethylsterol and oleanol (17). When the aglycons were subjected to GC-MS at least four peaks were found, which were identified as soyasapogenol B, soyasapogenol C, soyasapogenol D and adzukisapogenol based on the characteristic mass fragment ions reported in the literature (27) (Fig. 1). Among these, the major component was soyasapogenol B. The component sapogenin in purified saponin (Rf 0.16 compound) was only soyasapogenol B.

Sugar composition of glycosylsterols and saponin. The sugar composition of glycosylsterols and saponin from Adzuki beans is shown in Table 2. Considerable amounts of glucose were recognized in all glycosylsterols, while galactose was found in small amounts. When the methanolysates of the purified saponin were subjected to GLC, glucuronic acid was found together with glucose. The ratio of glucose to glucuronic acid was approximatery 1:0.6, but with the reduction of the saponins all the glucuronic acid was converted to glucose, and the amount found was almost twice that of the purified saponin. This suggests that the saponin isolated from Adzuki beans was of the diglycosyl type (G_2SA), with the saccharide chain consisting of glucose and glucuronic acid in equimolar proportions.

Anomeric nature of the glycosidic linkage. The recoveries of the methylglycosides after oxidation of acetyl oligoglycosylsterols with CrO_3 were 10, 18 and 13% in G_2ST, G_3ST and G_4ST, respectively. Therefore, all the glucose units in the oligoglycosylsterols should be linked by the β-glycosidic configuration (7,23). PMR of reduced G_2SA revealed two doublets at 4.40 ppm (J = 7.1 Hz) and 4.67 ppm (J = 7.2 Hz) due to the anomeric protons of the saccharide residues, which corresponded to the chemical shift (near 4.4 ppm) and coupling constant (J = 7.2 $\pm$ 0.2 Hz), indicating a β-glycosidic glucose linkage (20,24). This confirms that the glycosidic linkages in G_2SA have the β-configuration.

Position of the glycosidic linkages. Upon permethylation and subsequent methanolysis, oligoglycosylsterols from Adzuki beans gave four peaks of methylated

TABLE 1

Composition of 4-Desmethylsterols in Glycosylsterols from Adzuki Beans (%)

Sterol	Acylmonoglycosylsterol	Monoglycosylsterol	Diglycosylsterol	Triglycosylsterol	Tetraglycosylsterol
Cholesterol	0.2	0.2	5.3	2.5	7.9
24-Methylcholesterol[a]	2.4	2.3	3.7	1.8	1.2
Stigmasterol	34.2	32.7	35.7	49.5	41.2
Sitosterol	57.3	60.6	43.4	41.4	47.4
Isofucosterol	1.5	1.4	8.7	1.3	0.6
7-Stigmastenol	4.2	2.7	2.6	2.3	1.4
Avenasterol	0.3	0.2	0.7	1.1	0.3

[a]24-Methylcholesterol is probably an epimeric mixture of campesterol (24α)-epimer and dihydrobrasicasterol (24β)-epimer.

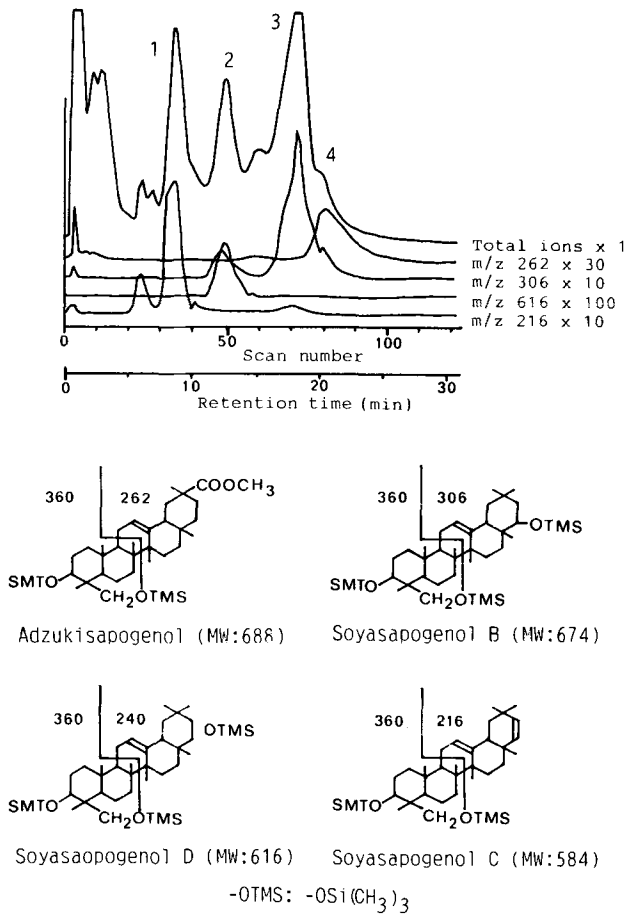

FIG. 1. Mass chromatograms of component sapogenins in the saponin fraction from Adzuki beans. Trimethylsilyl ether derivatives of sapogenins were analyzed by GC-MS with datalizer. A glass column, 0.3 × 100 cm, was packed with Diasolid ZT. Column temperature was 250°C. The energy level of the ion source was 20 eV and the ionizing current was 80 μA. The ion at *m/z* 616 is indicative of the molecular weight of soyasapogenol D (peak 1), those at *m/z* 216, 306 and 262 are characteristic of soyasapogenol C (peak 2), soyasapogenol B (peak 3) and adzukisapogenol (peak 4), respectively (25). The numerals on the right define amplication.

methylglycosides in GLC. By cochromatography with the authentic sample prepared from gentiobiose, these peaks were identified as α- and β-methyl-2,3,4,6-tetra-*O*-methylglucosides derived from the terminal glucopyranose unit, and α- and β-methyl 2,3,4-tri-*O*-methylglucosides derived from the internal 1,6-substituted glucopyranose unit, respectively (Table 3). The ratios of the terminal to the internal sugars were approximately 1.0:1.2 in G_2ST, 1.0:1.9 in G_3ST and 1.0:2.7 in G_4ST. This demonstrates that the $G_{2\text{-}4}ST$ possess two to four moles of glucose in each saccharide moiety. The identity of the peaks was confirmed by GC-MS analyses of partially methylated alditol acetates prepared from oligoglycosylsterols (28).

On the other hand, GLC and mass spectra of methylated methylglycosides prepared from reduced G_2SA showed the presence of four peaks, which were identified as α- and β-methyl-2,3,4,6-tetra-*O*-methylglucosides (derived from the terminal glucopyranose unit), and α- and β-methyl-3,4,6-tri-*O*-methylglucosides (derived from the internal glucopyranose unit with a 1,2-bond) (20,29). The latter two peaks were not found in GLC of methylated methylglycosides obtained from intact G_2SA. The results indicate that the internal sugar in G_2SA was glucuronic acid, to which the terminal glucose was linked by a 1-2 bond. The ratio of the terminal to the internal sugars can be assumed to be approximately 1:1.

Fatty acid composition of acylglycosylsterol. Seven fatty acids were found in the AG_1ST, while no fatty acids were present in the other polar steryl lipids of Adzuki beans. The composition of the fatty acid was as follows: Palmitic acid, 50%; linoleic acid, 26%; linolenic acid, 8%; oleic acid, 6%; and stearic acid, 5%.

FD-MS of glycosylsterols. FD-MS of G_2ST and G_3ST is shown in Figure 2. Prominent ions $(M + Na)^+$ indicative of the molecular weights were found at *m/z* 759 and 761 in the spectra of G_2ST, at *m/z* 921 and 923 in G_3ST. These ions confirm that G_2ST and G_3ST contain disaccharide and trisacchairde, mainly in combination with sitosterol or stigmasterol. This was also confirmed by the ions $(M + K)^+$ which were recognized in the mass spectra of G_2ST and G_3ST.

DISCUSSION

The present study shows that a series of sterylglycosides is present in Adzuki beans. Based on our results, and in light of available literature (1,7), the major components of each glycosylsterol class in Adzuki bean seeds were characterized as follows: AG_1ST, 6-*O*-palmitoyl-glucopyranosyl-(β1→3′)-sitosterol and -stigmasterol; G_1ST, glucopyranosyl-(β1→3′)-sitosterol and -stigmasterol; G_2ST, glucopyranosyl-(β1→6)-glucopyranosyl-(β1→3′)-sitosterol and -stigmasterol, i.e., gentiobiosylsterol; G_3ST, [glucopyranosyl-(β1→6)]$_2$-glucopyranosyl-(β1→3′)-sitosterol and -stigmasterol, i.e., gentiotriosylsterol; and

TABLE 2

Composition of Sugars in Glycosylsterols from Adzuki Beans (%)

Sugar	Acylmonoglycosylsterol	Monoglycosylsterol	Diglycosylsterol	Triglycosylsterol	Tetraglycosylsterol	Diglycosylsapogenol	
						Intact	Reduced
Galactose	<1	2	2	3	3	—	—
Glucose	100	98	98	97	97	62	100
Glucuronic acid	—	—	—	—	—	38	—

TABLE 3

Composition of Methylated Methylglycosides from Glycosylsterols in Adzuki Beans (%)

Glycoside	Diglycosylsterol	Triglycosylsterol	Tetraglycosylsterol	Reduced diglycosylsapogenol
Methyl-2,3,4,6-tetra-*O*-methylglucoside	46	34	27	56
Methyl-2,3,4-tri-*O*-methylglucoside	54	66	73	—
Methyl-3,4,6-tri-*O*-methylglucoside	—	—	—	44

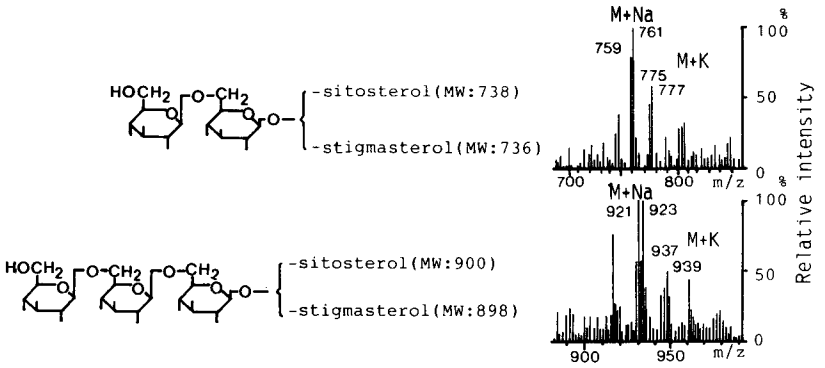

FIG. 2. Partial FD-MS of diglycosylsterol and triglycosylsterol from Adzuki beans.

G_4ST, [glucopyranosyl-(β1→6)]$_3$-glucopyranosyl-(β1→3′)-sitosterol and -stigmasterol, i.e., gentiotetraosylsterol. The major saponin isolated from Adzuki beans was glucopyranosyl-(β1→2)-glucopyranosyl-(β1→3′)-soyasapogenol B (20,27). The saccharide chains of the oligoglycosylsterols previously found in rice bran were shown to be the glucose units liked mainly by β1,4-bonds; their basic structures were concluded to be those of cellobiosyl-, cellotriosyl-, cellotetraosyl-, and cellopentaosylsterol (7,8). The presence of β1→4 bonds could not be detected in Adzuki bean glycosylsterols. Pentaglycosylsterol, which had been isolated from rice bran, was not found in Adzuki beans, as it had not been found in corn seeds (9). However, the existence of minor amounts of sterylglycoside in Adzuki beans cannot be completely excluded.

The glycosidic structures of tri- and tetraglycosylsterol found in Adzuki beans have not been reported previously, although gentiobiosylsterol was found in leaves of green tea (11) and *Musa paradisiaca* (14). The saccharide moieties of diglycosylsterol isolated from *Lindenbergia indica* (12) were reported to be rhamnosyl-(α1→4)-glucosyl- and rhamnosyl-(α1→4)-arabinosyl. Furthermore, a novel sterylglycoside containing the glucosyl-(β1→6)-inositol residue was found in *Musa paradisiaca* (14), which is an analogue of diglycosylsterol. Thus, the structural character of oligoglycosylsterols may differ according to plant species, although oligoglycosylsterols have not been well examined.

AG_1ST is considered to be widely distributed in the plant kingdom (2). Radwan *et al.* (30) reported the existence of oligoglycosylsterols containing fatty acid and sugar in equimolar proportions in plant tissue cultures, but their structures are not fully understood. Recently acyldiglycosylsterol has been reported to be present in *Musa paradisiaca* (14) and Acholeplasma (6). Therefore, a small amount of acylated oligoglycosylsterols may also exist in Adzuki bean seeds. However, their presence was not detected by TLC before alkaline treatment, as in the case of rice bran and maize seeds (7-9), because they may be present in very minor quantities.

Previously, five classes of saponins have been reported to occur in Adzuki beans (20,27). G_2SA, which was the major one, corresponded to the known saponin designated as Adzukisaponin I (20).

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Effect of Phosphatidylcholine on the α-Lactalbumin-Induced Fusion of Vesicles

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Previous studies on α-lactalbumin induced fusion of phosphatidylserine/phosphatidylethanolamine vesicles are extended to vesicles composed of various combinations of phosphatidylserine, phosphatidylethanolamine, phosphatidylcholine and cardiolipin. It was found that inclusion of phosphatidylcholine in the vesicles results in a depression of fusion. This depression of fusion appears to be caused by a reduction in the amount of irreversibly bound α-lactalbumin to vesicles containing phosphatidylcholine. It is suggested that in this system fusion is dependent upon the extent by which a particular protein segment penetrates the bilayer.
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Recently, there have been extensive investigations on protein-induced membrane fusion. Although many proteins were found to fuse model membranes under various conditions, the basic mechanism of the fusion process remains unknown. The general consensus that emerged from these investigations, however, appears to be that hydrophobic interactions between protein and lipid vesicles are a prerequisite for fusion (1-4). Since the hydrophobic interaction between lipid vesicles and a protein generally involves the interaction of the protein with phospholipid acyl chains, we investigated the penetration of the phosphatidylserine/phosphatidylethanolamine (PS/PE, 1:1) bilayer by several proteins including α-lactalbumin (α-LA) under conditions of fusion (5). It was found that at low pH, a small segment of α-LA in the presence of the vesicles is protected from proteolytic digestion, and that the extent of fusion is dependent on this interaction. Hydrophobic labeling of this segment with 3-(trifluoromethyl)-3-(m-[^{125}I]iodophenyl)diazirine ([^{125}I]-TID) and subsequent identification of labeled amino acid residues suggested that an α-helical form of the segment protrudes partially into the bilayer. One surface of the α-helix appears to be in contact with the hydrophobic acyl chain of the lipid while the opposite surface of the helix is exposed to the aqueous environment (6). The question of why this type of interaction should induce fusion is still to be answered.

The choice of PS/PE vesicles for these earlier investigations was made because such vesicles have extensively been used in studies of cation induced fusion and because PE is known to enhance the fusion process. Studies showed that Ca^{2+}-induced fusion of vesicles containing phosphatidylcholine (PC) was generally less pronounced than fusion of vesicles containing PE (7,8). Here we show a similar tendency for α-LA-induced fusion. It was found that the extent of fusion is proportional to the extent of protein binding, supporting the notion that binding and subsequent penetration of a segment of the proteins are responsible for fusion.

MATERIALS AND METHODS

Materials. PS (from bovine brain), PE (from bovine brain), PC (from egg yolk), α-LA (from bovine milk), trypsin (from bovine pancrease), and dipicolinic acid (DPA, pyridine-2,6-dicarboxylic acid) were purchased from Sigma Chemical Co. (St. Louis, MO). $TbCl_3 \times 6H_2O$ (99.99%) was obtained from Alfa (Danvers, MA). All chemicals were purchased in the highest purity available. The α-LA was purified by using Sephadex G-100 column chromatography. All phospholipids migrated as single spots in thin-layer chromatography on silica gel. The amount of lipid applied was 100 μg, and the solvent used was chloroform/methanol/water (65:25:4, v/v/v). Phospholipid concentration was determined according to the method of Vaskowsky *et al.* (9). Proteins were determined with a Gilford 260 UV-visible spectrophotometer.

Vesicle formation. Phospholipid vesicles composed of various phospholipids were prepared by the ether injection technique (10) followed by 15-s sonication. The multilamellar liposomes were removed by centrifugation at 6,500 rpm for 30 min, and the supernatant was collected. Relatively homogeneous unilamellar vesicles (~50 nm diameter) were obtained as determined after negative staining with uranyl acetate using a Japan Electron JEM 100 CX-II electron microscope.

Fusion. The fusion process was followed by the terbium-dipicolinic acid (Tb-DPA) method of Wilschut *et al.* (11). This method monitors the extent of mixing of vesicle contents upon fusion and has been widely used for model membrane fusion studies. One population of vesicles was prepared in a solution containing 2.5 mM $TbCl_3$ and 50 mM sodium citrate, and the other population of vesicles was prepared in a solution containing 50 mM DPA (sodium salt) and 20 mM NaCl. The vesicles were purified from nonencapsulated material by gel filtration on a Sephadex G-75 column, using a buffer solution containing 1.0 mM EDTA for elution. After equal concentrations of the two types of vesicles and protein at various concentrations were mixed, the fusion process was followed by using an Aminco-Bowman spectrofluorometer.

The leakage of the vesicles after the addition of the fusogenic agent was monitored by using PS/PE vesicles containing 1.25 mM $TbCl_3$, 25 mM sodium citrate, and 25 mM DPA (12). Again, the nonencapsulated material was removed by gel filtration (see above). The decrease in the fluorescence intensity after initiation of fusion resulted from the dissociation of the Tb-DPA complex when it leaked into the bulk phase containing

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Abbreviations: α-LA, α-lactalbumin; TID, 3-(trifluoromethyl)-3-(m-[^{125}I]iodophenyl) diazirine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; Tb, terbium; DPA, dipicolinic acid; PS, phosphatidylserine.

EDTA and from the leak-in of outside solution into the fused vesicles.

Protein binding to vesicles. The centrifugation method was used for the binding studies. After incubation of α-LA with vesicles at pH 4 (82 mM acetate, 18 mM Na-acetate, 41 mM NaCl), the vesicles were sedimented and the concentration of unbound α-LA in the supernatant was determined. Bound α-LA was estimated by a comparison of the protein concentration in the original solution with the remaining concentration in the supernatant. For these experiments it was essential that there was no sedimentation equilibrium redistribution of α-LA, and that all the vesicles were sedimented under centrifugation. The vesicles prepared at pH 4 were first centrifuged at 6,800 rpm to remove heavy liposomes. The vesicles in the supernatant were then sedimented eight times with progressively increasing angular velocity from 20,000 to 34,000 rpm, each operation lasting from 60–90 min, using a Beckman SW 41 swinging-bucket rotor. In this way, the size range of the vesicles was narrowed. After the final sedimentation, the supernatant was found to be optically clear as observed by the OD at 280 nm. Phosphate analysis confirmed that the phospholipid concentration of the supernatant was negligible. The last vesicle sediment was resuspended in a buffer solution, and an aliquot of α-LA solution was added to bring the concentration of both phospholipid and protein to predetermined values. After incubation at 18°C for 30 min, the vesicles were centerfuged at 35,000 rpm, and the supernatant was analyzed for the protein concentration by measuring OD values at 280 nm. Preliminary tests showed that at least 15 min of incubation were required prior to ultracentrifugation in order to reach binding equilibria.

To test the reversibility of the binding, the vesicles were incubated with α-LA at pH 4 at 18°C for 30 min, and centrifuged for 90 min at 35,000 rpm. The sediments were then resuspended in a pH 7 (2 mM Tes, 2 mM L-histidine, 100 mM NaCl) buffer solution and incubated for 48 hr at 18°C. The pH of this suspension remained constant at seven throughout this period. The suspension was centrifuged for 90 min at 35,000 rpm, and the protein concentration in the supernatant was determined (OD at 280 nm).

Proteolytic digestion of vesicle-bound proteins. The possibility of a segment of these proteins being inserted into the phospholipid vesicle membrane was checked by the treatment of the vesicle-protein complex with trypsin. In these experiments, 2 mg of protein were incubated with 10 ml of vesicles suspension (1 mM Pi) for 60 min at 18°C at pH 4. The vesicles with bound protein were sedimented by centrifuging for 90 min at 30,000 rpm using a Beckman SW 41 rotor. The pellets were resuspended in 5 ml of medium containing 150 mM KCl, 25 mM imidazole, and 100 μg of proteolytic enzyme, pH 7.5, and then incubated for one hr at 37°C. The digestion was stopped by the addition of PMSF as a freshly dissolved ethanolic solution to 3 mM final concentration followed by incubation of samples at 37°C for three min. In order to cleave -S-S linkages, β-mercaptoethanol was added to the sample solution at a 1% final concentration and incubated for 30 min at 18°C. The vesicles were then pelleted by centrifugation for 90 min at 30,000 rpm in a Beckman SW 41 rotor. The pellets were resuspended in 2 ml of buffer (25 mM imidazole, 1 mM EDTA; pH 7.5).

For molecular weight determination of the protected protein portion, the lipid was extracted in three volumes of chloroform-methanol (2:1) and the protein was precipitated in 10% trichloroacetic acid at 0°C followed by centrifugation for 15 min in an Eppendorf microfuge. After two acetone washings, part of the resulting pellet of the protein fragment was solubilized in a buffer of 40% glycerol, 2.5% SDS, 0.01 M H_3PO_4/Tris, 8 M urea, and 5% β-mercaptoethanol (pH 6.7). The dissolved protein was electrophoresed on 15% acrylamide gels in SDS. The remaining portion of the pellet was lyophilized. Amino acid sequencing of the extracted sample was done by the semi-micro manual Edman degradation method. Because of difficulties with the manual method, only 10 N-terminal residues of the segment were determined.

RESULTS AND DISCUSSION

The time-course of fusion for all the vesicle compositions studied here was similar to that described earlier (1, Fig. 1). The initial increase in the percent MAX fluorescence intensity due to fusion was followed by the decrease in the percent MAX fluorescence intensity caused by the leakage of vesicle content. The percent MAX fluorescence is defined as the fluorescence intensity measured during fusion divided by the fluorescence intensity after the vesicle-entrapped components were mixed following detergent treatment. The time-course of the percent MAX fluorescence, when corrected for leakage according to the method of Bentz *et al.* (12), approached a plateau. Figure 1 shows the

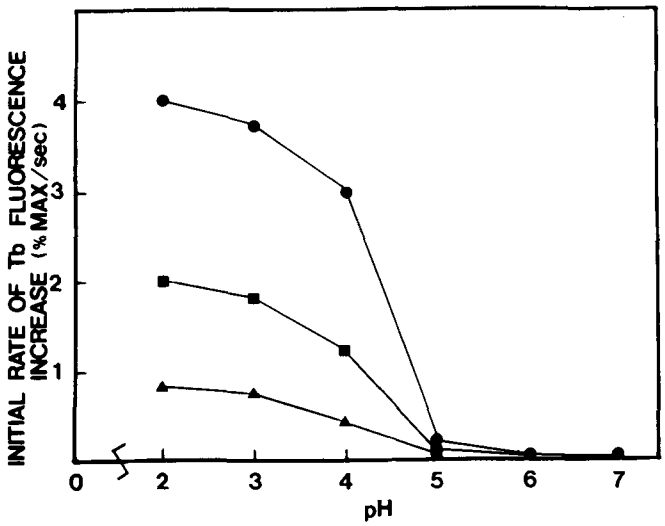

FIG. 1. pH dependence of initial rate of fusion of three types of vesicles. PS/PE (1:1) (●——●), PS/PC (1:1) (■——■), and PC/PE (1:1) (▲——▲). Fusion was initiated by adding α-LA solution to make up a final concentration of 100 μg/ml (18°C). Buffer solutions used are: pH 2 (5 mM KCl, 18 mM HCl, 43.4 mM NaCl); pH 3 (50 mM glycine, 11 mM HCl, 48 mM NaCl); pH 4 (82 mM acetate, 18 mM Na-acetate, 41 mM NaCl); pH 5 (14 mM acetate, 86 mM Na-acetate, 7 mM NaCl); pH 6 (2 mM Mes, 80 mM NaCl); pH 7 (2 mM Tes, 2 mM L-histidine, 100 mM NaCl).

initial rate of percent maximum fluorescence increase during the fusion of three kinds of vesicles as a function of pH. The fusion-pH profiles of these vesicles are similar, and the initial rates of fusion increase with decreasing pH. The initial rate of fusion of PS/PE vesicles is about twice that of PS/PC vesicles, and the initial rate of fusion of neutral PE/PC vesicles is much lower. In order to follow in detail the change in fusion behavior progressing from PS/PE to PS/PC vesicles, PE in the vesicles was gradually replaced by PC while maintaining PS constant. The time-course of α-LA-induced fusion of these vesicles after correcting for leakage (12) is shown in Figure 2. It can be seen that the fusion is complete within about two min. Figure 3 gives the initial rate of fusion plotted against PC content. It is of interest that the curve is sigmoidal.

Figure 4 gives the concentration dependent α-LA binding to the PS/PE (1:1), PS/PC (1:1) and PC/PE (1:1) vesicles. Essentially 100% of α-LA binds to both PS/PE (1:1) and PS/PC (1:1) vesicles regardless of the total amount of α-LA added. However, the amount of α-LA bound to PC/PE (1:1) vesicles is about 50% less than that bound to the other vesicles. These results suggest that simple binding per se has little bearing on fusion. The data points contain two components: reversible binding and irreversible binding. The irreversible portion of binding can be estimated by bringing the incubation pH to 7, where there is no initial binding, and by then determining the amount of α-LA still bound to the vesicles. Figure 5 gives the amount of irrevers-

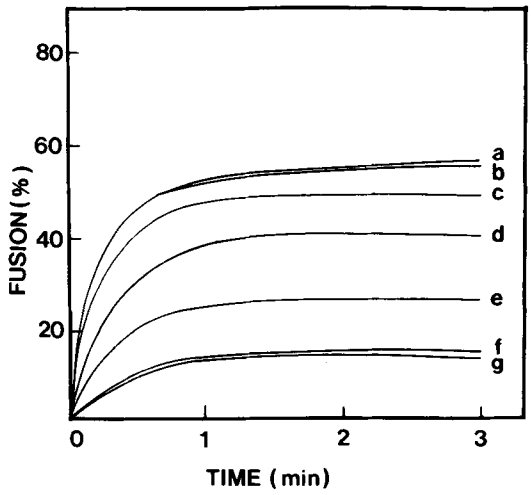

FIG. 2. Time course of fusion of vesicles composed of combinations of PS, PE and PC. Corrections for leakage were made according to the method of Bentz *et al.* (12). The vesicles are (a) PS/PE (1:1), (b) PS/PE/PC (5:4:1), (c) PS/PE/PC (10:7:3), (d) PS/PE/PC (5:3:2), (e) PS/PE/PC (5:2:3), (f) PS/PE/PC (5:1:4), and (g) PS/PC (1:1). At time zero, 0.1 ml of 1 mg/ml α-LA solution was added into the spectrofluorometer cuvette which contains 0.9 ml of vesicle suspension (0.05 mM Pi; 18°C, pH 4.0).

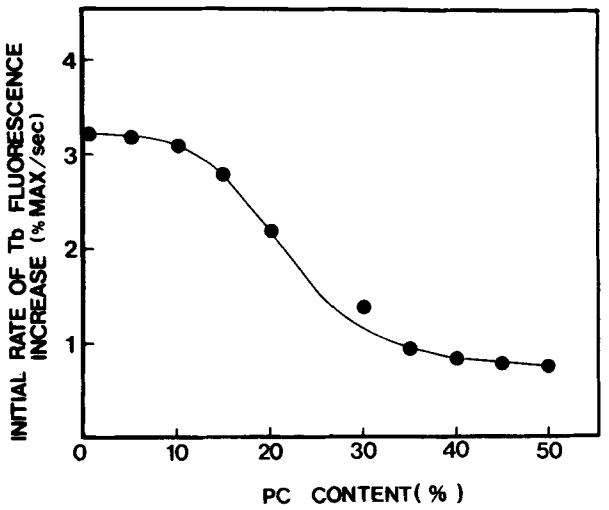

FIG. 3. Effect of PC content on α-LA-induced vesicle fusion. The experimental conditions are the same as in Figure 2. Data were obtained by replotting Figure 2.

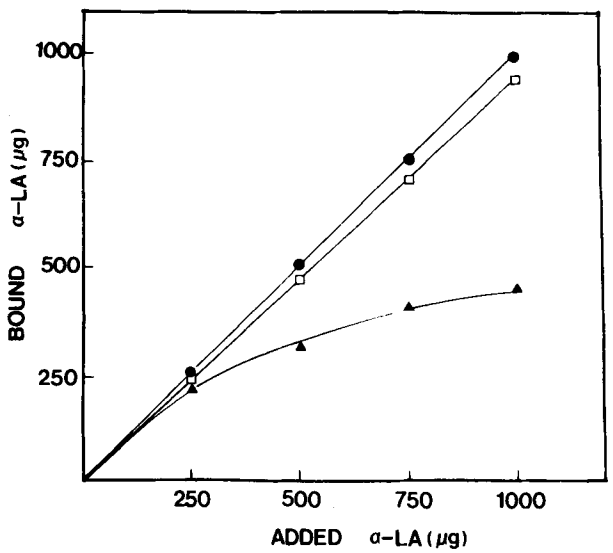

FIG. 4. Binding isotherms (18°C) of α-LA binding to three types of vesicles at various total protein concentrations. The vesicles are PS/PE (1:1) (●——●), PS/PC (1:1) (□—□), and PC/PE (1:1) (▲—▲).

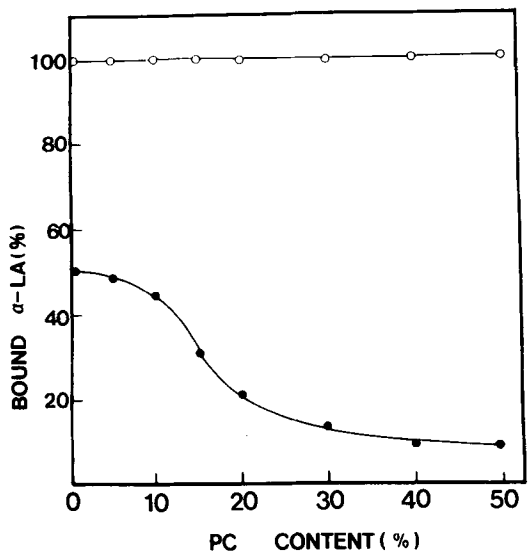

FIG. 5. The amount of irreversibly bound α-LA as a function of PC content. The compositions of the vesicles are the same as in Figure 2. (○—○), total binding. (●——●), the amount of irreversibly bound α-LA.

ibly bound α-LA plotted against PC content of the vesicles as the PE is slowly replaced by PC, while keeping the PS content constant. The curve is strikingly similar to the fusion profile shown in Figure 3. This strongly suggests that the inhibitory effect of PC on fusion is caused by a decrease in irreversible binding.

Figure 6 shows the SDS-PAGE bands of the α-LA segment which had been protected from tryptic digestion. α-LA was first incubated with vesicles of different PE/PC ratios at pH 4 and then treated with trypsin at pH 7.5 at 37°C. The lipids were extracted with chloroform-methanol, and the protein segment was precipitated with 10% trichloroacetic acid prior to the SDS-PAGE. A protein band with an estimated molecular weight of approximately 4,000 was observed for each vesicle system. Band intensities became stronger with increasing PE content and corresponded to the amount of α-LA segment protected from proteolytic digestion. It is likely that a portion of this M_r 4,000 unit is buried within the hydrophobic core of the vesicle bilayer regardless of phospholipid ratios in the PS/PE/PC vesicles. The amino acid sequence of the first 10 residues of this unit for all the phospholipid compositions was found to be the 80—89 stretch of α-LA. This, together with the observation that the M_r of all the segments were similar to the segment obtained previously (1), strongly suggests that the 80—108 segment of α-LA was protected from the tryptic digestion in all vesicles. The result suggests that the population of segment penetrating the bilayer is a key factor in bringing about fusion.

Table 1 shows the effect of phospholipids on α-LA-induced fusion of vesicles containing both acidic lipids and PC, and shows the amount of total binding and irreversible binding of α-LA to these vesicles. Here again, the extent of fusion and binding gradually decreases as the PC content increases.

It was reported that PC has an inhibitory effect on Ca^{2+}-induced fusion while PE has an enhancing effect (8). These contrasting effects were attributed to differences in phospholipid dehydration by Ca^{2+}. It was suggested that Ca^{2+} dehydrates the PE head group more readily than the PC head group, and thus would facilitate the approach of PE vesicles and vesicle fusion (8,13,14). Although the differential effect on dehydration may also be important for α-LA-induced fusion, it is clear that differences in α-LA binding behavior by these vesicles are a major reason for the discrepancies observed. The α-LA segments which penetrate the bilayer are identical regardless of whether or not the vesicles contain PC or PE. Thus, it appears that observed differences in fusion properties depend primarily on the extent by which a particular α-LA segment penetrates the bilayer.

The reason why more α-LA binds to PS/PE vesicles than to PS/PC vesicles is not altogether clear. PC and PE have similar charges within the pH range studied and the orientation of the head groups of these neutral phospholipids is parallel to the bilayer surface. The main differences between these head groups are their size and degree of hydration. PC has a larger head group with a higher affinity for water (15). It is possible that the PE head groups create less of a barrier for the α-LA segments to pass into the bilayer interior. Also, PE has a propensity for forming hexagonal H_{II} structures. Whether or not this is an important factor in the present context is not clear as yet.

TABLE 1

Effect of Phospholipids on Fusion, Binding, and Irreversible Binding

Vesicles	Percent		
	Fusion (%)[a]	Binding (%)	Irreversible Binding (%)
PC	—	20 ± 5	3 ± 1
PC/PS (9:1)	—	37 ± 4	4 ± 1
PC/PS (4:1)	1.5 ± 0.5	75 ± 5	11 ± 2
PC/PS (1:1)	15 ± 2	94 ± 4	19 ± 2
PC/CL (9:1)	2 ± 1	40 ± 3	4 ± 1
PC/CL (5:1)	16 ± 2	100 ± 4	20 ± 2
PC/CL (3:1)	42 ± 3	100 ± 4	35 ± 3

[a]The time-course of the percent MAX fluorescence, when corrected for leakage according to the method of Bentz *et al.* (12), approached plateaus and these limiting values were taken as percent fusion.

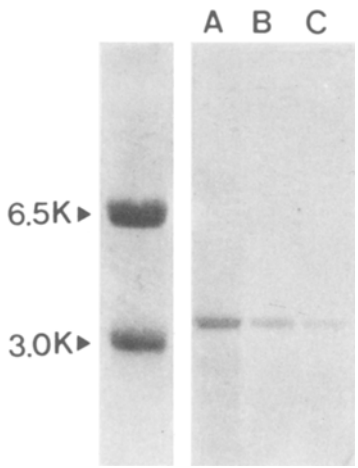

FIG. 6. SDS-PAGE patterns of the α-LA segment which had been protected in the α-LA/vesicle suspension from trypsin digestion. See text for experimental details. Lane A: PS/PE/PC (5:4:1). Lane B: PS/PE/PC (5:3:2). Lane C: (PS/PE/PC (5:1:4).

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Hepatic Biotransformation and Choleretic Effect of 7-Ketolithocholic Acid in the Rat

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Hepatic biotransformation and the effect on bile flow of 7-ketolithocholic acid (7-oxo-3α-hydroxy-5β-cholan-24-oic acid), in comparison to ursodeoxycholic acid, were examined in rats under conditions of continuous infusion of solutions of sodium salts of these bile acids (1.2 μmol/min/100 g body wt) for 2 hr. Both bile salts elevated the bile flow rate as well as the bile bicarbonate concentration to a similar degree. The minor difference observed was a transient (10–20 min) and subtle drop of bile flow during the first hour in rats given 7-ketolithocholate. In ursodeoxycholate infused rats, the major bile salt in the bile was its taurine conjugate, although excretion of tauroursodeoxycholate dropped considerably during the second hour. In 7-ketolithocholate infused rats, the major bile salt in the bile was again its taurine conjugate, but ursodeoxycholate and chenodeoxycholate and their conjugates were also excreted. In contrast to ursodeoxycholate infused rats, the drop in excretion of taurine conjugates and the increase of glycine conjugates in rats infused with 7-ketolithocholate were more rapid. In rats infused with 7-ketolithocholate, excretion of ursodeoxycholate and its conjugates was significantly higher than the corresponding values for chenodeoxycholate, suggesting that 7-ketolithocholate is reduced predominatly to the 7β-epimer in this species. However, the concentration of ursodeoxycholate and its conjugates excreted into the bile in rats infused with 7-ketolithocholate was only 10% of that of rats infused with ursodeoxycholate, yet the magnitude of choleresis and the rise in bile bicarbonate concentration were similar in both experiments. Therefore, it is suggested that the bicarbonate-rich bile, induced by 7-ketolithocholate infusion, is caused mainly by 7-ketolithocholate rather than by its metabolite, ursodeoxycholate.
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Although theories regarding the mechanism(s) of bile formation induced by bile acids have become much advanced in recent years, some aspects still remain unresolved. In particular, the demonstration by Dumont *et al.* (1) of an increase in the bicarbonate ion concentration in bile induced by I.V. infusion of ursodeoxycholic acid and 7-ketolithocholic acid (7-oxo-3α-hydroxy-5β-cholan-24-oic acid) and, more recently, by *nor* dihydroxy bile acids (2) in rats has stimulated further advances or modifications of theories concerning the mechanism for choleresis, since most other bile salts, including ursocholate (3), are known to decrease, rather than increase the bile bicarbonate concentration in this species.

Several hypotheses, such as "chole-hepatic recycling" (2) and "secretory choleresis" (4,5), have been proposed to explain this peculiar choleresis in the rat. However, there has been no direct proof of these hypotheses and the mechanism still remains to be elucidated. Since 7-ketolithocholic acid has been reported to be biotransformed into chenodeoxycholic acid and ursodeoxycholic acid in livers of different mammalian species (6–13) (including rats [10–13]), its choleretic effect may also reflect its biotransformation. Since pertinent data in this regard were lacking in the original report by Dumont *et al.* (1), we attempted in this study to determine (i) whether the choleretic pattern of 7-ketolithocholic acid is identical to or different from that of ursodeoxycholic acid, and (ii) whether possible differences are related to the biotransformation of 7-ketolithocholic acid in rat liver. Such information may also enhance our understanding of the physiology of this particular bile salt, which has been implicated recently as being effective in dissolving cholesterol gallstones in humans (9,14).

MATERIALS AND METHODS

Materials. 7-Ketolithocholic acid and ursodeoxycholic acid were generous gifts from Tokyo Tanabe Company Ltd. (Tokyo). The chemical purity of 7-ketolithocholic acid, as determined by high-performance liquid chromatography, was more than 98%. The major contaminant was chenodeoxycholic acid (less than 0.5%). These compounds were then converted to sodium salts. Taurine and glycine conjugates of 7-ketolithocholic acid were prepared according to the method previously reported for lithocholate (15) and were used as standards for the measurement of concentrations of conjugates of 7-ketolithocholic acid in bile and liver. 3α-Hydroxysteroid dehydrogenase (EC 1.1.1.50) was obtained from Worthington Biochemicals (Freehold, NJ). Other chemical agents for separating and measuring bile salts in the bile were all of analytical grade.

In vivo studies. Male Wistar rats (SLC, Shizuokajikken Dobutsu, Hamamatsu, Japan) were purchased at the age of 11 weeks and were kept for 2–3 weeks before use. The rats were given free access to food (CRF1, Oriental, Atsugi) and water (acidified, residual chlorine 10 ppm) under controlled lighting (0600–1800), humidity (55%) and temperature (23 ± 1°C).

Experimental protocols used were essentially the same as previously reported for ursodeoxycholic acid (4,16). In brief, the common bile duct was cannulated with PE-50 tubing (Clay-Adams, Parsippany, NJ) under sodium pentobarbital anesthesia (4.5 mg/100 g/body wt, Abbott, North Chicago, IL). A 10-min basal bile sample was obtained in a paraffin-sealed, bottom-tapered stock tube. Thereafter, a bile salt solution was infused by a Harvard pump (model 9446, Harvard Apparatus, Millis, MA) through a jugular vein catheter. The infusate was composed of bile salts (Na 7-ketolithocholate or Na ursodeoxycholate) dissolved in 3% bovine serum albumin solution. The pH (8.3 for both 7-ketolithocholate and ursodeoxycholate) and osmolality (300 mOsmol/kg) were adjusted as reported previously (4,16). The infusion rate was fixed at 1.2 μmol/min/100 g. Bile was collected every 10 min during the infusion period of 2 hr. Body temperature was continuously monitored by a rectal probe and was

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maintained throughout at 37.5 ± 0.5°C. All infusion studies were performed between 1000 to 1300.

Measurements of bile salt concentration. Total bile salt concentrations in the bile was determined enzymatically. Concentrations of individual bile salts in rats infused with ursodeoxycholate (ursodeoxycholate and its conjugates) were measured by high-performance liquid chromatography (Twinkle, Nihonbunko, Tokyo) coupled with 3α-hydroxysteroid dehydrogenase immobilized in a column (Bile Pack, Nihonbunko, Tokyo) as reported previously (4,16). In bile from rats infused with 7-ketolithocholate, nine different bile salts (i.e., three unconjugated bile salts: 7-ketolithocholate, ursodeoxycholate, and chenodeoxycholate, and their taurine and glycine conjugates) were measured. For this purpose, two different elution systems were used for the same samples. Solvent A (methanol/acetonitrile/30 mM ammonium acetate, 3:3:4) was used with unchanged content. Solvent B (methanol/acetonitrile/30 mM ammonium acetate, 1:1:3) was modified to 1:1:4 (hereafter called B′). The first run was performed with a gradient starting from 100% of B′ to 100% of A, taking 35 min. With this elution system, bile salts such as taurocholate, 7-ketolithocholate, glycochenodeoxycholate, taurochenodeoxycholate and chenodeoxycholate were separated and quantitated (Fig. 1a). In the second system, a solvent containing 17% of A and 83% of B′ was used without a gradient. With this system, glyco-, tauro-, and unconjugated ursodeoxycholate and glyco- and tauro-7-ketolithocholate were separated and quantitated (Fig. 1b). At the end of the experiments, the concentrations of bile salts in the liver were determined on extracts of liver specimens, as previously described (4,16).

Concentrations of chloride ions were measured by using electrodes (Photovolt, Analyzer PAV-4, Photovolt Co., New York). Bicarbonate concentration was determined with a Natelson microgasometer. For measurement of ion concentrations, two successive bile samples were pooled to obtain an adequate volume for these measurements.

Statistical analysis. All values are expressed as mean ± SD. The statistical comparisons were made using Student's *t*-test. P values lower than 0.05 are judged to be significant.

RESULTS

Sequential changes in bile flow and bile salt excretion. In general, the flow and excretion patterns were very similar for both bile salt infusion studies (Fig. 2). However, in many experiments of 7-ketolithocholate infused rats, the bile flow ceased to increase for 10 or 20 min during a period between 30 to 40 min after the start of infusion (Fig. 2a–c), or in some cases showed a slight decline (Fig. 2d,e). Thereafter, the flow rate began to rise rapidly again, peaked at 60–90 min, and then began to decline (Fig. 2a–c). Total bile salt excretion rate, on the other hand, showed a peak at 20–40 min after the start of infusion, which was followed by a gradual decline in most experiments. In contrast, in rats infused with ursodeoxycholate a more drastic decline in the bile salt excretion rate was observed at about 60 min after the start of infusion (Fig. 2f). Furthermore, the transient decline in bile flow observed in rats infused with 7-ketolithocholate was not observed in any of the rats infused with ursodeoxycholate.

Figure 3 shows examples of sequential changes in excretion rates of individual bile salts in rats infused with 7-ketolithocholate (Fig. 3a) and ursodeoxycholate (Fig. 3b). Sequential changes in excretion rates of infused bile salts and their metabolites in the bile are summarized in Tables 1 and 2. In rats infused with 7-ketolithocholate,

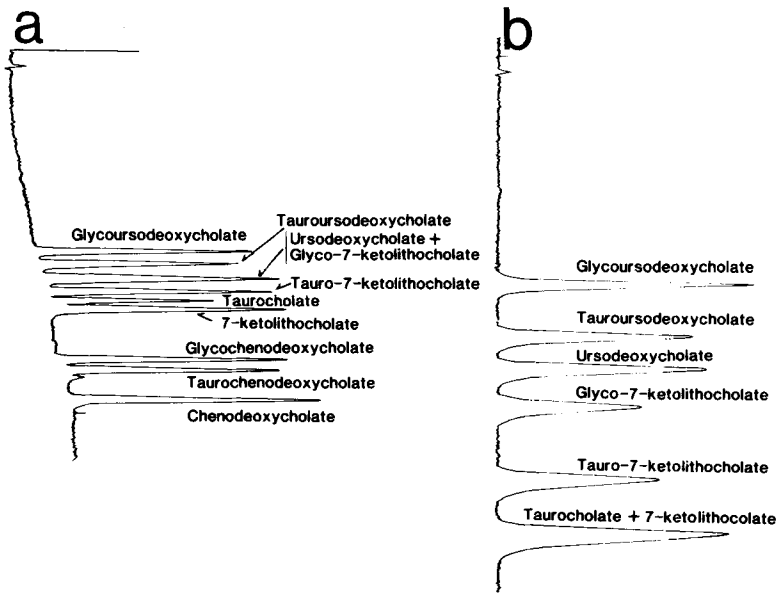

FIG. 1. High performance liquid chromatography profiles of different bile salts separated by system 1 (a) and system 2 (b). In system 1, concentrations of taurocholate, 7-ketolithocholate, glycochenodeoxycholate, taurochenodeoxycholate and chenodeoxycholate were determined. In system 2, glycoursodeoxycholate, tauroursodeoxycholate, ursodeoxycholate, glyco-7-ketolithocholate and tauro-7-ketolithocholate were determined. The elution systems are described in the text.

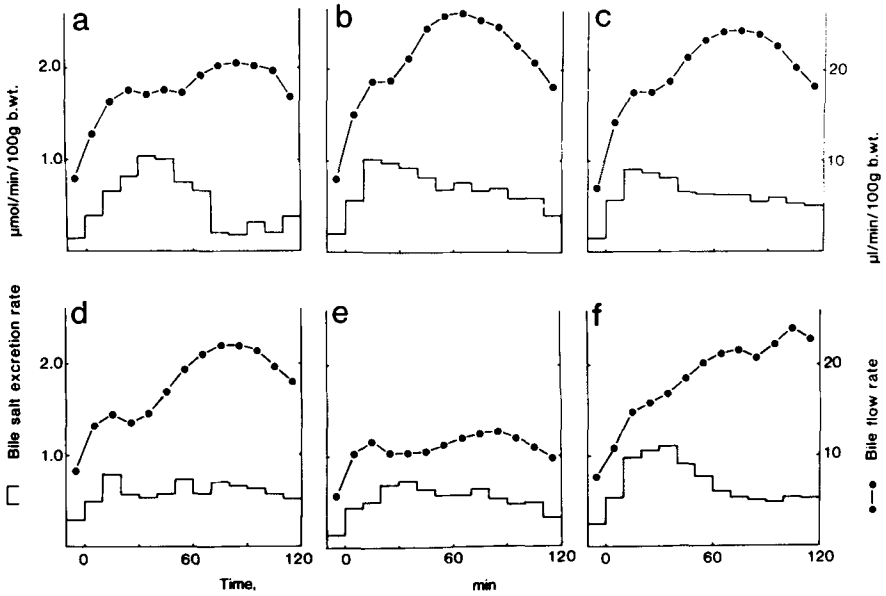

FIG. 2. Examples of sequential changes in the bile flow and total bile salt excretion rate in rats infused with 7-ketolithocholate (a–e) and in a rat infused with ursodeoxycholate (f). Closed circles with solid line indicate the bile flow rate and white columns the total bile salt excretion rate.

TABLE 1

Sequential Excretion of 7-Ketolithocholate and Ursodeoxycholate and Its Metabolites in Bile

Time period	Bile flow (μl/min/100 g)	Unconjugated bile acids (nmol/min/100 g)	Taurine conjugates (nmol/min/100 g)	Glycine conjugates (nmol/min/100 g)	Total (nmol/min/100 g)
10–20 min					
7-Ketolithocholate[a]	17.0 ± 2.1	14.4 ± 4.1	623.1 ± 123.4	98.2 ± 89.8	737.0 ± 94.9
Ursodeoxycholate[b]	16.6 ± 1.1	31.6 ± 5.1[c]	819.5 ± 71.7[c]	2.6 ± 0.1[c]	852.4 ± 76.8[c]
20–30 min					
7-Ketolithocholate	17.6 ± 2.2	18.6 ± 3.8	517.3 ± 210.9	162.9 ± 128.7	698.8 ± 128.0
Ursodeoxycholate	17.4 ± 1.4	45.7 ± 9.6[c]	993.8 ± 96.0[c]	9.6 ± 6.0[c]	1049.0 ± 110.1[c]
70–80 min					
7-Ketolithocholate	22.9 ± 3.5	33.1 ± 11.8	182.4 ± 92.8	243.6 ± 37.3	450.5 ± 122.5
Ursodeoxycholate	21.7 ± 0.6	53.7 ± 6.1[c]	350.7 ± 152.8[c]	184.4 ± 23.4[c]	588.8 ± 149.2
90–100 min					
7-Ketolithocholate	21.2 ± 2.7	66.2 ± 10.1	106.5 ± 40.2	190.5 ± 22.1	365.5 ± 60.4
Ursodeoxycholate	21.6 ± 1.4	41.7 ± 2.8[c]	118.4 ± 32.1	156.4 ± 16.5[c]	316.4 ± 45.4

All values are expressed as means ± SD.

[a] Sum of 7-ketolithocholate, ursodeoxycholate and chenodeoxycholate and their conjugates (n = 10).

[b] Sum of ursodeoxycholate and its conjugates (n = 4).

[c] Significantly different from corresponding values for 7-ketolithocholate infused rats ($P < 0.05$). Student *t*-test for unpaired values.

the major bile salts excreted in the bile were taurine and glycine conjugates of 7-ketolithocholate. The excretion of ursodeoxycholate and its conjugates was about 20% of that of 7-ketolithocholate. Chenodeoxycholate and its conjugates were minor components (Fig. 3a, Table 2).

In both groups of rats, taurine conjugates were the major components in the initial period which subsequently declined, reaching a lower plateau. However, a higher excretion rate of the taurine conjugate in the first 20 min and its slower decline were observed in ursodeoxycholate infused rats, in comparison to 7-ketolithocholate infused rats. In contrast, the increase in excretion of the glycine conjugate was slower in ursodeoxycholate experiments during the first hour and also, to some extent, during the second hour. This difference can be better appreciated by comparison of the taurine:glycine ratio (T:G ratio, Table 1). The excretion of unconjugated bile salt(s) was generally higher in ursodeoxycholate infused rats than in 7-ketolithocholate-infused rats, particularly during the first hour of infusion.

Table 3 summarizes concentrations of 7-ketolithocholate and its metabolites in the liver examined after 2-hr infusion of the bile salts. For comparison, corresponding values in rats infused with ursodeoxycholate, published

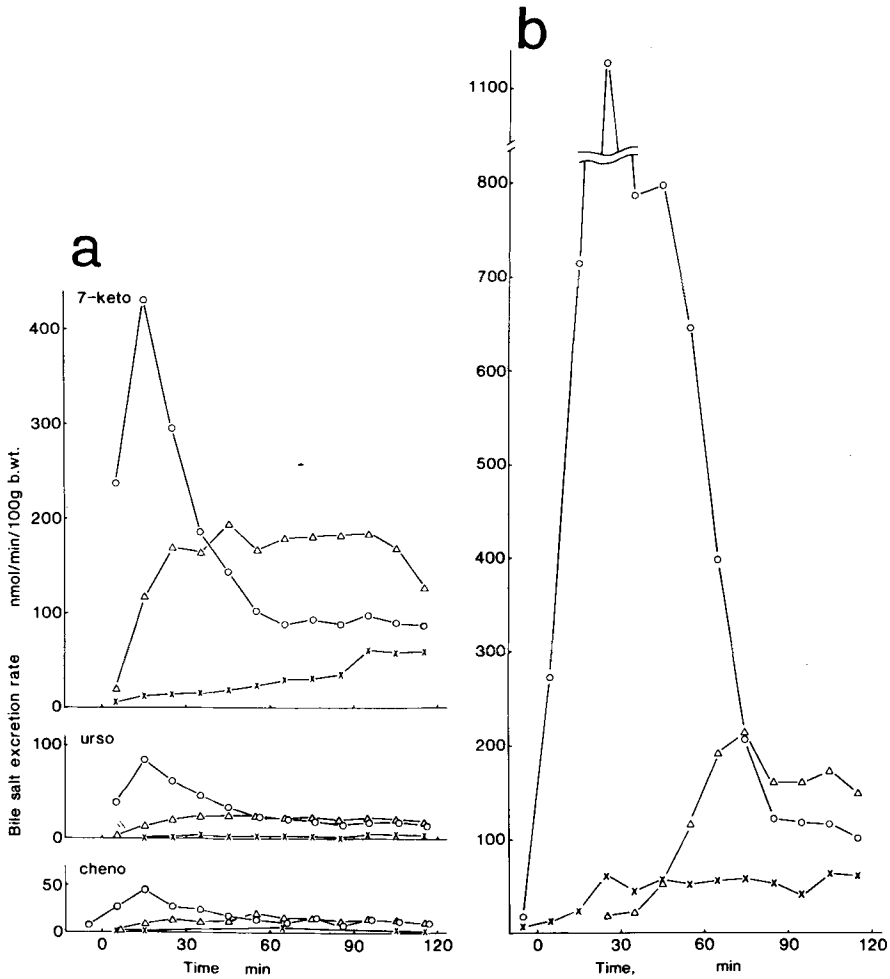

FIG. 3. Representative examples of sequential changes in excretion rates of individual bile salts in rats infused with 7-ketolithocholate (a) and ursodeoxycholate (b). Open circles indicate taurine conjugates, triangles glycine conjugates, and crosses unconjugated bile salts. In a, excretion rates of 7-ketolithocholate (top panel), ursodeoxycholate (middle panel) and chenodeoxycholate (bottom panel) and their conjugats are shown. In b, only excretion rates of ursodeoxycholate and its conjugates are shown.

TABLE 2

Sequential Excretion of 7-Ketolithocholate and Its Metabolites in Bile of Rats Infused with 7-Ketolithocholate

	Unconjugated bile acids (nmol/min/100 g)	Taurine conjugates (nmol/min/100 g)	Glycine conjugates (nmol/min/100 g)	Total (nmol/min/100 g)
10-20 min				
7-Ketolithocholate	12.3 ± 3.6	450.2 ± 74.2	73.4 ± 63.0	535.9 ± 48.6
Ursodeoxycholate	1.4 ± 0.7	115.1 ± 37.6	14.1 ± 12.6	127.7 ± 34.8
Chenodeoxycholate	1.5 ± 0.5	57.8 ± 18.2	10.0 ± 7.1	73.4 ± 33.7
20-30 min				
7-Ketolithocholate	15.8 ± 2.0	370.5 ± 143.7	127.3 ± 97.6	513.6 ± 79.9
Ursodeoxycholate	2.4 ± 2.9	97.0 ± 45.3	25.5 ± 21.2	112.3 ± 36.3
Chenodeoxycholate	1.5 ± 0.5	49.8 ± 24.7	15.9 ± 10.9	62.9 ± 19.9
70-80 min				
7-Ketolithocholate	30.5 ± 7.7	138.0 ± 71.3	197.9 ± 28.7	356.4 ± 99.4
Ursodeoxycholate	2.9 ± 3.5	30.6 ± 15.7	29.3 ± 9.4	62.8 ± 22.6
Chenodeoxycholate	2.4 ± 1.8	17.8 ± 9.3	16.4 ± 3.5	31.3 ± 11.1
90-100 min				
7-Ketolithocholate	53.7 ± 9.2	79.2 ± 31.3	147.0 ± 20.2	279.9 ± 48.9
Ursodeoxycholate	12.5 ± 1.8	19.5 ± 7.1	26.3 ± 5.9	58.2 ± 11.7
Chenodeoxycholate	1.0 ± 0.1	7.9 ± 3.4	17.3 ± 4.1	27.4 ± 9.1

All values are expressed as mean ± SD.

TABLE 3

Concentrations of 7-Ketolithocholate and Its Metabolites in the Liver After Two Hours Infusion of 7-Ketolithocholate

	Unconjugated bile acids (nmol/g/liver)	Taurine conjugates (nmol/g liver)	Glycine conjugates (nmol/g liver)	G:T	Total (nmol/g/liver)
7-Ketolithocholate	773.1 ± 232.3	213.8 ± 59.1	377.6 ± 103.4	1.8 (1.8)[b]	1364.4 ± 316.5
Ursodeoxycholate	600.9 ± 224.7	156.8 ± 71.6	294.7 ± 74.3	1.9 (1.3)	1052.4 ± 332.7
Chenodeoxycholate	547.4 ± 215.7	80.9 ± 31.7	182.2 ± 51.5	2.3 (2.4)	810.5 ± 282.2
Total	1921.4 ± 492.4	451.5 ± 156.2	854.4 ± 209.4	1.9 (1.8)	3227.3 ± 762.2
Ursodeoxycholate[a]	2402.5 ± 533.8	334.4 ± 130.6	616.6 ± 156.0	1.8 (1.2)	3353.2 ± 624.0

[a]Livers infused with ursodeoxycholate depicted from a previous study (10).

[b]G:T ratios in parentheses indicate the corresponding values in biles in times closest to the end of experiment (90–100 min).

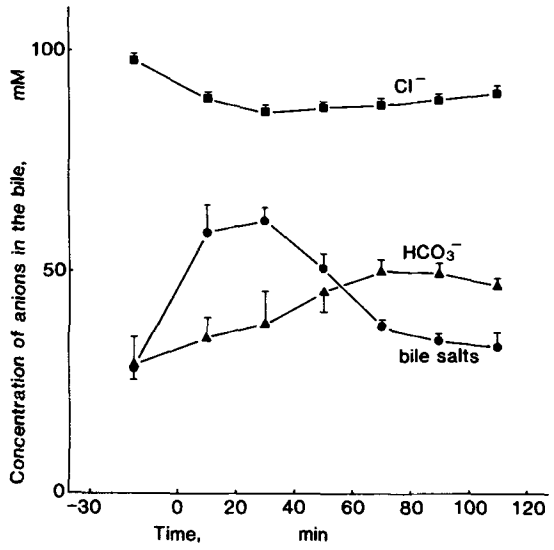

FIG. 4. Sequential changes in the concentrations of biliary anions (i.e., Cl^-, HCO_3^- and bile salts) in rats infused with 7-ketolithocholate.

in a previous study (16), are also shown. The glycine: taurine ratio (G:T) was mostly similar for the three bile salts. The ratios of the liver concentrations were very close to the corresponding values in bile obtained at the time point closest to the end of the experiments (90–100 min) for 7-ketolithocholate and chenodeoxycholate, whereas the ratio for ursodeoxycholate in the bile was lower than that in the liver. A similar discrepancy was also noted in rats infused with ursodeoxycholate in a previous study (16) (Table 3).

In Figure 4, sequential changes in concentrations of biliary anions (i.e., Cl^-, HCO_3^- and bile salts) are shown for rats infused with 7-ketolithocholate. The chloride ion concentration tended to decrease after the start of bile salt infusion, whereas the bicarbonate concentration began to rise immediately after the start of infusion and reached a higher plateau in the second hour. The total bile salt concentration also began to increase sharply at the start of infusion. However, it began to decline at 30 min of infusion, reaching a lower plateau in the second hour.

Figure 5 shows the relationships between the bile flow rate and excretion rates of anions (bicarbonate and bile salts) in rats infused with 7-ketolithocholate (Fig. 5a) and rats infused with ursodeoxycholate (Fig. 5b). The latter figure is based on data previously reported by the authors (16). In the experiments with either ursodeoxycholate or 7-ketolithocholate, there was no linear relationship between the bile flow and bile salt excretion rate. When the bile flow rate was plotted against the sum of excretion rates of bile salts and bicharbonate, a highly significant linear relationship was demonstrated for both experiments [$Y = (9.15 \pm 0.54)X + (3.10 \pm 1.58)$, $r = 0.96$, $n = 28$, $t = 16.95$, $P < 0.001$ for 7-ketolithocholate experiments: $Y = (10.49 \pm 0.52)X + (2.09 \pm 1.45)$, $r = 0.98$, $n = 20$, $t = 13.39$, $P < 0.001$ for ursodeoxycholate experiments]. As will be discussed later, the osmotic force of bicarbonate ions may be doubled by its counter cation, Na^+, while that of bile salts may be ignored since practically only an osmotic force of its counter cation Na^+ may be effective, because of the formation of mixed micelles. Therefore, we performed another calculation by using the sum of bile salt excretion rate and bicarbonate excretion rate times two. Again, a highly significant linear relation was obtained for 7-ketolithocholate experiments [$Y = (6.04 \pm 0.22)X + (3.22 \pm 0.89)$, $r = 0.98$, $t = 27.72$, $P < 0.001$] and for ursodeoxycholate experiments [$Y = (6.16 \pm 0.16)X + (2.73 \pm 0.77)$, $r = 0.99$, $t = 37.92$, $P < 0.001$].

DISCUSSION

Choleresis by 7-ketolithocholate. The increase in bile flow and bile bicarbonate concentration during the infusion of 7-ketolithocholate appeared similar to that in rats infused with ursodeoxycholate demonstrated by previous studies (4,16). However, the choleretic pattern was not identical for these two bile salts. Whereas in the ursodeoxycholate experiments the total bile salt excretion dropped rather sharply at 60–90 min after the start of infusion, the decline in bile salt excretion in 7-ketolithocholate infused rats was not so sharp in most cases. The gradual decline in total excretion rate was mostly due to more efficient excretion of the glycine conjugate of 7-ketolithocholate, as compared with the relatively limited excretion of glycoursodeoxycholate in rats infused with ursodeoxycholate.

Another subtle but interesting finding in 7-ketolithocholate-induced choleresis was a transient decline in bile flow during the first hour of infusion, which was followed by a second rapid increase in the bile flow rate. The transient decline in the bile flow was most likely caused by

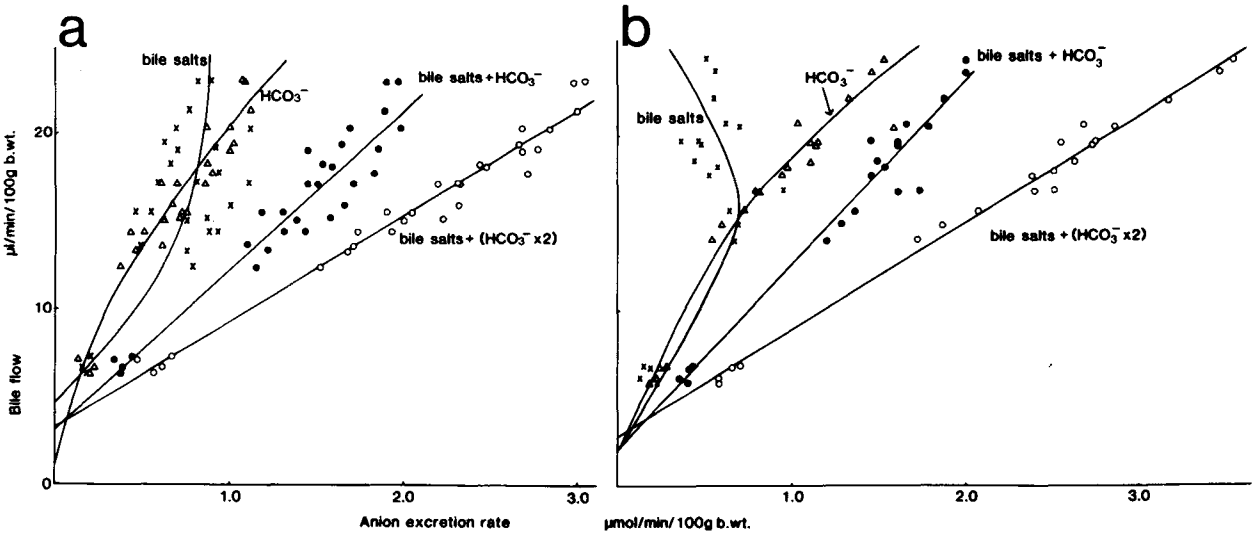

FIG. 5. Relationships between the bile flow and biliary excretion rates of anions (bile salts, bicarbonate ions) in rats infused with 7-ketolithocholate (a) and rats infused with ursodeoxycholate (b). The latter figure is based on previously published data (16). Crosses indicate the relation between the bile flow and bile salt excretion rate, open triangles with bicarbonate excretion rate, closed circles with the sum of bile salt and bicarbonate excretion rates, open circles that with the sum of excretion rate of bile salts and bicarbonate excretion rate times two. Relationships are described in the text.

a smaller excretion of conjugated bile salts in the initial choleretic period which may not have been fully compensated for by the bicarbonate-choleresis presumably caused by unconjugated 7-ketolithocholate.

The excretion of urosdeoxycholate and its conjugates in 7-ketolithocholate infused rats was about one tenth of that in ursodeoxycholate infused rats, while the extent of choleresis and the rise of bicarbonate concentration in 7-ketolithocholate-infused rats were fairly comparable to those in ursodeoxycholate-infused rats (4,16). These observations suggest not only ursodeoxycholate but also 7-ketolithocholate itself has the capacity to increase bile flow, with a concomitant rise in bicarbonate concentration in the bile.

A highly significant linear relation observed between the bile flow and excretion rates of bile salts and bicarbonate for both the 7-ketolithocholate and the ursodeoxycholate experiments (Fig. 4) suggests that part of the bile flow was osmotically generated by the excretion of bicarbonate ions, as suggested earlier by both Dumont *et al.* (1) and ourselves (4,16). With the assumption that the osmotic force of bicarbonate ions thus excreted is doubled by the excretion of its counter cation (Na^+), the correlations between the bile flow rate and the sum of the excretion rate of bile salts and twice that of bicarbonate were further improved. This calculation is based on the assumption that bicarbonate and its counter cation, Na^+, both have a similar choleretic efficiency. Furthermore, bile acids also bind the counterion to a considerable extent depending on concentration and critical micellar concentration of each bile salt which was ignored in the above calculation. Despite this simplication of our assumptions, it is interesting to note that, according to these calculations, the correlation coefficients further improved for both experiments.

Stereospecificity of metabolism of 7-ketolithocholate. Several earlier studies in humans (7), dogs (6,8) and rats (10) showed that 7-ketolithocholate is reduced to chenodeoxycholate to a greater extent than to ursodeoxycholate. However, most other studies in rats reported a dominant biotransformation of 7-ketolithocholate to ursodeoxycholate (11,12,13), which agrees with our present study. Our results, in conjunction with earlier studies in other species (6–9), suggest that a species difference exists in the stereospecificity of this reaction, as suggested previously by Nakagaki *et al.* (8).

Difference in amidation between ursodeoxycholate and 7-ketolithocholate. The excretion of tauro 7-ketolithocholate was more limited than that of tauroursodeoxycholate and, conversely, glyco-7-ketolithocholate excretion was much more efficient than that of glycoursodeoxycholate. It is usually assumed that one enzyme catalyzes conjugations of bile salts with both taurine and glycine (17,18). The ratio of taurine and glycine conjugates in the bile is generally thought to be determined by the hepatic taurine concentration (18). However, since the hepatic taurine concentration should be comparable between the two rat groups infused with 7-ketolithocholate and ursodeoxycholate, the lower excretion of tauro 7-ketolithocholate than tauroursodeoxycholate suggests that the relative affinity with 7-ketolithocholate for taurine conjugation is lower than with ursodeoxycholate. Bremer (17) previously showed that the efficacy of conjugation with taurine was in the order of cholic acid > deoxycholic acid > dehydrocholic acid in the rat, suggesting a difference in the affinity with different bile acids.

Furthermore, the lower excretion of glycoursodeoxycholate into the bile appears to be at least partly due to the limited excretion itself rather than to its limited availability, since comparison of the G:T ratio in liver and bile at the end of the experiments indicated that the G:T ratios were very close between the liver and bile with 7-ketolithocholate and chenodeoxycholate, whereas the ratio was lower in bile than in liver with ursodeoxycholate

(Table 3). We have previously shown that the maximal biliary excretion rate (Tm) of glycoursodeoxycholate (4) was comparable to that of tauroursodeoxycholate (19). Thus, the limited excretion of glycoursodeoxycholate in comparison to tauroursodeoxycholate or other glycine conjugates observed in this study may indicate that a difference exists in the affinity of different bile salts in the excretory process. This must, however, be clarified in more detail in the future by a different experimental set-up.

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Synthesis and Characterization of Triacylglycerols Containing Linoleate and Linolenate[1]

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Triacylglycerols containing linoleate and linolenate found in vegetable oils were synthesized in gram quantities for oxidation studies. Two acylation methods were examined to convert diacylglycerols or monoacylglycerols to the desired triacylglycerols. Acylations with fatty acid and 1,1′-dicyclohexylcarbodiimide in the presence of 4-dimethylaminopyridine were more rapid, gave triacylglycerols of better isomeric purities and generally better overall yields than the acylations with acid chloride in pyridine. The functional and isomeric purity of the synthetic triacylglycerols were investigated by thin-layer and gas-liquid chromatography of the methyl esters, by lipase hydrolysis, and by ^{13}C NMR. Quantitative ^{13}C NMR provided a valuable tool to determine isomeric structures of the unsaturated triacylglycerols and complemented the lipase hydrolysis method. The triacylglycerols purified by dry column chromatography were obtained in the following respective percent yields, functional and isomeric purities: LLLn, 92.1, 99.4, 98.1; LLnLn, 91.7, 99.2, 97.4; LLnL, 84.2, 99.7, 98.9; and LnLLn, 77.5, 97.8, 99.0 (where L = linoleoyl and Ln = linolenoyl glycerol residues). These synthetic triacylglycerols are valuable models to elucidate the interrelationship of unsaturated fatty acids on the oxidative stability of polyunsaturated vegetable oils.
Lipids 24, 866–872 (1989).

Considerable research has been directed on the oxidation of pure fatty acids and esters, but relatively scant attention has been given to the effects of mixed fatty acids, their interactions, and how their relative positions in the triacylglycerol molecule influence oxidative and flavor stability. Previous studies indicate that the relative positions of the unsaturated fatty acids in triacylglycerols containing palmitate, stearate, oleate and linoleate, may influence their relative oxidation rates (1–3). No information is available, however, on the effect of the relative positions of linoleate and linolenate in mixed polyunsaturated triacylglycerols. Much attention has been given recently to the use of low-erucic acid rapeseed oil as a replacement for soybean oil. Although both soybean oil and low-erucic acid rapeseed oil contain significant amounts of linolenic acid (7—10%), which is the most readily oxidized fatty acid component, the rapeseed oil has been claimed to be more stable than soybean oil, but the literature on this issue is controversial (4–6). To better understand the effect of triacylglycerol structure on the oxidative stability of polyunsaturated fats, a program was initiated in this laboratory to synthesize and evaluate a number of model triacylglycerols containing linoleate and linolenate in known positions.

Syntheses of triacylglycerols containing saturated, oleic and linoleic acids have been described in the literature (7–12), but little has been reported on the more difficult syntheses of triacylglycerols containing linolenic acid. This paper describes the syntheses and characterizations of all possible diacyl triacylglycerols containing linoleate and linolenate for oxidation studies.

EXPERIMENTAL

Materials. All solvents were high performance liquid chromatography (HPLC) grade or analyzed reagent grade. Triacylglycerols and fatty acids and their acid chlorides were purchased (99%+ purity) from NuChek Prep, Inc. (Elysian, MN). Mono- and diacylglycerols (>98%) were purchased from either NuChek or Sigma Chemical Co. (St. Louis, MO) or synthesized as described below. Trilinolein (ca 99%), TRIZMA, sodium cholate, and pancreatic lipase were purchased from Sigma Chemical Co., 1,2(2,3)-isopropylidene glycerol ("Solketal"), dihydroxyacetone, boric acid, trimethyl borate, 4-dimethylaminopyridine, and 1,1′-dicyclohexylcarbodiimide, from Aldrich Chemical Co. (Milwaukee, WI). 4-Dimethylaminopyridine on polystyrene was purchased from Fluka Chemical Corp. (Ronkonkoma, NY). 1,3-Benzylidene glycerol was prepared as previously described (13), or purchased from Serdary Research Laboratories (London, Ontario, Canada) and recrystallized. Silica gel (Grade 60 F254, 70–230 mesh; EM Science, Cherry Hill, NJ) and Omnisolv chloroform, stabilized with hydrocarbon, were purchased from Bodman Chemicals (Algonquin, IL), and Nylon tubing (QE film, 1-5/8 mil thick, 2″ or 3″ flat dia) for dry column chromatography, from M & Q Plastic Product (Freehold, NJ).

Methods. ^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR) spectra were determined as previously reported (7). GLC of fatty acid methyl esters was carried out on a Hewlett Packard 5700 instrument with flame ionization detection, using a capillary column (DB-225, 30 m × 0.32 mm, J & W Co., Rancho Cordova, CA), splitless mode; injection port, 250°C; detector, 250°C; isothermal, 190°C or programmed, 160–200°C at 4°C/min and hold 20 min. For gas-liquid chromatography (GLC), acylglycerols were transesterified with 0.5 N KOH in methanol. For thin-layer chromatography (TLC) analyses of monoacylglycerols and diacylglycerols, the method of Thomas *et al.* (14) was followed. For triacylglycerols, silica gel plates were developed with hexane/diethyl ether (80:20, v/v) and visualized by UV and I_2 absorption and by charring with sulfuric acid.

Lipase analyses were carried out essentially by the method of Yoshida (15), except that the monoacylglyc-

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[1]Presented in part at the AOCS meeting in Phoenix, AZ in May 1988.

Abbreviations: L, linoleoylglycerol; Ln, linolenoylglycerol; LLLn, 1,2-dilinoleoyl-3-linolenoyl-*rac*-glycerol; LLnLn, 1-linoleoyl-2,3-dilinolenoyl-*rac*-glycerol; LLnL, 1,3-dilinoleoyl-2-linolenoylglycerol; LnLLn, 1,3-dilinolenoyl-2-linoleoylglycerol; GLC, gas-liquid chromatography; HPLC, high performance liquid chromatography; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography.

erols were isolated by TLC (silica; hexane/diethyl ether/formic acid, 60:40:1, v/v/v) and then transesterified for GLC with 0.5 N KOH in methanol.

For dry column chromatography, only chromatographic grade solvents were used. The butylated hydroxytoluene stabilizer in diethyl ether was removed by passing through a column of basic, activated alumina just before use. Activity of the silica gel was adjusted according to the procedure of Loev and Goodman (16-18). A Nylon column was packed with 300 g adsorbent/g of crude sample to be purified. For the purifications of monoacylglycerols and diacylglycerols by dry column chromatography, silica gel was impregnated with 10% boric acid by adding a 15% solution of boric acid in methanol to 480 g of silica gel in a round-bottom flask and then removing the methanol on a rotary evaporator by gradually reducing the pressure and increasing the temperature to 65°C. Activity was again checked and adjusted as necessary. The silica gel was equilibrated with 10% (v/w) of the mixed solvent on a rotary evaporator for several hours until free-flowing (19), and then checked again for activity and adjusted, if necessary. The solvent mixtures used to separate different crude products were: hexane/diethyl ether (80:20, v/v) for triacylglycerols; chloroform/acetone (96:4, v/v) for diacylglycerols; chloroform/acetone (90:10, v/v) for monoacylglycerols; hexane/ethyl acetate (80:20, v/v) for dihydroxyacetone esters; hexane/diethyl ether (60:40, v/v) for isopropylidene glycerol esters and benzylidene glycerol esters. The band of the desired component on the column was located by TLC (20). Every 1 or 2 cm along the chromatographic column, a 1-2 mm section of silica gel was cut out, inserted into a Pasteur pipette, extracted with methanol, and analyzed by TLC. The desired band was cut out, transferred and extracted with diethyl ether in an Airlessware filter (Kontes, NJ), filtered under N_2, and concentrated under vacuum on a rotary evaporator and then in an amber glass vial under N_2. To remove any boric acid, filtrates of the monoacylglycerols and diacylglycerols were washed with ice-water before concentration.

Monoacylglycerols and diacylglycerols were acylated to diacyl triacylglycerols by one of the two methods described below. All reactions with unsaturated compounds were conducted under N_2 or argon.

1,2-Dilinoleoyl-3-linolenoyl-rac-glycerol (LLLn) by acid acylation. According to the procedure of Kodali *et al.* (21), 1.00 g (1.6 mmol) of 1,2-dilinoleoyl-*rac*-glycerol was acylated in CCl_4 under N_2 with linolenic acid, and 1,1′-dicyclohexylcarbodiimide, which was warmed under N_2 until liquid (22) in the presence of 4-dimethylaminopyridine. TLC of the isolated crude triacylglycerol (1.72 g) showed traces of diacylglycerol, linolenic acid, 4-dimethylaminopyridine, and 1,1′-dicyclohexylcarbodiimide remaining as impurities. Crude LLLn was purified by dry column chromatography (yield, 92.1%, sample 3, Table 1): ^{1}H NMR ($CDCl_3$) δ5.2-5.4 (*m*,15) olefinic H + glycerol H(2′); δ4.0-4.3(*m*,4) glycerol H(1′,3′); δ2.7-2.8(*m*,8) diallylic CH_2; δ2.2-2.3 (*t*,6) -C<u>H</u>$_2$CO; δ1.9-2.1 (*m*,12) allylic CH_2; δ1.5-1.6 (*m*,6) -CH_2C<u>H</u>$_2$CO; δ1.2-1.3(*m*,36) chain CH_2; δ0.90-0.99(*t*,3) CH_3- (Ln); δ0.81-0.90(*t*,6) CH_3- (L).

1-Linoleoyl-2,3-dilinolenoyl-rac-glycerol (LLnLn) by acid chloride acylation. 1-Monolinoleoyl-*rac*-glycerol, 857 mg (2.4 mmol) was acylated with linolenoyl chloride in pyridine (7,9-11), using airless transfer techniques (23). The crude LLnLn product (2.3 g) was purified by dry column chromatography (yield, 58%, sample 10, Table 1): ^{1}H NMR ($CDCl_3$) δ5.30-5.38(*m*,17) olefinic H + glycerol H(2′); δ4.14-4.29 (*m*,4) glycerol H(1′,3′); δ2.75-2.81(*m*,10) diallylic CH_2; δ2.27-2.33 (*t*,6) -CH_2CO; δ2.02-2.09(*m*,12) allylic CH_2; δ1.59-1.62(*m*,6) -C<u>H</u>$_2$CH$_2$CO; δ1.29-1.36 (*m*,30) chain CH_2; δ0.91-1.00(*t*,6) CH_3- (Ln); δ0.82-0.91(*t*,3) CH_3-(L).

Typical examples of monoacylglycerol syntheses starting with 1,2-isopropylidene-*rac*-glycerol or 1,3-benzylidene glycerol (7) follow.

1-Linolenoyl-2,3-isopropylidene-rac-glycerol. Isopropylidene glycerol, 0.95 g (7.2 mmol), was acylated with linolenoyl chloride in dry pyridine (7). The crude product was obtained as a clear, yellow liquid (yield, 97.5%). Linolenic acid and monoacylglycerol impurities were removed by silica gel column chromatography developing with a solvent gradient of hexane-diethyl ether. The resulting linolenoyl isopropylidene glycerol was functionally pure by TLC (yield, 66.8%): ^{1}H NMR ($CDCl_3$) δ5.22-5.43 (*m*,6) olefinic H; δ4.26-4.29 (*m*,1) glycerol H(2′); δ4.02-4.17(*m*,4) glycerol H(1′,3′); δ3.68-3.75(*m*,1) glycerol H(1′ or 3′); δ2.76-2.80 (*m*,4) diallylic CH_2; δ2.29-2.34(*t*,2) -CH_2CO; δ2.01-2.08(*m*,4) allylic CH_2; δ1.58-1.60(*m*,2) -C<u>H</u>$_2$CH$_2$CO; δ1.28-1.41 (*m*,14) chain CH_2 + ketal CH_3 δ0.92-0.97(*t*,3) CH_3.

1-Monolinolenoyl-rac-glycerol. The above linolenoyl isopropylidene glycerol, 1.51 g, was hydrolyzed with boric acid in trimethyl borate (7) to produce the 1-monoacylglycerol (24) (in crude yield of 82.3%), which was purified by dry column chromatography using boric acid-silica gel. Yield: 35.2% (column recovery, 59.4%). TLC analysis (boric acid-silica gel) of crude linolenoyl isopropylidene glycerol showed a trace of the 2-isomer and other impurities. To minimize isomerization, the 1-monoacylglycerol kept under dry N_2 in a freezer, was acylated as soon as possible with linoleoyl chloride to prepare LLLn in 73.6% yield (sample 1, Table 1).

2-Linoleoyl-1,3-benzylidene glycerol and 2-linolenoyl-1,3-benzylidene glycerol. The procedure of Jensen and Pitas (7) for 2-oleoyl benzylidene glycerol was followed generally, except that the crude linoleoyl benzylidene glycerol was purified by dry column chromatography, with silica gel and hexane/diethyl ether (60:40, v/v) in yield of 69.9%. ^{1}H NMR($CDCl_3$) δ7.35-7.52(*m*,5) phenyl H; δ5.55(*s*,1) Ph-CH; δ5.35-5.37(*m*,4) olefinic H; δ4.63-4.80 (*br.s*,1) glycerol H(2′); δ4.12-4.29(*d-d*,4) glycerol H_2(1′,3′); δ2.68-2.89 (*m*,2) diallylic CH_2; δ2.37-2.51(*t*,2) -CH_2CO; δ2.04-2.06 (*m*,4) allylic CH_2; δ1.57-1.80(*m*,2) -C<u>H</u>$_2$CH$_2$CO; δ1.18-1.52(*m*,16) chain CH_2; δ0.87-0.92(*t*,3) CH_3.

Linolenoyl benzylidene glycerol was prepared in a similar manner in about 75% yield. ^{1}H NMR ($CDCl_3$) δ7.28-7.60 (*m*,5) Ph H; δ5.55(*s*,1) Ph-CH; δ5.29-5.49(*m*,6) olefinic H; δ4.63-4.78(*br.s*,1) glycerol H(2′); δ4.08-4.32(*d-d*,4) glycerol H (1′,3′); δ2.72-2.90(*m*,4)diallylic CH_2; δ2.39-2.49(*t*,2) -CH_2CO; δ2.00-2.18(*m*,4) allylic CH_2; δ1.58-1.73(*m*,2) -C<u>H</u>$_2$CH$_2$CO; δ1.28-1.42(*m*,8) chain CH_2; δ0.90-1.04(*t*,3) CH_3.

2-Monolinolenoyl and 2-monolinoleoylglycerol. The 2-monoacylglycerols were prepared from the benzylidene

TABLE 1

Synthesis of Triacylglycerols Containing Linoleate (L) and Linolenate (Ln)

Run number	Tracylglycerols	Acylation procedure[a]	Starting material[a]	Yield %[b]	Functional purity, %[c]	Isomeric purity, %[d]
1	LLLn	Ac Chloride	1-MLn	73.6	99.5	91.3
2	LLLn	Ac Chloride	1-MLn	39.1	98.3	93.7
3	LLLn	Acid	1,2-DiL	92.1	99.4	98.1
4	LLnL	Ac Chloride	1,3-DiL	82.5	98.9	93.8
5	LLnL	Ac Chloride	1,3-DiL	87.2	99.5	95.0
6	LLnL	Ac Chloride	1,3-DiL	41.0	99.0	96.7
7	LLnL	Ac Chloride	2-MLn	42.0	99.6	92.6
8	LLnL	Acid	1,3-DiL	80.5	99.3	96.8
9	LLnL	Acid	1,3-DiL	84.2	99.7	98.9
10	LLnLn	Ac Chloride	1-ML	58.0	99.4	98.1
11	LLnLn	Acid	1-ML	91.7	99.2	97.4
12	LnLLn	Ac Chloride	2-ML	71.6	99.3	93.0
13	LnLLn	Acid	1,3-DiLn	84.0	99.1	97.0
14	LnLLn	Acid	1,3-DiLn	77.5	97.8	99.0
15	LLLn/ LLnL	Interester-ification	TL + TLn	n.d.	99.8	67.2

[a]Abbreviations: Ac = acyl, L = linoleoyl, Ln = linolenoyl, MLn = monolinolenoylglycerol, DiL = dilinoleoylglycerol, ML = monolinoleoylglycerol, DiLn = dilinolenoylglycerol, TL = trilinoleoylglycerol, TLn = trilinolenoylglycerol, n.d. = not determined.

[b]After purification by dry column chromatography.

[c]Based on GLC analysis of fatty esters after transesterification. Standard deviations from duplicate or triplicate analyses ranged between ± 0.01 and ± 0.48, averaging ± 0.26.

[d]Positional purity (not optical) based on GLC analysis of fatty esters after lipolysis, isolation of 2-monoacylglycerols, and transesterification. Standard deviations from duplicate or triplicate analyses ranged between ± 0.05 and ± 1.05, averaging ± 0.34.

glycerol esters by the procedure of Jensen and Pitas (7) for 2-oleoylglycerol, except that purification was by dry column chromatography with a boric acid-silica gel column in yield of 58.9%. ^{1}H NMR($CDCl_3$) δ5.21-5.38(*m*,4) olefinic H; δ4.80-4.89(*m*,1) glycerol H(2′); δ3.67-3.76(*m*,4) glycerol H (1′,3′); δ3.48-3.54 (*m*,2) glycerol OH; δ2.69-2.76(*m*,2) dialylic CH_2; δ2.25-2.34(*t*,2) -CH_2CO; δ1.94-2.05(*m*,4) allylic CH_2; δ1.52-1.62(*m*,2) -$C\underline{H}_2CH_2CO$; δ1.18-1.38 (*m*,15) chain CH_2; δ0.80-0.88(*t*,3) CH_3. An additional 11.4% yield of 1- and 2-monoacylglycerol mixture was isolated from the dry column as a lower R_f band. The total column recovery of mononoacylglycerols was 70.3%. The 2-monoacylglycerol was acylated immediately with linolenoyl chloride in pyridine to give 1,3-dilinolenoyl-2-linoleoylglycerol (LnLLn) in 71.6% yield (sample 12, Table 1).

2-Monolinolenoylglycerol was synthesized and purified the same way in 41% yield, and acylated with linoleoyl chloride in pyridine to prepare LLnL in 42% yield (sample 7, Table 1).

1,3-Dihydroxypropan-2-one-1,3-dilinoleate. 1,3-Dihydroxyacetone (0.90 g, 10 mmol) was acylated in CCl_4 (20 ml) with linoleic acid and 1,1′-dicyclohexylcarbodiimide in the presence of 4-dimethylaminopyridine (8) using a Firestone valve. The reaction was complete in 2.5 hr, according to TLC (hexane/ethyl acetate, 4:1, v/v). The crude keto diacylglycerol, 6.77 g, was isolated as a clear, dark amber liquid. After purification by dry column chromatography on a silica gel column, minor impurities were still indicated by TLC. The once-chromatographed product was used without further purification. ^{1}H NMR ($CDCl_3$) δ5.22-5.42 (*m*,8) olefinic H; δ4.73(*s*,4) CH_2(dihydroxyacetone 1′,3′); δ2.69-2.80 (*m*,4) diallylic CH_2; δ2.29-2.45(*m*,4) -CH_2CO; 1.92-2.11 (*m*,8) allylic CH_2; δ1.52-1.72(*m*,4) -$C\underline{H}_2CH_2CO$; δ1.18-1.45(*m*,31) chain CH_2; δ0.80-0.92 (*t*,6) CH_3.

1,3-Dilinoleoylglycerol. 1,3-Dihydroxypropan-2-one-1,3-dilinoleate, 2.0 g (3.25 mmol) was reduced to 1,3-dilinoleoylglycerol with neutral sodium borohydride (8). The crude product (2.12 g) was purified by dry column chromatography on a boric acid-silica gel column, dried over Drierite (0.66 g, 32.9% yield) and acylated with linolenic acid and 1,1′-dicyclohexylcarbodiimide in the presence of 4-dimethylaminopyridine to give triacylglycerol 1,3-dilinoleoyl-2-linoleaoylglycerol (LLnL) in 80.5% yield (sample 8, Table 1).

Dilinoleoyl-linolenoylglycerol isomeric mixtures by interesterification. Trilinoleoylglycerol, 8.8 g (10.2 mmol), and trilinolenoylglycerol, 2.1 g (2.38 mmol), were reacted with dry sodium methoxide (13), 0.024 g, in a little anhydrous diethyl ether under N_2, at 90-97°C for 1.76 hr and the reaction was monitored by TLC. More sodium methoxide (0.034 g) was added after 105 min. After 135 min at reaction temperature, TLC indicated that only a trace of trilinolenoylglycerol remained. After cooling to approximately 60°C and adding 0.5 ml of glacial acetic acid, the mixture was stirred for 15 min, cooled to about 35°C, and 5 ml of hexane was added. A clear, yellow liquid product (10.8 g) was obtained after washing (water) and drying (Na_2SO_4). Analysis by reversed phase TLC (development, 10% $AgNO_3$ in acetone/acetonitrile, 4:1, v/v) showed mostly trilinoleoylglycerol and isomeric LLLn/LLnL mixture. The LLLn/LLnL fraction was isolated by preparative HPLC (Waters Prep 500 Liquid Chromatograph, Prep Pak-500/C_{18} column, refractive index detector), solvent: acetonitrile/acetone, 1:1, v/v. Combined LLLn/LLnL HPLC fractions (1.22 g) were purified further by dry column

chromatography (silica gel, hexame/diethyl ether, 80:20, v/v); 91.3% yield (sample 15, Table 1). Both lipase analysis and quantitative ^{13}C NMR showed a 2:1 LLLn/LLnL isomer mixture. ^1H NMR ($CDCl_3$) δ 5.20–5.42 (m,15) olefinic H + glycerol H(2′); δ4.08–4.31 (m,4) glycerol H (1′,3′); δ2.67–2.85(m,8) diallylic CH_2; δ2.21–2.37(t,6) -CH_2CO; δ1.92–2.11(m,12) allylic CH_2; δ1.50–1.68(m,6) -$C\underline{H}_2CH_2CO$; δ1.19–1.41 (m,39) chain CH_2; δ0.92–1.00(t,3) CH_3(Ln); δ0.85–0.91(t,6) CH_3 (L).

RESULTS AND DISCUSSION

To synthesize triacylglycerols, monoacylglycerol or diacylglycerol intermediates are usually acylated with an acid chloride or anhydride in the presence of pyridine for 3–4 days at room temperature (7,9–12). Acyl migrations of the monoacylglycerol or diacylglycerol intermediates may, however, occur during acylation, workup, and purification (25,26), and may increase during prolonged reactions. More recently, rapid acylation (2–3 hr) of monoacylglycerol and diacylglycerol was achieved at room temperature with the reagents 1,1′-dicyclohexylcarbodiimide and 4-dimethylaminopyridine (21,27–29). It was of interest, therefore, to investigate whether or not the isomeric purity of synthetic triacylglycerols can be improved with this new acylation procedure. We also evaluated dry column chromatography (16–20) to purify our synthetic triacylglycerols, because it was expected to give better yields than conventional adsorption column chromatography and low-temperature crystallization.

Synthesis of diacyl triacylglycerols. All triacylglycerols in Table 1 were synthesized by acylation of the respective monoacyl mono- and diacylglycerols. The acylglycerol intermediates were acylated at ambient temperature either with the acid chloride in pyridine (7), or the appropriate fatty acid and 1,1′-dicyclohexylcarbodiimide in the presence of 4-dimethylaminopyridine (21,27–29). All crude synthetic triacylglycerols were purified by dry column chromatography on silica gel. Functional purities of the triacylglycerols based on GLC analyses ranged from 97.8 to 99.7% after purification by dry column chromatography to remove more polar (e.g., mono- and diacylglycerols) and less polar (e.g., some reagents) impurities. Positional isomeric purities, determined by pancreatic lipolysis and GLC analyses of the fatty acids on the 2-monoacylglycerols, ranged from 91.3 to 99.0% (Table 1).

Acylations by the acid method in the presence of 1,1′-dicyclohexylcarbodiimide and 4-dimethylaminopyridine were generally superior to the acid chloride method in the overall yields and isomeric purities of purified triacylglycerols. A triacylglycerol of higher purity was produced when commercial 1,2-dilinoleoyl-*rac*-glycerol was acylated with linolenic acid by the acid method (run 3, Table 1), than when 1-monolinolenoyl-*rac*-glycerol was acylated with the acid chloride (runs 1 and 2, Table 1). Acylation of 1,3-dilinoleoylglycerol from dihydroxyacetone by the acid method in runs 8 and 9, gave a much better yield with the same isomeric purity than did the acid chloride acylation in run 6, and gave similar yields but much better isomeric purity than acid chloride runs 4 and 5 (Table 1). Acylation of 1-monolinoleoyl-*rac*-glycerol by the acid method gave a much better yield, but the isomeric purity was slightly lower than by the acid chloride method (runs 10 and 11). Acid chloride acylations of 2-monolinoleoylglycerol and 2-monolinolenoylglycerol (runs 7 and 12, Table 1), prepared from their respective benzylidene glycerol esters, gave lower yields, and the isomeric purities of the products were unsatisfactory. For the synthesis of LLnLn and LnLLn, 1,3-diacylglycerols generally were much more promising intermediates than the 2-monoacylglycerols, and better sources of the diacylglycerol intermediates were available. Acylation of 1,3-dilinolenoylglycerol from dihydroxyacetone by the acid method gave a satisfactory yield and purity for LnLLn, and an equivalent isomeric purity (runs 13 and 14, Table 1). An insoluble polymeric dimethylaminopyridine catalyst was investigated because it can be recovered by simple filtration, regenerated, and recycled (30). Acylation of 1,3-dilinoleoylglycerol with linolenic acid in the presence of this polymeric reagent was slow, and did not go to completion after two days, allowing a yield of only 34%.

Trilinoleoylglycerol and trilinolenoylglycerol were interesterified to prepare a LLLn/LLnL isomeric mixture (run 15, Table 1) to serve as a reference for comparison with the pure isomers.

NMR Spectroscopy. ^1H NMR confirmed the structure and functional purity of the synthetic triacylglycerols by showing the chemical shifts and proton counts for the olefinic, secondary glycerol, primary glycerol, diallylic, methylene β and α to ester carbonyl, allylic, chain methylene, and terminal methyl protons. The terminal methyl protons of linoleate (0.80–0.91 ppm) and linolenate (0.91–1.00 ppm) were observed as two triplets in a 2:1 ratio corresponding to the respective diacyl triacylglycerol. Also, the expected complex spin-spin coupling pattern typical of the glycerol backbone of a triacylglycerol was found in the 4–5.4 ppm region (31) in all spectra of synthetic triacylglycerols listed in Table 1.

Quantitative ^{13}C NMR has previously been used to estimate the fatty acid composition of triacylglycerol mixtures; to show the position of the butyryl group in butter oil triacylglycerols (32); to distinguish the carbonyl carbons of saturated, oleic, and linoleic acids of triacylglycerols (33); to differentiate the fatty acyl chains substituted on glycerol carbons one and three (29); to determine the fatty acid composition of palm oil (34), and to estimate the isomeric composition of mixtures of unsaturated triacylglycerols containing a cyclic fatty acid (13). In the present study, a quantitative ^{13}C NMR method was also employed to identify linoleate and linolenate triacylglycerol isomers and to estimate the composition of isomer mixtures.

By examining quantitative ^{13}C NMR resonances of olefinic carbons, we were able to distinguish between the isomeric 1,2- and 1,3-dilinoleoyl, and between the 1,2- and 1,3-dilinolenoyl triacylglycerols. In the expanded ^{13}C NMR spectrum of the LLLn isomer, each olefinic carbon of the linoleate and linolenate moieties gave a distinguishable resonance, except for the C-9 of the 3-substituted linolenate and the C-13s of the 1- and 2-substituted linoleate at 130.13 ppm (Fig. 1). The resonance pattern for the olefinic carbons of the LnLnL isomer is similar to that of the LLLn isomer,

but all olefinic carbons were distinguished and identified, except C-13 of the 3-substituted linoleate and C-9s of the 1- and 2-substituted linolenate resonating at 130.09–130.12 ppm (Fig. 2).

The spectrum of the olefinic carbon resonances of LLnL isomer shows that the resonances of the linoleoyl olefinic carbons are twice those for the linolenoyl olefinic carbons, and all of the chemical shifts are distinguishable and identifiable (Fig. 3). This spectrum is simpler than that of LLLn and is characteristic of the 1,3-dilinoleoyl isomer. The olefinic carbon resonances in the expanded spectrum of LnLLn were also distinctive (Fig. 4). Here all carbon resonances except those of C-9 for the 1,3-substituted linolenate and C-13 of the

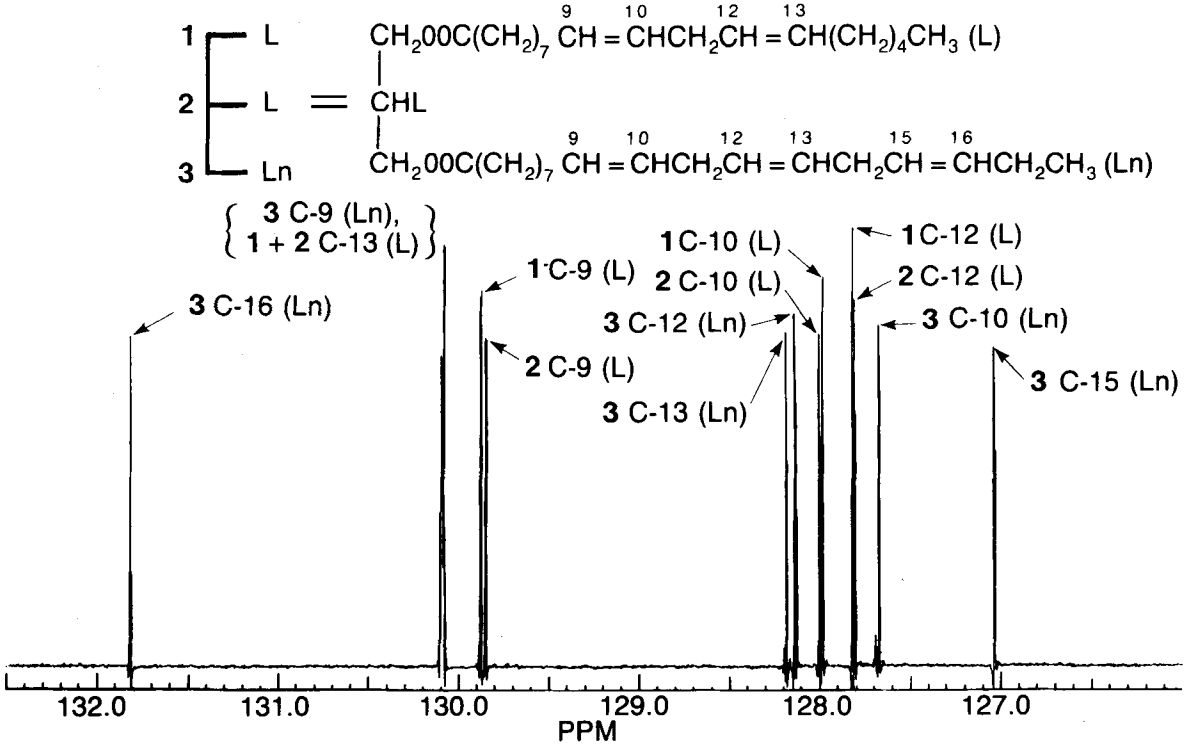

FIG. 1. Partial ^{13}C NMR spectrum of olefinic carbons of 1,2-dilinoleoyl-3-linolenoyl-*rac*-glycerol (LLLn).

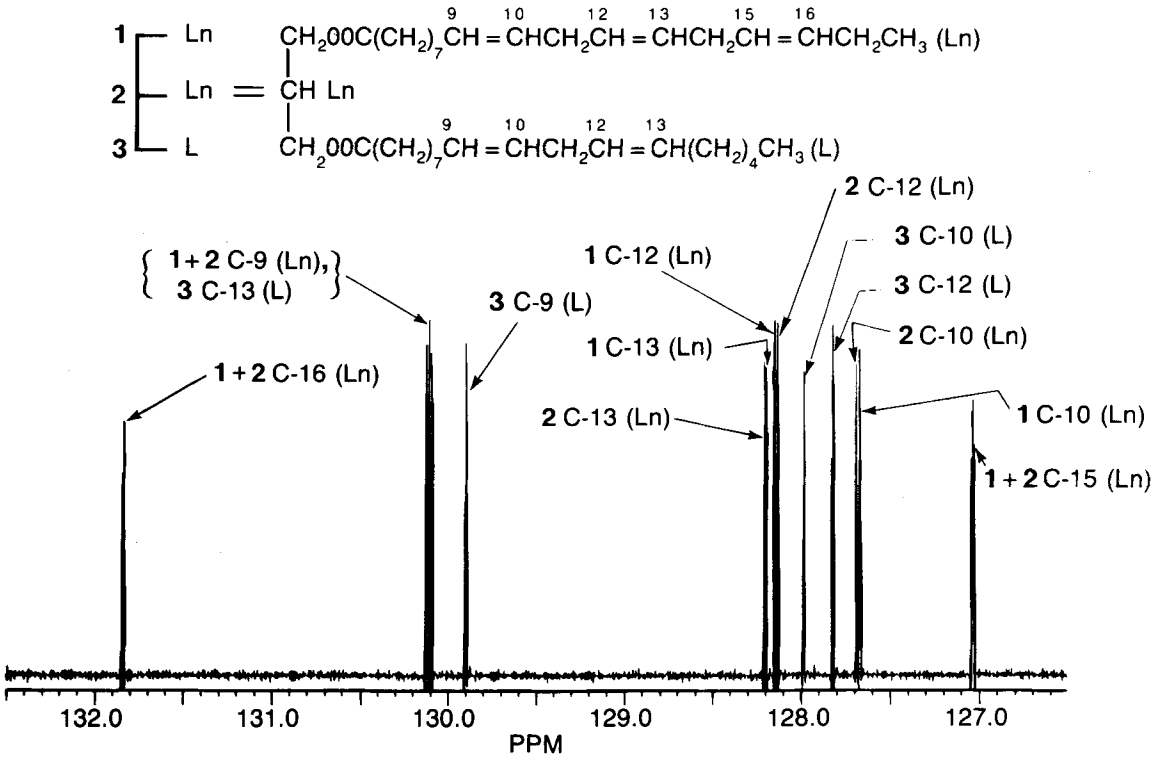

FIG. 2. Partial ^{13}C NMR spectrum of olefinic carbons of 1,2-dilinolenoyl-3-linoleoyl-*rac*-glycerol (LnLnL).

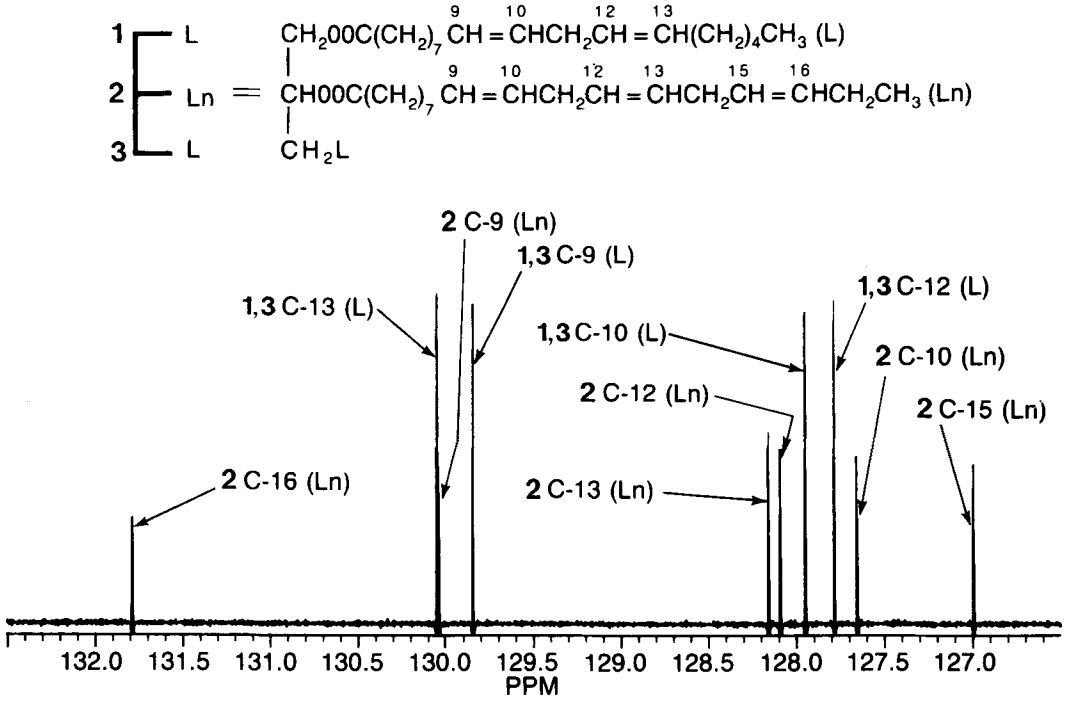

FIG. 3. Partial ^{13}C NMR spectrum of olefinic carbons of 1,3-dilinoleoyl-2-linolenoylglycerol (LLnL).

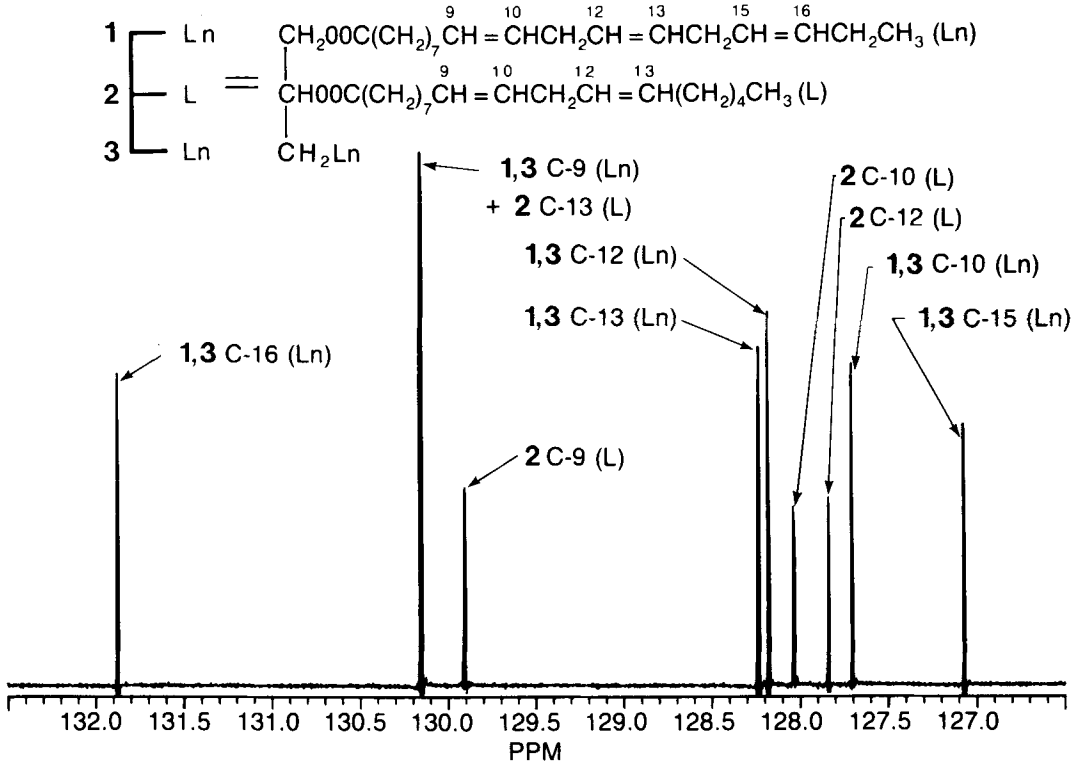

FIG. 4. Partial ^{13}C NMR spectrum of olefinic carbons of 1,3-dilinolenoyl-2-linoleoylglycerol (LnLLn).

2-substituted linoleate were distinguished. ^{13}C NMR analysis of a mixture of LLLn and LLnL isolated by preparative HPLC from interesterified trilinoleoylglycerol and trilinolenoylglycerol (run 15, Table 1) gave an isomer ratio of 2:1, which is in good agreement with lipase analyses showing a mixture of 67.7% LLLn isomer and 33.3% LLnL isomer. Quantitative ^{13}C NMR spectroscopy is not only a powerful structural method, but can also effectively complement the lipase method for characterizing and distinguishing between positional diacyl triacylglycerol isomers. Apparently, the lipase analysis method cannot detect positional isomeric im-

purities of less than 1-2%, and certainly the ^{13}C NMR method cannot be expected to do as well (perhaps only 5% under the best conditions).

The synthetic triacylglycerols described in this paper have been used in a study that will be reported in a future paper comparing their relative rates of autoxidation and the nature of the oxidation products. HPLC procedures, which were used to analyze and remove impurities and oxidation products below 1-2% immediately before oxidizing the synthetic triacylglycerols, will also be reported in the later paper on oxidation studies.

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Thiobarbituric Acid-Reactive Substances from Peroxidized Lipids

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The thiobarbituric acid (TBA) reaction was performed on linoleic acid 13-monohydroperoxide, autoxidized fatty esters, edible fats and oils, rat liver microsomal lipids, and on human erythrocyte ghost lipids in order to determine which substances from peroxidized lipids are TBA-reactive. The reaction was carried out in 2% acetic acid containing butylated hydroxytoluene using two different reaction modes: a one-step mode which involves heating at 100°C, and a two-step mode which involves first treatment at 5°C and subsequent heating at 100°C. Yields of the red 1:2 malonaldehyde/TBA adduct, as estimated by absorbance, fluorescence intensity and high-performance liquid chromatography, were much higher than the malonaldehyde content as determined by direct chemical analysis. Yields of red pigment obtained by the two-step mode were slightly higher than those obtained by the one-step mode. Pigment yields were dramatically increased by addition of *t*-butyl hydroperoxide. Red pigment formation from alkenals and alkadienals was similarly enhanced by the two-step mode or by addition of *t*-butyl hydroperoxide, whereas pigment formation from malonaldehyde was not. It appears likely that a component of the total red pigment formed from the peroxidized lipids was due to aldehyde species other than malonaldehyde.
Lipids 24, 873-881 (1989).

The thiobarbituric acid (TBA) test has been commonly used to measure lipid peroxidation ever since it was introduced by Kohn and Liversedge (1-3). Heating of peroxidized lipids with TBA under acidic conditions produces red pigment with an absorption maximum at 532 nm (2). The pigment has been considered to be the 1:2 adduct of malonaldehyde and TBA (4,5). For many years the test was thought to be specific for malonaldehyde. Recently, it has been demonstrated that other lipid oxidation products such as alkanals, alkenals and alkadienals also produce the pigment (6-16). However, it has been shown that malonaldehyde is the aldehyde most reactive with TBA (11,12).

Our recent studies have demonstrated that TBA produces the pigment by reaction with alkanals (13), alkenals (14) and alkadienals (15) via the intermediary 1:1 adducts between TBA and the aldehydes in the presence of water and oxygen. Furthermore, it has been found that mixtures of an alkenal, an alkadienal and an organic hydroperoxide produce unexpectedly large amounts of the pigment (16). The pigment formation from alkenals and alkadienals is markedly enhanced by organic hydroperoxides, and the pigment formation from alkadienals is dependent on the reaction modes: a two-step mode which involves an initial treatment at 5°C and subsequent heating at 100°C produces a much greater amount of the pigment than a one-step mode which involves immediate heating at 100°C (15,16). On the contrary, malonaldehyde produces the pigment without being affected by the presence of other components or hydroperoxides and by the reaction modes.

In the present investigation, the TBA test was performed on various peroxidized lipids in order to elucidate the contributions of various aldehyde species to red pigment formation in the TBA reaction. Yields of the pigment were estimated by the one- and two-step modes, and the effect of an organic hydroperoxide was examined. For comparison, the contents of malonaldehyde, other aldehydes and intrinsic hydroperoxides in the peroxidized lipids were determined by the Hantzsch method (17), the 2,4-dinitrophenyl-hydrazine (11) and dimedone (18) methods, and the sesamol dimer method (19), respectively.

MATERIALS AND METHODS

Materials. TBA, 2,4-dinitrophenylhydrazine (DNPH), 5,5-dimethyl-1,3-cyclohexanedione (dimedone) and 1-hexanal were obtained from Wako Pure Chemical Industries (Osaka, Japan). 1-Propanal, 1-pentanal, 2-hexenal, 2,4-hexadienal, tetramethoxypropane, methyl linoleate and methyl oleate were obtained from Tokyo Kasei Kogyo Co. (Tokyo, Japan). 2-Heptenal, 2-octenal, 2,4-heptadienal and 2,4-nonadienal were from Aldrich Chemical Co. (Milwaukee, WI). *t*-Butyl hydroperoxide (*t*-BuOOH) (70% in water) was purchased from Nakarai Chemicals (Kyoto, Japan). Butylated hydroxytoluene (BHT) was from Nikki Universal Co. (Tokyo, Japan). Adenosine-5′-diphosphate monopotassium salt (ADP) was the product of Oriental Yeast Co. (Tokyo, Japan). Glacial acetic acid was a special reagent-grade product of Wako Pure Chemical Industries. Silica gel column chromatography was performed with silica gel for chromatography from Kanto Chemical Co. (Tokyo, Japan).

Soybean oil was a product of Showa-Sangyo Co. (Tokyo, Japan), and sesame oil was Japan Pharmacopoeia grade. Subcutaneous lipids of hog and chicken and whole lipids of sardine meat were extracted and prepared as described by Kosugi and Kikugawa (20). Linoleic acid 13-monohydroperoxide (13-LOOH) (21) was prepared from linoleic acid (Nippon Oil and Fats Co., Tokyo, Japan) and lipoxidase (Type 1: 130,000 units/mg, Sigma Chemical Co., St. Louis, MO). The purity of the hydroperoxide was 96% as estimated on the basis of the molar extinction coefficient (24,500) at 234 nm (22).

Rat liver microsomal suspension was prepared according to the method of Albro *et al.* (23). As detailed therein, livers weighing 120 g from twelve Spraque-Dawley male rats were homogenized, and a microsomal suspension (60 ml) in 25 mM morpholinopropane sulfonic acid buffer (pH 7.4) −0.25 M mannitol was

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Abbreviations: TBA, thiobarbituric acid; *t*-BuOOH, *t*-butyl hydroperoxide; ADP, adenosine-5′-diphosphate; BHT, butylated hydroxytoluene; AOM, active oxygen method, HPLC, high-performance liquid chromatography.

obtained. The suspension was frozen at −20°C until used.

Human erythrocyte ghosts were prepared according to the method of Steck (24) and stored at −20°C in aliquots until used (25).

Analyses. Absorption spectra were measured on a Shimadzu UV-240 UV-visible or a Hitachi U-2000 spectrophotometer. Fluorescence spectra were measured on a Hitachi 650-60 fluorescence spectrophotometer. High-performance liquid chromatography (HPLC) was carried out with a Shimadzu LC-6A liquid chromatograph equipped with a stainless-steel column (4.6 mm i.d. × 25 cm) of YMC A-303 ODS (Yamamura Chemical Laboratories, Kyoto, Japan). For determination of malonaldehyde, the column was eluted with methanol/0.025 M phosphate buffer, pH 7.5 (1:1, v/v) at a flow rate of 0.5 ml/min. The fluorescent peak was detected at 400 nm (excitation) and 465 nm (emission) with a Shimadzu RF-530 fluorescence spectrofluorometer. For determination of red pigment, the column was eluted with methanol/0.04 M acetate buffer (pH 5.5) (4:6 v/v) at a flow rate of 0.8 ml/min. The peak was detected at 532 nm with a Shimadzu SPD-6A spectrophotometer. All analyses were carried out within the linear response ranges of the detectors.

Oxidation of lipid samples. Methyl linoleate, methyl oleate, soybean oil, sesame oil, hog fat and chicken oil were oxidized at 98°C by the active oxygen method (AOM) (26). Sardine oil was oxidized by ultraviolet irradiation as previously described (20).

Microsomal suspension (60 ml) was mixed with an equal volume of 0.05 M Tris-0.15 M KCl (pH 7.5) containing 2.0 mM ADP, 2.0 mM sodium ascorbate and 24 μM $FeCl_2$, and the mixture was incubated at 37°C for 30 min (27). Lipids of the oxidized microsomes were extracted with chloroform/methanol according to the method of Bligh and Dyer (28), and yielded 243 mg of lipid.

Human erythrocyte ghost suspension (40 ml) was mixed with 20 ml of 0.1 M phosphate buffer (pH 7.0) containing 10 μM carbonmonoxy hemoglobin, and the mixture was kept at room temperature for 30 min. A 20-ml solution of 1.0 mM *t*-BuOOH in phosphate buffer was added (29). The mixture was incubated at 37°C for 30 min. Lipids of the oxidized ghosts were extracted and obtained in a yield of 119 mg.

Determination of hydroperoxides. Hydroperoxide content of the peroxidized lipid samples was determined by peroxide value (neq/mg) (30), conjugated diene (31) and the sesamol dimer method (19). For determination by sesamol dimer, 6 mg of the lipid sample was used, and hydroperoxide content was expressed as nmol of 13-LOOH.

Determination of total aldehydes with 2,4-dinitrophenylhydrazine (DNPH) (11). DNPH (50 mg) was dissolved in 100 ml of 1 N hydrochloric acid, and the solution was extracted twice with 50 ml of *n*-hexane to remove impurities. To 2.0 ml of the solution were added 1.8 ml of water and 0.2 ml of glacial acetic acid containing lipid sample (2-4 mg) or standard aldehyde (0-0.2 μmol). The mixture was vigorously shaken at room temperature for five min in the dark and extracted six times with 3.0 ml of benzene. The organic layers were combined and evaporated at 30°C *in vacuo*, and the residue was redissolved in 0.5 ml of benzene. The solution was applied to a column (9 mm i.d. × 3.5 cm) of silica gel, and the column was eluted with benzene. Hydrazone fractions (3-15 ml) and DNPH fractions (19-27 ml) were separated. The hydrazone fractions were combined and evaporated *in vacuo*, and the residue was dissolved in 10.0 ml of methanol in order to measure the absorption spectrum.

Absorption spectra of the hydrazone fractions from the standard aldehydes, 1-hexanal, 2-hexenal and 2,4-hexadienal, showed respective maxima at 358, 373 and 390 nm. Calibration curves of the absorbance at 370 nm of the fractions were linear. Yields of the hydrazones were 55% for 1-hexanal, 103% for 2-hexenal and 118% for 2,4-hexadienal on the basis of the average molar extinction coefficient (26,000) of the various hydrazones at 370 nm (32).

Absorption spectra of the fractions from 13-LOOH, oxidized methyl linoleate, microsomal lipids and ghost lipids showed maxima at 355-380 nm. The amount of total aldehyde in the peroxidized lipid samples was estimated by the absorbance at 370 nm and by comparison with the calibration curve of 2-hexenal. The amount of total aldehyde was expressed as nmol of 2-hexenal.

Determination of total aldehyde with 5,5-dimethyl-1,3-cyclohexanedione (dimedone) (18). 0.5 ml of 2-propanol containing lipid sample (0.5-4.0 mg) or standard aldehyde (0-0.4 μmol) was added to a mixture of 0.5 ml of 0.75 mM dimedone in 2-propanol and 1.0 ml of 1.5 mM ammonium acetate in water. The mixture was heated at 80°C for 60 min after removal of dissolved oxygen. The fluorescence spectrum of each mixture was taken, and fluorescence intensity was determined against blank solution containing no lipid or aldehyde.

Alkanals (1-propanal, 1-pentanal and 1-hexanal) exhibited fluorescence with excitation maxima at 385 nm and emission maxima at 450 nm and displayed similar fluorescence intensities. Alkenals (2-hexenal, 2-heptenal and 2-octenal) showed fluorescence with excitation maxima at 367 nm and emission maxima at 450 nm and displayed similar intensities. Alkadienals (2,4-hexadienal, 2,4-heptadienal and 2,4-nonadienal) showed fluorescence with excitation maxima at 378 nm and emission maxima at 450 nm, and displayed similar intensities. Relative fluorescence intensities measured at 385 nm (excitation) and 450 nm (emission) were 1.0 ± 0.002 for the alkanals, 0.2 ± 0.01 for the alkenals, and 0.34 ± 0.02 for the alkadienals.

Fluorescence spectra of the reaction mixtures of 13-LOOH, oxidized methyl linoleate, microsomal lipids and ghost lipids showed excitation maxima at 380-385 nm and emission maxima at 450-455 nm. The amount of total aldehyde was estimated by the fluorescence intensity relative to that of the reaction of 1-hexanal. The amount of total aldehyde was expressed as nmol of 1-hexanal.

Determination of malonaldehyde. Malonaldehyde content of peroxidized lipid samples was determined by the Hantzsch method after treatment of the samples with acetic acid (17). The lipid sample (5-50 mg) was dissolved in 0.5 ml of 70% acetic acid and heated at 100°C for 30 min, and the mixture was neutralized

by addition of 0.5 ml of 10 N NaOH. Eight ml of 2-propanol, 1.0 ml of 0.1 M methylamine hydrochloride in water and 1.0 ml of 0.1 M acetaldehyde in methanol were added to the above mentioned solution. The mixture was heated at 80°C for two hr after removal of dissolved oxygen. The reaction mixture was subjected to HPLC. The amount of 1,4-dimethyl-1,4-dihydropyridine-3,5-dicarbaldehyde was determined by the peak height of the fluorescent peak at a retention time of 6.8 min. The malonaldehyde content of the sample was calculated from the calibration curve of the dihydropyridine versus standard malonaldehyde sodium salt (0–20 nmol).

TBA test. The TBA test was performed according to a previously reported method (16) with slight modification. A TBA solution (0.4%) in water (5.0 ml) was placed into a 13-ml screw-cap tube. For the one-step reaction, the tube was preheated at 100°C, and 0.1 ml of glacial acetic acid containing the lipid sample (0.5–4 mg), 0.5% BHT and 10 μmol *t*-BuOOH were then added. The mixture was vigorously shaken and immediately heated at 100°C for 20 min. For the two-step reaction, the tube containing the TBA solution was precooled to 5°C and 0.1 ml of the sample solution containing BHT and *t*-BuOOH was added. The mixture was kept at 5°C for 60 min and subsequently heated at 100°C for 20 min. Insoluble material in the reaction mixtures was removed by extraction with 1.0 ml of chloroform or 3.0 ml of chloroform/methanol (5:1, v/v). The absorption spectrum of each aqueous phase was recorded, and the amount of red pigment was calculated from the absorbance at 532 nm using the molar extinction coefficient (156,000) of the red 1:2 malonaldehyde/TBA adduct (4).

For measurement of the pigment by fluorometry, fluorescence intensity of each aqueous phase was determined at 515 nm (excitation) and 550 nm (emission) (33) after 50-fold dilution with water. The amount of pigment was calculated from its intensity relative to that of a standard reaction mixture containing tetramethoxypropane.

On HPLC, the peak due to the red pigment with absorption at 532 nm was detected at a retention time of 7.5 min, and the amount of the pigment was calculated by comparing the peak area with that of a standard reaction mixture containing tetramethoxypropane.

RESULTS

Reaction conditions for the TBA test of peroxidized lipids. TBA reaction mixtures in the present experiments contained 0.4% TBA, 2% acetic acid and the large amount of water that is necessary for effective red pigment formation from alkenals and alkadienals (14–16). Although the presence of a large amount of water prevented complete dissolution of lipid samples, the pigment formation was not affected, regardless of whether the reaction was conducted by the one-step or the two-step mode (15,16).

While oxidized methyl linoleate (1350 neq hydroperoxides/mg) produced 5 nmol red pigment/mg at 20-min heating and 8.5 nmol at 90-min heating by the two-step mode, the amount of pigment did not exceed 5 nmol in the presence of 0.01% BHT. The amount of red pigment from unoxidized methyl linoleate at 20-min heating increased from 1.4 to 2.7 nmol by addition of 10 μmol *t*-BuOOH, but the effect of the added hydroperoxide was completely abolished in the presence of 0.01% BHT. The methyl linoleate could be oxidized by the intrinsic hydroperoxides or the exogenously-added hydroperoxide during the TBA reaction, and this oxidation could be prevented by BHT. Since BHT only slightly affected the pigment formation from alkenals and alkadienals (16) and malonaldehyde (34), 0.01% BHT was routinely included in the TBA test as carried out in the present study.

The effect of unoxidized methyl linoleate on red pigment formation from a mixture of tetramethoxypropane, 2-hexenal, 2,4-hexadienal and *t*-BuOOH was examined (Fig. 1). The pigment formation was dependent upon the amount of mixture reacted and was slightly higher by the two-step than by the one-step mode. Most of the red pigment was derived from the combined effect of 2-hexenal, 2,4-hexadienal and *t*-BuOOH (16). The red pigment formation was little affected by methyl linoleate (Fig. 1).

Red pigment formation from linoleic acid 13-monohydroperoxide (13-LOOH). The TBA test of enzymatically-synthesized 13-LOOH (0.5–2.0 mg) in either the one- or the two-step mode produced red pigment. Absorption and fluorescence spectra of the reaction mixtures (Fig. 2B, dotted curves) were similar to those of a standard reaction mixture of tetramethoxypropane (Fig. 2A). The amount of pigment estimated by absorbance and fluorescence intensities increased linearly with increasing amounts of hydroperoxide. The yields of red pigment were slightly higher by the two-step than by the one-step mode, and were estimated to be 12 nmol (one-step) and 13 nmol pigment (two-step)/1.0 mg (or 3.2 μmol) of hydroperoxide (Table 1). When the amount of malonaldehyde produced from the hydroperoxide by acid treatment was determined by the Hantzsch reaction (17), it was less than 1 nmol. A large amount of aldehydes including alkenals and alkadi-

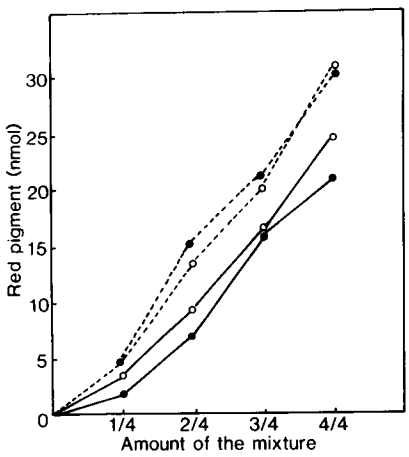

FIG. 1. Effect of unoxidized methyl linoleate on the red pigment formation in the TBA reaction from a mixture of various aldehydes and *t*-BuOOH. The red pigment formation in a mixture of 2.5 nmol tetramethoxypropane, 0.98 μmol 2-hexenal, 0.092 μmol 2,4-hexadienal and 9.4 μmol *t*-BuOOH in the presence (●) and absence (○) of 2.0 mg of unoxidized methyl linoleate is shown. The TBA reaction was performed in the presence of 0.01% BHT by the one-(——) and the two-step (-----) modes.

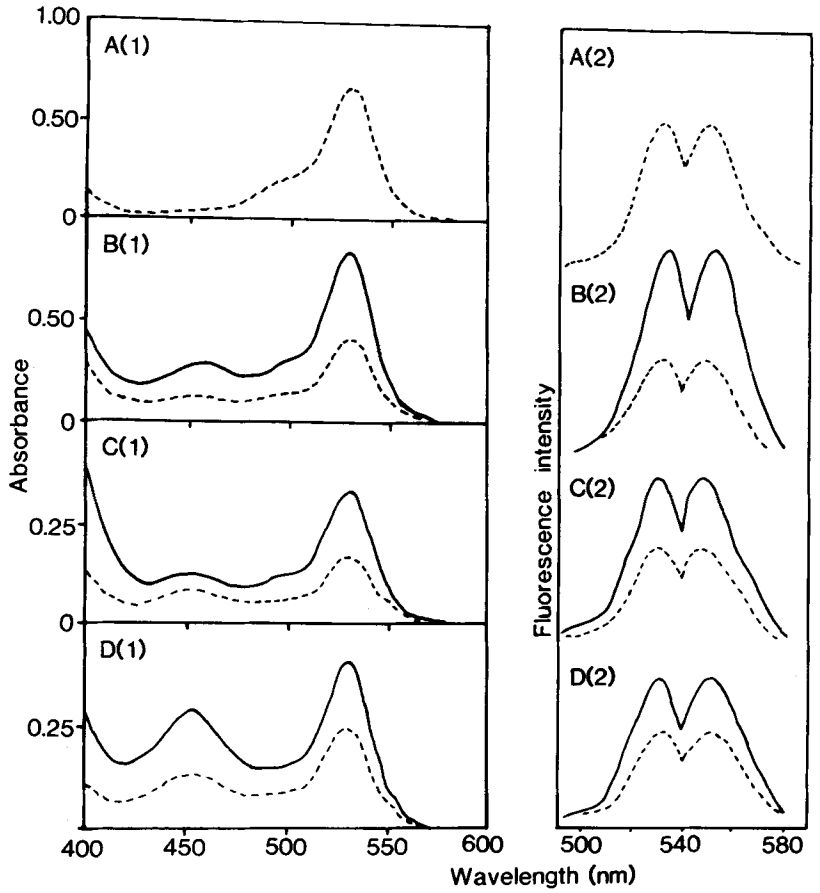

FIG. 2. Absorption (left) and fluorescence (right) spectra of the TBA reaction mixtures. The TBA reaction of tetramethoxypropane (24.4 nmol) (A), 13-LOOH (1.0 mg) (B), oxidized rat liver microsomal lipids (1.8 mg) (C) and human erythrocyte ghost lipids (3.9 mg) (D) with (——) and without (- - - -) 10 μmol *t*-BuOOH was performed in the presence of 0.01% BHT by the two-step mode. Absorption spectra of the reaction mixtures were recorded directly and fluorescence spectra were recorded after dilution into water.

TABLE 1

Amounts of Red Pigment, Total Aldehydes, Malonaldehyde and Hydroperoxides Formed from 13-LOOH and Oxidized Fatty Esters

	Red pigment (nmol/mg lipid) in the TBA test				Total aldehyde (nmol/mg lipid)		Malonaldehyde (nmol/mg lipid)	Hydroperoxide (μmol/mg lipid)
	One-step mode		Two-step mode					
Sample	None	+ *t*-BuOOH (10 μmol)	None	+ *t*-BuOOH (10 μmol)	DNPH	Dimedone	Hantzsch	Peroxide value
13-LOOH	11.4[a]	31.8[a]	13.4[a]	27.5[a]	87 ± 58*	231 ± 119*	<1.0	3.21
	11.8[b]	30.8[b]	13.0[b]	25.9[b]				
Methyl linoleate AOM-12 hr	7.7[a]	20.0[a]	8.9[a]	21.9[a]	67	202	0.7	0.81
	7.3[b]	22.1[b]	11.1[b]	23.2[b]				
	7.0[c]	19.6[c]	8.6[c]	21.2[c]				
Methyl oleate								
AOM-3 hr	1.2[a]	2.9[a]	2.4[a]	3.4[a]				0.11
27 hr	1.5[a]	4.5[a]	3.3[a]	4.3[a]				0.49

The TBA reactivity of each sample was assessed in the presence of 0.01% BHT. The amounts of red pigment are expressed as the mean values ± 1.0 (S.D.) of more than two different experiments. The amounts of total aldehyde liberated from 13-LOOH varied from experiment to experiment, and are expressed as the mean values ± S.D. of four different experiments (*).

[a]The amount of red pigment was determined by absorbance.

[b]The amount of red pigment was determined by fluorescence intensity.

[c]The amount of red pigment was determined by HPLC.

enals were detected by the DNPH method (about 87 nmol) and by the dimedone method (about 231 nmol) (Table 1). Addition of about 3 equivalent amounts (10 μmol) of *t*-BuOOH to the hydroperoxide dramatically increased the yields of the pigment. Absorption and fluorescence spectra of the reaction mixtures with *t*-BuOOH (Fig. 2B, solid curves) were similar to those without *t*-BuOOH (Fig. 2B, dotted curves). The yields of the pigment were 31 nmol (one-step) and 27 nmol (two-step) (Table 1). The enhanced pigment formation by *t*-BuOOH is characteristic to alkenals and alkadienals (16). The red pigment produced in the TBA reaction with 13-LOOH was most likely derived from the combined effect of alkenals, alkadienals and the intrinsic hydroperoxide content of the sample.

Red pigment formation from autoxidized fatty esters. Methyl linoleate was oxidized by the active oxygen method (AOM) for up to 24 hr (Fig. 3). The peroxide values of the ester increased during the first 12 hr and decreased thereafter (Fig. 3A). The amount of total aldehyde, as estimated by the DNPH and dimedone methods, increased during the first 12 hr and then decreased (Fig. 3B). Since volatile aldehydes may be removed during the oxidation, the aldehydes thus estimated appear to be those nonvolatile species liberated from the hydroperoxide compounds. The amount of total aldehyde was lower than that of the amount of hydroperoxide at any stage of autoxidation. The TBA reaction by the one- and the two-step modes showed that amounts of the pigment estimated by absorbance increased during the first 15 hr of oxidation and then decreased (Fig. 3C). Addition of 10 μmol *t*-BuOOH dramatically increased the amount of the pigment at any stage of autoxidation. No further pigment increases were observed by addition of 20 μmol *t*-BuOOH.

The reaction mixtures of the 12 hr-oxidized methyl linoleate with and without added *t*-BuOOH exhibited absorption spectra, fluorescence spectra and HPLC peaks identical with those due to a standard reaction mixture of tetramethoxypropane. The yields of red pigment from the 12 hr-oxidized methyl linoleate, estimated by absorbance, fluorescence intensity and HPLC, were much greater than the amount of malonaldehyde (Table 1). Aldehydes other than malonaldehyde and the hydroperoxide derivatives must have produced the pigment.

Time courses of peroxide values and red pigment formation associated with AOM-oxidized methyl oleate are shown in Figure 4. Red pigment formation was much greater by the two-step mode than by the one-step mode at all stages of autoxidation. The amounts of the pigment from the 3 hr and 23 hr-oxidized methyl oleate were markedly increased by addition of *t*-BuOOH (Table 1). The pigment derived from the oxidized methyl oleate may be due to the combined effect of aldehyde species other than malonaldehyde and the intrinsic hydroperoxide components.

Red pigment formation from autoxidized edible fats and oils. Soybean oil, sesame oil, hog fat and chicken oil were oxidized by AOM, and sardine oil was oxidized by ultraviolet light irradiation. The time courses of peroxide values and red pigment formation are shown in Figure 5. All the fats and oils showed similar parallel increases and decreases in the peroxide value and amount of red pigment. The red pigment formation from soybean, sesame, hog and chicken oils was slightly higher by the two-step TBA-reaction than by the one-step mode at all stages of autoxidation. The amounts of red pigment from soybean, sesame, chicken and sardine oils were significantly increased by addition

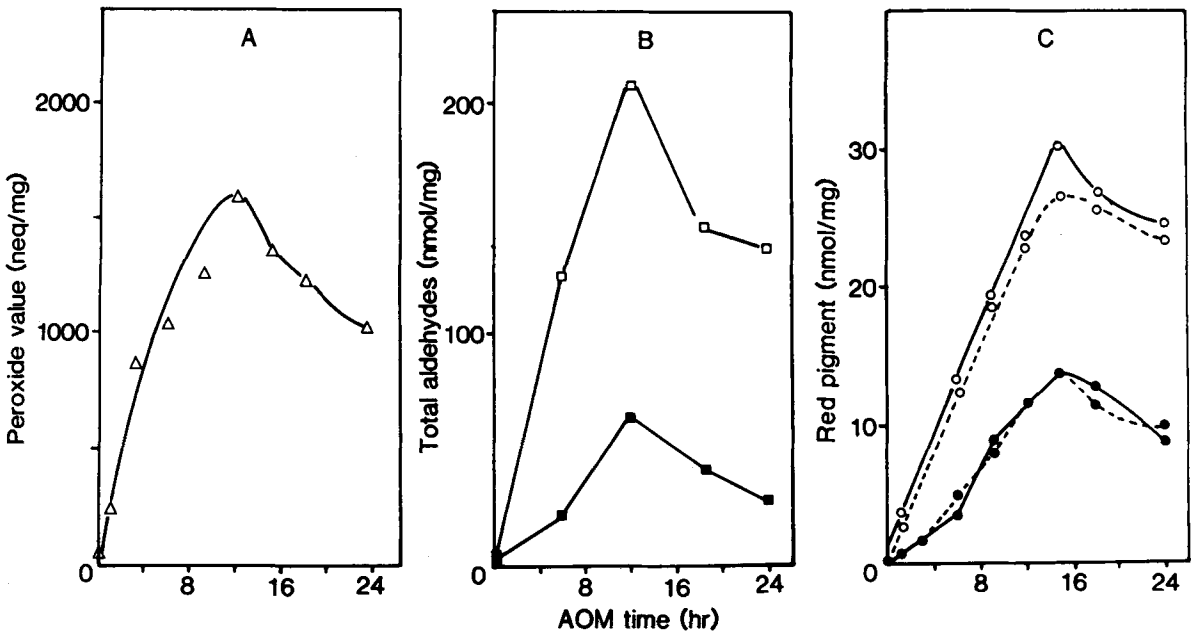

FIG. 3. Time courses of peroxide value (A), total aldehyde formation (B) and red pigment formation (C) of the AOM-oxidized methyl linoleate. Peroxide value (△) and amount of total aldehyde determined by DNPH (■) and dimedone (□) are presented. The TBA test was performed in the presence of 0.01% BHT by the one- (——) and the two-step (- - - -) modes. Absorbance at 532 nm of each sample linearly increased with the sample amount of 0.5–2.5 mg. Red pigment produced in the absence (●) and presence (○) of 10 μmol *t*-BuOOH was estimated by absorbance.

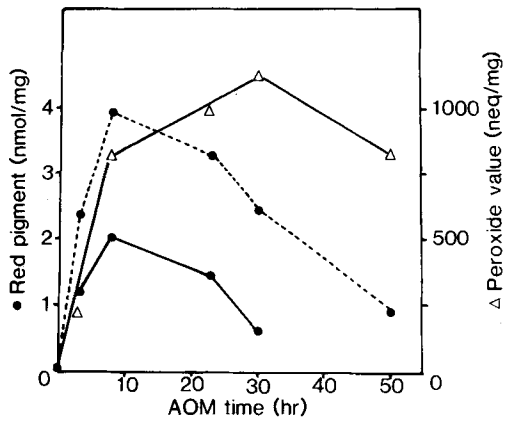

FIG. 4. Time courses of peroxide value and red pigment formation from the AOM-oxidized methyl oleate. The TBA test was performed in the presence of 0.01% BHT by the one- (——) and the two-step (— — —) modes. Absorbance of each sample linearly increased with the sample amount of 0.5–2.5 mg.

of *t*-BuOOH (Table 2). The results suggest that red pigment observed was derived from the combined effect of alkenals, alkadienals and intrinsic hydroperoxides.

Red pigment formation from oxidized rat liver microsomal lipid and oxidized human erythrocyte-ghost lipid. Rat liver microsomes were oxidized with ADP-ascorbate-Fe^{2+}, and human erythrocyte ghosts were oxidized with *t*-BuOOH in the presence of catalytic hemoglobin. The one- and the two-step modes of the TBA-reaction with either the extracted microsomal or ghost lipids yielded absorption spectra, fluorescence spectra (Fig. 2, C and D, dotted curves) and HPLC peaks identical with those of a standard reaction mixture of tetramethoxypropane. The microsomal lipids yielded 3 nmol pigment/mg by the one-step mode, and a little higher amount by the two-step mode (Table 3). The malonaldehyde content was lower than 1 nmol, but a larger amount of other aldehydes (more than 15 nmol) and hydroperoxide derivatives (about 10 nmol) were detected. The ghost lipids yielded 2 nmol pigment/mg by the one-and the two-step modes (Table 3). The malonaldehyde content was about 10% of the pigment content. A large amount of other aldehydes (more than 8 nmol) and hydroperoxide derivatives (about 25 nmol) were detected.

Addition of *t*-BuOOH to the microsomal or the ghost lipids yielded absorption and fluorescence spectra (Fig. 2, C and D, solid curves) and HPLC peaks identical with those of a standard reaction mixture of tetramethoxypropane, but the pigment yield in each case was greatly increased (Table 3). In the TBA reaction of these biological lipids, aldehydes other than malonaldehyde must have contributed to pigment formation.

DISCUSSION

Red pigment produced in the TBA test has been used as a measure of lipid peroxidation (1–3). It has been long held that the red pigment is derived from malonaldehyde generated from peroxidized lipid samples, since Sinnhuber *et al.* (4) and Nair and Turner (5) demon-

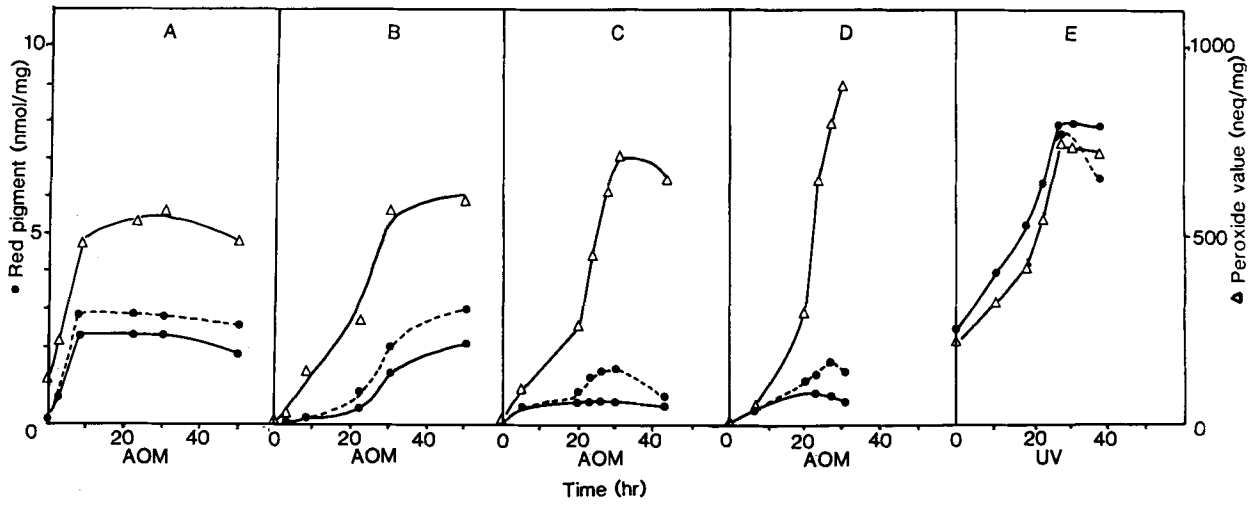

FIG. 5. Time courses of peroxide value and red pigment formation from oxidized soybean oil (A), sesame oil (B), hog fat (C), chicken oil (D) and sardine oil (E). The TBA test was performed in the presence of 0.01% BHT by the one- (——) and the two-step (— — —) modes. Absorbance of each sample linearly increased with the sample amount of 0.5–2.5 mg.

TABLE 2

Red Pigment Formation from Autoxidized Fats and Oils in the TBA Test

Sample	Red pigment (nmol/mg lipid) in the TBA test				Hydroperoxide (μmol/mg lipid)
	One-step mode		Two-step mode		
	None	+ *t*-BuOOH (10 μmol)	None	+ *t*-BuOOH (10 μmol)	Peroxide value
Soybean oil					
AOM-3 hr	0.7	1.3	0.8	1.3	0.11
AOM-30 hr	2.3	4.1	3.0	4.6	0.29
Sesame oil					
AOM-23 hr	0.5	0.9	0.8	1.0	0.14
AOM-50 hr	2.0	3.6	3.1	4.2	0.29
Hog fat					
AOM-5 hr	0.4	0.4	0.5	0.6	0.05
AOM-27 hr	0.6	1.2	1.5	1.5	0.31
Chicken oil					
AOM-27 hr	0.8	1.2	1.7	1.9	0.40
Sardine oil					
UV-37 hr	8.0	9.2	6.5	9.2	0.36

Fats and oils were autoxidized by AOM or ultraviolet (UV) irradiation. The TBA reaction of the sample was performed in the presence of 0.01% BHT, and the amount of red pigment formed was quantified by its absorbance.

TABLE 3

Red Pigment, Total Aldehyde, Malonaldehyde and Hydroperoxide Formation from Peroxidized Microsomal and Erythrocyte-Ghost Lipids

Sample	Red pigment (nmol/mg lipid) in the TBA test				Total aldehyde (nmol/mg lipid)		Malonaldehyde (nmol/mg lipid)	Hydroperoxide (nmol/mg lipid)	
	One-step mode		Two-step mode						
	None	+ *t*-BuOOH (10 μmol)	None	+ *t*-BuOOH (10 μmol)	DNPH	Dimedone	Hantzsch	*Sesamol* dimer	*Conjugated* diene
Peroxidized rat liver microsomal lipid	2.6[a] 2.6[b] 2.5[c]	5.1[a] 6.1[b] 5.0[c]	2.9[a] 2.8[b] 2.7[c]	4.8[a] 6.0[b] 4.4[c]	15.2	16.9	<1.0	9.9	11.8
Peroxidized human erythrocyte-ghost lipid	1.8[a] 1.6[b]	3.6[a] 3.4[b]	1.8[a] 1.7[b]	3.7[a] 3.3[b]	60.3	8.2	0.23	26.7	23.3

Rat liver microsomes and human erythrocyte ghosts were oxidized, and the oxidized membrane lipids were extracted. The TBA reactivity of each oxidized lipid sample was performed in the presence of 0.01% BHT. The amounts of red pigment are expressed as the mean values ± 0.5 (S.D.) of more than two different experiments.

[a]The amount of red pigment was determined by absorbance.

[b]The amount of red pigment was determined by flourescence.

[c]The amount of red pigment was determined by HPLC.

strated that the pigment is the 1:2 adduct of malonaldehyde and TBA. The amount of pigment can be determined by absorbance (4) and fluorescence intensity (33) and, more specifically, by HPLC (35). However, malonaldehyde determination by various methods, i.e., acid decomposition-acetylation (36), dansyl hydrazine (37), direct HPLC (38–40), aromatic amine-fluorometry (41) and Hantzsch method (17), have consistently shown that the malonaldehyde contents of various peroxidized lipid and membrane samples are much lower than those estimated by the TBA test. Hence, the TBA test can now be considered nonspecific for malonaldehyde and nonquantitative as a measure of malonaldehyde.

Standard aldehydes other than malonaldehyde may form red pigment in the TBA test (6–16). However, according to the experiments of Esterbauer *et al.* (11) and Witz *et al.* (12), yields of red pigment from the other aldehydes are much lower than that from malonaldehyde when each of them is reacted with TBA. We have previously reported that there are significant differences in the pigment formation between malonaldehyde and the other aldehydes (14–16). Although malonaldehyde produces the pigment without being affected by the reaction modes and by the presence of other aldehydes and organic hydroperoxides, alkenals and alkadienals produce red pigment depending on the reaction modes, the presence of oxygen and water, and the presence of other aldehydes and organic hydroperoxides (14–16). Pigment formation from alkenals and alkadienals is synergistically enhanced, and it is mark-

edly enhanced by organic hydroperoxides. While the reaction of each alkenal or alkadienal produces 0.2-0.5% (14) and 5-10% (15) pigment, respectively, yields of the pigment are greatly increased (by >30%) when they are combined with each other and with organic hydroperoxides (15,16). The organic hydroperoxides may act as a strong oxidant to promote the red pigment formation (15,16).

The present study was undertaken in order to elucidate which components of peroxidized lipid samples (i.e., peroxidized fatty esters, 13-LOOH, peroxidized edible fats and oils, and peroxidized microsomal and erythrocyte ghost lipids) are TBA-reactive. For this purpose, yields of red pigment formed by two TBA-reaction modes, one-step and two-step, were compared. Yields of the pigment in the absence and presence of *t*-BuOOH were also compared. Yields of the pigment were higher by the two-step mode than by the one-step mode, and much higher in the presence of *t*-BuOOH than in the absence of the agent. These characteristics of red pigment formation from the peroxidized lipid samples were similar to those from alkenals and alkadienals and different from that from malonaldehyde (15,16). Total aldehyde content of the peroxidized lipid samples estimated by the DNPH (11,32) and the dimedone (18) methods was much higher than the malonaldehyde content of the same sample, as estimate by the Hantzsch method (17). Under these TBA-test conditions, red pigment produced in the TBA test from peroxidized lipid samples could largely reflect aldehydes other than malonaldehyde.

Ohkawa *et al.* (42,43) used the TBA test for estimation of hydroperoxides in peroxidized lipid samples, but did not offer mechanistic details. In this regard, the red pigment formation from pure 13-LOOH in the present study is interesting. 13-LOOH by itself may not produce the pigment, since organic hydroperoxides, i.e., *t*-BuOOH and cumene hydroperoxide, do not (16). In the present study, 13-LOOH decomposed into various aldehyde products (3% of the hydroperoxide), including malonaldehyde (less than 0.03% of the hydroperoxide). Yield of red pigment from 13-LOOH (0.4% of the hydroperoxide) greatly exceeded the malonaldehyde content. Thus, aldehyde species other than malonaldehyde must have produced the pigment in concert with the intrinsic hydroperoxide function of 13-LOOH.

The TBA test may be a useful method for estimating a broad range of lipid peroxidation products. The red pigment formed in the reaction should be regarded as reflecting some combination of aldehydes and lipid hydroperoxides. Since the presence of *t*-BuOOH in the TBA reaction mixtures used maximized pigment formation, inclusion of *t*-BuOOH is desirable. The degree of lipid peroxidation estimated by the TBA test should be expressed as red pigment and not as malonaldehyde.

For determination of aldehyde species in peroxidized lipids, the DNPH method (11,32) and the dimedone (18) or cyclohexanedione (44) methods are often used. However, it was found in the present experiments that the total aldehyde content of pure 13-LOOH estimated by these methods was surprisingly high, indicating that aldehydes were liberated during the brief treatment of the hydroperoxide under the strongly acidic or heating conditions required by the methodology. These findings indicate that some of the aldehydes estimated by these methods are those liberated from lipid hydroperoxides as a direct result of the analytical procedures themselves.

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Effects of Dietary Fats on Fatty Acid Composition and Δ5 Desaturase in Normal and Diabetic Rats

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We have studied the effect of various diets on the phospholipid fatty acid composition and *in vitro* Δ5 desaturase activity of hepatic microsomes derived either from the normal or streptozotocin-induced diabetic rat. The diets studied were the standard rat chow diet and a basal fat-free diet supplemented either with 20 percent saturated fat, 20 percent unsaturated fat, or 20 percent menhaden oil. Phospholipid fatty acid composition analysis revealed that the normal rat fed the saturated fat or menhaden oil diet had significantly decreased arachidonate levels, consistent with decreased Δ5 desaturase activities and decreased 18:2n-6 intake. On the contrary, the unsaturated fat diet decreased dihomo-γ-linolenate and increased arachidonate levels, without increased Δ5 desaturase activity. Streptozotocin-induced diabetes resulted in decreased arachidonate and Δ5 desaturase activity. The unsaturated fat diet fed to the diabetic rat also failed to correct this decreased Δ5 desaturase activity. The unsaturated fatty acids in this diet also displaced a substantial amount of n-3 fatty acids in both normal and diabetic microsomes, due to the competition between these two fatty acid families for incorporation into the membrane phospholipids. Conversely, the menhaden oil diet fed to the normal and diabetic rats displaced n-6 fatty acids, reduced Δ5 desaturase activity, and enhanced 22:6n-3 incorporation into diabetic microsomes.

Lipids 24, 882–889 (1989).

Several studies have shown that the *in vitro* Δ9 (1–4) and Δ6 (2–5) fatty acid desaturase activities are depressed in liver microsomes of experimentally diabetic rats. This has been correlated with increased levels of linoleate and dihomo-γ-linolenate and decreased levels of palmitoleate, oleate, and arachidonate in the membrane fatty acid profile of several tissues (5–8). While few studies have included actual assays of the *in vitro* activity of Δ5 desaturase, the altered membrane fatty acid profile, i.e., the increased dihomo-γ-linolenate and the decreased arachidonate, suggests a defect in this enzyme in experimental diabetes.

Furthermore, treatment with insulin has been shown to correct or overcorrect the defects in Δ9 and Δ6 desaturases in diabetic rats (1–4). The effect of diabetes and insulin on Δ5 desaturase activity deserves further study. Poisson *et al.* (9) reported a decrease in Δ5 desaturase activity in streptozotocin diabetic rats fed either a balanced or fat-free diet. In addition, Wilder and Coniglio (8) reported decreased testicular Δ5 desaturase activity in streptozotocin diabetic rats.

Other factors, including diet, hormones, duration and severity of diabetes, may affect Δ9, Δ6, and Δ5 desaturase activities (10). For example, it has been reported that dietary supplementation with polyunsaturated fatty acids may stimulate Δ5 desaturase activity in normal rats, while inhibiting Δ9 and Δ6 desaturases (10, 11). In contrast, a fat-free diet has been found to decrease Δ5 desaturase activity in normal rats, while inducing Δ9 and Δ6 desaturases (10,12). On the other hand, Pugh and Kates (13) reported a stimulation of Δ5 desaturase in normal rats starved and refed a fat-free diet. Also, partially hydrogenated marine oils or fish oils are reported to decrease both Δ6 and Δ5 desaturase activities (14–16) in normal rats. This is of particular importance since dietary supplementation with marine oils has been reported to decrease plasma triglycerides and may reduce plasma cholesterol in human subjects, which may provide a protective effect from atherosclerosis and coronary heart disease (17–20). There are few studies investigating the effect of dietary manipulation on Δ5 desaturase in diabetic rats, however.

In the present communication, we report the fatty acid composition of liver microsomal phospholipids and the *in vitro* activity of Δ5 desaturase in control, diabetic, and insulin-treated diabetic rats fed a standard chow diet. We also describe the effect of dietary supplementation with saturated fat, n-6 polyunsaturated fat, or n-3 polyunsaturated fat on the fatty acid composition of liver microsomal phospholipids and on the *in vitro* activity of Δ5 desaturase in control and diabetic rats.

MATERIALS AND METHODS

Precoated silica gel G thin-layer chromatography plates were purchased from Eastman Kodak Company, Rochester, NY. Streptozotocin was kindly donated by Dr. A.Y. Chang, Upjohn Co., Kalamazoo, MI. Boron trifluoride in methanol (14 percent) was purchased from Sigma Chemical Co., St. Louis, MO. 8,11,14-[2-^{14}C]Eicosatrienoic acid was purchased from Amersham, Arlington Heights, IL. The experimental diets were obtained from ICN Biochemicals, Cleveland, OH. The fat-free diet (Cat. No. 901683) contained 58.5 percent sucrose, 21.1 percent vitamin-free casein, 16.5 percent alphacel and 4 percent salt mixture USP XIV and was supplemented with either 20 percent saturated fat, polyunsaturated fat, or menhaden oil. All 3 supplemental diets contained equal amounts (0.12 percent) of DL-α-Tocopherol powder (250 IU/gm) as an antioxidant agent. Authentic fatty acid methyl ester standards for gas-liquid chromatography identification were purchased from Sigma Chemical Co. and Nu-Check Prep, Inc., Elysian, MN. Protamine zinc insulin (PZI) was purchased from Eli Lilly, Indianapolis, IN. Organic solvents such as methanol, chloroform, hexane, and ben-

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Abbreviations: $AgNO_3$, silver nitrate; ANOVA, analysis of variance; ATP, adenosine triphosphate; GLC-MS, gas-liquid chromatography-mass spectrometry; KOH, potassium hydroxide; NADH, nicotinamide adenine dinucleotide; PZI, protamine zinc insulin; TLC, thin-layer chromatography.

zene were reagent grade and commercially available.

Animals and their treatment. White, male, Sprague-Dawley rats, strain CR1:CD(SD)BR, 2 months old, from Charles River Breeding Laboratories were maintained on a Purina chow diet and water *ad libitum*. Rats were divided into diabetic and nondiabetic control groups, each containing 18 rats. Rats were made diabetic by the intravenous injection of 75 mg/kg of streptozotocin dissolved in sterile 25 mM citrate buffer (pH 4.5). After several days, blood glucose obtained by tail vein aspirate was estimated using a commercial glucose oxidase method (Sigma Kit 510).

All rats received a Purina Laboratory Chow No. 5001C (5 percent fat) diet for 2 weeks following the induction of diabetes, after which each group was divided into three equal subgroups which received diets supplemented with either saturated fat (20 percent partially hydrogenated coconut oil), n-6 polyunsaturated fat (20 percent safflower oil) or n-3 polyunsaturated fat (20 percent menhaden oil). Since the menhaden oil contained 0.5 percent cholesterol (i.e., 0.1 percent in the menhaden oil supplemented diet), 0.1 percent cholesterol was added to the other two diets as well. These diets were kept refrigerated at 4°C throughout the experimental period to avoid rancidity. The cage food was changed at least twice a week. The fatty acid composition of the diets was analyzed by gas-liquid chromatography at the beginning and end of the study and no alteration was found. A record of each rat's food consumption and body weight throughout the study was maintained.

After receiving dietary supplementation for one month, the rats were sacrificed by decapitation. Blood was collected for glucose determination and livers were removed, washed in normal saline and stored at −70°C. The livers were then homogenized in 4 ml of 0.25 M sucrose/g of liver and the homogenate was centrifuged at 1000 × *g* for 10 minutes. The supernatant was collected and centrifuged at 16,000 × *g* for 20 minutes, after which the resulting supernatant was centrifuged at 100,000 × *g* for one hour. The microsomal pellet was washed 3 times and resuspended in 0.25 M sucrose. Protein concentration was determined by a modification of the Lowry method as described by Hartree (21), using bovine serum albumin as standard.

In one experiment, 2-month-old rats were maintained on the Purina chow diet during the entire time and were divided into three groups of 6–8 rats each. One group served as nondiabetic controls. The other two groups were made diabetic for 6 weeks before sacrificing. Animals in the third group received injections of 8 units/day of protamine zinc insulin for 4–5 days before sacrifice. A record of body weight was maintained and blood glucose was determined at the time of sacrifice. Liver microsomal isolation and protein determination were performed as described above.

Enzyme assays. The *in vitro* activity of Δ5 desaturase was determined by a modification of the method of Yamaoka *et al.* (22). A typical reaction mixture contained 0.75 mM NADH, 2 mM ATP, 0.2 mM coenzyme A, 10 mM $MgCl_2$, 50 mM potassium phosphate buffer (pH 6.8), 62 mM sucrose, 30 μM 8,11,14-[2-^{14}C]eicosatrienoic acid, and 3 mg of microsomal protein in a final volume of 0.8 ml. (Determination of enzyme activities at different protein concentrations showed that optimal amounts were from 2–4 mg.) Reactions were incubated for 30 minutes at 37°C and terminated with 2 ml of 25 percent (v/v) 10 N KOH in ethanol. The reaction mixtures were then hydrolyzed at 60°C for 1 hour and titrated to a pH of 3–4 using bromophenol blue as indicator. The fatty acids were extracted with petroleum ether and methylated with boron trifluoride in methanol as described previously (23). After methylation, the fatty acid methyl esters were extracted twice with hexane, evaporated to dryness, and resuspended in 200 μl of chloroform/methanol (2:1). Ten to fifteen μl (or about 2 mg) of authentic (nonradioactive) 8,11,14-eicosatrienoic acid and arachidonic acid methyl esters were added to the samples as carriers. The fatty acid methyl esters were separated on silica gel TLC plates impregnated with 20 percent (w/v) $AgNO_3$ in acetonitrile using a benzene/ethyl acetate/acetic acid (90/10/1) solvent system (22). Spots were identified using 2′,7′-dichlorofluorescein indicator. Under these conditions, 8,11,14-eicosatrienoic acid and arachidonic acid methyl esters were totally separated (Rf = 0.40 and 0.21, respectively). Their identification was checked by gas-liquid chromatography. Those spots corresponding to arachidonic acid were scraped and their radioactivity determined in a scintillation counter. [^{14}C]-Substrate incorporation into product was determined by dividing the counts in the arachidonic acid spot by the specific activity of 8,11,14-[2-^{14}C]eicosatrienoate applied to the plate, followed by correction for incomplete recovery and quenching. The percent recovery of radioactivity was determined by dividing the counts from spotting 50 μl of the reaction mixture (without microsomal protein) to the TLC plates directly, scraping, and counting in a scintillation counter by the counts of 50 μl of the reaction mixture (without microsomal protein) counted directly. This averaged 30 percent.

Phospholipid fatty acid composition. To 1 ml of liver microsomes (15 mg protein) was added 19 ml of chloroform:methanol (2:1 v/v) to extract microsomal lipid. The extracts were allowed to stand for more than 2 hours at 4°C, after which 4 ml of water was added, the extracts vortexed, and the aqueous (upper) phase removed. Using thin-layer chromatography silica gel G plates with a n-hexane/diethyl ether/acetic acid (80:20:1 v/v/v) solvent system, the phospholipid fraction, which remained at origin, was scraped and placed in a tube containing 0.5 ml benzene and 1 ml 14 percent trifluoroboron in methanol. The tubes were flushed with nitrogen and capped tightly. The transesterification reactions were carried out by heating the samples to 95–100°C for one hour. Resulting fatty acyl methyl esters were extracted with 2 ml n-hexane twice. The hexane was subsequently evaporated and the samples resuspended in 50 μl of benzene. Phospholipid fatty acid composition was determined using a Hewlett-Packard gas-liquid chromatograph, model 5710 A equipped with a 6 foot column packed with chromosorb W AW 100/200 mesh containing 12 percent EGSS-X + 2 percent polyvinyl-pyrrolidone, a flame ionization detector, and a model 3390A Hewlett-Packard reporting integrator. Chromatography was carried out isothermally at 180°C. Identification of fatty acids was

based on retention times of authentic standards, except for the 4,7,10,13-16-docosapentaenoate peak which was based on the high level (greater than 16 percent) of this fatty acid in lipid extracts from rat testis (5). This system resolves all the major normally occurring tissue fatty acids but does not separate 20:3n-6 from 20:3n-9. Therefore, for these two fatty acid methyl esters, we have used GLC-MS [Hewlett-Packard 5890A (GC) and 5988 (MS)] equipped with a DB-wax capillary column (J & W Scientific) and HP 59970 MS Chem station to resolve them into separate peaks and reevaluated their compositions. Chromatography was carried out with a gradient temperature, (T), initial T=180°C for 2 min, the 20°C/min rate and final T=240°C, injection port T=250°C.

Statistical analysis. Results of replicate gas-liquid chromatograms were used for statistical analysis. Values shown in the tables are the means ± SD (n=), with n values indicating the numbers of animals per each experimental group. The statistical analysis was performed by using VMS/SAS software (1986 SAS Institute Inc., Cary, NC). The analysis was based on Duncan's Multiple Range Test preceded by Analysis of Variance (ANOVA). The significant differences between groups were evaluated with the alpha level set at 0.05 and 0.01.

RESULTS

Rat treatment and diet. The fatty acid composition of the dietary supplements is shown in Table 1. The saturated fat diet contained 99.0 percent total saturated fatty acids, of which myristate, stearate and palmitate comprised 44.8 percent, 28.3 percent and 26.9 percent, respectively. This diet contained 0.5 percent linoleate. The n-6 unsaturated fat diet contained 90.8 percent total unsaturated fatty acids of which 83.7 percent was linoleate. The menhaden oil diet contained 33.2 percent n-3 unsaturated fat, of which eicosapentaenoate and docosahexaenoate comprised 56.3 percent and 31 percent, respectively. Total unsaturated fat in this diet comprised 67.3 percent, with linoleate (n-6) comprising only 5.2 percent of total fatty acids. The distribution of fatty acids in these diets allowed comparison between the effects of saturated, n-6 unsaturated, and n-3 unsaturated fat on the activity of Δ5 desaturase. The chow diet contained 27.1 percent saturated fatty acids, 27.2 percent oleate (n-9), 40.5 percent linoleate (n-6), and 1.7 percent linolenate (n-3). Thus, the chow diet provided a balanced amount of saturated, n-9 and n-6 unsaturated fatty acids, although the amount of n-3 fatty acid was only 4.5 percent.

An adequate diabetic state was established by IV injection of streptozotocin as evidenced by the mean blood glucose level of 525 ± 130 mg/dl in all groups of diabetic animals not treated with insulin compared to a mean blood glucose level of 138 ± 38 mg/dl in control animals fed the experimental diets and 112 ± 16 mg/dl in control animals fed a chow diet. Insulin treatment resulted in less severe diabetes with a mean blood glucose level of 165 ± 89 mg/dl. Diabetic rats demonstrated significantly less weight gain in both the chow diet group (Fig. 1) and the experimental diet groups (Fig. 2) as compared to the corresponding control groups, although the amounts of food consumed by the diabetic rats, which averaged above 25 g/day with all three experimental diets, were not significantly different from that of the control rats fed the same diets. Thus, the diabetic rats showed significant increases of food intake per gram of body weight.

In general, diabetic rats supplemented with menhaden oil had a greater severity of illness despite a blood glucose level that was not significantly different from other groups of diabetic rats. Menhaden oil-fed diabetic rats had poorer coat texture and weight gain and a more reduced activity level than other diabetic rats. Three diabetic rats in the group fed menhaden oil died before completion of the study, while two diabetic rats in each of the saturated and unsaturated fat groups failed to survive until completion of the study. Although the diabetic rats fed the experimental diets appeared ill, the combination of diabetes and dietary

TABLE 1

Fatty Acid Composition of the Diets

	Chow Diet	Saturated	Unsaturated	Menhaden Oil
14:0	1.8	44.3	—	—
16:0	24.1	26.6	7.3	27.4
16:1	0.3	—	—	10.4
18:0	1.2	28.1	1.9	5.3
18:1n-9	27.2	—	13.5	15.5
18:2n-6	40.5	0.5	76.1	5.2
18:3n-3	1.7	0.4	0.3	1.3
20:4n-6	0.2	—	0.2	1.1
20:5n-3	1.3	—	0.1	18.7
22:4n-6	0.2	0.1	0.1	1.2
22:5n-6	—	—	0.2	0.7
22:5n-3	0.1	—	0.3	2.9
22:6n-3	1.4	—	—	10.3

[a]Total lipids were extracted from the diets as described in the Methods section. Transesterifications were carried out in the presence of benzene and 14 percent trifluoroboron in methanol.

[b]Fatty acid composition was determined by GLC and values were expressed as percentages of each fatty acid over total fatty acids identifiable.

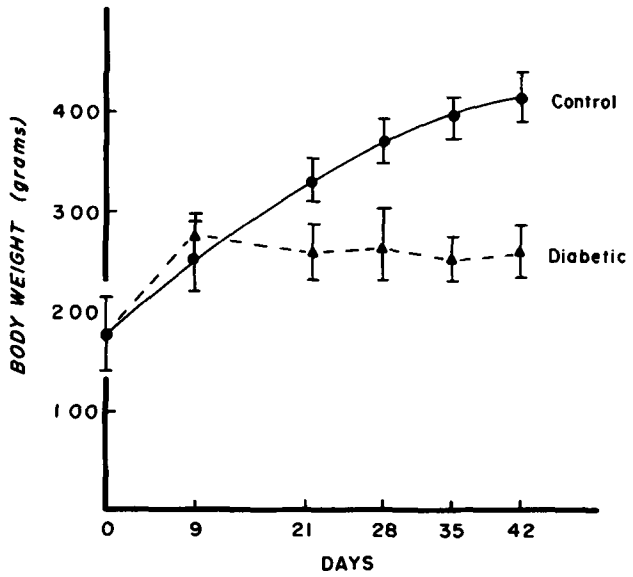

FIG. 1. Body weights of control and diabetic rats fed the chow diet. Normal control rats and streptozotocin-induced diabetic rats fed the chow diet were weighed periodically as indicated in the abscissa. Body weights were expressed as means ± SD with number of rats (n) in each group = 8.

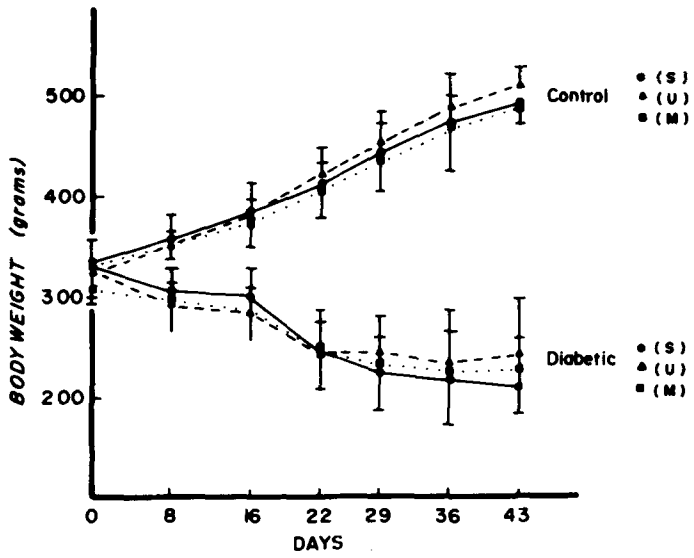

FIG. 2. Body weights of control and diabetic rats fed experimental diets. Normal control rats and diabetic rats fed different experimental diets were weighed periodically as indicated in the abscissa. Body weights were expressed as means ± SD with number of rats (n) = (3-6); (S) = saturated fat diet; (U) = unsaturated fat diet; (M) menhaden oil diet.

supplementation with menhaden oil appeared to increase the severity of the disease.

Microsomal phospholipid fatty acid composition: effects of diets. The fatty acid composition of phospholipids derived from normal rats fed the chow or different experimental diets, i.e. the diet supplemented with saturated fat, unsaturated fat, or menhaden oil is shown in Tables 2 and 3. The saturated fat diet resulted in significantly increased levels of 16:1n-7 (290 percent), 18:1n-9 (44 percent), and 20:3n-6 and 20:3n-9 (62 percent), but a significantly decreased level of 20:4n-6 (21 percent) as compared to chow-fed diet reference (Table 2). The decreased 20:4n-6 and increased 20:3n-6 would correlate with the decreased Δ5 desaturase activity (56 percent) shown in Table 4. The saturated fat diet, which contained very little linoleate, also led to a significantly decreased level of 18:2n-6 (49 percent), a major component as well as a precursor for the biosynthesis of the n-6 fatty acid family, including 20:4n-6. Thus, the low 20:4n-6 may be partly due to an inadequate supply of the precursor 18:2n-6. The decrease of 18:2n-6 was replaced by a proportionally increased level of 22:6n-3 (44 percent).

On the other hand, the unsaturated diet caused significantly decreased 20:3n-6 (38 percent), 16:1n-7 (80 percent), and 18:1n-9 (22 percent) and increased 20:4n-6 (38 percent). Interestingly, the level of 16:0 was also significantly decreased (15 percent) by the unsaturated diet, although this may simply be due to the low 16:0 content of the diet. A competitiveness for the incorporation of n-6 and n-3 fatty acids into the phospholipids was also observed. Thus, while feeding the unsaturated diet caused significantly increased 20:4n-6 (38 percent), a major product formed from 18:2n-6, this diet displaced a substantial amount of 22:5n-3 and 22:6n-3 (82 percent and 45 percent, respectively), two major fatty acids in the n-3 fatty acid family in microsomal phospholipids.

On the contrary, when normal rats were fed the menhaden oil diet, significantly increased incorporation of the n-3 fatty acids, such as 20:5, 22:5, and 22:6 (800 percent, 164 percent, and 85 percent, respectively) into phospholipid membranes occurred at the expense of n-6 fatty acids such as 18:2, 20:3, and 20:4 (67 percent, 46 percent, and 37 percent, respectively). Levels of 16:0 and 16:1n-7 were increased (22 percent and 240 percent, respectively), while 18:0 was decreased (19 percent) and 18:1n-9 unaffected.

Microsomal phospholipid fatty acid composition: effects of diabetes. The effect of diabetes in chow-fed rats and the effects of the different experimental diets on membrane fatty acid composition in diabetic rats are shown in Table 3. As previously observed, diabetic rats fed the chow diet had increased 18:2n-6 (28 percent) and 22:6n-3 (65 percent) and decreased 20:4n-6 (39 percent) as compared to the nondiabetic rat control (2,4,6,7). Similar to the results obtained from the control rat, the diabetic rat fed saturated fat had significantly increased 18:1n-9 levels, 124 percent more than the diabetic rat fed the chow diet and 37 percent more than the normal rat fed the saturated fat diet even though the level of 16:1n-7 did not increase as it did in the control. The saturated diet also increased the 20:3n-6 and 20:3n-9 levels (88 percent), but the level of 20:4n-6 was not further reduced as compared to the diabetic rat fed the chow diet.

Although the unsaturated diet fed to the diabetic rat increased the 20:4n-6 level (74 percent) over that of the control rat fed the chow diet, these levels were still lower than that of the control rat fed the unsaturated diet. Furthermore, this diet caused a build-up in levels of 18:0 and 18:2n-6 in the diabetic rat to much

TABLE 2

Fatty Acid Composition of Control Rats Fed Chow or Experimental Diets

Fatty Acids	Chow (n=6)	Saturated (n=4)	Unsaturated (n=4)	Menhaden (n=3)
16:0	17.1 ± 0.9	16.0 ± 1.2	14.7 ± 0.7[b]	20.8 ± 1.5[c]
16:1n-7	1.0 ± 0.1	3.9 ± 0.8[a]	0.2 ± 0.1	3.4 ± 1.4[c]
18:0	21.5 ± 0.3	21.7 ± 1.6	21.0 ± 1.7	17.4 ± 2.4[c]
18:1n-9	9.8 ± 0.8	14.0 ± 1.0[a]	7.6 ± 0.7	9.4 ± 0.8
18:2n-6	15.7 ± 1.5	8.0 ± 0.5[a]	15.2 ± 1.4	5.2 ± 1.0[c]
20:2n-6	ND	2.3 ± 0.6	1.9 ± 0.5	0.1 ± 0.1[c]
20:3n-6 and 20:3n-9	1.3 ± 0.3	2.1 ± 0.3[a]	0.5 ± 0.2[b]	0.7 ± 0.3[c]
20:4n-6	21.8 ± 1.3	17.2 ± 0.7[a]	30.2 ± 1.3[b]	13.9 ± 0.7[c]
20:5n-3	1.1 ± 0.1	0.5 ± 0.2	0.1 ± 0.2	9.9 ± 1.2[c]
22:4n-6	1.1 ± 0.3	0.6 ± 0.2[a]	1.0 ± 0.3	0.3 ± 0.1[c]
22:5n-6	ND	1.4 ± 0.3	2.7 ± 0.6	0.3 ± 0.1
22:5n-3	1.1 ± 0.3	0.2 ± 0.1[a]	0.2 ± 0.6[b]	2.9 ± 0.5[c]
22:6n-3	8.5 ± 1.5	12.1 ± 2.4[a]	4.7 ± 0.4[b]	15.7 ± 2.4[c]

[a]Phospholipid fatty acid composition of liver microsomes isolated from control rats fed either chow, saturated, unsaturated, or menhaden oil diets.

[b]Fatty acid composition is expressed as percentages of each individual fatty acid (means ± SD, with number of rats (n) as indicated in the table), per total fatty acid content as determined and identified by GLC.

[c]Statistical significant differences between saturated, unsaturated, or menhaden versus chow diet fed to the control rats are shown as letters *a*, *b*, and *c*, respectively.

TABLE 3

Fatty Acid Composition of Diabetic Rats Fed Chow or Experimental Diets

Fatty Acids	Chow		Saturated (n=4)	Unsaturated (n=4)	Menhaden (n=3)
	Diabetic (n=6)	Diabetic + Insulin (n=6)			
16:0	17.8 ± 1.2	18.7 ± 1.1	15.2 ± 1.0[a]	11.8 ± 1.4[b]	18.4 ± 1.5
16:1n-7	0.5 ± 0.1	2.7 ± 0.7[e,f]	0.4 ± 0.3	0.1 ± 0.1[v]	0.6 ± 0.1[d]
18:0	21.3 ± 1.4	21.4 ± 1.8	23.2 ± 2.3	26.9 ± 2.8[b,d]	22.5 ± 1.8[d]
18:1n-9	8.6 ± 0.8	13.7 ± 1.9[e,f]	19.2 ± 4.2[a,d]	4.0 ± 2.3[b,d]	7.7 ± 0.4
18:2n-6	20.0 ± 1.4[d]	10.8 ± 1.1[e,f]	7.8 ± 0.6[a]	23.7 ± 2.9[b,d]	5.5 ± 0.2[c]
20:2n-6	ND	ND	3.5 ± 1.5	0.7 ± 0.1[d]	0.1 ± 0.1
20:3n-6 and 20:3n-9	1.6 ± 0.8	2.6 ± 0.2[e,f]	3.0 ± 0.4[a,d]	0.5 ± 0.3	0.5 ± 0.1
20:4n-6	13.4 ± 2.9[d]	15.2 ± 1.5[e,d]	13.2 ± 1.3	23.4 ± 4.7[b,d]	14.3 ± 2.6
20:5n-3	0.9 ± 0.2	1.2 ± 0.5	0.4 ± 0.1	0.2 ± 0.1	5.7 ± 2.5[c,d]
22:4n-6	0.8 ± 0.2[d*]	1.2 ± 0.5[f*]	0.7 ± 0.1	0.8 ± 0.3	0.2 ± 0.1
22:5n-6	ND	ND	2.2 ± 0.8	2.1 ± 1.1	0.7 ± 0.3
22:5n-3	1.1 ± 0.2	1.3 ± 0.4	ND	0.1 ± 0.1[b]	1.9 ± 0.3[c,d]
22:6n-3	14.0 ± 3.1[d]	11.2 ± 0.5	11.3 ± 1.9	5.7 ± 0.9[b]	19.9 ± 2.7[e,d]

[a]Phospholipid fatty acid composition of liver microsomes isolated from diabetic rats fed either chow, saturated, unsaturated, or menhaden oil diets. Included in the table is phospholipid fatty acid composition of insulin-treated diabetic rats fed the chow diet.

[b]Fatty acid composition is expressed as percentages of each individual fatty acid (mean ± SD, with number of rats (n) as indicated in the table, per total fatty acid content as determined and identified by GLC.

[c]Statistically significant differences between fatty acids of control and diabetic rats fed the same diets (i.e., Table 2 vs Table 3) are shown by a letter *d*. Also, statistically significant differences between fatty acids of insulin-treated diabetic versus control or diabetic rats fed the chow diet are shown as letters *e* and *f*, respectively. Statistical analyses in Tables 2 and 3 were performed by DUNCAN's multiple range test preceded by ANOVA set alpha level = 0.01, except where indicated by an *, indicating the alpha level set at 0.05.

higher levels than the control rat fed either the chow (25 and 28 percent, respectively) or the unsaturated diet (50 and 56 percent, respectively). Similarly, 16:1n-7 and 18:1n-9 were reduced 80 and 53 percent from those in the chow-fed diet. The unsaturated diet, however, significantly displaced n-3 fatty acid levels such as 22:5n-3 (91 percent) and 22:6n-3 (59 percent), similar to observations in the control rat.

The diabetic rat fed the menhaden oil diet also had significantly increased incorporation of the n-3 fatty acids, 20:5 (533 percent), 22:5 (73 percent), and 22:6 (57 percent) into phospholipids at the expense of the n-6 fatty acids, 18:2 (73 percent) and 20:3 (69 percent). 20:4n-6 was already low in the diabetic rat, and was not further reduced. Most importantly, it was observed that there was a significantly increased level of 22:6n-3

TABLE 4

Effects of Diabetes and Various Diets on the Hepatic Microsomal Δ5 Desaturase Activity

Groups (n)	Diets	Mean ± SD: Activities (nmoles of 20:4n-6 formed/ 3 mg protein/30 min)	Change
Control (8)	Chow	8.1 ± 1.6	0
Control (6)	Saturated	*3.5 ± 1.0	−56
Control (6)	Unsaturated	7.7 ± 5.0	−4
Control (6)	Menhaden	*4.6 ± 1.5	−44
Diabetic (8)	Chow	*2.5 ± 0.9	−69
Diabetic + Insulin (6)	Chow	*3.6 ± 2.5	−55
Diabetic (4)	Saturated	*3.4 ± 1.5	−57
Diabetic (4)	Unsaturated	*4.3 ± 3.1	−47
Diabetic (3)	Menhaden	*2.2 ± 0.4	−72

[a]Δ5 Desaturase activities were determined in liver microsomes prepared from different groups of rats fed different diets as indicated in the table. Enzyme assays were carried out in replicate at 37°C for 30 min with 3 mg microsomal protein. Enzyme activities were expressed as means ± SE (n = number of rats per group). Included in the Table are percentage changes as compared to the normal rat fed the chow diet as the control.

[b]*Signifies differences between experimental Δ5 desaturase activity and that of control fed the chow diet.

(40 percent), while the levels of 20:5n-3 and 22:5n-3 were significantly decreased in the diabetic rats fed the menhaden oil diet (42 and 34 percent, respectively) as compared to that of the controls fed the same diet.

Hepatic microsomal Δ5 desaturase activity. The *in vitro* activity of Δ5 desaturase in rats fed the chow and the various experimental diets is shown in Table 4. Compared to the control rats fed the chow diet or unsaturated fat diet, rats fed the saturated fat or menhaden oil diet showed significantly decreased Δ5 desaturase activity (56 percent and 44 percent, respectively). However, since rats fed the unsaturated fat diet showed considerable variation in Δ5 desaturase activity, this diet caused no significant increase in Δ5 desaturase activity as compared to the control fed the chow diet.

When diabetic rats fed the chow or experimental diets were compared to control rats fed the chow diet, diabetic rats had significantly lower Δ5 desaturase activities (Table 4), especially in the menhaden oil-fed rats (72 percent). The saturated fat diet decreased Δ5 desaturase activity in normal rats (56 percent), but did not further reduce its activity in the diabetic rat (57 percent).

DISCUSSION

In this investigation, an attempt has been made to study the effects of various diets on the fatty acid composition of hepatic microsomal phospholipids isolated from normal and streptozotocin-induced diabetic rats. The diets studied were a fat-free basal diet supplemented with either 20 percent saturated, n-6 or n-3 polyunsaturated fatty acids as compared to a rat chow control diet containing 5 percent fat. These effects were then correlated with activities of fatty acid desaturases obtained from the same tissues with the purpose of defining mechanisms by which the various diets alter the fatty acid composition of phospholipids of the normal and diabetic liver. Since Δ9 and Δ6 desaturase activities have been previously investigated and reported from our laboratories (3,4) and elsewhere (1,2), only Δ5 desaturase activity was studied here. We also attempted to determine if the altered fatty acid composition of phospholipids or the decreased Δ5 desaturase activity in the diabetic liver (Table 4) could be reversed by insulin therapy or by feeding such diets.

As shown in Table 2, the saturated fat diet fed to the normal rat resulted in significantly increased levels of 16:1n-7, 18:1n-9, 20:3n-6 and 20:3n-9, but significantly decreased levels of 20:4n-6. Thus, the increases of 16:1n-7, 18:1n-9, and 20:3n-9 are consistent with the inducement of this diet on Δ9 desaturase as previously described (24); however, the increased 20:3n-6 and decreased 20:4n-6 are consistent with the decreased Δ5 desaturase activity induced by this diet as shown in Table 4. On the contrary, although the unsaturated fat diet fed to the normal rat caused a significant 20:4n-6 increase and a significant 20:3n-6 decrease, suggesting that the unsaturated diet might have induced Δ5 desaturase activity, the *in vitro* enzyme activity did not show a significant increase. This discrepancy could be due to large variations in the desaturase activities obtained in the *in vitro* assays (Table 4), indicating that the unsaturated fat diet may stimulate Δ5 desaturase activity in some rats, but not in others. Another possible explanation for our failure to see a significant induction of Δ5 desaturase by the unsaturated diet is that the inclusion of 0.1 percent cholesterol in the experimental diet may inhibit and/or prevent this induction (25). The diet supplemented with menhaden oil fed to the normal rat, on the other hand, caused significant decreases of all n-6 fatty acids such as 18:2, 20:3, and 20:4. Thus, the decreased 20:4n-6 level is consistent with the decreased Δ5 desaturase activity induced by this diet (Table 4), or a decrease in both Δ5 and Δ6 desaturases, similar to studies previously described (15,16). Since the decreased proportions of n-6 fatty acids were replaced by an increase in n-3 fatty acids,

it seems that in addition to the decreased Δ5 desaturase described above there is a competitive mechanism for the incorporation of fatty acids into phospholipids (catalyzed by the acyltranferases such as fatty acyl-CoA: 1-acylglycerol-3-phosphocholine acyltransferase). Both of these mechanisms are probably responsible for the decreased n-6 fatty acids seen with the menhaden oil supplemented diet.

Streptozotocin-induced diabetes in rats results in decreases of both Δ6 and Δ9 desaturases in the liver (2,4). Results in Table 4 also indicate that Δ5 desaturase activity was significantly decreased in the diabetic rat liver as compared to that of the normal rat fed the same diet (chow-fed control). The decreased Δ5 and Δ6 desaturases were also evidenced by an increase in 18:2n-6 and a decrease in 20:4n-6 levels in the fatty acid composition analysis (Table 3). The diabetic rat fed either the saturated fat or menhaden oil supplemented diets did not cause a further decrease in Δ5 desaturase activity as compared to the chow-fed diet (Table 4). This suggests that diabetes has depressed the Δ5 desaturase to its minimum level and the saturated or menhaden oil supplemented diets caused no further effects. Furthermore, the diet supplemented with n-6 polyunsaturated fatty acids failed to induce Δ5 desaturase activity to the level seen in the normal rat fed a chow diet, although fatty acid composition analysis indicated that 20:4n-6 levels were similar to that of the normal rat fed the chow diet. However, 18:2n-6 levels in the diabetic rat liver remained higher than those of normal rats fed either chow or n-6 polyunsaturated diets (23.7 percent vs 15.8 and 15.2 percent, respectively), indicating that the Δ6 and/or Δ5 desaturase activities were still partially blocked, resulting in an accumulation of the substrate level.

Previous studies in our laboratories (4,7) indicated that insulin treatment to diabetic rats resulted in a superinduction of the Δ9 and, to a lesser extent, Δ6 desaturase activities. This superinduction is consistent with the results shown in Table 3, which indicate that the insulin treatment resulted in significant increases of 16:1n-7 and 18:1n-9 (from 0.5 percent to 2.7 percent and 8.6 percent to 13.7 percent, respectively) which are even higher than those obtained from the normal rat fed the same diet (2.7 percent vs 1 percent and 13.7 percent vs 9.8 percent, respectively). However, our previous studies (4,7) indicated that insulin treatment to the diabetic rat failed to correct the decreased level of 20:4n-6. This also is consistent with the results shown in Table 3, with the 20:4n-6 level in the insulin-treated diabetic rat remaining decreased as compared to that in the normal rat fed the same diet (15.2 percent vs 22.0 percent). This suggests that insulin treatment of the diabetic rat failed to correct the Δ5 desaturase activity. Indeed, the data presented in Table 4 show that after insulin treatment this enzyme activity still remained much lower than that of the control (3.5 vs 8.1 nmoles/3 mg protein/30 min). However, these results are not generally applicable since the study with diabetic patients indicates that if insulin is continuously administered for several days, it results in normalizing the Δ5 desaturase activity (26). Thus, higher or longer doses of insulin treatments may be necessary to correct for the activity of this enzyme.

Our earlier studies (4,7) and that of others (6) indicated that diabetic rats have elevated levels of 22:6n-3 in hepatic microsomal membranes as compared to control rats. This may be partially due to the increased acyltransferase activities in diabetes (27). However, the results in Table 3 show that when diabetic rats were fed the menhaden oil supplemented diet there was a significantly increased level of 22:6n-3, while levels of 20:5n-3 and 22:5n-3 were significantly decreased. Thus, it appears that Δ4 desaturase activity may have been induced in the diabetic rat, producing a rapid conversion of 20:5n-3 and 22:5n-3 to 22:6n-3 through the elongation and Δ4 desaturase steps. Further studies are needed to elucidate the mechanism of this observation in diabetes.

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Turnover of Fatty Acyl-CoA Oxidase in the Liver of Rats Fed on a Partially Hydrogenated Marine Oil

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The change in turnover of fatty acyl-CoA oxidase (FAO), the rate-limiting enzyme of the peroxisomal β-oxidation system, was investigated in rats fed a 30% (w/w) partially hydrogenated marine oil (PHMO) diet. The FAO activity increased five-fold after two weeks of PHMO feeding, and decreased after withdrawal of the diet. Based on *in vivo* experiments using L-[4,5-^{3}H]leucine and an immunoprecipitation technique, the increase in the activity of FAO could be accounted for by a 1.6-fold higher rate of FAO synthesis and a 3.4-fold slower rate of FAO degradation as compared to controls. In the same PHMO-fed rats, the rates of synthesis and degradation of carnitine palmitoyltransferase were 1.8-fold higher and 2.0-fold slower, respectively, as compared to controls. The results indicate that the observed increase in the activity of the enzymes of peroxisomal β-oxidation is mainly due to a reduced rate of FAO degradation in the liver of rats fed the PHMO diet.
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Several hypolipidemic agents such as clofibrate and a number of structurally unrelated compounds, including a plasticizer, di-(2-ethylhexyl)phthalate (DEHP), have been shown to increase both hepatic peroxisome numbers as well as the activities of several enzymes, especially those involved in the peroxisomal β-oxidation of fatty acids (1-6). These changes have been well studied, and the enhancement of peroxisomal enzyme activities after treatment with DEHP (7-9) was shown to involve increased synthesis of the enzymes, based on increased levels of mRNAs coding the respective enzymes (8,9). On the other hand, it is also well known that high-fat diets can induce a proliferation of peroxisomes accompanied by an increase in the activity of the enzymes of peroxisomal β-oxidation (10-13). The effects observed in rats given a partially hydrogenated marine oil (PHMO) in a high-fat diet are very pronounced compared with the responses seen in rats fed peanut or safflower oil diets (13,14-17). The reason for the increase in hepatic peroxisomal β-oxidation in rats given a PHMO-diet is still not clear, although it has been speculated that it may be triggered by an accumulation of CoA esters of long-chain and very long-chain unsaturated fatty acids (12,18) which are poorly oxidized by mitochondria (19). No kinetic studies on the turnover of peroxisomal β-oxidation enzymes in a high-fat diet condition have been performed.

In order to understand the mechanism of enhancement of peroxisomal β-oxidation in the liver of rats fed PHMO, we examined the changes in the turnover rates of FAO and carnitine palmitoyltransferase (CPT), a mitochondrial key enzyme which is thought to be involved in the transfer of long-chain acyl groups across the membrane.

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Abbreviations used: FAO, fatty acyl-CoA oxidase; PHMO, partially hydrogenated marine oil; DEHP, di-(2-ethylhexyl)phthalate; CPT, carnitine palmitoyltransferase; SDS, sodium dodecyl sulfate.

EXPERIMENTAL

Materials. PHMO was provided by Nippon Oil and Fats Co., Ltd. Control diet was purchased from Nippon Clea Co. Ltd. L-[4,5-^{3}H]Leucine (150 Ci/mmol) was purchased from Amersham, Japan. L-Carnitine hydrochloride was provided by Otsuka Pharmaceutical Co., Japan. Palmitoyl-CoA, bovine serum albumin (fatty acid-free) and FAD were purchased from Sigma (St. Louis, MO). Freund's complete adjuvant was from Difco Laboratories (Detroit, MI). Aquasol-2 was from NEN Research Products (Boston, MA).

Animals and treatments. Male Wistar rats (approximately 150 g initial body weight) were housed in groups in a climate-controlled room (temperature, 23 ± 1°C; humidity, 55 ± 5%) with a light/dark cycle of 12 hr. They had free access to water and powdered chow (high-fat diet or control diet). The high-fat diet contained 30% (w/w) PHMO from sardine (average increasing melting point, 32.9°C; acid value, 0.11; iodine value, 78.0; peroxide value, 0.0; saponification value, 187.4), and was given to groups of rats for various periods as described in the figures and tables. A separate PHMO-treated group was placed on the control diet after 14 days of feeding with the PHMO-diet. The compositions of the semisynthetic diets were, in weight percentage of the total: dietary oil, 4.6% safflower oil for the control and 30.0% PHMO oil for the treated groups; corn starch, 55.4% for the control and 30.0% for the treated groups; milk casein, 25.0%; granulated sugar, 5.0%; minerals, 4.0%; cellulose powder, 3.9%; vitamin mixture, 2.0%; and choline chloride, 0.1%. The fatty acid compositions of the diets are given in Table 1.

Purifications of FAO and CPT. FAO was purified from the liver of rats treated with clofibrate. The procedure was based on the method described by Osumi *et al.* (20), with some modifications. The fraction sedimenting between 6000 × *g* and 250,000 × *g* was used as the enzyme source and was homogenized with 20 mM potassium phosphate (pH 7.8). After fractionation with ammonium sulfate, DEAE-cellulose, hydroxyapatite, and Sephadex G-150 column chromatography were carried out. Purification of CPT was done according to the method of Miyazawa *et al.* (21), with slight modifications. Column chromatography with hydroxyapatite and Sephadex G-200 were used instead of those with calcium phosphate gel-cellulose and Sephadex G-100, respectively, and DEAE-cellulose column chromatography was repeated after the hydroxyapatite column chromatography. Both enzymes were free from other proteins as judged by SDS-polyacrylamide gel electrophoresis.

Preparations of antiobides. Antibodies against purified FAO and CPT were prepared by injection of the respective enzyme with an equal volume of Freund's complete adjuvant into Japanese white female rabbits. The antibodies were purified by ammonium sulfate precipitations and DEAE-cellulose column chromatography. The immunotitration experiments were carried out with the purified enzymes and the supernatant fractions obtained

TABLE 1

Fatty Acid Compositions of Diets

Fatty acid	Control	PHMO[a]
12:0	0	0.3
14:0	0.4	7.8
14:1	0	0.1
15:0	0.1	0.6
16:0	16.5	20.7
16:1	0.7	11.4
17:0	0.2	0.6
17:1	0.2	1.2
16:4(ω1)	0	0.1
18:0	2.2	6.4
18:1(ω9)		16.0
18:1(ω7)	23.2	6.4
18:1(ω5)		1.0
18:2(ω6)	50.2	0.4
18:3(ω3)	4.1	0
20:0	0.2	1.8
20:1(ω11)	0.8	2.9
20:1(ω9)		10.1
22:0	0.4	0.8
22:1(ω13 + 11)	0	3.8
22:1(ω9)		4.2
24:0	0	0.8

Values are percentages of total fatty acids.

[a]The PHMO was derived from sardine.

from solubilized samples of homogenates (see Measurements), and equivalent amounts of the respective antibodies were used. When we performed SDS-polyacrylamide gel electrophoresis followed by fluorography of the immunoprecipitation products, the resulting bands coincided with those of purified FAO and CPT, although the bands were faint.

Measurements. L-[4,5-^{3}H]Leucine (150 μCi in saline/100 g of body weight) was injected into the tail vein of rats and the animals were killed either 2 hr, or 1, 2 or 4 days after the injection. Livers were frozen at −40°C for more than one day before use. Twenty percent liver homogenate was prepared with 50 mM potassium phosphate buffer (pH 7.8) containing 1% (w/w) Triton X-100 in a Potter-Elvehjem type homogenizer. The homogenate was centrifuged at 105,000 × *g* for 1 hr, and the supernatant was stored. The extracts prepared from 0.2 g of the control and PHMO-treated rat livers were used for precipitation of FAO and CPT with the corresponding antibodies. After the addition of antibody (two or more equivalents), the mixture was incubated at 30°C for 1 hr and kept in chilled water. The mixture was then centrifuged and the precipitate was washed with a solution containing 0.15 M NaCl-10 mM potassium phosphate (pH 7.8)-1% (w/w) Triton X-100. This was repeated three times. The precipitate was dissolved in a small amount of 0.2 N NaOH and neutralized with acetic acid. After the addition of 10% (w/w) ascorbic acid the solution was mixed with Aquasol-2 and the radioactivity was measured.

Analytical methods. FAO and CPT activities were determined as previously described (22). Protein content was determined by the method of Lowry *et al.* (23). The fatty acid composition of PHMO was analyzed by GC separation of the methyl esters employing a Hewlett Packard gas-chromatograph (model 5890A) equipped with a capillary column (0.24 mm × 50 m) packed with Fused Silica Thermon 3000A at a column temperature of 160°C. The column temperature was raised to 220°C at a rate of 5°C/min.

RESULTS AND DISCUSSION

The treated group of animals showed no difference from the control group in daily body weight gain except for the first day after the start of treatment. The ratio of liver weight to body weight was increased slightly ($p > 0.05$, by Student's t-test) at the 7th and 14th days after the start of treatment, but the increase disappeared after the diet was changed back to the control diet (data not shown). Feeding the rats the PHMO-diet for two weeks resulted in approximately five- and three-fold increases in FAO and CPT activities, respectively, compared with controls. In order to pinpoint the reasons for the increased activities, we first examined the time course of changes in the activities of both enzymes (Fig. 1).

In general, the relationship between synthesis and degradation of an enzyme is formulated by dE/dt =

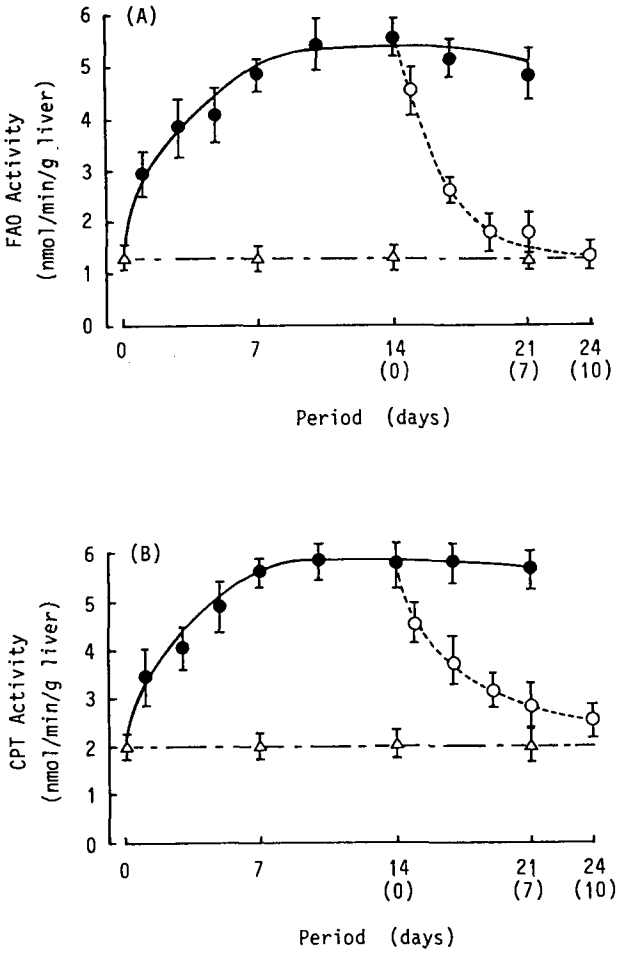

FIG. 1. Time course of changes in activities of FAO (A) and CPT (B). Rats were fed a diet with or without 30% (w/w) PHMO. Each value represents the mean ± S.D. of four rats. Dotted lines indicate the course of change in activities of a separate PHMO-treated group after changing the diet back to the control. △, control; ●, PHMO; ○, change of diet back from PHMO to the control.

$K_s - K_dE$ (E: the content of enzyme; K_s: a zero-order rate constant of synthesis; K_d: a first-order rate constant for degradation) (24). As can be seen in Figure 1, it was thought that the feeding of PHMO caused prompt changes of K_s and K_d to K'_s and K'_d, respectively, and that K'_s and K'_d returned rapidly to K_s and K_d, respectively, after removal of PHMO. The validity of estimation by this method has been established by Swick (25). The integrated formulation of the above equation shows that the time course of the enzyme induction is determined only by the rate constant of degradation, K'_d. Therefore, according to previous studies (7,26), it can be assumed that the time required to reach the half-maximal induction of the enzyme during PHMO treatment is the same as the half-life of the enzyme during the treatment. On changing the PHMO diet to the control one, K_d can be obtained from the rate of decrease in the enzyme activity. Since the plots of the data fit the equations derived by assuming that the turnover of the enzymes is random (24), the apparent rates of synthesis and degradation were estimated from semilogarithmic plots (26). The data calculated from the results of Figure 1 are summarized in Table 2. The time required for the amounts of FAO and CPT to exhibit half-maximal induction during PHMO feeding were approximately 6 and 4.5 days, respectively. The respective values for half-recovery after withdrawal of PHMO were approximately 2 and 3 days. Thus, the half-life of FAO was extended about three-fold upon administration of PHMO-diet.

In order to ascertain the turnover rates of FAO and CPT during PHMO-treatment, we applied an immunoprecipitation technique after the i.v. injection of radioactive leucine into the rats. The radioactivities incorporated into FAO and CPT 2 hr after injection of L-[4,5-^{3}H]-leucine were taken as representative of apparent K_s (Table 3). The apparent K_s values of FAO and CPT were increased 1.6- and 1.8-fold, respectively, by the treatment with PHMO-diet. However, the increase of FAO was one order of magnitude lower than that observed in rats treated with DEHP (16.2-fold) (8). Figure 2 shows the decay of radioactivities of FAO and CPT after a single injection of L-[4,5-^{3}H]leucine. The average half-lives of both enzymes were as follows: FAO, 2.1 days for the control and 7.1 days for the PHMO group; CPT, 2.4 days for the control and 4.8 days for the PHMO group. These

TABLE 2

Apparent Turnover Rates of FAO and CPT

	Half-time in days		
	Induction	Degradation	(Ind/Deg)
FAO	6.0 ± 0.7	2.0 ± 0.3	3.0
CPT	4.5 ± 0.5	3.0 ± 0.3	1.5

Results are expressed as means ± S.D. of 4 samples. Values were estimated from semilogarithmic plots of the data in Figure 1.

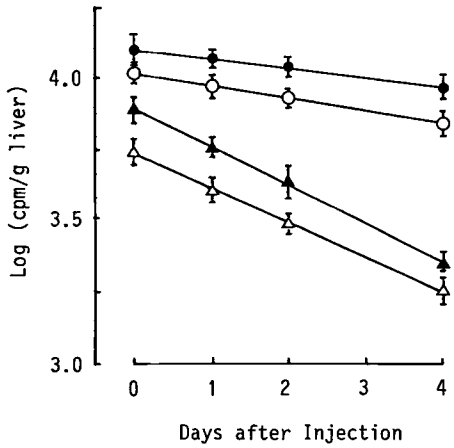

FIG. 2. Decay curves of radioactivity in FAO and CPT. Each value is the mean ± S.D. of four samples. Rats were fed a diet with or without 30% (w/w) PHMO for 14 days and injected with radioactive leucine. Rats received the same diet subsequently until the indicated time. Values for 0 day were those 2 hr after the injection. ●, FAO from PHMO-diet; ○, CPT from PHMO-diet; ▲, FAO from the control diet; △, CPT from the control diet.

TABLE 3

Activities of FAO and CPT and Incorporation of L-[4,5-^{3}H]Leucine into Soluble Protein, FAO and CPT, and Their Half-lives

	Soluble protein	FAO	CPT
Total activity (nmol/min/g liver)			
Control		1.09 ± 0.12	2.36 ± 0.31
PHMO		5.35 ± 0.61	6.92 ± 0.59
Total R.A. (cpm/g liver)[a]			
Control	1,535,790 ± 141,621	7,884 ± 1,162	5,648 ± 915
PHMO	1,614,590 ± 132,683	12,798 ± 1,056	10,241 ± 819
	(1.05)	(1.62)	(1.81)
Half-life (days)[b]			
Control	3.8	2.1	2.4
PHMO	5.7	7.1	4.8
	(1.5)	(3.4)	(2.0)

Values represent means (± S.D.) of four samples.

[a] Radioactivities (cpm/g liver) represent values incorporated into protein 2 hr after the injection of L-[4,5-^{3}H]leucine (150 μCi/100 g body weight).

[b] Half-lives of FAO and CPT were obtained from Figure 2. The control/PHMO ratios are shown in parentheses.

values are similar to the values obtained from the time course of enzyme activities (Fig. 1). As can be seen in Table 3, the half-lives of FAO and CPT were 3.4- and 2.0-fold longer in the PHMO-treated group. In addition, it was found that the treatment with PHMO increased the half-life of hepatic soluble protein by 1.5-fold. Hydrolytic activities of lysomal proteases are thought to be an important regulatory mechanism for protein degradation (27). The results obtained here might support the speculation that feeding of a high-fat diet causes disorder of cellular proteolytic metabolism, probably based on the inhibition of lysosomal function due to the accumulation of some lipids (unpublished data). Ozasa *et al.* (9) reported that the half-life of CPT did not change, whereas the rate of synthesis was 5-fold higher after the administration of DEHP. The activity of FAO was not increased very much by feeding of PHMO compared to that seen in the rat treated with DEHP or clofibrate. However, in contrast to the result of DEHP-treatment (8,9), it is clear that the translatable mRNA levels of FAO and CPT do not increase substantially in rats fed PHMO. As the amount of an enzyme is regulated by changes in the rates of synthesis and degradation of the enzyme, the change in the amount of an enzyme caused by a treatment can be estimated by multiplying K_s by K_d of the enzyme. The calculated values of FAO and CPT *in vivo* were 5.5- and 3.6-fold, respectively. These values are comparable to the increases in the activities of FAO and CPT obtained by *in vitro* measurement under these conditions (Table 3).

Elucidation of the pattern of turnover of various enzymes in peroxisomes of rats treated with PHMO is important to get information about the mechanism of peroxisomal proliferation under such conditions. From the time courses of changes in the activities of the whole peroxisomal β-oxidation system and carnitine octanoyltransferase, a peroxisomal matrix enzyme, we found that the degradation rates of the enzymes were apparently reduced. For example, the ratio of induction to degradation of carnitine octanoyltransferase activities was 2.8 (estimated in the same way as in Table 2). On the other hand, the increases in the activities of other peroxisomal enzymes such as catalase, urate oxidase and D-amino acid oxidase were small (data not shown), being different from that of FAO under same conditions. Therefore, it is possible to presume that newly synthesized peroxisomes, which contain different proportions of enzymes to the control peroxisomes, are relatively resistant to breakdown. This phenomenon may be due to the alteration of fatty acid and/or amino acid compositions of the membrane under the high-fat diet condition (28).

Results presented here show that the increase in the quantity of FAO induced by administration of PHMO is due predominantly to a decrease in the rate of degradation rather than an increase in the rate of synthesis. This is in contrast to FAO stimulation induced by clofibrate or DEHP (the latter can be interepreted mainly in terms of the increase in the mRNA level and transcription rate [8,29]). This may imply that a peroxisome proliferator-binding protein of the kind proposed by Lalwani *et al.* (30,31) does not mediate the response in the liver of PHMO (high-fat diet)-treated rats. However, care is necessary in evaluating the turnover of FAO in the PHMO-treated rats because leucine is not a suitable precursor to estimate the turnover of proteins with long half-lives (32). Nevertheless, when we calculated the half-life of FAO under the PHMO-fed condition in another way (the increased ratio of the FAO activity to the control, 4.91, which is shown in Table 3, was divided by the increased ratio of FAO synthesis to the control, 1.62, also shown in Table 3), a value of 3 was obtained. This value again indicates a prolonged half-life of FAO under the PHMO-diet-fed condition. As a next step, it will be important to study the transcription rate and translation activity of mRNA for FAO protein under the PHMO-fed condition. Very recently, Flatmark *et al.* (33) reported that feeding rats a PHMO diet evoked a 12-fold increase in the level of mRNA for the peroxisomal bifunctional enzyme, enoyl-CoA hydratase-3-hydroxyacyl-CoA thiolase. They rejected the previously proposed mechanism (12,18) that the increase in peroxisomal β-oxidation enzyme activity in response to PHMO feeding might occur in a substrate-induced manner. However, translation of the bifunctional enzyme is not referred to in their report (33), and in any case the bifunctional enzyme is not the rate-limiting enzyme of the peroxisomal β-oxidation system (34). Experiments on translation for FAO are presently underway in our laboratory.

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METHODS

Gas Chromatographic Analysis of Free and Bound Malonaldehyde in Rat Liver Homogenates

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A gas chromatographic method for the measurement of free and bound malonaldehyde (MA) in rat liver homogenate was developed to investigate biological damage caused by lipid peroxidation. Free MA was derivatized by reaction with *N*-methylhydrazine (NMH) to form 1-methylpyrazole (1-MP), which was subsequently analyzed by capillary gas chromatography (GC). Bound forms of MA were determined by first hydrolyzing samples in acetic acid, then measuring the free MA as 1-MP. Bound MA in rat liver was released within 15 min by acid hydrolysis. A slight increase in bound MA levels in rat liver was observed 1 hr after CCl_4 treatment, with bound MA then returning to control levels. Free MA levels and thiobarbituric acid assay (TBA) values did not show appreciable changes over a 24-hr period after CCl_4 administration. However, the TBA values were approximately ten times the MA values in most samples.
***Lipids 24,* 895-898 (1989).**

Malonaldehyde (MA) is one of the most studied products of lipid peroxidation. It is believed to form as a decomposition product of certain lipid hydroperoxides (1,2). MA has been implicated in aging (3), mutagenesis (4), and carcinogenesis (5). The toxicity of MA is believed to be the result of its reactivity with biological nucleophiles such as amino acids (6,7) and thiols (8), and its ability to induce cross-linking of proteins and nucleic acids (7).

Several methods have been developed to estimate the MA formed by lipid peroxidation either *in vivo* or *in vitro*. The most widely used method is the thiobarbituric acid (TBA) assay. In this method, TBA gives a red complex with certain compounds, including MA, which is measured by colorimetry. However, the TBA method is not specific for MA and often overestimates MA levels (9,10) because some chemicals, such as aldehydic compounds, react with TBA to produce absorbance spectra similar to those of the TBA-MA complex (11,12). MA may exist in both free and in hydrolyzable bound forms in biological samples. Bound forms of MA may include Schiff's base-type adducts to protein, nucleic acids and other biological nucleophiles. Under acidic, basic, or high temperature conditions, these adducts may release free MA. The TBA methods can only measure total MA (free + bound), because bound MA is released during sample preparation. More specific methods for MA determination have been developed by using high performance liquid chromatography (HPLC) (9,10,13,14) and gas chromatography (GC) (2,15).

Free MA in animal tissues has been reacted with 4,4'-sulfonyldianiline to form a MA fluorescent derivative which was subsequently determined by HPLC (16). The lowest amount of MA determined by this method was approximately 1 ng per injection. Later, the same method was applied to investigate the role of vitamin E in formation of free and bound MA in rat liver tissues (14,17). Recently, a capillary GC method has been developed to allow determination of free MA (18). Free MA formed in UV irradiated lipids was derivatized to 1-methylpyrazole (1-MP) and subsequently analyzed by GC with a nitrogen/phosphorus specific detector (NPD).

It is useful to separately measure the levels of both free and bound MA formed by lipid peroxidation in order to better understand the activity of MA and to accurately assess the impact of MA upon biological systems. In the present study, this GC method was applied to the determination of both free and bound MA formed in CCl_4-treated rat liver. CCl_4 was chosen because it has been widely used to induce experimental lipid peroxidation either in *in vivo* or *in vitro*. CCl_4 forms the trichloro radical (CCl_3·) by action of cytochrome P-450 in liver microsomes and the radical, in turn, is thought to induce lipid peroxidation (19,20).

MATERIALS AND METHODS

Reagents. Malonaldehyde *bis*(dimethylacetal), TBA, and *N*-methylhydrazine (NMH) were purchased from Aldrich Chemical Co. (Milwaukee, WI). 1-MP was synthesized by the method reported previously (14).

Treatment of animals and preparation of liver homogenates. Male Sprague-Dawley rats (280-300 g), 2.5 months old, were used in all experiments. CCl_4 (3 ml/kg body weight) was administered either as a neat liquid by intraperitoneal (ip) injection or as a solution in 50% olive oil by gavage in separate experiments. Control rats for gavage experiments were given olive oil only. Animals were sacrificed at various times after administration of CCl_4, and the livers were perfused via the right ventricle with saline. The livers were stored at −84°C until analysis. The livers were homogenized using a Polytron homogenizer and a 50% homogenate (w/v) prepared by addition of 50 mM Na-, K-phosphate buffer (pH = 7.5).

Analysis of free MA. One ml of the 50% liver homogenate (w/v) prepared from a control rat was mixed with 3 ml of methanol to precipitate proteins which were removed by centrifugation at 3000 × *g* for 10 min. The supernatant was mixed with 20 ml of deionized water and the pH was adjusted to 9 with 6N NaOH solution. A pH of 9 maximized the yield of 1-MP from the reaction of

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Abbreviations: MA, malonaldehyde; 1-MP, 1-methylpyrazole; NMH, N-methylhydrazine, TBA, 2-thiobarbituric acid; GC, gas chromatography; HPLC, high performance liquid chromatography; ip, intraperitoneal injection; NPD, nitrogen-phosphorus detector; BHT, butylated hydroxytoluene; SDS, sodium dodecyl sulfate.

METHODS

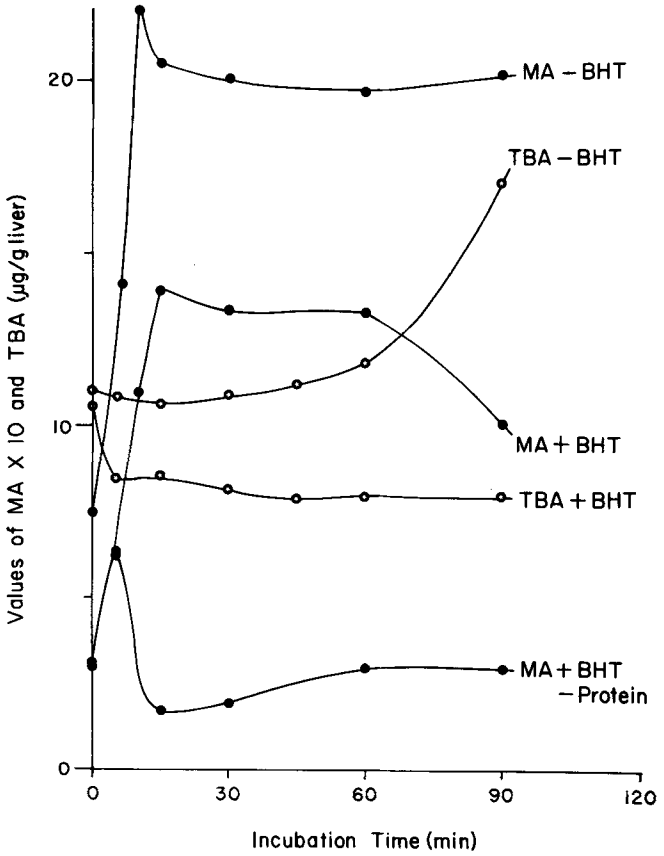

FIG. 1. Values of MA × 10 and TBA obtained from acid hydrolysis of liver homogenates after various incubation times.

NMH and MA. Derivatization of MA to 1-MP was, therefore, conducted at pH 9 for all experiments. NMH (50 μl) was added to the solution, which was allowed to stand for 1 hr at 25°C with or without 0.04 mg of butylated hydroxytoluene (BHT). BHT was added to avoid any further oxidation of lipid. The pH of the reaction mixture was adjusted to 1 with 6N HCl solution, and then washed with two 5 ml portions of hexane. The aqueous reaction mixture was then mixed with 20 ml of 10% NaCl, and the pH was adjusted to 12 with 6N NaOH. The mixture was extracted with 5 ml of dichloromethane using a liquid-liquid continuous extractor for 3 hr. The extract was analyzed for free MA by GC-NPD with 2-methylpyrazine as an internal standard.

Effect of BHT on MA determination. Possible effects of the addition of BHT on the determination of MA was examined using model solutions. An aqueous MA solution prepared by hydrolysis of tetramethoxypropane (2.5 μg as MA) was added to 20 ml of acetic acid buffer solution (pH 3) containing 0.04 mg of BHT and the solution was incubated for 0, 15, 30, and 60 min at 60°C. NMH (50 μl) was added to the solution and the resulting 1-MP was analyzed as described above.

Effect of hydrolysis time on determination of total MA. Fifty percent rat liver homogenate (0.5 ml) prepared from a control rat was incubated with 2 ml of 20% acetic acid (pH 3) with or without BHT for various periods of time at 60°C. NMH was added to the solution and the resulting 1-MP was analyzed as described above. Results are shown in Figure 1. Bound MA was released within 15 min. Therefore, 20 min incubation time was used for further experiments.

Determination of MA in the liver from CCl_4-treated rats. One ml of 50% liver homogenate prepared from a rat administered CCl_4 either orally or by intraperitoneal injection (ip) was analyzed for free and total MA by the same procedure used for liver homogenate from a control rat. The results are shown in Tables 1 and 2.

TBA assay. Parallel experiments were conducted to obtain TBA values of each sample according to the method described by Ohkawa and co-workers (21). The rat liver homogenate (0.1 ml) was mixed with 0.2 ml of 8% sodium dodecyl sulfate (SDS), 1.5 ml of acetic acid buffer solution

TABLE 1

MA and TBA Values for Rat Liver After Intraperitoneal Injection of CCl_4

Time after injection[a]	MA values (μg/g liver)[b]			TBA values (μg/g liver)[b]
	Total	Free	Bound[c]	
Control	1.34	0.23	1.11	11.4
1 hr	1.39	0.25	1.14	13.6
2 hr	0.98	0.17	0.77	11.2
3 hr	1.10	0.21	0.90	12.0
4 hr	0.78	0.26	0.52	11.1

[a] Time indicates when animals were killed and liver removed.

[b] Values are expressed as means (n = 2).

[c] Bound MA determined as the difference between total MA and free MA.

TABLE 2

Total MA and TBA Values for Rat Liver After Oral Administration of CCl_4

Time after CCl_4 administration	Total MA (μg/g liver)	Free MA (μg/g liver)	Bound MA (μg/g liver)	TBA values (μg/g liver)
Control	1.25 ± 0.08[b]	0.17 ± 0.02	1.08 ± 0.09	8.9 ± 1.9
1 hr	2.55 ± 0.42	0.22 ± 0.02	2.31 ± 0.39	8.9 ± 1.6
3 hr	1.62 ± 0.60	0.18 ± 0.03	1.43 ± 0.59	9.9 ± 1.8
12 hr	0.94 ± 0.11	0.17 ± 0.03	0.77 ± 0.08	9.2 ± 0.4
24 hr	0.85 ± 0.34	0.18 ± 0.06	0.67 ± 0.26	10.2 ± 1.6

[a] Time indicates when animals were killed and liver removed. CCl_4 was administered as a 50% solution in olive oil.

[b] All values are mean ± standard deviation (n = 3).

(pH 3.5) containing 0.04 mg of BHT, and 1.5 ml of 0.68% aqueous solution of TBA. The mixture was finally made up to 4.0 ml with distilled water, and heated at 95°C for 60 min. After the reaction mixture cooled, 5 ml of n-butanol was added. After centrifugation at 3000 rpm for 10 min, the absorbance of the organic layer was measured at 532 nm.

Instrumental methods. A Model 5880A GC equipped with an NPD and a 30 m × 0.25 i.d. bonded phase DB-WAX fused silica capillary column (J & W Scientific, Folsom, CA) was used for routine quantitative analysis. GC peak areas were calculated with a Hewlett-Packard 5880A series GC terminal. The column temperature was programmed from 35°C to 180°C (15 min hold) at a rate of 4°C/min. Injector and detector temperatures were 300°C. The structural confirmation of 1-MP in the samples was performed by a ZAB-HS high resolution magnetic sector mass spectrometer interfaced with a Hewlett-Packard Model 5790 GC. The TBA assay was conducted using a Shimadzu model UV-160 spectrophotometer.

RESULTS AND DISCUSSION

The lowest detection level of 1-MP by NPD was 8.9 pg, equivalent to 7.8 pg of MA. This value was determined by the generally recognized definition of GC detection limit; the amount of analyte which produces a peak height more than three times the noise level. The percent recoveries of spiked 1-MP standard (2.84 ppm) from aqueous solution and from liver homogenates were 91.0 ± 0.6% and 89.5 ± 2.5%, respectively. The values are mean ± standard deviation for three replicates.

Figure 1 shows the level of MA obtained by acetic acid hydrolysis at different incubation times with or without BHT, along with measured TBA values. When the pH of the sample was adjusted from 1 to 6 with acetic acid, the maximum formation of MA was observed at a pH around 2-4. Between pH 2 and 4, no significant differences in MA formation was observed. These results are consistent with those previously reported (22). If HCl was used for hydrolysis, many additional peaks appeared on GC even though the amount of MA determined was consistent with that obtained by the acetic acid hydrolysis. The same phenomenon was observed in alkaline hydrolysis conducted with NaOH. Consequently, acetic acid was used to obtain total MA in the present study. However, it is quite tedious to adjust the pH of many samples for routine analysis. To eliminate this need, unless specific pH adjustment was required, the hydrolysis was performed using 2 ml of 20% acetic acid, which gives a solution pH of 3.0-3.2.

If incubation was conducted at temperatures lower than 55°C, formation of the products was quite slow. If the temperature was raised over 65°C, some polymeric materials, which reduced MA recovery, were produced. Therefore, 60°C incubation temperature was chosen for further experiments. Incubation of reaction mixtures with or without BHT for various periods of time produced no significant change in the recovery of MA.

The results indicate that MA bound to protein and other nucleophiles is released within 15 min during acetic acid hydrolysis. In the absence of BHT, levels of MA and TBA reactants were much higher. These results suggest that BHT prevented lipid peroxidation during the incubation with acid. The difference between values from (MA − BHT) and (MA + BHT) is the amount of MA formed from lipid peroxidation during hydrolysis.

Amount of free and bound MA determined by HPLC in the vitamin E supplemented rat liver hydrolyzed at pH 13 and 60°C for 30 min were reportedly 0.25 ± 0.06 μg/liver and 1.49 ± 0.27 μg/liver, respectively (14). The amounts of free and bound MA determined in the normal diet rat liver were not different from those of the vitamin E supplemented rat liver (14). These values are quite consistent with those determined in the present study. Referring to the values at 30 min incubation in

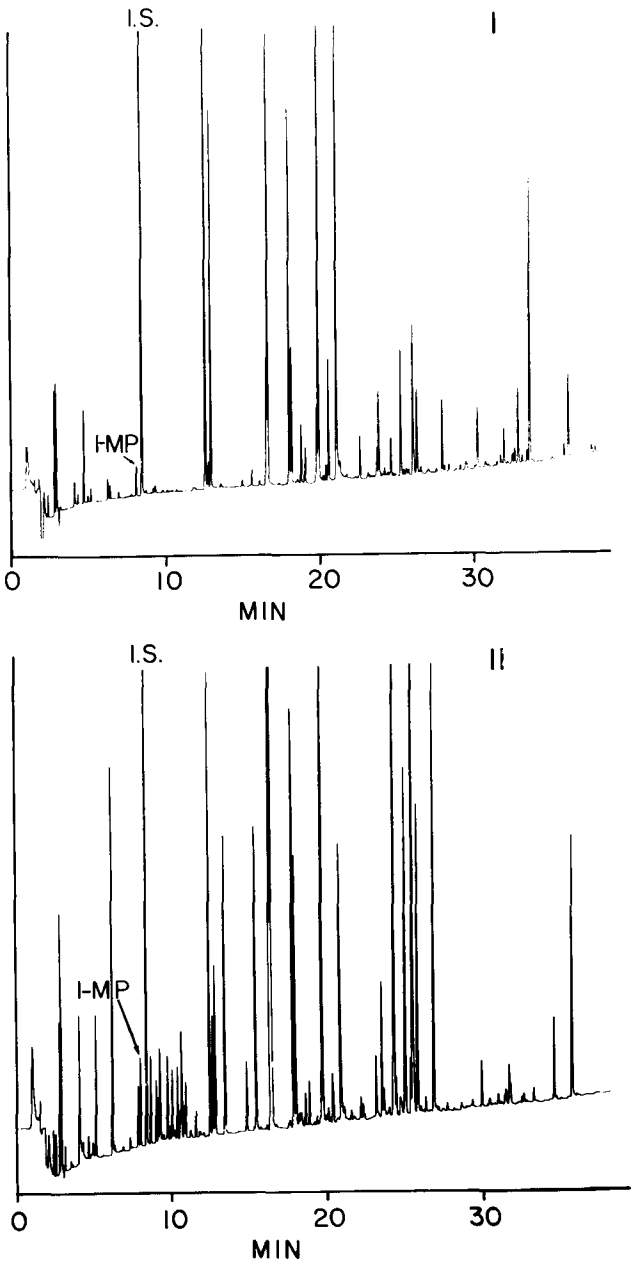

FIG. 2. Gas chromatogram of a dichloromethane extract of liver homogenate from CCl_4-nontreated (I) and CCl_4-treated rats (II).

Figure 1, the amounts of free MA (MA + BHT − protein) was 0.23 μg/g liver and bound MA [MA + BHT (total) − free] was 1.27 μg/g liver in the present study.

Figure 2 shows typical gas chromatograms of the samples prepared from livers of CCl_4-nontreated (control) and CCl_4-treated rats. Some major peaks were identified by GC/MS. They are hydrazones, 1-methylpyrazolines, and 1-methylpyrazoles which were derived from n-aldehydes, α,β-unsaturated aldehydes, and β-diketones, respectively. However, reporting on those compounds, which were formed by lipid peroxidation, is not within the scope of this study.

Tables 1 and 2 show TBA and MA values obtained from rat liver at different periods of time after intraperitoneal (ip) and oral administration of CCl_4 (3 ml/kg), respectively.

As shown in Table 1, following ip CCl_4 administration, levels of free and bound MA did appear to increase slightly within 60 min. With oral administration, bound MA values approximately doubled and free MA increased by a factor of 1.3 over control values after 1 hr (Table 2). Previous studies have shown that conjugated dienes increase significantly in first 30 min of CCl_4-induced peroxidation in the rat (23) or mouse (24) liver.

It was previously reported that TBA values increased more than six-fold in mouse liver 24 hr after ip CCl_4 administration (24). In the present study, TBA values increased slightly, but slight decreases in the bound MA were observed 24 hr after oral CCl_4 administration. The present MA and TBA values did not correlate well with the time after ip administration within the time examined.

One hr after ip CCl_4 administration, the livers from animals were visibly damaged, showing swelling, increased weight, and change in color from white to red. However, following oral CCl_4 administration, there was no change in liver condition from that of the control rats after 1 hr. This observation indicates that the time required for CCl_4 intoxication of the liver is longer for oral than for ip administration.

The GC method used in the present study determined the levels of bound MA, which may be one indicator of damage to proteins, nucleic acids and other nucleophiles.

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COMMUNICATIONS

Linoleic Acid Hydroperoxide Concentration in Relation to Mutagenicity of Repeatedly Used Deep-Frying Fats

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A high-performance liquid chromatography procedure was developed to measure linoleic acid hydroperoxides (LAHPO) in 19 used, deep-frying fat samples that were screened for mutagenicity. The detection limit of the method was found to be 10 nmol of LAHPO/g of fat. In eight samples, LAHPO were not detected; levels in the other samples ranged from 17 to 267 nmol/g of fat. LAHPO were not detectable in unusued hydrogenated frying fat samples. Concentrations of LAHPO correlated positively with mutagenicity to Salmonella tester strains TA97 and TA100, in presence of S9 mix only; coefficients of correlation were, respectively, $r = 0.48$ ($p < 0.05$) and $r = 0.24$ (n.s.). Without metabolic activation no significant associations were observed. These results suggest that metabolites or secondary autoxidation products of linoleic acid generated in presence of liver S9 mix may contribute to mutagenicity of some of the used deep-frying fat samples.
Lipids 24, 899-902 (1989).

Thermal and oxidative deterioration of fats and oils during deep-fat frying is of major concern with regard to possible adverse health effects, as well as the quality of foods prepared. When fed to laboratory animals, heated oils and fats have been reported to cause growth retardation, increase relative liver and kidney weights, induce detoxifying enzyme activities and lipid peroxidation in the liver, and produce cellular damage to liver, kidneys and epididymides (1-4). Heating of fats and oils causes degradation of triglycerides, which may involve hydrolysis into free fatty acids as well as free radical mediated oxidation reactions of polyunsaturated fatty acids. Ultimately, di- and polymeric triglycerides and low molecular weight decomposition products are formed, both volatile and non-volatile (5).

Isolated lipid oxidation products, autoxidation products of methyl linoleate and methyl linolenate, hydroperoxides of methyl linoleate, malondialdehyde, and various other aldehydes and carbonyls have been found mutagenic in the Salmonella/microsome assay (6-10). Recently, we have also observed mutagenic activity in repeatedly used deep-frying fats, sampled from local snack bars and restaurants (11). In our study, highest mutagenic activity has been found in the polar fraction of these fats, separated by means of silica gel column chromatography, which indicates that polar oxidation and degradation products are involved. The contribution of lipid oxidation products to mutagenicity of these used fats has also been implicated by the positive correlation between mutagenicity of the polar fraction to strains TA97 and TA100 and levels of thiobarbituric acid-reactive substances (TBA-RS) of the fats (11). The thiobarbituric acid assay, however, is of limited value since it not only detects malondialdehyde but also other saturated aldehydes (12), as well as derivatives of amino acids, carbohydrates and nucleic acids that have been damaged by free radicals (13).

Since linoleic acid hydroperoxides (LAHPO) have been reported to exert mutagenic activity in the Salmonella/microsome assay using tester strains TA97 and TA100 (6), the objective of the present study was to evaluate further the contribution of lipid oxidation products to mutagenicity of used deep-frying fats by quantifying the concentrations of LAHPO and relating these to mutagenicity of the fats. Therefore, we developed a high-performance liquid chromatography (HPLC) assay measuring the primary oxidation products LAHPO, which has been modified from the method described by Teng and Smith (14).

EXPERIMENTAL

Reagents. Linoleic acid and soybean lipoxygenase, type V (EC 1.13.11.12), were obtained from Sigma Chemical Co. (St. Louis, MO). Sodium borate and analytical grade glacial acetic acid were purchased from Merck (Darmstadt, Federal Republic of Germany). Chromatography solvents, all of the highest purity available, were obtained from Fisons (Loughborough, England).

Preparation of LAHPO. Hydroperoxides of linoleic acid were enzymatically prepared using linoleic acid as substrate, and purified by thin-layer chromatography as described by Teng and Smith (14). Hydroperoxides were visualized using *N,N*-dimethyl-*p*-phenilenediamine spray and recovered from simultaneously developed chromatoplates by scraping off the correspondent areas. LAHPO were extracted from the silica gel using diethyl ether. After evaporating the diethyl ether under a stream of nitrogen, LAHPO were dissolved in hexane and their concentration was determined spectrophotometrically at 234 nm using a molar extinction coefficient of 2.7×10^4 l/mol cm (15).

Determination of LAHPO in deep-frying fat samples. Twenty deep-frying fat samples that had been screened for mutagenic activity were stored under refrigeration until analysis. Unused control samples were stored and processed under the same conditions. One sample was withdrawn from the experiment because the amount of material left after mutagenicity testing was not sufficient for further analyses. Deep-frying fat samples of 50 mg were dissolved in 0.5 ml hexane, after which the mixture was extracted three times with 0.5 ml acetonitrile/water

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Abbreviations: HPLC, high performance liquid chromatography; LAHPO, linoleic acid hydroperoxides; P/S, polyunsaturated/saturated; TBA-RS, thiobarbituric acid-reactive substances.

(1:1, v/v). The combined aqueous extracts were subjected to HPLC analysis. Aliquots of 20 μl were separated on a Chromsep LiChrosorb RP18 column, 200 × 3.0 i.d. with guard column (Chrompack, Middelburg, The Netherlands) applying a quaternary eluent composed of tetrahydrofurane/acetonitrile/water/acetic acid (45:100:100:1, v/v/v/v) at a flow rate of 0.2 ml/min. Peaks were detected by an UV-detector (Spectroflow 783 UV, Kratos Analytical, Ramsey, NJ) set at 234 nm. Positive identification of LAHPO was based on retention time and spiking of fat samples with a known amount of purified LAHPO. A calibration curve was obtained using enzymatically prepared and purified LAHPO, which were added to 50 mg of unused deep-frying fat that contained no detectable LAHPO.

Mutagenicity testing of deep-frying fat samples. Sixty deep-frying fat samples were obtained from local snack bars and restaurants. Twenty were selected based on presence of di- and polymeric triglycerides, 10 samples had concentrations exceeding 10% (by weight). Samples were separated into two major fractions by means of silica gel column chromatography (11); a non-polar petroleum ether fraction, which has been reported to consist of non-polar degradation products of fatty acids and unaltered triglycerides, and a polar acidified diethyl ether fraction, which has been found to contain polar oxidation and degradation products (16). Non-polar and polar fractions were screened for mutagenicity using *Salmonella typhimurium* tester strains TA97 (20 samples) and TA100 (14 samples) in absence and presence of liver S9 mix obtained from Aroclor induced rats. Samples were dissolved in tetrahydrofuran and tested at doses ranging from 0.1 to 10 mg/plate using a liquid preincubation of 20 min at 37°C (17). Unused fat samples, as well as solvent controls, were also included. A more detailed description of the protocols used for mutagenicity testing has been given elsewhere (11). The mutagenic activity of the non-polar and polar fractions was estimated by extrapolation of the linear, non-toxic portion of dose-response curves applying linear regression. Mutagenicity per g of frying fat was calculated by summation of mutagenic activity of non-polar and polar fractions.

RESULTS AND DISCUSSION

Chromatograms of enzymatically obtained and purified LAHPO and of LAHPO extracted from a deep-frying fat sample are given in Figure 1. Isomers of LAHPO, *cis-trans* or *trans-trans* stereo isomers of 9-hydroperoxy-10,12-octadecadienoate and 13-hydroperoxy-10,12-octadecadienoate elute in three peaks, of which the exact conformation is not known. Confirmation of the hydroperoxide identity has been obtained by analysis of aliquots of the mixture of linoleic acid and lipoxygenase after 0, 10, 20 and 30 min of incubation; heights of the three peaks have been found to increase as the reaction proceeded. The total amount of LAHPO is calculated from the chromatograms by summation of the three peak heights.

Figure 2 shows the calibration curve of LAHPO in deep-frying fat. Detection limit of the method is 10 nmol/g of fat. Purified LAHPO dissolved in hexane are detectable at concentrations of 0.1 nmol/ml. Recovery of added LAHPO is determined for four concentrations of the calibration curve. Mean recovery (± SD) is 94 ± 6%. Reproducibility of the method, as determined by triplicate

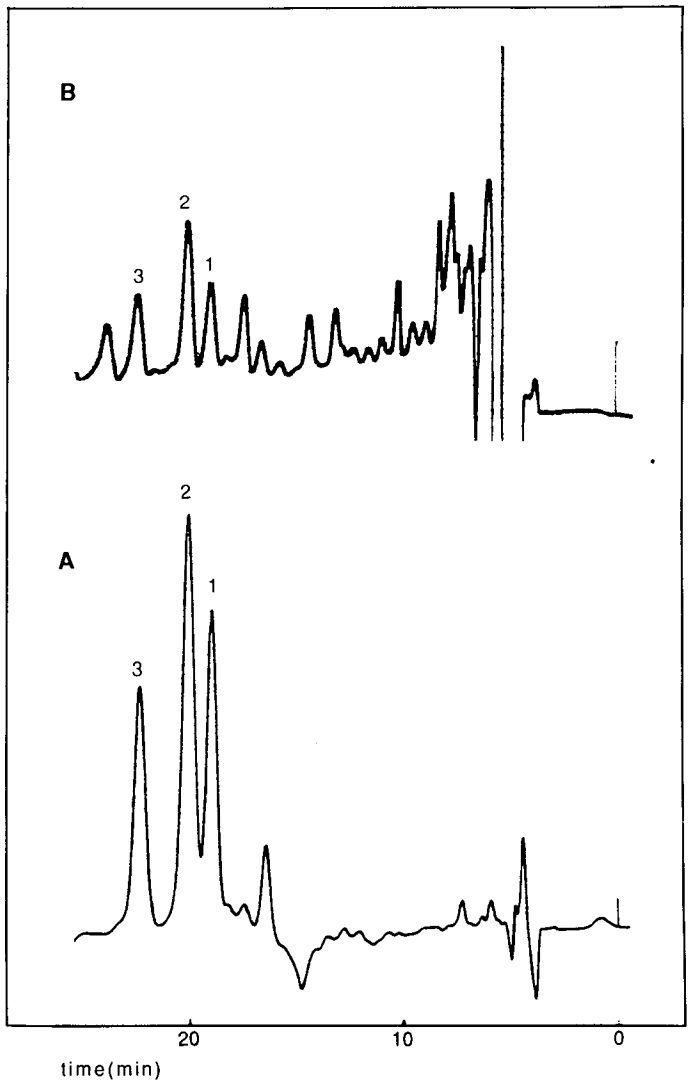

FIG. 1. High-performance liquid chromatograms of hydroperoxides of linoleic acid (peaks 1, 2 and 3); A) enzymatically prepared and purified hydroperoxides of linoleic acid; B) hydroperoxides of linoleic acid extracted from a repeatedly used deep-frying fat sample.

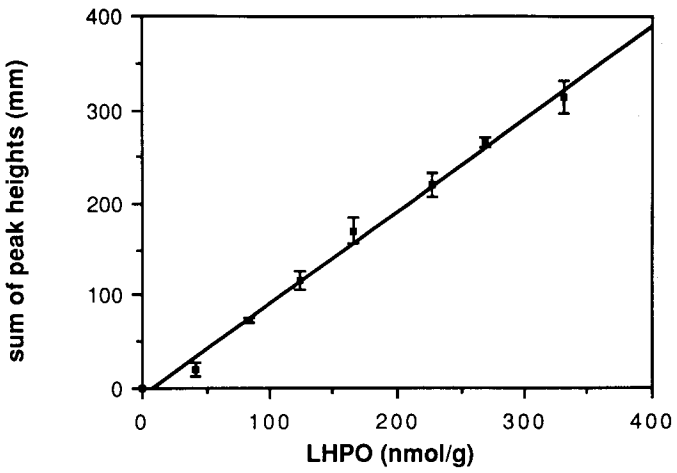

FIG. 2. Calibration curve of enzymatically prepared and purified LAHPO added to an unused deep-frying fat sample and extracted with acetonitrile:water (1:1, v/v). The detection level is 10 nmol/g of fat. Results are expressed as mean ± SD.

TABLE 1

Linoleic Acid Hydroperoxide (LAHPO) Concentrations of 19 Used Deep-Frying Fat Samples (Mean ± SD)

Sample	LAHPO (nmol/g)
1	65 ± 1
2	78 ± 4
13	<10
14	200 ± 5
17	<10
18	170 ± 12
19	<10
21	20 ± 8
22	17 ± 1
25	<10
30	257 ± 10
35	160 ± 16
43	107 ± 3
45	267 ± 2
49	49 ± 1
50	<10
55	<10
56	<10
58	<10

extraction and analysis of 39.5 nmol LAHPO added to 1 g of deep-frying fat, yields an intra-assay coefficient of variation of 3%, and an inter-assay coefficient of variation of 6%.

Levels of LAHPO measured in repeatedly used deep-frying fat samples ranged from below the detection level to 267 nmol/g (Table 1). LAHPO were below the detection level in two different brands of unused hydrogenated deep-frying fat (data not given).

Seventeen out of 19 samples tested were mutagenic; mean mutagenicity in strain TA97 without S9 mix is 18128 revertants/g (range: 943-86065 revertants/g). In presence of S9 mix 12 samples showed mutagenic activity; mean mutagenicity was 16415 revertants/g (range: 5875-35671 revertants/g). Twelve out of 14 samples tested with strain TA100 showed mutagenic activity without S9 mix; mean mutagenicity was 8319 revertants/g (range: 1760-12210 revertants/g). In presence of S9 mix, 7 samples were mutagenic; mean mutagenicity was 10558 revertants/g (range: 3740-17010 revertants/g).

Since LAHPO have been found to be present in non-polar as well as in polar fat fractions separated by silica gel column chromatography, mutagenic activity calculated per g of frying fat has been compared with levels of LAHPO analyzed in unfractionated fat samples. LAHPO concentrations of repeatedly used deep-frying fat samples correlate positively with mutagenicity to *Salmonella typhimurium* tester strain TA97 in presence of S9 mix ($r = 0.48$, $p < 0.05$, $n = 19$). In absence of S9 mix, no statistically significant association is observed ($r = 0.02$). Correlations between LAHPO levels and mutagenicity to strain TA100 show the same tendency—without S9 mix, mutagenicity of the fats to strain TA100 is not associated with LAHPO levels ($r = 0.07$, $n = 14$), while in presence of S9 mix a weak, but statistically insignificant, positive correlation has been found ($r = 0.24$). In addition, LAHPO concentrations and mutagenic activity to both strains TA97 and TA100, added together, reflecting the overall mutagenic potency to induce frameshift as well as base pair mutations, revealed a similar relationship. In absence of S9 mix, total mutagenic potency and LAHPO concentrations showed no correlation ($r = 0.05$, $n = 14$), whereas in presence of S9 mix a positive, but statistically insignificant, correlation was found ($r = 0.48$, $n = 14$).

Fatty acid hydroperoxides are the first isolatable oxidation products of polyunsaturated fatty acids, which now are reported to be present in used deep-frying fat samples. Although most brands of deep-frying fats consist of hydrogenated oils, and thus contain low amounts of polunsaturated fatty acids, during deep-fat frying of various foods polyunsaturated fatty acids migrate from the food into the fat, thereby increasing the P/S ratio (18). During heating of the frying fat, hydroperoxides of linoleic acid and other polyunsaturated fatty acids may be formed and remain in the frying fat in detectable amounts after cooling down. This has actually been demonstrated in a separate experiment in our laboratory, in which increased levels of LAHPO have been observed after repeated use in a frying oil containing 60% linoleic acid. LAHPO are not detected in unused frying oil of this particular type, while after 10, 20 and 30 hr of frying, LAHPO have been found to be present in a concentration of 17, 55 and 62 nmol/g, respectively. In this experiment it was also found that LAHPO in used deep-frying fats are stable for at least two weeks while stored under refrigeration. Stored fat samples analyzed within three days after frying and again after two weeks showed no systematically increased or decreased concentrations of LAHPO.

Whereas in general, concentrations of TBA-RS (19) or the conjugated diene-fraction (20) are determined as an indication of lipid oxidation of fats or foods, identification of lipid oxidation products in relation to mutagenic activity requires more specific assays. In addition to determination of LAHPO, assays detecting unsaturated aldehydes and carbonyls appear to be of greater relevance (7). These oxidation products have been reported to possess mutagenic activity in Salmonella tester strain TA104 (7), and have also been detected in lipid oxidation studies *in vitro* using rat liver microsome preparations (21).

The positive correlation observed in the present study between LAHPO levels and mutagenic activity with S9 mix is in accordance with mutagenicity data obtained by other authors (6,9) who report mutagenic activity of hydroperoxides of methyl linoleate and oxidized linoleic acid solutions with high peroxide values in presence of S9 mix. The positive correlation found between mutagenicity of repeatedly used deep-frying fat samples in presence of S9 mix and LAHPO concentrations may be explained by three different mechanistic routes. First, autoxidation of LAHPO and concomitant oxidation of other polyunsaturated fatty acids, is very likely to occur during incubation in the Salmonella mutagenicity assay due to the presence of trace amounts of iron or other transition metals (22), especially with S9 mix. It is not clear, however, to what extent autoxidation may take place in S9 mix, since S9 homogenate also possesses free radical scavenging activities. Second, mutagenic metabolites of LAHPO may be derived as a result of degradation by cytochrome P-450 activities of LAHPO. Third, oxidation

of polyunsaturated fatty acids, present in fat samples or in microsomal membranes, by cyclooxygenase or lipoxygenase resulting in mutagenic intermediates, may be enhanced in presence of LAHPO, which specifically has been demonstrated by Marshall *et al.* (23) for the oxidation of arachidonic acid by cyclooxygenase in plasma.

Although used deep-frying fats have been observed to exert potent direct-acting mutagenic activity, in some of these samples mutagenicity in presence of S9 mix appears to be induced by metabolites or secondary autoxidation products of LAHPO as well. Therefore, future studies will be concentrated on the isolation and identification of direct-acting mutagenic compounds as well as possible mutagenic metabolites or secondary oxidation products of LAHPO in used deep-frying fats.

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The Occurrence of Estrone and Estriol in *Trichostrongylus colubriformis* (Nematoda)

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Mammalian sex steroids and cholesterol wre isolated from the lipid extract of the zooparasitic nematode *Trichostrongylus colubriformis*. In addition to the previously identified sex steroids progesterone and testosterone, estrone and estriol were detected and isolated from the mixture. The steroids were analyzed by thin-layer, gas-liquid and high-performance liquid chromatography, and their structures confirmed by proton nuclear magnetic resonance and mass spectroscopy.
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The occurrence and importance of steroids for the growth and development of a number of nematode species has been demonstrated (1-3). Several reports have described the physiological effects of mammalian sex steroids on nematodes. It has been shown that testosterone and diethylstilbesterol influence the fecundity of *Trichinella spiralis* (4), and that testosterone increased the recovery of *Nippostrongylus brasiliensis* from mice and caused a dose-dependent production of eggs (5). In addition, progesterone was found at picogram per milligram levels in the free-living nematode *Panagrellus redivisus*, and enzymes involved in androgen biosynthesis occur in *Caenorhabditis elegans* (6).

Recently, we have reported the presence of sex steroid receptors for progesterone, testosterone, and 17β-estradiol in an extract from the ruminant nematode *Trichostrongylus colubriformis* (7). These results led to the isolation of progesterone and testosterone from the same organism (8). In companion studies, we have demonstrated steroid receptors for progesterone, testosterone, and 17β-estradiol in an extract from the rodent nematode *N. brasiliensis*, which has led to the development of new steroid anthelmintic agents using sex steroids as models (9).

In our studies on the sex steroid content of nematodes, we have extended our search for additional members of this steroid class. In the present communication, we wish to report that re-examination of lipid extract obtained from *T. colubriformis* has resulted in the isolation and identification of estrone and estriol as significant components in the steroid mixture.

EXPERIMENTAL

The nematode, *T. colubriformis*, was removed from the small intestine of male goats sacrificed at 21 days post-infection (7,8). Frozen batches of nematodes were thawed and homogenized with chloroform/methanol (2:1, v/v) to a final 1:20 dilution (w/v). The aqueous phase was reextracted with chloroform/methanol and the combined organic phase dried with 0.02% magnesium chloride (10,11). After solvent evaporation, residues were dissolved in a small amount of methanol and eluted through a reverse phase Sep-Pak cartridge (Waters Associates, Milford, CT) with water and methanol as mobile phases (12). The solution was concentrated under nitrogen at 45°C and dried in a vacuum desiccator. The residue was dissolved in 2 ml of absolute ethanol. Steroids in this solution were analyzed by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). The dry weight of worms was determined by taking 10% of their wet volume and drying at 90°C prior to weighing the sample.

Gas liquid chromatography (GLC) analyses were conducted using a Shimadzu (GC-9A) gas chromatograph equipped with a flame ionization detector and a Chromatop Ac computer operating at a temperature gradient 220°C (1 min) to 290°C (4°C/min). Glass columns (6 ft) packed with Gas-Chrom Q (100/200 mesh) containing 2% SE-30 (13,14) were used with a nitrogen flow rate of 20 ml/min. Conditions for TLC and HPLC analysis have been described (8). TLC solvent systems were ether/toluene (35:65), hexane/diethyl ether (50:50) and chloroform/diethyl ether (25:75).

Steroids were isolated for melting point (MP), mass spectrometry (MS) and proton nuclear magnetic resonance (PMR) analysis by TLC (20 × 20 cm silica gel G glass plates; Analtech, Newark, DE), which had been activated at 105°C for 40 min (15,16). Plates were developed with hexane/diethyl ether (1:1), and steroids were visualized by iodine vapor (17). Fractions were scraped off, extracted with chloroform/methanol (2:1), the solvent was evaporated, and the sample was dried prior to analysis. Conditions for MP determinations, MS and PMR analysis have been described (18).

Authenic standards of cholesterol (after purification [19]), progesterone, testosterone, estrone, and estriol were obtained from Sigma Chemical Co. (St. Louis, MO).

RESULTS AND DISCUSSION

Examination of the lipid extract obtained from *T. colubriformis* by GLC, TLC and HPLC analysis revealed the presence of a complement of mammalian sex steroids. In these experiments the presence of progesterone, testosterone, estrone, and estriol was detected. Cholesterol was also detected and was found to be the major steroid component of this mixture. The presence of these steroids was confirmed by comparison with authentic standards which had identical retention times in GLC and HPLC analysis and identical mobilities in TLC in three solvent systems (Experimental section). The presence of progesterone, testosterone, and cholesterol was described in a previous communication (8), but the opportunity to examine larger samples has allowed us to identify the presence of two additional sex steroids, estrone and estriol. This is the first report describing the presence of estrone or estriol in a zooparasitic nematode species.

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Abbreviations: GLC, gas liquid chromatography; HPLC, high performance liquid chromatography; MP, melting point; MS, mass spectrometry; PMR, proton nuclear magnetic resonance; TLC, thin-layer chromatography.

Table 1 gives the steroid content of *T. colubriformis* as measured by GLC analysis of the lipid extract using internal standards (11,20). None of the described sex steroids was detected in a five gram sample of goat chyme, while cholesterol was found in chyme from the host.

The steroids described in Table 1 were isolated by TLC and subjected to PMR and MS analysis. The spectra of each isolated steroid were identical in all respects to those obtained from the respective standard compounds. An analysis of the spectra obtained from each steroid isolated from *T. colubriformis* is presented in Table 2. These results are consistent in all respects with the published spectra (21,22). Finally, each steroid had a MP similar to the authentic standards, which corresponded to literature values (23).

The results reported herein add further support to the hypothesis that nematode endocrine control may involve some compounds similar in structure to vertebrate steroid hormones. Other helminths are reported to utilize steroidal hormones. *Schistosoma mansoni* metabolized androstenedione, cortisone, estrone, 17α-hydroxyprogesterone, 17α-hydroxypregnenolone and cholesterol (24). Willett cited unpublished results which indicated that *Caenorhabditis elegans* had some enzymes of androgen biosynthesis (6). Our own studies have shown that progesterone is enzymatically converted to 17α-hydroxyprogesterone in *T. colubriformis* (8). In the latter work we have indicated that the probable origin of progesterone and testosterone may result from direct sequestering of these hormones from the host. Continued research may clarify the role of steroidal hormones and their metabolism in parasite physiology.

TABLE 1

Steroid Content of *Trichostrongylus colubriformis*

Steroid	Wt % in dry worms	Wt % in lipid
Cholesterol	0.082 ± 0.010	0.65 ± 0.08
Progesterone	0.0084 ± 0.0004	0.066 ± 0.003
Testosterone	0.031 ± 0.007	0.24 ± 0.01
Estrone	0.0012 ± 0.0002	0.009 ± 0.002
Estriol	0.0029 ± 0.0002	0.023 ± 0.002

Values reported are based on GLC analysis of the lipid extract using internal standards (five analyses were conducted). A total of 50 g (dry weight) of *T. colubriformis* was used in these experiments.

TABLE 2

Proton Nuclear Magnetic Resonance (PMR) and Mass Spectral (MS) Data of Steroids Isolated from *Trichostrongylus colubriformis*

Steroid	PMR[a]	MS (relative intensity of major ions)
Cholesterol	0.80 (C-18 CH_3), 1.01 (C-19 CH_3), 2.50 (C-3 H) 5.37 (C-6 H, vinyl)	386 (100%), 368 (19%), 353 (9%), 326 (4%), 301 (18%), 275 (22%), 255 (10%)
Progesterone	0.70 (C-18 CH_3), 1.21 (C-19 CH_3), 2.13 (C-21 CH_3) 5.70 (C-4 H, vinyl)	314 (91%), 272 (79%), 244 (34%), 229 (58%), 191 (28%), 173 (15%), 191 (28%), 173 (15%), 147 (30%), 124 (100%)
Testosterone	0.81 (C-18 CH_3), 1.21 (C-19 CH_3), 3.62 (C-17 H), 5.69 (C-4 H, vinyl)	288 (28%), 273 (5%), 246 (43%), 228 (14%), 203 (19%), 165 (13%), 147 (34%), 124 (100%)
Estrone	0.91 (C-18 CH_3), 4.67 (C-3 OH), 6.56 (C-4 H, aromatic), 6.60 (C-2 H, aromatic), 7.12 (C-1 H, aromatic)	270 (100%), 213 (19%), 185 (28%), 172 (21%), 160 (13%), 140 (17%)
Estriol	0.75 (C-18 CH_3), 3.39 (C-17 H), 3.95 (C-16 H), 6.45 (C-4 H, aromatic), 6.50 (C-2 H, aromatic), 8.60 (C-3 OH)	288 (100%), 213 (26%), 185 (11%), 172 (10%), 160 (29%), 133 (25%)

[a]$CDCl_3$ served as solvent, except for estriol, where acetone-d_6 was used.

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Time Dependent Changes Occurring in Rat Liver Microsomes upon Lipid Peroxidation

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Steady-state fluorescence anisotropy of diphenylhexatriene and n-(9-anthroyloxy)stearic acids (n=2,12) in rat liver microsomes showed a marked increase in the early stages of enzymatically or non-enzymatically induced lipid peroxidation. The changes in fluorescence anisotropy occurred in parallel with the formation of thiobarbituric acid-reactive substances (TBA-RS). Parallel to these changes, the fluorescence emitted from peroxidized microsomes increased markedly in the early stages of lipid peroxidation. In contrast to the changes in the fluorescence anisotropy and in the formation of TBA-RS, the fluorescence showed a continuing increase over the three hr period of lipid peroxidation. Glucose-6-phosphatase was inactivated in the early stages of lipid peroxidation, whereas NADH-cytochrome b_5 reductase underwent a slow deactivation over three hr. The apparently slow deactivation of the peripheral protein may be explained by the formation of fluorescent substances.
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Lipid peroxidation of biological membranes causes the chemical transformation of lipids resulting in the generation of products such as short-chain hydrocarbons, aldehydes, ketones and fluorescent substances, including the age pigment lipofuscin (1-5). Accompanying the formation of these substances, biochemical changes occur such as changes in the activities of membrane-bound enzymes and in membrane permeability (6-8).

Lipid peroxidation does affect membrane structure. Dobretsov *et al.* (9) reported that lipid peroxidation decreases the membrane fluidity of liposomes, microsomes and mitochondria. Eichenberger *et al.* (10) and Gut *et al.* (11) found that lipid peroxidation decreases the fluidity of the microsomal membrane lipid based on steady-state fluorescence anisotropy measurements with diphenylhexatriene (DPH). Spin label studies, however, gave different results, indicating that γ radiation-induced lipid peroxidation decreases the degree of order of membrane lipids (12). Utsumi *et al.* (13) suggest that carbontetrachloride or its metabolites do increase the membrane fluidity of liver microsomes. The mode of generation of fluorescent substances in peroxidized microsomes was substantially different from that of thiobarbituric acid-reactive substances (TBA-RS). Whereas the fluorescent substances are thought to be retained in the membrane matrix (14), the amount of TBA-RS bound to the membrane is insignificant relative to the total amount of TBA-RS produced in the course of lipid peroxidation (15).

The present study was undertaken to follow the time course of membrane lipid peroxidation by simultaneously monitoring chemical, physical and biochemical indices of lipid peroxidation.

MATERIALS AND METHODS

Materials. Glucose 6-phosphate, cytochrome C (from horse heart, type VI), *t*-butylhydroperoxide, butylated hydroxytoluene and 1,6-diphenyl-1,3,5-hexatriene (DPH) were purchased from Sigma Chemical Co. (St. Louis, MO). $FeSO_4 \cdot 7H_2O$, EDTA-Na, thiobarbituric acid, L-ascorbic acid, 2-(9-anthroyloxy)stearic acid (2-AS) and 12-(9-anthroyloxy)stearic acid (12-AS) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). β-NADPH and β-NADH were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). All other reagents were of analytical grade.

Microsomes. Liver microsomes were prepared from male Wistar rats (170-250 g) as described elsewhere (14). The protein concentration of the microsomes was determined according to Lowry *et al.* (16). Phenobarbital-treatment of the rats was performed according to Gut *et al.* (11).

Lipid peroxidation. The microsomal suspension (1 mg protein/ml) was first incubated at 37°C for five min before starting the lipid peroxidation reaction. Microsomal lipid peroxidation was induced at 37°C by the addition to the microsomal suspension of 0.2 mM NADPH and 20 μM $FeSO_4$, and the reaction was terminated at the times indicated by adding 1 mM EDTA. The samples were centrifuged at 105,000 × g for 30 min to remove fluorescent NADPH from the peroxidized microsomes and then were resuspended in the buffer containing 0.1 M sodium phosphate and 1 mM EDTA, pH 7.4 (11). Microsomal lipid peroxidation was alternatively induced with 2 mM *t*-butylhydroperoxide, and the reaction was stopped by adding 0.1 mM butylated hydroxytoluene (14). Microsomal lipid peroxidation, induced with 50 μM ascorbic acid and 2.5 μM Fe^{2+}, was terminated by adding 1 mM EDTA (8).

Fluorescent probe labelling of microsomes. Microsomal membranes were labelled with fluorescent probes after termination of lipid peroxidation. One half ml of the microsomal suspension was incubated at 25°C for one hr with 4.5 ml of 5 μM DPH solution containing 1 mM EDTA in phosphate buffer. Two ml of the microsomal suspension was also incubated at 25°C for one hr with 2 ml of 10 μM 2-AS or 12-AS solution containing 1 mM EDTA in phosphate buffer. In the case of microsomes that were peroxidized with *t*-butylhydroperoxide, butylated hydroxytoluene was used throughout instead of EDTA. Following removal of the free probes by centrifugation at 105,000 × g for 30 min, the fluorescent-labelled microsomes were resuspended in the buffer containing EDTA or butylated hydroxytoluene.

Fluorescence anisotropy measurements. Steady-state fluorescence anisotropy was measured at 25°C with a Hitachi fluorescence spectrophotometer 650-60.

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Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; 2-AS, 2-(9-anthroyloxy)stearic acid; 12-AS, 12-(9-anthroyloxy)stearic acid; TBA-RS, thiobarbituric acid-reactive substances; MDA, malondialdehyde.

The samples (0.1 mg protein/ml) labelled with fluorescent probe were excited by vertically polarized light at 360 nm for DPH and 368 nm for 2- and 12-AS. The fluorescence of the probes associated with the microsomes was analyzed through a cut-off filter at 450 nm for DPH, and at 460 nm for 2- and 12-AS as the vertically and horizontally polarized components, I_V and I_H, respectively. The steady-state anisotropy, r^s, was:

$$r^s = (I_V - GI_H)/(I_V + 2GI_H)$$

where G is the ratio of the sensitivity of the detection system for vertically and horizontally polarized light being taken as equal to I_V/I_H obtained by horizontally polarized excitation.

Measurement of fluorescent substances. The fluorescence emitted from peroxidized microsomes was measured directly on the membrane preparations as previously described (14).

Thiobarbituric acid assay. Malondialydehyde (MDA) formed in the lipid peroxidation reaction was assayed as previously described (14) and according to Buege and Aust (17).

Microsomal enzyme assay. Glucose 6-phosphatase was measured according to Baginski *et al.* (18). NADH-cytochrome b_5 reductase and NADPH-cytochrome C reductase were determined according to Takesue and Omura (19) and Omura and Takesue (20), respectively.

RESULTS AND DISCUSSION

The time course of chemical and physical changes occurring in rat liver microsomes due to enzymatic (NADPH and Fe^{2+}) and non-enzymatic (*t*-butylhydroperoxide, ascorbic acid and Fe^{2+}) lipid peroxidation were examined over three hr periods (Fig. 1). Steady-state fluorescence anisotropy of DPH labelled microsomes increased markedly in the early stages of lipid peroxidation. Interestingly, in both the enzymatic and non-enzymatic peroxidation systems, the time-dependent decrease in fluidity of the microsomal membranes occurred in parallel with the formation of TBA-RS. Both anisotropy and TBA-RS simultaneously showed saturable profiles. The results observed in the early stages of lipid peroxidation are consistent with those reported by Eichenberger *et al.* (10) and Gut *et al.* (11), who examined lipid peroxidation induced by NADPH and Fe^{2+}. Other fluorescent probes used to monitor membrane fluidity gave similar results.

A set of n-AS (n=2,12) was used to monitor the environment at different depths in peroxidized microsomal membranes (21). An example of NADPH- and Fe^{2+}-catalyzed lipid peroxidation is shown in Figure 2. 12-AS reporting the environment near the core of the bilayer showed a marked increase in fluorescence anisotropy in the early stages of lipid peroxidation. The same was noted for 2-AS, which monitors the lipid environment closer to the surface of the membrane. Parallel to the formation of TBA-RS, the fluidity of the microsomal membrane decreased.

It is well recognized that the prime targets of lipid peroxidation are the constituent polyunsaturated fatty acids of the phospholipids that form the membrane matrix (1,22), which leads to the formation of peroxidized fatty acids and low molecular weight products. It could readily be accepted that the degradative processes that occur in the early stages of lipid peroxidation would cause drastic microsomal membrane perturbations, resulting in a decrease in membrane fluidity. The time course of the fluorescence emitted from peroxidized microsomes is quite characteristic. The fluorescence increased markedly in the early stages of lipid peroxidation (Fig. 1A), and then showed a continuing increase. Why fluorescence continues to increase over longer reaction times is unknown at present. At least, two explanations appear possible: (a) A quantitative

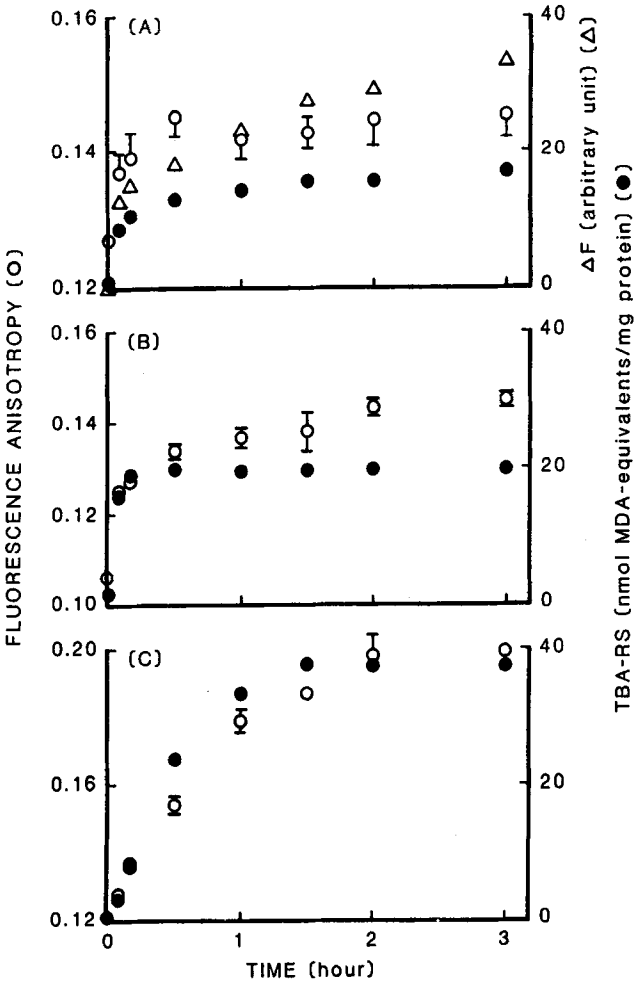

FIG. 1. Lipid peroxidation and steady-state fluorescence anisotropy of DPH in rat liver microsomal membranes. Steady-state fluorescence anisotropy of DPH in microsomes undergoing enzymatic or nonenzymatic lipid peroxidation was measured (O). The formation of TBA-RS was used as an index of lipid peroxidation (●). The increase of fluorescence intensity (ΔF) = (fluorescence intensity of peroxidized microsomes) — (fluorescence intensity of nonperoxidized microsomes at time zero) (Δ). The data points of the fluorescence anisotropy represent the mean values ± SEM of five determinations. The data points of TBA-RS represent the mean values ± SEM of three determinations expressed as MDA-equivalents per mg protein of microsomes. Where absent, SEMs were smaller than the symbols. Lipid peroxidation systems: [A] NADPH and Fe^{2+}, [B] t-butylhydroperoxide, [C] ascorbic acid and Fe^{2+}.

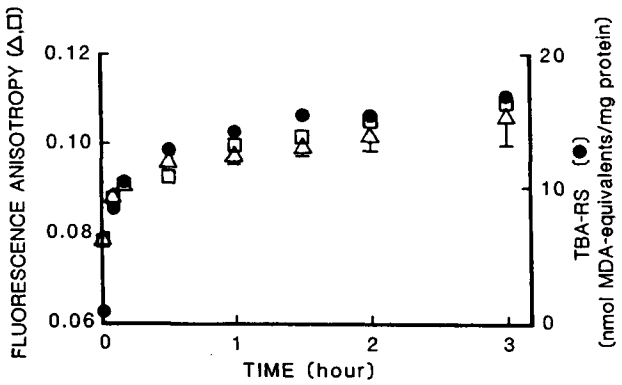

FIG. 2. Lipid peroxidation and steady-state fluorescence anisotropy of 2- and 12-AS in rat liver microsomal membranes. Lipid peroxidation was induced by NADPH and Fe^{2+}. △, Steady-state fluorescence anisotropy of 2-AS; □, Steady-state fluorescence anisotropy of 12-AS; ●, TBA-RS formation. The data points of anisotropy represent the mean values ± SEM of five determinations. The data points of TBA-RS represent the mean values ± SEM of three determinations. Where absent, SEMs were smaller than the symbols.

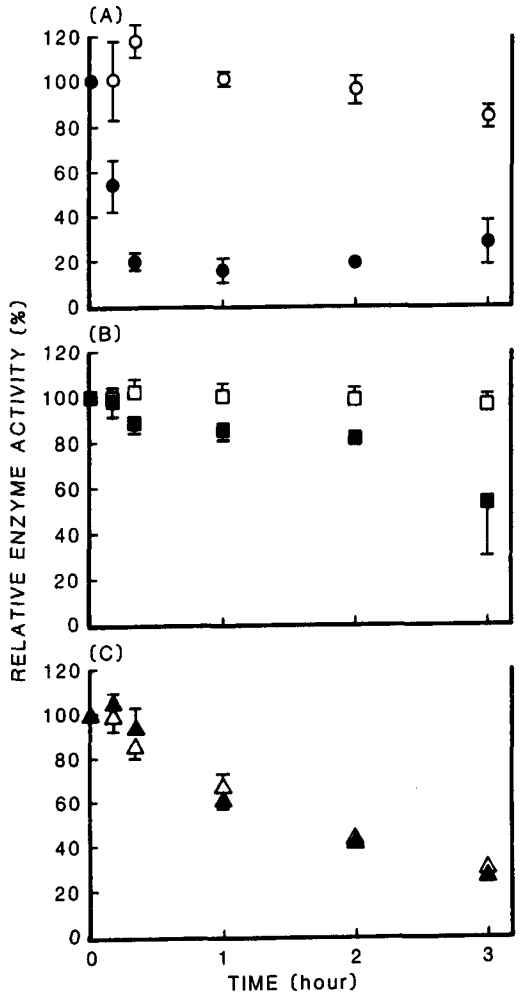

FIG. 3. Effects of lipid peroxidation on the microsomal membrane bound enzymes. Lipid peroxidation was induced by ascorbic acid and Fe^{2+}. The relative enzyme activity (%) represents the ratio of the enzyme activity in the microsomes at the indicated time to that at time zero. Open symbols show the data of microsomes incubated at 37°C without peroxidation system. Closed symbols show the data incubated at 37°C with peroxidation system. The data points represent the mean values ± SEM of three determinations. Where absent, SEMs were smaller than the symbols. [A] glucose 6-phosphatase, [B] NADH-cytochrome b_5 reductase, [C] NADPH-cytochrome C reductase.

increase of fluorescent substances, or (b) a qualitative change in fluorescent substances. Secondary reactions of peroxidation products also affect the membrane, as is observed in various changes in membranes, e.g., upon crosslinking with amines (2,4,23). The time-dependent formation of high molecular weight protein adducts in microsomes over two hr of lipid peroxidation was reported by Koster and Slee (2). Such secondary reactions, including the formation of fluorescent substances, seem to proceed rather slowly. We have recently found that the fluorescence emitted from the peroxidized microsomes exposed to ascorbic acid and Fe^{2+} for 24 hr is composed of at least three species of fluorophores by measuring the fluorescence lifetimes of the peroxidized microsomes (unpublished data). Further analysis of the fluorescence lifetimes may explain the continuing increase in fluorescence. This is being investigated further.

Lipid peroxidation inactivates membrane bound microsomal enzymes such as glucose 6-phosphatase and UDP glucuronyltransferase (24). The time course of the effect of lipid peroxidation on membrane bound microsomal enzymes were examined (Fig. 3), with ascorbic acid and Fe^{2+} being used as peroxidation system. The peroxidation system NADPH and Fe^{2+} disturbed the assay of enzymes, such as NADPH-cytochrome C reductase. Glucose 6-phosphatase, an integral protein which is located on the lumenal side of the microsomal lost its activity at early stages of lipid peroxidation (Fig. 3A). Groot *et al.* (24) suggested that lipid peroxidation induced inactivation of glucose 6-phosphatase was due to irreversible damage of the active site and also to alterations of the transporter components of the glucose 6-phosphatase system. In addition, the membrane perturbation as observed by DPH and n-AS fluorescence anisotropy is also likely to contribute to its rapid inactivation. Gardia and Brenner (25) had reported that glucose 6-phosphatase activity decreased by incorporation of cholesterol into liver microsomes which caused an increase in the DPH fluorescence anisotropy. On the other hand, NADH-cytochrome b_5 reductase and NADPH-cytochrome C reductase, peripheral proteins which are located on the cytosol side, were affected by lipid peroxidation either very slowly or almost not at all) (Fig. 3B and C).

The marked changes in fluorescence anisotropy of the probes, even 2-AS, occurred foremost in the early stages of lipid peroxidation. Therefore, slow inactivation of peripheral proteins, such as NADH-cytochrome b_5 reductase, could be attributed to secondary reactions of the peroxidation products, particularly to the formation of fluorescent substances rather than to membrane perturbation per se.

ACKNOWLEDGMENTS

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Reduced Glutathione Effects on α-Tocopherol Concentration of Rat Liver Microsomes Undergoing NADPH-Dependent Lipid Peroxidation

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Factors involved in reduced glutathione (GSH) and vitamin E-mediated inhibition of NADPH-dependent rat liver microsomal lipid peroxidation were examined. Lipid peroxidation was monitored over a time-course of 180 min by thiobarbituric acid reactive product formation. The addition of 5 mM GSH to the reaction system containing microsomes from rats fed a diet supplemented with 150 IU/kg of α-tocopherol acetate for eight weeks produced a lag in peroxidation of >30 min. This effect was not observed for microsomes prepared from rats fed a diet deficient in vitamin E. Indeed, a prooxidant effect of 5 mM GSH was observed in assays containing microsomes from rats fed a diet deficient in vitamin E. The inhibition by GSH of lipid peroxidation in microsomes prepared from livers of vitamin E supplemented rats was not restricted by its availability, for it was found that approximately 92% of the GSH remained in the reduced form after 60 min. Additional experiments revealed that the α-tocopherol content of peroxidizing microsomes decreased rapidly in the absence of GSH. The addition of 5 mM GSH to the assay system markedly depressed the loss of microsomal α-tocopherol. The results of *in vivo* labeling of liver microsomes with [^{14}C]α-tocopherol demonstrated that i) GSH addition to the *in vitro* peroxidizing medium reduced the disappearance of α-tocopherol, and ii) a compound that interfered with the determination of α-tocopherol was separated by HPLC and was not an oxidation product of α-tocopherol. A portion of the microsomal ^{14}C-labeled α-tocopherol was converted to an unidentified product with HPLC retention characteristics that was similar, but not identical, to α-tocopherol quinone.
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Several groups of investigators have identified glutathione (GSH) dependent systems that inhibit *in vitro* lipid peroxidation (1–5). The system reported by McCay *et al.* (1) and Gibson *et al.* (2) contained a cytosolic factor that inhibited both NADPH-dependent enzymatic and ascorbate-dependent nonenzymatic lipid peroxidation in rat liver microsomes in the presence of GSH. This factor was not associated with either selenium-dependent or selenium-independent glutathione peroxidase activity. Ursini *et al.* (3) recently purified a protein from pig liver cytosol that inhibited lipid peroxidation in the presence of GSH; however, protection against iron-ascorbate induced peroxidation by this protein required the presence of α-tocopherol. Reddy *et al.* (4,5) have described a rat liver microsomal system that inhibited both enzymatic and nonenzymatic lipid peroxidation in the presence of GSH and microsomal α-tocopherol. The GSH-dependent inhibition of lipid peroxidation was not observed in microsomes obtained from vitamin E deficient rats and was independent of the selenium status of the animals. This effect was specific in its requirement for GSH—other thiol compounds tested were not effective when substituted for GSH. These investigators suggested that a microsomal heat labile protein was likely involved in the mediation of vitamin E dependent GSH inhibition of lipid peroxidation. Burk (6) has also reported the presence of a GSH dependent liver microsomal factor that inhibited iron-ascorbate initiated lipid peroxidation. This factor was inactivated by both trypsin digestion and heat denaturation of the microsomes.

Franco and Jenkins (7) showed that the addition of GSH inhibited lipid peroxidation when it was added to a reaction system containing rat lung microsomes derived from animals adequate in vitamin E. This effect was not observed with microsomes derived from vitamin E deficient animals. The inhibition was neither heat labile nor specific for GSH—other thiols could readily be substituted. These investigators suggested the presence of a nonenzymatic thiol-vitamin E dependent system that inhibited lipid peroxidation. Thirot *et al.* (8) observed that GSH did not effectively inhibit lipid prooxidation of liver microsomes derived from rats exposed to whole body γ radiation. The liver microsomal vitamin E content of the rats exposed to γ radiation in their studies was markedly reduced, supporting Reddy *et al.'s* observation (4,5) that GSH is only effective in inhibiting lipid peroxidation of liver microsomes which contain adequate amounts of vitamin E. Wefers and Sies (9) have shown that GSH is capable of extending the lag period before lipid peroxidation only when microsomes derived from animals containing adequate levels of vitamin E are used. Scholich *et al.* (10) have recently reported that the antioxidant effect they observed with dihydrolipoate against microsimal lipid peroxidation was also dependent upon α-tocopherol. These investigators also demonstrated that dihydrolipoate prolonged the lag phase before the onset of microsomal α-tocopherol depletion.

In light of these recent findings, an understanding of the association of vitamin E and GSH in antioxidant defense systems is of importance. Vitamin E has been reported to interact closely with polyunsaturated fatty acids (PUFA) having three or more double bonds in cellular and subcellular membranes (11–14). It has been shown that there is a direct correlation between vitamin E content of membranes and their resistance to lipid peroxidation (6,7,15). Additionally, the molar ratio of α-tocopherol to peroxidizable PUFA (PUFA > 18:2) in liver tissue is on the order of 1:1200 in rats fed a diet containing 20% corn oil (16). This low ratio of α-tocopherol to PUFA suggests that for vitamin E to exert a significant effect as an antioxidant some mechanism for its regeneration may exist *in vivo*. Two systems have been identified in which GSH interacts synergistically with vitamin E to inhibit lipid peroxidation: a nonenzymatic system in rat lung microsomes (7) and an enzymatic system in rat liver microsomes (4,5). It is possible that one or both of

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Abbreviations used: BSA, bovine serum albumin; GSH, reduced glutathione; GSSG, oxidized glutathione; HPLC, high performance liquid chromatography; PUFA, polyunsaturated fatty acids; TBA, thiobarbituric acid.

these systems function via the regeneration of vitamin E. The experiments reported here were designed to investigate the relationship between GSH and vitamin E in the inhibition of rat liver microsomal peroxidation.

METHODS

Animals and diets. Weanling male Long-Evans Hooded rats were purchased from Charles River (Wilmington, MA) and fed chemically defined torula yeast-based diets for eight weeks (4). Tocopherol-stripped corn oil (10%) and lard (6%) were included to formulate diets in which fat comprised 36% of the total calories. Animals were divided into two groups differening in their dietary vitamin E status (0 and 150 IU vitamin E as dl-α-tocopheryl acetate/kg diet).

Preparation of microsomes. The rats were killed by decapitation and their livers homogenized with 9 vol (w/v) of 0.15 M Tris-HCl buffer, pH 7.4. The preparation of microsomes was as previously described (4).

Lipid peroxidation assays. Lipid peroxidation assays were always conducted on freshly prepared liver microsomes and were monitored by the formation of thiobarbituric acid (TBA) reactive products. The TBA reactive products were quantitated at 535 nm using an extinction value of 1.56×10^5 M^{-1} cm^{-1} (17).

Lipid peroxidation was initiated using a modification of the method described by McCay *et al.* (1). The reaction mixture contained 0.012 mM $FeSO_4$, 0.25 mM NADPH, 0.05 M Tris-HCl buffer (pH 7.4) and 0 or 5 mM GSH. Approximately 1.6 mg of microsomal protein/ml of reaction mixture for the determination of vitamin E and 0.16 mg of microsomal protein/ml for determination of TBA reactive products or GSH concentration were used in these experiments. The reaction was stopped by removing a 1 ml aliquot at a specified time from the incubation mixture and pipetting it into a tube containing 100 μl of 0.5 mg/ml bovine serum albumin (BSA), 400 μl of 30% TCA and 0.2% BHT and 600 μl of 0.05 M TBA. Aliquots for the determination of GSH were added to a tube containing 1 ml of ice cold 6% perchloric acid. Aliquots for the determination of vitamin E were pipetted into tubes containing 1.0 ml ethanol, 1 μl γ-tocopherol as internal standard, and 0.5 ml of a 25% solution of ascorbate.

Determination of glutathione. Reduced and oxidized glutathione were determined using a modification of the assay system devised by Reed *et al.* (18), and contained 5 mM γ-glutamyl-glutamate in the reactions as an internal standard. A 250 mm × 4.6 mm Dupont Zorbax NH_2 column fitted with a 50 mm × 4.6 mm Permaphase ETH precolumn was used for the analysis which was monitored at 365 nm.

Determination of α-tocopherol. The HPLC method for α-tocopherol determination is a modification of the method of Bieri *et al.* (19), and employed both UV and fluorometric detection. A 250 mm × 4.6 mm DuPont Zorbax ODS column fitted with a 50 mm × 4.6 mm Permaphase ODS precolumn was used for the separations. The mobile phase was 97.2% methanol and 2.8% water (v/v) at a flow rate of 2.4 ml/min. All samples for HPLC were extracted from a solution containing 1.0 ml ethanol, 1 μl γ-tocopherol as internal standard, 0.5 ml 25% ascorbate and 1.0 ml of reaction mixture.

In vivo *labeling of liver microsomes with [^{14}C]α-tocopherol.* Two animals that had been maintained on the vitamin E supplemented diet for eight weeks were given two 5 μCi doses of [^{14}C]α-tocopherol acetate (s.a. 85 μCi/mg, Hoffmann LaRoche, Nutley, NJ) by gastric intubation. One ml of intubation mixture contained 10 μCi of α-tocopherol acetate labeled with ^{14}C in positions 3 and 4, 0.2 ml of ethanol and 0.8 ml of Tween 80. The animals were fasted 12 hours prior to each intubation, which were administered 24 hours apart. At all other times the animals had free access to food and water. The rats were killed by decapitation 24 hours after the second intubation, and their livers were removed for the preparation of microsomes.

Microsomes labeled with [^{14}C]α-tocopherol were subjected to NADPH-dependent lipid peroxidation assays and extraction of α-tocopherol as previously described. Changes in α-tocopherol were monitored by HPLC and 30 fractions (2.4 ml) were collected from each chromatogram for liquid scintillation counting.

RESULTS

The α-tocopherol concentration of liver microsomes prepared from rats fed diets supplemented or deficient in vitamin E are shown in Table 1. Weanling rats that were fed the diet deficient in vitamin E for eight weeks had a liver microsomal α-tocopherol concentration that was approximately 3% of the control level.

Lipid peroxidation of liver microsomes was monitored during a 180 min time course by the formation of thiobarbituric acid (TBA) reactive products (Fig. 1). As shown in Figure 1A, the addition of 5 mM GSH to the reaction system containing microsomes from rats supplemented with vitamin E resulted in an inhibition of lipid peroxidation that lasted for >30 min. GSH did not produce a lag in peroxidizing microsomes prepared from rats fed the diet deficient in vitamin E (Fig. 1B). Indeed, GSH appeared to produce a prooxidant effect in liver microsomes that were essentially devoid of α-tocopherol. The strong inhibitory effect of GSH on lipid peroxidation of liver microsomes containing adequate α-tocopherol was removed after approximately 60 min; and it was of interest to determine whether either GSH or α-tocopherol was limiting to account for this observation.

The data presented in Figure 2 show changes in GSH and GSSG as a function of time during peroxidation of liver microsomes from rats fed the vitamin E supplemented diet. GSH was oxidized to a limited extent during the time course of peroxidation and was accompanied by an increase in the formation of GSSG. After 60 min, however, approximately 92% of the GSH initially present remained in the reduced form. Companion incubations

TABLE 1

Diet	α-Tocopherol
+ Vitamin E	0.31 ± 0.03
− Vitamin E	0.01 ± 0.002

The values for α-tocopherol content (μg/mg microsomal protein) are means ± SEM for six animals and are statistically different ($P < 0.01$) from each other.

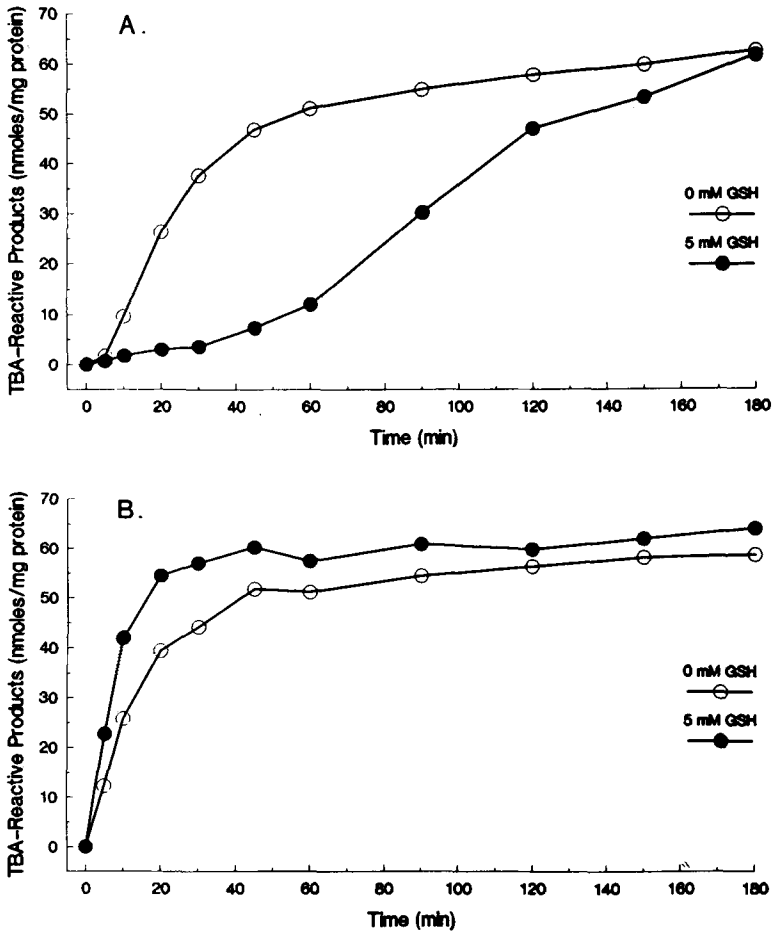

FIG. 1. Time-course of NADPH-dependent lipid peroxidation of rat liver microsomes. A) Microsomes obtained from rats fed a diet containing 150 IU/kg dl-α-tocopheryl acetate and contained 0.31 μg α-tocopherol/mg microsomal protein; ●—●, +GSH; ○—○, −GSH. The pooled SEM for these data is 2.5. B) Microsomes obtained from rats fed a diet deficient in vitamin E and contained 0.01 μg α-tocopherol/mg microsomal protein; ●—●, +GSH; ○—○, −GSH. The pooled SEM for these data is 4.2. The concentration of GSH (when included in the reaction mixture) was 5 mM. Each point represents a mean value obtained from six animals.

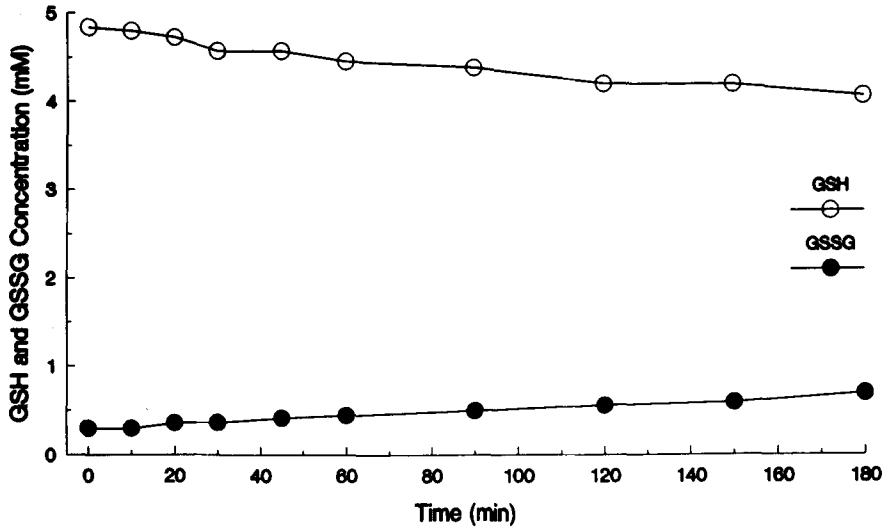

FIG. 2. Time-course of changes in GSH and GSSG concentration during NADPH-Fe^{++}-dependent lipid peroxidation of liver microsomes from rats fed diets adequate in vitamin E. GSH was added at a concentration of 5 mM. Points on this figure are mean values obtained from six animals. The pooled SEM for these data is 0.05.

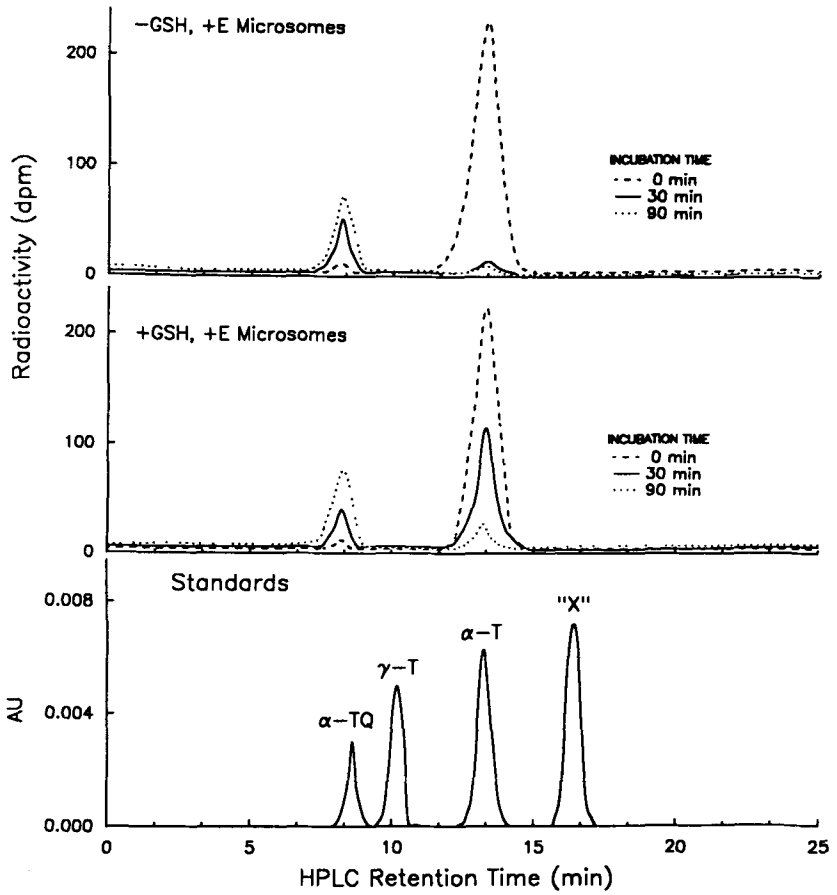

FIG. 3. The distribution of radioactivity following HPLC analysis of rat liver microsomes containing [^{14}C]α-tocopherol before and after being subjected to NADPH-Fe^{++}-dependent lipid peroxidation in the presence (+GSH) and absence (−GSH) of 5 mM reduced glutathione. Fractions of 2.4 ml each were taken at 1 min intervals for determination of radioactivity. α-TQ = α-tocopherol quinone, γ-T = γ-tocopherol, α-T = α-tocopherol, "X" = unknown compounds.

without microsomes or with heat denatured microsomes revealed results similar to those shown in the figure, indicating spontaneous GSH oxidation in this assay system (data not shown). Nevertheless, the data in Figure 2 show that the reduction in GSH concentration during the time course of lipid peroxidation was not a limiting factor in these experiments.

Initial attempts to quantitate changes in microsomal α-tocopherol concentration during peroxidation were unsuccessful. In these experiments (data not shown) microsomes were prepared for α-tocopherol analysis employing alkaline hydrolysis and hexane extraction procedures, and were found to contain a compound that had a fluorescence intensity greater than that which could be attributed to the total α-tocopherol in the preparations. We also observed (data not shown) that this compound co-eluted with α-tocopherol on a 10 μm particle size 250 mm × 4.6 mm ODS HPLC column. In subsequent experiments we employed a 250 mm × 4.6 mm HPLC column packed with 5 μm particle size Dupont Zorbax ODS, which effectively separated α-tocopherol from the interfering compound (designated as "x") as shown in Figure 3. In this experiment, rats fed the diet supplemented with vitamin E were administered [^{14}C]α-tocopherol acetate by gavage to label liver microsomes *in vivo*. The microsomes prepared from these animals wre subjected to NADPH-dependent lipid peroxidation in the presence or absence of GSH and the results are also presented in the figure. Prior to initiating lipid peroxidation, >98% of the radioactivity in the microsomes was associated with α-tocopherol and the remainder eluted in a peak immediately before α-tocopherol quinone. In the absence of GSH, most of the radioactivity associated with α-tocopherol had disappeared within a 30 min interval of peroxidation. In the presence of 5 mM GSH, however, approximately 50% of the radioactivity remained associated with α-tocopherol after 30 min. No radioactivity was recovered in the peak corresponding to "X," either in the presence or absence of GSH, demonstrating that this compound was not an oxidation product of α-tocopherol. As the radioactivity associated with α-tocopherol decreased during lipid peroxidation, however, there was an increase in radioactivity that eluted in a peak close to that of α-tocopherol quinone. We did not identify this compound, but were able to confirm that it was not α-tocopherol quinone by its failure to co-elute on HPLC with the authentic compound. Additionally, a net loss of hexane extractable radioactivity was observed as a result of peroxidation, indicating that

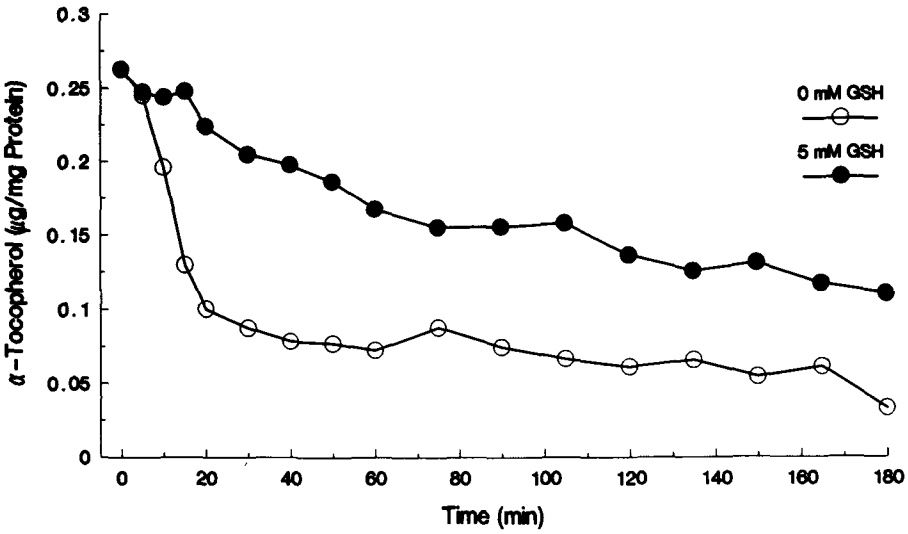

FIG. 4. α-Tocopherol concentration in rat liver microsomes undergoing NADPH-Fe^{++}-dependent lipid peroxidation in the presence (5 mM) and absence of added GSH. Aliquots (1 ml) containing approximately 1.6 mg of microsomal protein were removed from the incubation vessels for determination of α-tocopherol. Points on the figure are mean values obtained from six animals. The pooled SEM for these data is 0.018.

the formation of oxidation products could not be identified by the procedures employed. Compound "X" has a UV absorbance maximum at 277 nm (in methanol) and fluorescence excitation and emission maxima of 288 nm and 321 nm, respectively. It is this compound that interfered with our earlier attempts to monitor fluorometrical changes in microsomal α-tocopherol concentration during lipid peroxidation.

The data in Figure 4 illustrate changes in α-tocopherol concentration during lipid peroxidation of liver microsomes from rats supplemented with vitamin E as influenced by the presence or absence of GSH. α-Tocopherol depletion was slowed in the presence of 5 mM GSH in that, after 20 min of peroxidation >85% remained, whereas in the absence of GSH, <40% of the original α-tocopherol remained in the liver microsomes. In this experiment, the disappearance of α-tocopherol during lipid peroxidation was somewhat slower than that observed for the experiment with [^{14}C]α-tocopherol (Fig. 3). The reason for this discrepancy is not precisely known, but may represent differences in the methods employed or perhaps incomplete equilibration with [^{14}C]α-tocopherol of microsomal α-tocopherol pool(s) during the *in vivo* labeling period. Nevertheless, the general trend is similar and shows a strong inhibitory effect of GSH on microsomal α-tocopherol depletion during lipid peroxidation. Interestingly, the rapid formation of TBA-reactive products (Fig. 1) in the absence of GSH occurred at a time when α-tocopherol content was also declining rapidly. In the presence of GSH, TBA-reactive product formation was delayed, as was the decline in α-tocopherol.

DISCUSSION

It has been postulated that vitamin E functions as an antioxidant and undergoes oxidation to prevent the peroxidation of PUFA (20,21). Indeed, research has demonstrated that susceptibility of lipid membranes to peroxidation is directly related to the vitamin E content of the membranes (6,7,15). However, in all tissues studied, the ratio of α-tocopherol to peroxidizable PUFA is low (16). The large molar excess of PUFA to α-tocopherol suggests that membrane associated α-tocopherol functions very efficiently as an antioxidant, and that perhaps some means of regeneration exists at the cellular level.

Since Tappel (22) originally suggested that vitamin C, in concert with GSH, could regenerate vitamin E from its oxidized forms, several groups have examined interrelationships among these compounds. Packer *et al.* (23) and Niki *et al.* (24) demonstrated that in homogeneous solution, vitamin C could regenerate vitamin E from the vitamin E radical. Niki *et al.* (24) also showed that GSH in homogeneous solution could regenerate vitamin E from the vitamin E radical. Other investigations using model membrane systems revealed that although vitamin C alone could not inhibit lipid peroxidation, it did act synergistically with vitamin E to extend its inhibitory effects (25-28). A recent report by Liebler *et al.* (28) showed that unless α-tocopherol was presnt at a threshold level, ascorbate would act as a prooxidant. Furthermore, these investigators demonstrated that GSH functioned only as a prooxidant and was unable to extend the antioxidant effects of α-tocopherol in a liposomal system. This observation, in light of the research reported by Niki *et al.* (24), suggests that although GSH can regenerate vitamin E in a homogenous solution it may require an enzyme or enzyme system to accommodate this process with membrane associated vitamin E. It is also relevant to indicate that cytosolic concentrations favor GSH rather than ascorbic acid as a better reducing agent *in vivo* (29).

The NADPH-independent liver microsomal lipid peroxidation system employed in the present experiments demonstrated a GSH-vitamin E dependent lag in peroxidation. Previously this effect was shown to be associated with a heat labile factor or factors (4). The present study has demonstrated that although GSH is oxidized to GSSG during lipid peroxidation, it is not a limiting factor in its inhibitory effect. We also found that in the

absence of GSH, microsomal α-tocopherol concentration was rapidly decreased at a time when TBA-reactive product formation was increased. In the presence of 5 mM GSH, however, the decline in α-tocopherol concentration was markedly reduced. Murphy and Kehrer (30), using an ADP/ascorbate system to initiate lipid peroxidation in rat liver microsomes *in vitro,* have previously demonstrated an inhibitory effect of 1 mM GSH on α-tocopherol depletion. These results suggest the possibility that GSH through a heat labile factor(s) either delays the oxidation of α-tocopherol or functions to regenerate it from its oxidized forms.

Haenen and Bast (31) have reported that the GSH dependent, heat labile factor is inactivated during peroxidation and that the duration of the lag is dependent upon the concentration of GSH. Thes investigators also proposed that the factor prevented peroxidation by inhibiting radical formation and found no direct evidence for vitamin E dependence. It was suggested by Haenen and Bast (31) that vitamin E deficient rats lack this activity because the factor is inactivated *in vivo.* It should be noted, however, that their experiments were conducted using animals that were adequate in vitamin E nutrition. Hill and Burk (32) have reported that GSH produced a lag in peroxidation with liver microsomes prepared from rats deficient in vitamin E. These investigators suggested the presence of a GSH dependent, radical scavenging protein that was independent of the presnce of vitamin E.

Previous reports from our laboratories (4,5) suggested the existence of a GSH dependent, heat labile factor that regenerates vitamin E from its oxidized forms, allowing it to extend its antioxidant property in a system undergoing lipid peroxidation. Hill and Burk (32) observed a GSH dependent lag in lipid peroxidation of approximately 3 min in microsomes prepared from vitamin E deficient rats, which was significantly shorter than the lag they observed for microsomes prepared from rats fed vitamin E. Our results indicate that 5 mM GSH produced a >30 min lag in peroxidation with liver microsomes from rats supplemented with vitamin E and no lag with vitamin E deficient microsomes. In the latter case, GSH actually exhibited a prooxidant effect. We have observed during the course of our investigations that the extent of the GSH dependent lag in lipid peroxidation is directly related to the vitamin E (α-tocopherol) content of the microsomes. It is only when the vitamin E content of rat liver microsomes is essentially zero that the GSH dependent lag in peroxidation will be absent.

A recent report by McCay *et al.* (33) lends additional support to the existence of a GSH dependent factor that regenerates vitamin E during lipid peroxidation. These investigators have shown that vitamin E can be incorporated into vitamin E deficient microsomes and is able to restore the GSH dependent lag in peroxidation. This finding casts doubt on the suggestion of Haenen and Bast (31) that the GSH dependent factor is inactive in vitamin E deficient microsomes. Furthermore, McCay *et al.* (33) have indicated (by employing ESR techniques) that the liver microsomal factor and GSH will reduce the α-tocopherol radical. This factor has not been identified but it is possible that it may be capable of reducing other radical species. In this event, the GSH dependent factor could have far reaching implications in the prevention of free radical chain reactions in biological systems.

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Detergent Induced Changes in Serum Lipid Composition in Rats

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The influence of *in vivo* administration of detergents on serum lipid composition was studied in rats. Male Wistar rats received 50 mg Emulgen 913 (polyoxyethylene nonylphenylether, a nonionic detergent) or SDS (sodium dodecylsulfate, an anionic detergent) per kg of body weight intraperitoneally for 3 consecutive days. Emulgen 913 and SDS administration increased the level of cholesterol esters and phospholipids, respectively. But Emulgen 913 administration reduced the level of triglycerides in the serum, and SDS administration reduced also the levels of triglycerides and cholesterol esters. In spite of the changes in serum lipid composition, the administration of these detergents did not affect the amount of total lipids in rat serum. The proportion of palmitic, oleic, and docosahexaenoic acids in phospholipids was decreased by the administration of Emulgen 913 while the level of arachidonic acid was raised. However, the level SDS administration had no effect on the fatty acid composition of the serum phospholipids. On the other hand, both Emulgen 913 and SDS administration showed an effect on the fatty acid composition of triglycerides. It is postulated that liver damage due to administration of detergents is responsible for the changes in serum lipid and fatty acid composition in detergent-treated rats.
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Detergents have been shown to possess stimulatory or inhibitory effects on various enzymatic reactions (1–4). In previous papers (5,6), we reported on the *in vitro* and *in vivo* effects of Emulgen 913 (polyoxyethylene nonylphenylether, a nonionic detergent) and SDS (sodium dodecylsulfate, an anionic detergent), the most common synthetic detergents, on the hepatic microsomal cytochrome P-450 system in rats and frogs. It was demonstrated that the hepatic microsomal cytochrome P-450-dependent fatty acid hydroxylase system from different sources did not show the same response to the detergents, and that cytochrome P-450 content in liver microsomes was significantly decreased, to 70–85% of control, by the administration of Emulgen 913 or SDS to rats. On the other hand, it has also been reported that detergents such as Tween 20, Triton WR-1339 and fatty acid derivatives of aminimides (the detergent aminimides) possess hyperlipidemic or hypolipidemic activity in animals (7–10). However, little information is available concerning the *in vivo* effect of Emulgen 913 and SDS on lipid metabolism in animals. Recently much attention has been paid to water pollution by detergents as they are released from factories and household laundries (11). Considering the effects of detergents on the metabolism in living organisms, we decided to focus our attention on how detergents may affect lipid metabolism in animals. We describe here the effects of *in vivo* administration of Emulgen 913 and SDS on serum lipid composition in rats. The results obtained were compared to those reported for Triton WR-1339.

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Abbreviations: B.W., body weight; CE, cholesterol ester; Cho, cholesterol; FFA, free fatty acid; GLC, gas-liquid chromatography; LCAT, lecithin-cholesterol acyltransferase; MS, microsomal; PL, phospholipid; SDS, sodium dodecylsulfate; TG, triglyceride.

MATERIALS AND METHODS

Chemicals. Emulgen 913 was kindly supplied by Kao-Atlas Co. (Tokyo, Japan). SDS and silica gel were purchased from Wako Pure Chemical Industries Ltd. (Tokyo, Japan). SDS had a purity of more than 98%. Standard lipids (cholesterol, oleic acid, triolein and cholesteryl oleate) used as reference compounds for thin-layer chromatography were obtained from Sigma Chemical Company (St. Louis, MO). Organic solvents were reagent grade and freshly distilled before use.

Treatment of animals. Male Wistar Imamichi rats (6 weeks, 140 ~ 150 g) were obtained from Imamichi Institute for Animal Reproduction (Ohmiya-shi, Japan) and housed in the same environmentally controlled animal room (24 ± 1°C; 50 ± 10% humidity; 12 hr light cycles). Rats were randomly assigned to 3 groups each containing 10 animals. Each group was allowed free access to tap-water and was fed *ad libitum* on the standard diet (CE-2 from Clea Inc., Tokyo, Japan) containing 24.9% proteins, 4.5% lipids, 50.2% carbohydrates, 7.5% minerals, 4.1% cellulose, 1.0% vitamin mix and 7.8% water. Detergent-treated animals received 50 mg Emulgen 913 or SDS per kg of body weight, dissolved in 0.9% saline, intraperitoneally for 3 consecutive days. Control animals received intraperitoneal saline injections at the same time. The amount of food consumed by animals was comparable for all groups and there was no significant difference between control and detergent-treated rats in the body weight of the animals at the end of the experiments. The animals were starved for 24 hr after the last administration. Ca. 4 ml of blood was withdrawn from the retro-ocular plexes of each animal in each group 24 hr after the last dose of detergents or 0.9% saline. Blood samples were centrifuged and serum was separated. The animals were killed by exsanguination, and the livers were perfused *in situ* with ice-cold 0.9% saline and excised. Liver microsomes were prepared as described previously (5). The microsomes were resuspended in 0.25M sucrose solution and stored at −80°C. The protein concentrations of microsomal suspensions were determined by the method of Lowry *et al.* (12) and microsomal content of cytochrome P-450 was determined by the method of Omura and Sato (13). All experiments were done in duplicate.

Assay for laurate hydroxylation and aminopyrine N-demethylation. The hydroxylation of laurate was assayed by the method described previously (14,15). Aminopyrine N-demethylation activity was measured by determining formaldehyde production using the Nash method (16).

Extraction of the total lipids and separation of lipid fractions by column chromatography and thin-layer chromatography. Total lipids were extracted from the serum or from liver microsomes by the method of Folch *et al.* (17). The lipids were separated into major classes (neutral lipids and phospholipids) by passage through a silicic acid column (1 × 15 cm column, silica gel). Neutral lipids were eluted with diethyl ether while phospholipids were eluted with chloroform/methanol (2:1, v/v) and then with methanol. Recovery of lipids from the column was essentially quantitative. Relative neutral lipid composition was determined by thin-layer chromatography, as described by Kaneko *et al.* (18). Silica gel sintered rods (Chromarods, 0.9 × 150 mm) and a hydrogen flame ionization detector (Iatroscan TH-10 TLC-FID analyzer, Iatron Lab., Tokyo) were used. The solvent system was n-hexane/diethyl ether/acetic acid (80:20:2, v/v/v).

Gas-liquid chromatographic analysis. A portion of the separated phospholipids was subjected to methanolysis by heating with 3% HCl in methanol at 100°C for 3 hr. The fatty acid methyl esters were extracted three times with n-hexane. For analysis of the fatty acid composition of each neutral lipid fraction, separation was achieved by preparative thin-layer chromatography as described previously (19), except that the solvent system was n-hexane/diethyl ether/acetic acid (60:40:1, v/v/v). Separated cholesterol esters, triglycerides and free fatty acids were subjected to methanolysis as described above. The fatty acid methyl esters obtained by transesterification from the lipid fractions (cholesterol esters, triglycerides, free fatty acids and phospholipids) were routinely analyzed by gas-liquid chromatography (GLC) on a Shimadzu Model GC-R1A gas chromatograph equipped with dual flame ionization detectors and a Model C-R5A digital integrator. All analyses were carried out on coiled columns (3 mm × 2 m) packed with 15% EGSS-X on Celite 545 (60–80 mesh; Gasukuro Kogyo, Tokyo, Japan) in duplicate. The flow rate of the carrier gas (nitrogen) was 60 ml/min at a column temperature of 190°C. Identification of fatty acid methyl ester peaks was based on the comparison of retention times with known standards. Peak areas from GLC were determined with a digital integrator and the percentage of each acid was calculated from the ratio of the area of its peak to the total area of all peaks.

RESULTS

Body weight of rat and cytochrome P-450 content, and laurate hydroxylation and aminopyrine demethylation in liver microsomes. Table 1 shows the body weight of rats before and after administration of detergents, cytochrome P-450 content, laurate hydroxylation activity, and aminopyrine (AP) demethylation activity in the liver microsomes of control and detergent-treated rats. There was no significant difference in body weights between control and detergent-treated rats. However, the cytochrome P-450 content, laurate hydroxylation activity and AP demethylation activity in liver microsomes decreased significantly in detergent-treated rats except for AP demethylation activity in SDS-treated rats. A similar result was reported in a previous paper (6).

Lipid composition of the total lipids from rat serum. Table 2 shows the amount of serum total lipids in control and detergent-treated rats and changes in serum lipid composition due to detergent treatment. Several points should be noted: there was no significant difference between control and detergent-treated rats in the amount of serum total lipids. The phospholipid level in serum was unchanged in Emulgen 913-treated rats, but the triglyceride level decreased significantly while the cholesterol ester level was elevated in the same rats. On the other hand, the level of serum phospholipids was increased to 158% of controls by the administration of SDS, while serum triglyceride and cholesterol ester levels were reduced to 77% and 79% of the corresponding controls, respectively.

Fatty acid composition of lipid fractions. Since it was found that the detergent administration to rats could affect the serum lipid composition, the effect on the fatty acid composition of lipid fractions in rat serum was also examined. Table 3 shows the fatty acid composition of the serum phospholipids. The proportion of palmitic, oleic, and docosahexaenoic acids was decreased by the administration of Emulgen 913 while that of arachidonic acid was raised. The administration of SDS had no effect on the fatty acid composition of the serum phospholipids. Table 4 shows the fatty acid composition of the serum triglycerides. There was a decline in the percentage of palmitic acid and a rise in the percentage of arachidonic acid in Emulgen 913-treated rats. Although the percentage of oleic and arachidonic acids was also elevated in SDS-treated rats, that of docosahexaenoic acid was decreased. On the other hand, the administration of Emulgen 913 and SDS did not affect the fatty acid composition of the serum free fatty acids and cholesterol esters (data not shown).

DISCUSSION

Although several studies on the effect of detergents, other than Emulgen 913 and SDS, on serum lipid composition have been reported in recent years (7–10), little information is available concerning the effect of *in vivo* administration of Emulgen 913 and SDS on serum lipid composition. In this present study, we demonstrated several characteristic effects of Emulgen 913 and SDS on serum lipid composition in rats. First, our results demonstrated that Emulgen 913 and SDS administration increased both the levels of cholesterol esters and phospholipids. In contrast to the increase in the cholesterol ester and phospholipid levels, Emulgen 913 administration reduced the level of triglycerides in serum, and SDS administration reduced also the level of triglycerides and cholesterol esters. Moreover, it was demonstrated that administration of the detergents did not affect the amount of the total lipids in serum, in spite of the changes in serum lipid composition. It has been reported that Triton WR-1339 (polyoxyethylene ether of alkyl phenol, a nonionic detergent) is a hyperlipidemic agent in animals; the levels of all the lipid fractions (cholesterol, cholesterol esters, triglycerides and phospholipids) in serum were elevated and the serum lipid composition was changed by Triton WR-1339 administration (9,20).

Secondly, *in vivo* administration of Emulgen 913 and SDS showed also different effects on the fatty acid composition of lipid fractions in rat serum, compared to those of Triton WR-1339 (20). Although Triton WR-1339

TABLE 1

Changes in Body Weight (B.W.), Cytochrome P-450 Content, Laurate Hydroxylation and Aminopyrine (AP) Demethylation Activities by Acute Emulgen 913 and Sodium Dodecylsulfate Treatment

Dose of detergent (mg/kg B.W.)		Body weight (g)		Cytochrome P-450 (nmol/mg MS protein)	Laurate hydroxylation (nmol products/MS protein/15 min)	AP demethylation (nmol products/ MS protein/15 min)
0 (control)		142 ± 2[a]	147 ± 5[b]	1.02 ± 0.11 (100)	59.2 ± 3.0 (100)	37.0 ± 5.0 (100)
Emulgen 913	50	142 ± 3	148 ± 7	0.85 ± 0.06 (83)[c]	47.2 ± 3.4 (80[c]	26.0 ± 4.0 (70)[c]
SDS	50	145 ± 3	142 ± 6	0.72 ± 0.04 (71)[c]	38.9 ± 7.0 (66)[c]	35.2 ± 6.0 (95)

[a]Body weight before administration.

[b]Body weight at the end of experiments.

Each value represents the mean ± S.D. for 10 animals. The numbers in parentheses are percent to corresponding controls. Significance ([c]$p < 0.001$) of the difference between means of control and detergent-treated animals was determined by standard Student's t-test.

TABLE 2

Changes in Serum Lipid Composition by Acute Emulgen 913 and Sodium Dodecylsulfate Treatment

Dose of detergent (mg/kg B.W.)		Total lipids (mg/ml serum)	Lipid composition (mg/ml serum)				
			PL	Cho	FFA	TG	CE
0 (Control)		3.1 ± 0.5	0.85 ± 0.10 (100)	0.19 ± 0.05	0.12 ± 0.04	0.77 ± 0.14 (100)	1.02 ± 0.10 (100)
Emulgen 913	50	3.2 ± 0.4	0.86 ± 0.05	0.23 ± 0.03	0.13 ± 0.02	0.64 ± 0.10 (83)[a]	1.23 ± 0.14 (121)[b]
SDS	50	3.2 ± 0.6	1.34 ± 0.07 (158)[c]	0.19 ± 0.03	0.12 ± 0.02	0.59 ± 0.05 (77)[b]	0.81 ± 0.06 (79)[c]

Each value represents the mean ± S.D. for 10 animals. The numbers in parentheses are percent to corresponding controls. Significantly different from control animals; [a]$p < 0.05$; [b]$p < 0.01$; [c]$p < 0.001$.

TABLE 3

Fatty Acid Composition of Serum Phospholipids of Control and Detergent-treated Rats

Fatty acid	Control	Dose of detergent (mg/kg B.W.)	
		Emulgen 913 (50)	SDS (50)
14:0	trace	0.2 ± 0.1	0.4 ± 0.2
16:0	26.8 ± 1.9	25.3 ± 1.1[a]	28.2 ± 1.7
16:1	trace	—	—
18:0	21.2 ± 1.0	22.2 ± 1.6	21.7 ± 1.2
18:1	7.7 ± 1.0	6.7 ± 0.5[b]	8.4 ± 1.5
18:2	14.0 ± 1.2	14.0 ± 1.0	13.2 ± 1.5
20:4	14.9 ± 2.7	18.0 ± 2.4[b]	16.9 ± 2.6
22:5	2.8 ± 0.3	2.5 ± 0.5	2.4 ± 0.4
22:6	7.7 ± 0.9	6.2 ± 0.4[c]	7.0 ± 0.7

Expressed as a percentage of the total fatty acids. Each value represents the mean ± S.D. for 10 animals. Fatty acids are designated as number of carbon atoms:number of double bonds. Significantly different from control animals; [a]$p < 0.05$; [b]$p < 0.02$; [c]$p < 0.001$.

TABLE 4

Fatty Acid Composition of Serum Triglycerides of Control and Detergent-treated Rats

Fatty acid	Control	Dose of detergent (mg/kg B.W.)	
		Emulgen 913 (50)	SDS (50)
14:0	0.7 ± 0.3	0.9 ± 0.4	0.8 ± 0.2
16:0	23.1 ± 3.2	19.3 ± 1.2[a]	21.6 ± 3.2
16:1	4.3 ± 1.2	3.4 ± 1.0	4.5 ± 1.3
18:0	2.8 ± 1.1	3.1 ± 0.8	2.2 ± 0.4
18:1	18.5 ± 1.3	18.5 ± 1.4	21.7 ± 2.6[a]
18:2	31.2 ± 3.5	28.9 ± 2.8	31.6 ± 2.1
20:4	4.7 ± 0.9	6.8 ± 1.5[a]	6.6 ± 1.8[a]
20:5	2.7 ± 0.6	3.3 ± 0.4	2.5 ± 1.0
22:6	12.2 ± 2.2	12.7 ± 2.3	7.3 ± 1.7[b]

Expressed as a percentage of the total fatty acids. Each value represents the mean ± S.D. for 10 animals. Fatty acids are designated as number of carbon atoms:number of double bonds. Significantly different from control animals; [a]$p < 0.01$; [b]$p < 0.001$.

increased the percentage of unsaturated fatty acids (oleic, linoleic and arachidonic acids) in the phospholipid fraction 24 hr after administration, Emulgen 913 administration increased only the percentage of arachidonic acid in the serum phospholipids and rather reduced the levels of other unsaturated fatty acids (oleic and docosahexaenoic acids). On the other hand, SDS administration had no effect on the fatty acid composition of the serum phospholipids. All Triton WR-1339, Emulgen 913 and SDS administration caused an increase in the percentage of arachidonic acid in the serum triglycerides, but the effect of these detergents on the percentage of other fatty acids such as palmitic, linoleic and docosahexaenoic acids was quite different (Table 3) (20). Moreover, Triton WR-1339 administration caused changes in the fatty acid composition of the serum cholesterol esters, but Emulgen 913 and SDS administration had no effect. Thus, our results demonstrated that the effect of Emulgen 913 and SDS was quite different from that of Triton WR-1339 on serum lipid and fatty acid compositions in rats.

Orbán *et al.* reported that altered fatty acid composition induced by Triton WR-1339 is presumably due to inhibited lecithin-cholesterol acyltransferase (LCAT) activity (20). However, the mechanisms by which Emulgen 913 and SDS altered fatty acid composition in serum lipid components is not known. It could be possible that differences in food intake between control and detergent-treated rats could affect the serum and fatty acid composition in rats. However, this possibility can be eliminated because food consumption by the animals was quite similar for control and detergent-treated groups, and all animals had similar weights at the end of the experiments (Table 1). There are several reports concerning the accumulation, degradation and toxicity of metabolites from alkylsulfates and nonionic detergents in liver (11,21,22). We have demonstrated previously, and also here, that the cytochrome P-450 content, laurate hydroxylation activity and AP demethylation activity decreased in detergent-treated rats (6) (Table 1). Therefore, it may be postulated that liver damage, followed by some metabolic changes in rat livers due to the detergents, is responsible for the changes in serum lipid and fatty acid compositions in detergent-treated animals.

Since Emulgen 913 and Triton WR-1339 are both nonionic detergents and their structures are very similar, it is noteworthy that administration of these detergents showed quite different effects on serum lipid content and composition. Although we assumed that the differences between our data and those reported by Orbán *et al.* (20) are mainly due to a decrease in the liver functions mentioned above, the differences may also in part be due to the different rat strains used (Wistar strain in this present study, and CFY strain in their study). Further experimental work is needed to elucidate the mechanism of the changes in serum lipid composition in rats due to the administration of Emulgen 913 and SDS.

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Effect of L-Triiodothyronine on Δ9 Desaturase Activity in Liver Microsomes of Male Rats

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Male rats injected with a single saturating dose of L-triiodothyronine (T_3) showed, after a lag time of approximately eight hr, a sharp rise in Δ9 desaturase activity. Desaturase activity reached a plateau which was 1–1.2 times above the base line levels of rats which were not hormone-treated. The plateau was maintained for five days in animals which were kept on daily hormone-treatment. The increase in Δ9 desaturase activity by T_3 required ongoing protein synthesis, because the increase in enzymatic activity due to hormone treatment was completely abolished in the presence of cycloheximide. These findings suggest that cycloheximide may block the induction of Δ9 desaturase by T_3 and/or inhibit the synthesis of protein(s) essential to the desaturation-response to T_3. Modifications observed in liver microsomal fatty acid composition in T_3 treated rats were independent of the effect on desaturation. It is suggested that other factors, such as diet, membrane lipid synthesis and degradation, as well as fatty acid turnover and oxidation, could be involved in affecting the fatty acid composition of thyroid hormone-treated rats.
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The activity of liver microsomal Δ9 desaturase, which is responsible for the conversion of palmitic and stearic acid into palmitoleic and oleic acid, respectively, can be modulated by both hormonal (1–4) and dietary factors (5–10). Starvation causes a dramatic decrease in the enzyme activity that can be recovered by refeeding (6). On the other hand, carbohydrate-rich (5,7,11) and fat-free (11,12) diets (lipogenic diets) lead to increased activity compared to normal chow-fed animals. The state of thyroid activity of the rat has profound effects on the activity of this membrane-bound enzyme. Thyroidectomized animals fed a normal chow diet have a reduced level of Δ9 desaturase activity, which is restored by triiodothyronine administration (13). In thyroidectomized rats fed a low-iodine diet (14) and in propyl thiouracil (hypothyroid agent) treated animals (2), the enzymatic activity remains unchanged. On the other hand, thyroid hormone-treated rats have increased levels of enzymatic activity when compared to untreated animals (2,4,15). However, in all these studies, thyroid hormones were administered for several days or even weeks before changes were observed.

Δ9 Desaturase behaves similarly to a group of enzymes involved in lipogenesis (lipogenic enzymes), such as fatty acid synthetase, acetyl-CoA carboxylase, and enzymes essential for the production of necessary cofactors to support the process as their activity is induced by thyroid hormones and lipogenic diets (16,17). The changes in enzyme activity due to thyroid or dietary conditions have been shown to be due to changes in the amount of the enzyme under study (16,17).

The aim of the present investigation was to study the time course of the Δ9 desaturase activity as it is increased by triiodothyronine and the significance of ongoing protein synthesis in the process. Liver microsomal Δ9 desaturase activity was, therefore, measured at different time points after triiodothyronine treatment as well as in animals which were injected with triiodothyronine plus cycloheximide after having received daily injections of cycloheximide for two days prior to the experiments. We also measured microsomal lipid fatty acid composition, as it can reflect early changes in fatty acid desaturation.

MATERIALS AND METHODS

Material. [1-^{14}C]Palmitic acid (53.8 mCi/mmol, 99% radiopurity) and [1-^{14}C]stearic acid (56 mCi/mmol, 97.4% radiopurity) were purchased from Amersham International (Buckinghamshire, England). Cofactors used for the enzymatic reactions were provided by Sigma Chemical Co. (St. Louis, MO) and unlabeled fatty acids were from Nu-Chek Prep, Inc. (Elysian, MN). L-Triiodothyronine was a gift from Glaxo Laboratories (Munro, Argentina). The fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH) was purchased from Aldrich Chemical Co. (Milwaukee, WI).

Animals. Male Wistar rats of 60–70 days of age weighing 180–200 g were maintained on a commercial standard pellet diet (Nutrimento rat chow 3, Escobar, Argentina) and tap water *ad libitum.* The diet contained 4.0% of total lipid with a fatty acid composition of 16.7% 16:0 (palmitic acid), 0.8% 16:1 (palmitoleic acid), 4.9% 18:0 (stearic acid), 21.8% 18:1 (oleic acid), 52.4% 18:2 n-6 (linoleic acid) and 4.3% 18:3 n-3 (α-linolenic acid). All animals were subjected to a daily light-period of 12 hr, and then to 12 hr of darkness. Rats received a daily intraperitoneal injection of 1000 μg T_3/100 g body weight dissolved in 4 mM NaOH saline solution. The hormone dose used in all our experiments is, according to Oppenheimer *et al.* (18), a saturating nuclear T_3 receptor dose. Cycloheximide dissolved in saline solution was injected intraperitoneally, alone or with triiodothyronine, at doses of 20 μg, 60 μg and 90 μg/100 g body weight. Two control groups were used: one received a daily intraperitoneal injection of an equivalent volume of the whole vehicle and the other received only saline solution. There was no difference between the two groups in desaturation activity or fatty acid composition. When the time course of the T_3 effect was studied, pairs of T_3 hormone-treated and control rat groups were sacrified at different times

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Abbreviations used: DPH, 1,6-diphenyl-1,3,5-hexatriene; T_3, L-triiodothyronine.

after T_3 administration. In the rest of the experiments, 24 hr after the final administration, the animals were killed by decapitation at eight a.m. to equalize circadian effects (19). Liver microsomes were isolated as described previously (20).

Enzymatic assays. The Δ9 (16:0 → 16:1 and 18:0 → 18:1) desaturase enzyme assays were carried out by measuring the conversion of the [1-^{14}C]-labeled fatty acid substrate to the corresponding [1-^{14}C]-labeled fatty acid product. Reactions were initiated by adding 4 mg of microsomal protein to preincubated flasks containing 5 nmol [1-^{14}C]-labeled acid and 45 nmol unlabeled acid plus the necessary cofactors in a final volume of 1.6 ml according to procedures described previously (20). Palmitic and stearic acids were used as substrates. Microsomal protein was determined by the Lowry procedure using crystalline bovine serum albumin as standard (21). Fatty acid methyl esters were obtained by saponification of the incubation mixture (at 82°C for 45 min under N_2), acidification, extraction with petroleum ether (b.p. 30-40°C under N_2) and esterification with methanolic 3 N HCl (at 62°C for one hr). Radioactive fatty acid methyl esters were separated and quantified by gas-liquid radiochromatography using an Acromat CG-100 equipped with a Packard proportional counter Model 893. The column was packed with 10% SP 2330 on Chromosorb WAW 100-120 mesh.

Fatty acid analysis. Methyl esters were prepared from total microsomes as described previously (20) and analyzed by gas-liquid chromatography in a Hewlett-Packard 5840A gas-chromatograph with a terminal computer integrator system using a 6-ft column filled with 10% SP 2330 on 100/120 mesh Chromosorb WAW.

Fluorescence anisotropy. An Aminco-Bowman Spectrofluorometer equipped with two Glan Thompson polarizers was used for fluorescence measurements. The polarization ratio P is defined as $I_{||}/I_{\perp}$. G. $I_{||}$ and $I_{\perp}$ are referred to intensities emitted parallel and perpendicular, respectively, to the vertically polarized excitation. G is a factor for instrument correction (22).

Excitation wavelengths of 360 nm and emission wavelengths of 430 nm were used for fluorescence polarization of DPH. A 2.0 M $NaNO_2$ solution placed between the emission monochromator and photomultipler was used as a cut-off filter for the wavelengths below 390 nm. Light scattering was less than 5%. Unlabeled samples at the same concentrations as those of the labeled samples were used as reference blanks to correct for excitant light-scattering and other contributions to the fluorescence signal.

The steady-state fluorescence anisotropy (r_s) of DPH was calculated using the equation $r_s = P-1/P+2$, where P is the polarization ratio defined previously.

Temperature was monitored by a thermocouple which was inserted into the cuvette; experiments were done at 32°C. Fluorescence quenching and uncorrected emission spectra were measured in 10 × 10 cuvettes. Corrections by the inner filter-effect never exceeded 10% of the fluorescence intensity.

Microsomes (final concentration 50 μg of protein/ml) suspended in 0.25 M sucrose and 50 mM potassium phosphate buffer, pH 7 were labeled by adding 3-5 μl of DPH in tetrahydrofuran. The concentration of DPH in stock solution was determined in hexane (23). The molar ratio of DPH to lipids was about 1:300.

Statistical analysis. All results were tested for statistical significance by Student's *t* test for paired comparison with control.

RESULTS

Time course of Δ9 desaturase activity increased by T_3. Δ9 Desaturase activity was measured in hepatic microsomes of rats given a single receptor-saturating dose of 1000 μg T_3/100 g body weight (18). Animals were killed in groups of four at sequential time points after hormone injection. The activity of each point was expressed as a percentage of its own time control because Δ9 desaturase activity has a circadian rhythm (Fig. 1). We observed that the T_3-induced rise in enzymatic activity was preceded by a lag phase of eight hr. The initial delay was followed by a sharp rise in Δ9 desaturase activity that reached a plateau within one hr (nine

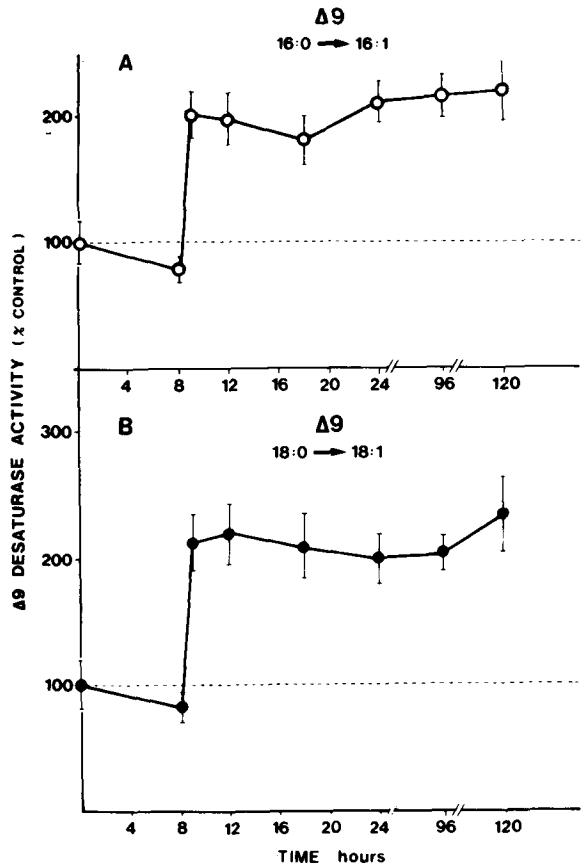

FIG. 1. Time course of the Δ9 desaturase activity increase caused by T_3. Enzymatic activity was measured in hepatic microsomes of rats given a single receptor-saturating dose of 1000 μg T_3/100 g body weight and then killed groups of four at sequential time points after hormone injection. Groups killed at 96 and 120 hr were treated daily with the same hormonal dose. Controls were also killed at each time point to eliminate circadian effects and results are expressed as the mean ± S.E. of the group referred to as percent of its control. Mean ± S.E. control Δ9 (16:0) and Δ9 (18:0) activities of zero time was 0.072 ± 0.006 (n=10) and 0.082 ± 0.006 (n=10) nmol.desat.mg^{-1}.min^{-1}., respectively. Palmitic (A) and stearic (B) acids were used as substrates in the incubations. Significantly different from control after nine hr of T_3 treatment: P<0.01.

hr after T_3 injection). The plateau was maintained for five days when the animals were kept on daily hormone treatment. There was a 1-1.2-fold increase above the base level of Δ9 desaturase activity after T_3 stimulation compared to base-line control value.

Effect of cycloheximide on Δ9 desaturase activity increased by T_3. As the increase in lipogenic enzyme activities by thyroid hormones is due to changes in the amount of the enzymes (16,17), we tested whether ongoing protein synthesis was necessary for the increase in Δ9 desaturase activity due to T_3. Nine hr after T_3 injection, Δ9 desaturase activity reached a plateau that was maintained over five days of daily hormone treatment (Fig. 1). In order to clarify possible problems arising from the comparison of enzyme specific activity during non-steady state conditions, we decided to examine this parameter under Δ9 desaturase steady-state conditions. Consequently, rats were administered different doses of cycloheximide (20, 60 or 90 μg/100 g body weight per day) intraperitoneally during the first two days, and on the third day with the same cycloheximide dose plus a single saturating dose of 1000 μg T_3/100 g body weight. Another group received intraperitoneally a single saturating dose of 1000 μg T_3/100 g body weight. The animals of all groups were sacrificed 24 hr after the last administration. Gellhorn and Benjamin (24) have shown that the half life of Δ9 desaturase is approximately four hr. This represents at least 12 half-lives during the first two days of cycloheximide treatment and six half-lives for the enhanced T_3 state. Therefore, these treatments should be long enough to re-establish steady states for both conditions, the inhibited (cycloheximide treatment) and the increased (T_3 treatment). We found that cycloheximide given at a dose of 60 (Fig. 2) and 90 μg/100 g body weight (results not shown) prevented induction of Δ9 desaturase activity by T_3. On the other hand, at a dose of 20 μg cycloheximide/100 g body weight, the increase in enzymatic activity still occurred. Results showed that the increase in Δ9 desaturase activity caused by T_3 required ongoing protein synthesis.

Fatty acid composition of total microsomal hepatic lipids of male rats treated with T_3 or cycloheximide. Table 1 shows the effect of a single saturating dose of 1000 μg T_3/100 g body weight, of 60 μg cycloheximide/100 g body weight administered daily for three days, and of an injection of T_3 plus cycloheximide in rats treated with the same cycloheximide dose for two days. T_3 caused an increase in 18:0 that was independent of Δ9 desaturase activity since it is raised by thyroid hormones (2,4,15) (Fig. 1). This could be due to the increase in *de novo* fatty acid synthesis produced by the increased activity of acetyl-CoA carboxylase (25-28), fatty acid synthetase (15,25-29,30) and especially by chain elongation (30,31). On the other hand, in the n-6 fatty acid family there was a decrease in 18:2 and 20:3 and an increase in 20:4. These results in liver microsomal fatty acid composition are in agreement with those reported with different doses and duration of thyroid hormone treatment in liver, plasma and heart (2,4,32,33).

The fatty acid composition of the liver microsomes remained, in general, unchanged after cycloheximide treatment. However, in the T_3 plus cycloheximide-treated animals we observe the same modification in fatty acid composition as in those rats given only T_3. This occurred in spite of their different enzymatic activities (Fig. 2).

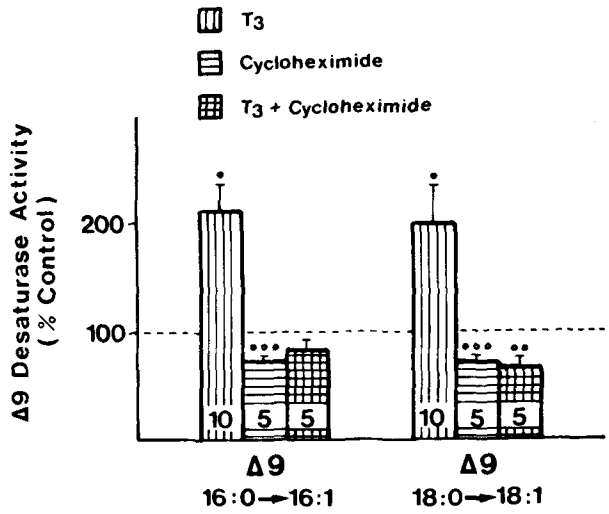

FIG. 2. Effect of cycloheximide on the Δ9 desaturase activity increase caused by T_3. One group of animals received the whole vehicle solution for two days, and on the third day a single saturating dose of 1000 μg T_3/100 g body weight intraperitoneally (▥); another group, a daily injection of 60 μg cycloheximide/100 g body weight intraperitoneally for three days (▤); and the last group, a single injection of 1000 μg T_3 plus 60 μg cycloheximide/100 g body weight intraperitoneally in rats injected the previous two days with the same daily dose of cycloheximide (▦). Control rats received the whole vehicle solution. Animals were sacrificed 24 hr after the final administration. Each bar represents the mean ± S.E. The number of animals of each group is given within the bars. Mean ± S.E. control Δ9 (16:0) and Δ9 (18:0) activities were 0.095 ± 0.010 (n=30) and 0.080 ± 0.006 (n=10) nmol. desat. $mg^{-1}.min^{-1}$, respectively. Significantly different from controls; ∘, P<0.001; ∘∘, P<0.01; ∘∘∘, P<0.05.

DISCUSSION

It has been established that Δ9 desaturase activity is sensitive to thyroid activity. Hypothyroid rats show a reduced enzymatic activity (13), whereas animals made hyperthyroid by the administration of thyroid hormones show an increased Δ9 desaturase activity (2,4,15).

The lag time of 8-9 hr for the increase in Δ9 desaturase activity caused by T_3 administration (Fig. 1) seems to be a response pattern to the hormone. Both the induction by T_3 of mRNA-S11, which codes for a protein of pI 6.1 and an M_r of 22,500, and the induction of the mRNA coding for hepatic malic enzyme were preceded by a lag time of six hr (34-36).

Results using cycloheximide showed that ongoing protein synthesis was required for the Δ9 desaturase activity increase after T_3 treatment, since this increase was abolished by cycloheximide (Fig. 2). These findings suggest that cycloheximide might act by blocking the induction of Δ9 desaturase by T_3 and/or by inhibiting the synthesis of protein/s essential in the desaturation process and the syn-

TABLE 1

Effect of L-Triiodothyronine and Cycloheximide on Liver Microsomal Fatty Acid Composition[a]

Fatty acid %	Control (n = 10)	T_3 (n = 9)	Cycloheximide (n = 5)	T_3 + Cycloheximide (n = 10)
16:0	15.8 ± 0.3	15.3 ± 0.2	15.7 ± 0.5	16.5 ± 0.2
16:1	1.2 ± 0.1	1.0 ± 0.1[e]	0.4 ± 0.1[c]	0.6 ± 0.1[c]
18:0	22.9 ± 0.4	25.0 ± 0.6[d]	24.1 ± 0.3[e]	25.6 ± 0.2[c]
18:1	10.8 ± 0.3	9.8 ± 0.4[e]	10.1 ± 0.1	9.0 ± 0.2[c]
18:2 (n-6)	15.2 ± 0.3	13.9 ± 0.2[d]	16.2 ± 0.3	13.0 ± 0.4[c]
20:3 (n-6)	2.2 ± 0.2	1.4 ± 0.1[d]	2.1 ± 0.1	1.4 ± 0.1[c]
20:4 (n-6)	24.1 ± 0.5	26.9 ± 0.2[c]	23.7 ± 0.4	26.0 ± 0.4[d]
22:5 (n-3)	1.5 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	1.4 ± 0.2
22:6 (n-3)	6.2 ± 0.3	5.6 ± 0.3	6.3 ± 0.2	6.6 ± 0.2
Unsaturation index[b]	1.90 ± 0.02	1.85 ± 0.05	1.88 ± 0.02	1.90 ± 0.02

[a]Fatty acid composition was determined by GLC as described in the Materials and Methods Section. Animals of all groups were injected intraperitoneally at eight a.m. as follows: Control, whole vehicle solution for three days; T_3, whole vehicle solution for two days, and on the third day a single saturating-receptor dose of 1000 μg T_3/100 g body weight; cycloheximide, 60 μg/100 g body weight daily for three days; T_3 + cycloheximide, 60 μg cycloheximide/100 g body weight daily for two days, and on the third day 1000 μg T_3/100 g body weight plus the same daily dose of cycloheximide. Only main fatty acids were considered. Results are shown as the mean ± S.E.

[b]Unsaturation index: Σ number unsaturated mol × number double bonds/Σ number total mol.

[c]Significantly different from controls: P<0.001.

[d]Significantly different from controls: P<0.01.

[e]Significantly different from controls: P<0.05.

thesis of which may have been stimulated by the hormone.

Supporting this view, Thiede and Strittmatter have shown that the increase in Δ9 desaturase activity in animals fed a high carbohydrate and fat-free diet is due to an induction of the enzyme (37). This response mechanism appears to be similar to that of lipogenic enzymes such as acetyl-CoA carboxylase, fatty acid synthetase, glucose 6-phosphate dehydrogenase and malic enzyme. The increment in these enzymatic activities, produced both by T_3 and a rich-carbohydrate diet, is due to an increased amount of these enzymes (16).

There are factors other than enzyme synthesis, such as the fluidity of the microsomal membrane, that regulate the activities of desaturases (membrane-bound enzymes) not only in animals, but also in yeast and Tetrahymena (38). For this reason, we measured the fluorescence anisotropy of microsomal membranes from animals that had received a single saturating dose of T_3 24 hr before. We found no difference between the r_s of T_3 treated (r_s = 0.126 ± 0.001) and that of untreated (r_s = 0.125 ± 0.001) liver microsomes. These results are consistent with the unchanged unsaturation index observed in Table 1. These preliminary data indicate that membrane physicochemical properties were not modified by T_3 administration and, therefore, were not involved in the Δ9 desaturase activity increase due to hormonal treatment.

The modifications observed in liver microsomal fatty acid composition in T_3 treated rats were similar to those reported with other doses of thyroid hormones in studies of lipid fatty acid composition of liver, plasma and heart (2,4,32,33). Although Δ9 desaturase activity was increased by T_3, the level of 18:0 increased instead of decreased. This could be due to the increase in *de novo* fatty acid synthesis noted by others (15,25-31). In the n-6 fatty acid family only the decreased 20:3 might be related to the decrease of Δ6 desaturase reported in T_3 treated rats (2,4), since opposite changes would have been expected in 18:2 and 20:4 [Δ6 desaturase is the rate-limiting enzyme in arachidonate synthesis (11)]. Factors other than desaturation activity, such as diet, membrane lipid synthesis and degradation, fatty acid turnover and oxidation must be involved in the determination of fatty acid composition of thyroid hormone-treated rats.

These results do not permit us to determine whether the mechanism by which T_3 acts on Δ9 desaturase is a direct hormone hepatic effect, an extrahepatic effect or a combination of these. However, our findings do suggest that the increase in Δ9 desaturase activity produced by T_3 requires the ongoing protein synthesis.

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Kinetic Study of the Reaction Between Tocopheroxyl Radical and Unsaturated Fatty Acid Esters in Benzene

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A kinetic study of the reaction between a tocopheroxyl radical and unsaturated fatty acid esters has been undertaken. The rates of allylic hydrogen abstraction from various unsaturated fatty acid esters (ethyl oleate *2*, ethyl linoleate *3*, ethyl linolenate *4*, and ethyl arachidonate *5*) by the tocopheroxyl radical (5,7-diisopropyltocopheroxyl *6*) in benzene have been determined spectrophotometrically. The second-order rate constants, k_3, obtained are 1.04×10^{-5} $M^{-1}s^{-1}$ for *2*, 1.82×10^{-2} $M^{-1}s^{-1}$ for *3*, 3.84×10^{-2} $M^{-1}s^{-1}$ for *4*, and 4.83×10^{-2} $M^{-1}s^{-1}$ for *5* at 25.0°C. Thus, the rate constants, k_{abstr}/H, given on an available hydrogen basis are $k_3/4 = 2.60 \times 10^{-6}$ $M^{-1}s^{-1}$ for *2*, $k_3/2 = 9.10 \times 10^{-3}$ $M^{-1}s^{-1}$ for *3*, $k_3/4 = 9.60 \times 10^{-3}$ $M^{-1}s^{-1}$ for *4*, and $k_3/6 = 8.05 \times 10^{-3} M^{-1}s^{-1}$ for *5*. The k_{abstr}/H values obtained for the polyunsaturated fatty acid esters *3*, *4*, and *5* containing H-atoms activated by two π-electron systems are similar to each other, and are about three orders of magnitude higher than that for the ethyl oleate *2* containing H-atoms activated by a single π-system. From these results, it is suggested that the prooxidant effect of α-tocopherol in edible oils and fats may be induced by the above hydrogen abstraction reaction.
***Lipids* 24, 936-939 (1989).**

It is well known that vitamin E (α-,β-,γ-, and δ-tocopherols) is present in cellular membranes and edible oils and functions as an antioxidant by protecting polyunsaturated lipids or fatty acids from peroxidation. The antioxidant properties of tocopherols have been ascribed to the initial oxidation by a peroxyl radical of the phenolic hydroxyl group, producing a tocopheroxyl radical (reaction [1]), which, in turn, combines with another peroxyl radical (reaction [2]) (1,2).

$$LOO\bullet + TocH \rightarrow LOOH + Toc\bullet$$

$$LOO\bullet + Toc\bullet \rightarrow \text{Non-radical products}$$

On the other hand, several investigators demonstrated that α-tocopherol at high concentrations acts as a prooxidant during the autoxidation of polyunsaturated fatty acids in an aqueous medium and bulk phase (3-9). This prooxidant effect of α-tocopherol leads to an increase of the level of hydroperoxides having conjugated diene structure. Loury *et al.* (3) and Terao *et al.* (9) have proposed that tocopheroxyl radicals participate in this prooxidant effect through the following reactions ([3] and [4]):

$$Toc\bullet + LH \xrightarrow{k_3} TocH + L\bullet \quad [3]$$

$$Toc\bullet + LOOH \xrightarrow{k_{-1}} TocH + LOO\bullet \quad [4]$$

where [3] is the chain transfer reaction and [4] is the reverse reaction of [1]. However, the kinetic studies of reactions [3] and [4] have not been undertaken, as far as we know [the reactions of phenoxyl radicals with some hydrocarbons and their hydroperoxides are very important in determining the kinetics of phenol-inhibited autoxidations and the overall efficiency of the inhibitor. However, there have been relatively few absolute (as opposed to relative) rate constant measurements on these reactions (10-12)]. Therefore, we have recently measured the rate constants, k_{-1}, for reaction of alkyl hydroperoxides (*n*-butyl, *sec*-butyl, and *tert*-butyl hydroperoxides) with the tocopheroxyl radical (5,7-diisopropyl-tocopheroxyl 6) in benzene solution at 25.0°C (13); the rates, k_{-1}, observed were $(1.34\text{-}3.65) \times 10^{-1}$ $M^{-1}s^{-1}$, and were found to be about seven orders of magnitude lower than those, k_1, for the reaction [1] of α-tocopherol with peroxyl radical (1,14).

In the present work, we have measured the second-order rate constant, k_3, for the reaction of fatty acid esters (ethyl stearate 1, ethyl oleate 2, ethyl linoleate 3, ethyl linolenate 4, and ethyl arachidonate 5) with tocopheroxyl radical (5,7-diisopropyl-tocopheroxyl 6) in benzene at 25.0°C (Fig. 1). The rate constant, k_3, for the above reaction has been determined by following the decrease in absorbance at 417 nm of the tocopheroxyl radical 6 (15). As reported in a previous paper, vitamin E radicals (α-,β-,γ-, and δ-tocopheroxyls) are not stable, and thus the stable 5,7-diisopropyltocopheroxyl radical 6 was used for the present work (16,17). The rate, k_3, observed was compared with the rate, k_{-1}, reported for the reaction between tocopheroxyl radical and alkyl hydroperoxides.

MATERIALS AND METHODS

Ethyl stearate 1, ethyl oleate 2, ethyl linoleate 3, ethyl linolenate 4, and ethyl arachidonate 5 (>99%, respectively) were purchased from Sigma Chemical Co. (St. Louis, MO), and used as received. Preparation of 5,7-diisopropyl-tocopherol was reported in a previous paper (13). The 5,7-diisopropyl-tocopheroxyl radical 6 was prepared by the PbO_2 oxidation of corresponding 5,7-diisopropyltocopherol in benzene under nitrogen atmosphere.

The kinetic data were obtained on a JASCO spectrophotometer Model UVIDEC-660 by mixing equal volumes of benzene solutions of tocopheroxyl and fatty acid esters under nitrogen atmosphere. The oxidation rate constant, k_{obsd}, was calculated in the usual way using a standard least-square analysis. All the measurements were performed at 25.0 ± 0.5°C.

RESULTS AND DISCUSSION

The 5,7-diisopropyl-tocopheroxyl 6 is comparatively stable in the absence of unsaturated fatty acid ester and shows absorption peaks at λ_{max} = 417 nm and 397 nm in benzene solution (Fig. 1) (15). By adding a ben-

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zene solution of excess fatty acid esters to a benzene solution of tocopheroxyl, the absorption spectrum of the tocopheroxyl gradually disappears. Figure 1 shows an example of the result of interaction between 5,7-diisopropyl-tocopheroxyl 6 (ca 0.20 mM) and ethyl linoleate 3 (75.0 mM) in benzene. The time dependence of the decrease in absorbance at 417 nm observed when ca 0.34 mM benzene solution of tocopheroxyl is mixed with 150 mM benzene solution of ethyl linoleate 3 (1:1 in volume, final concentration of 3 is 75.0 mM) is shown in Figure 2. Under these conditions, the rate of disappearance of tocopheroxyl in the presence of a constant concentration of ethyl linoleate 3 is accurately first-order in tocopheroxyl. The pseudo-first-order rate constant, k_{obsd}, was obtained by varying the concentration of ethyl linoleate 3. The tocopheroxyl 6 shows very slow natural decay in a similar solution. Therefore, k_{obsd}, the pseudo-first-order rate constant for tocopheroxyl bleaching is given by equation [5]

$$k_{obsd} = k_0 + k_3 [LH] \qquad [5]$$

where k_0 is the rate constant for natural decay of tocopheroxyl in the medium and k_3 is the second-order rate constant for reaction of tocopheroxyl with added fatty acid ester. These rate parameters are obtained by plotting k_{obsd} against [LH], as shown in Figure 3. The second-order rate constant k_3 obtained for ethyl linoleate 3 is 1.82×10^{-2} $M^{-1}s^{-1}$, and $k_0 = 2.56 \times 10^{-5}$ s^{-1}. Similar measurements were performed for the reaction of tocopheroxyl with unsaturated fatty acid esters (2,4, and 5) in benzene solution. On the other hand, in the case of ethyl stearate 1 containing no double bond, the hydrogen-atom abstraction reaction between tocopheroxyl and ethyl stearate 1 has not been observed. The k_3 values obtained are summarized in Table 1. As is clear from both Figure 3 and Table 1, k_0 values are small and negligible for the polyunsaturated fatty acid esters 3, 4, and 5.

As listed in Table 1, k_3, the rate constants, increase as the number of the double bond in fatty acid esters increases (1<2<3<4<5). By comparing the second-order rate constants (k_3) obtained for ethyl oleate 2 and ethyl linoleate 3, the k_3 value (1.04×10^{-5} $M^{-1}s^{-1}$) of the former is about three orders of magnitude lower than that (1.82×10^{-2} $M^{-1}s^{-1}$) of the latter. The ethyl oleate 2 has four hydrogen atoms activated by a single π-

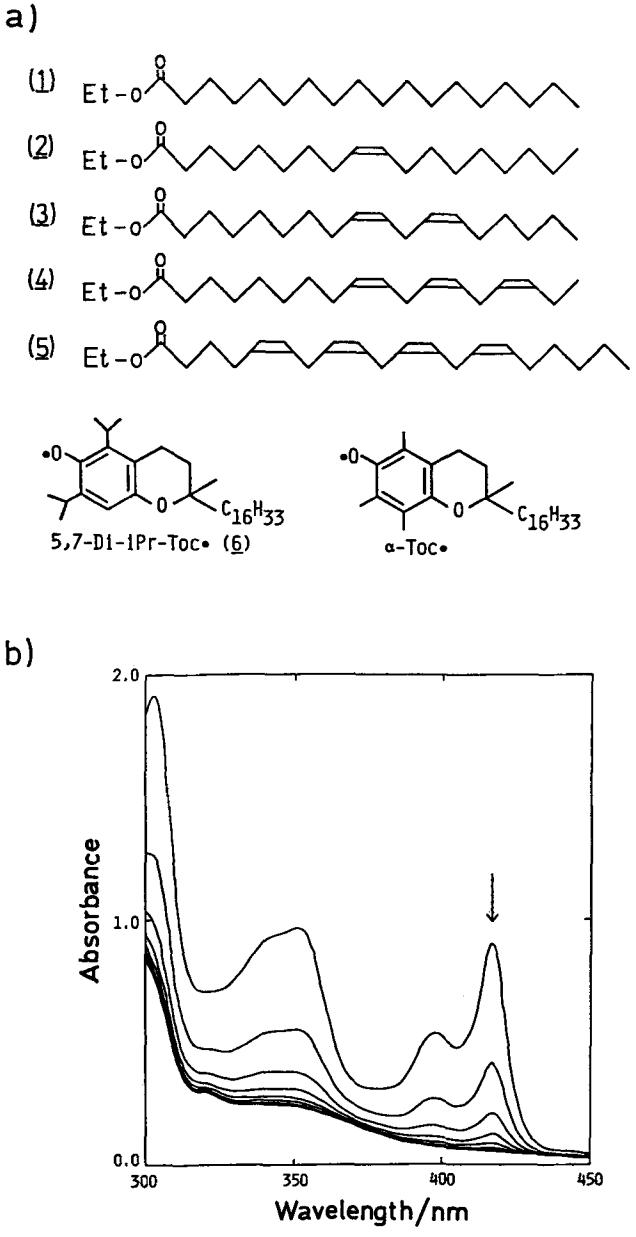

FIG. 1. a) Molecular structures of fatty acid ethyl esters (ethyl stearate *1*, ethyl oleate *2*, ethyl linoleate *3*, ethyl linolenate *4*, and ethyl arachidonate *5*) and vitamin E radicals (5,7-diisopropyl-tocopheroxyl *6* and α-tocopheroxyl). b) Change of electronic absorption spectrum of 5,7-diisopropyltocopheroxyl radical *6* for the reaction of tocopheroyl *6* with ethyl linoleate *3* in benzene at 25.0°C. $[Toc•]_{t=0}$ ca 0.20 mM and $[LH]_{t=0}$ 75.0 mM. The spectra were recorded at every 10-min interval. Arrow indicates decrease (↓) of absorbance with time.

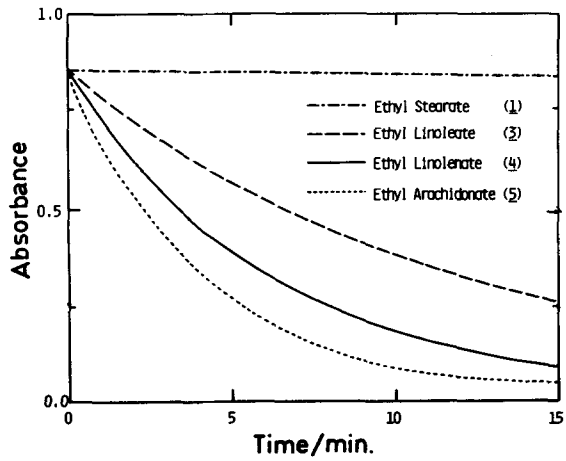

FIG. 2. The decay of 5,7-diisopropyl-tocopheroxyl radical *6* for the reaction of tocopheroxyl *6* with fatty acid esters *1*, *3*, *4*, and *5* observed at 417 nm in benzene at 25.0°C. $[Toc•]_{t=0}$ ca 0.17 mM and $[LH]_{t=0}$ 75.0 mM.

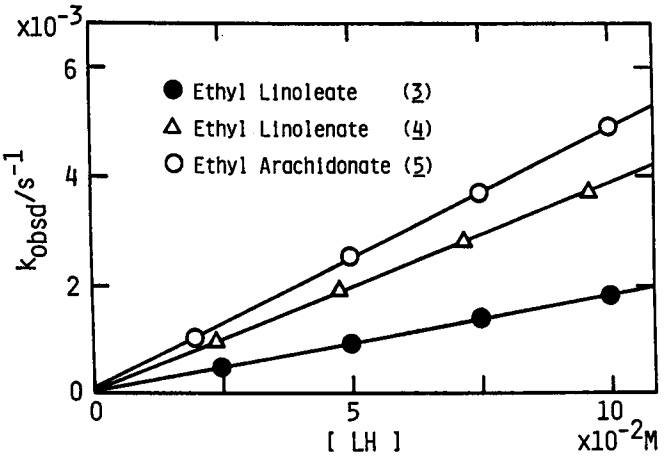

FIG. 3. The dependence of the pseudo-first-order rate constant, k_{obsd}, on the concentration of polyunsaturated fatty acid ethyl esters *3, 4,* and *5* in benzene at 25.0°C.

TABLE 1

Rate Constants for Reaction of Fatty Acid Ethyl Esters *1,2,3,4,* and *5* with Tocopheroxyl Radical *6* in Benzene at 25.0°C

	[LH] (mM)	k_{obsd} (s^{-1})	k_0 (s^{-1})	k_3[a] ($M^{-1}s^{-1}$)	k_{abstr}/H ($M^{-1}s^{-1}$)
Ethyl stearate 1	500.0	—	—	$<< 10^{-5}$	
Ethyl oleate 2	99.9	3.96×10^{-5}	3.85×10^{-5}	$(1.04 \pm 0.03) \times 10^{-5}$	2.60×10^{-6}
	149.9	4.01			
	203.8	4.05			
	254.7	4.12			
Ethyl linoleate 3	24.9	4.78×10^{-4}	2.56×10^{-5}	$(1.82 \pm 0.01) \times 10^{-2}$	9.10×10^{-3}
	49.8	9.27			
	74.7	13.94			
	99.6	18.32			
Ethyl linolenate 4	24.0	9.63×10^{-4}	5.07×10^{-5}	$(3.84 \pm 0.01) \times 10^{-2}$	9.60×10^{-3}
	47.9	18.98			
	71.9	28.23			
	95.9	37.22			
Ethyl arachidonate 5	20.0	10.22×10^{-4}	7.54×10^{-5}	$(4.83 \pm 0.02) \times 10^{-2}$	8.05×10^{-3}
	50.0	25.22			
	75.0	36.93			
	100.1	48.97			

[a]Errors are standard deviations.

electron system. On the other hand, the ethyl linoleate 3 has two hydrogen atoms activated by two π-electron systems. Consequently, these two hydrogen atoms will contribute to the high reactivity of ethyl linoleate 3. In fact, as listed in Table 1, the polyunsaturated fatty acid esters 3,4, and 5 containing hydrogen atoms activated by two π-electron systems show similar reactivity with tocopheroxyl 6. These fatty acid esters 3, 4, and 5 have two, four, and six hydrogen atoms activated by two π-electron systems, respectively, and thus the rate constants, k_{abstr}/H, given on an available hydrogen basis, are $k_3/2 = 9.10 \times 10^{-3}$ $M^{-1}s^{-1}$ for 3, $k_3/4 = 9.60 \times 10^{-3}$ $M^{-1}s^{-1}$ for 4, and $k_3/6 = 8.05 \times 10^{-3}$ $M^{-1}s^{-1}$ for 5. These values are similar to each other. The results suggest that tocopheroxyl radical produced in edible oils can react with polyunsaturated lipids by abstracting hydrogen atoms activated by two π-electron systems. Since ethyl stearate 1, which does not contain allylic hydrogens, did not react with the tocopheroxyl radical, we estimated that k_3 of ethyl stearate 1 must be $<<10^{-5}$ $M^{-1}s^{-1}$.

As described in a previous section, we have recently succeeded in measuring the rate constants (k_{-1}) for the reaction of alkyl hydroperoxides with 5,7-

diisopropyl-tocopheroxyl radical 6, i.e., the reverse of reaction [1]. k_1 the observed rates, were (1.34~3.65) × 10^{-1} $M^{-1}s^{-1}$ in benzene solution at 25.0°C. In the present work, we have measured the rate constants, k_3, for the reaction of polyunsaturated fatty acid esters 3, 4, and 5 with tocopheroxyl radical 6. The k_3 values obtained are (1.82~4.83) × 10^{-2} $M^{-1}s^{-1}$ in benzene at 25.0°C, as listed in Table 1. By comparing these values, the values of k_{-1} are only about one order of magnitude higher than those of k_3. The results suggest that the reactions [3] and [4] may both relate to the prooxidant effect of α-tocopherol at high concentration. Therefore, if the polyunsaturated lipids coexist with the hydroperoxides in edible oils or membranes, the rate of disappearance of tocopheroxyl radical will be represented by the following equation [6]:

$$-d[Toc\bullet]/dt = k_3\,[LH]\,[Toc\bullet] + k_{-1}\,[LOOH]\,[Toc\bullet] \qquad [6]$$

The above radical decay reactions [3] and [4] may happen competitively with reaction [2] or with radical dimer formation (reaction [7]) (10). However, both the reactions [2] and [7]

$$Toc\bullet + Toc\bullet \xrightarrow{k_7} \textit{Non-radical products} \qquad [7]$$

do not participate in the prooxidant effect of α-tocopherol, and, conversely, the reaction [2] contributes to the antioxidant effect of α-tocopherol.

In the initial stage of lipid degradation, the concentration of hydroperoxides will be much lower than that of polyunsaturated fatty acids, and thus the second term in equation [6] is negligible. Consequently, the prooxidant effect of α-tocopherol in edible oils and fats will be induced by the hydrogen-atom abstraction reaction [3] between tocopheroxyl radical and polyunsaturated fatty acids. On the other hand, if the autoxidation of edible oils and fats proceeds, the level of lipid hydroperoxides increases. When the concentration of lipid hydroperoxides approaches approximately 10% of that of polyunsaturated fatty acids, the radical decay reactions [3] and [4] will happen at a similar rate. These facts suggest that not only the chain transfer reaction [3] due to tocopheroxyl but also the reaction [4] between tocopheroxyl and hydroperoxides participates in the prooxidant effect of α-tocopherol.

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Fatty Acid and β-Amino Acid Syntheses in Strains of *Bacillus subtilis* Producing Iturinic Antibiotics

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Iturinic antibiotics, produced by different strains of *Bacillus subtilis*, contain long-chain β-amino acids (β-AA). The regulation of the synthesis of fatty acids (FA) and β-AA was studied by modifying the culture medium. Addition of possible precursors, branched-chain α-amino acids, to the medium affected the FA and β-AA compositions. According to this, the *B. subtilis* strains can be divided into two groups. The first contains the producers of mycosubtilin and bacillomycin F which synthesize a high level of *iso* C_{16} chains; the second contains the producers of bacillomycin D, bacillomycin L and iturin which synthesize a high level of *n* carbon chains.

The incorporation of radioactive sodium acetate into FA and β-AA showed rapid FA synthesis followed by a second synthetic step. Although the detailed mechanism has not yet been elucidated, this second step, corresponding to the β-AA synthesis, seemed to be a key step in determining the alkyl chain of β-AA.
***Lipids* 24, 940-944 (1989).**

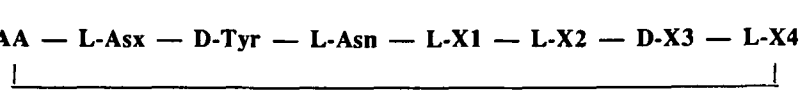
Bacillomycins, iturin and mycosubtilin are antifungal peptidolipids from *Bacillus subtilis*. They belong to the iturin group, which is characterized by the presence of β-AA with 14 to 17 carbon atoms (1,2) (Fig. 1). The relationship between β-AA biosynthesis and FA biosynthesis had previously been studied for strains producing iturin and bacillomycin F (3,4). It had been shown that valine was the precursor of even *iso* β-AA and FA, leucine that of odd *iso* β-AA and FA for both *B. subtilis*, while isoleucine was the precursor of odd *anteiso* β-AA only in the case of the bacillomycin F producer. The β-AA of bacillomycin F differ from those of iturin by chain length and type of carbon chain (3,4). Thus, in order to understand the differences in the properties of these two strains, the relationship between β-AA and FA biosyntheses was studied in the case of *B. subtilis* strains producing three other antibiotics (bacillomycin D, bacillomycin L and mycosubtilin). β-AA and FA were analyzed after branched-chain α-amino acids were added to the culture medium of these three strains. The relationship between β-AA and FA synthesis was also determined by following the incorporation of radioactive sodium acetate into both compounds.

MATERIALS AND METHODS

Strains and culture conditions. B. subtilis producing bacillomycin D (*B. subtilis* BD) was kindly supplied by Dr. F.R. Raubitschek, Rothschild Hadaash Medical Organization, Jerusalem, Israel; *B. subtilis* NICIB 8872 producing bacillomycin L (*B. subtilis* BL) by Dr. J.B. Barr, Royal Victoria Hospital, Belfast, U.K.; *B. subtilis* producing mycosubtilin (*B. subtilis* MS) by Dr. M.C. Mynard, Bio-Mérieux, Charbonnières-les-Bains, France; and *B. subtilis* producing iturin (*B. subtilis* IT) by Dr. L. Delcambe, C.N.P.E.M., Liège, Belgium.

These strains were grown at 35°C in a basal medium containing L-glutamic acid and glucose (5) to which DL-isoleucine, leucine or valine was added. In some experiments, sodium [1-^{14}C]acetate (0.56 GBq/mmol, CEA, Saclay, France) was added at a concentration of 18.5 MBq per ml of culture medium. After 100 hr of growth, cultures were centrifuged at 10,000 × *g* for 20 min.

Purification of antibiotics and preparation of β-AA (6). Crude antibiotic preparation was obtained by acidification (pH 2) of the cell supernatant. The precipitate was purified by column chromatography on silicic acid. β-AA were obtained after hydrolysis of pure antibiotic



Iturin A : Asx=Asn, X1=Gln, X2=Pro, X3=Asn, X4=Ser
Bacillomycin L : Asx=Asp, X1=Ser, X2=Gln, X3=Ser, X4=Thr
Bacillomycin D : Asx=Asn, X1=Pro, X2=Glu, X3=Ser, X4=Thr
Bacillomycin F : Asx=Asn, X1=Gln, X2=Pro, X3=Asn, X4=Thr
Mycosubtilin : Asx=Asn, X1=Gln, X2=Pro, X3=Ser, X4=Asn.

FIG. 1. Structure of iturinic antibiotics.

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Abbreviations used: β-AA, β-amino acids; FA, fatty acids; GC, gas chromatography; i, *iso*; ai, *anteiso; B. subtilis* BD, strain producing bacillomycin D; BF, bacillomycin F; BL, bacillomycin L; IT, iturin; MS, mycosubtilin.

with 6N HCl for 8 hr at 150°C and extraction with chloroform.

Purification of FA. Cellular lipids were extracted with ethanol/diethyl ether (3:1, v/v) and FA were purified according to the Ito procedure (7).

Gas chromatography (GC). FA, as methyl esters and β-AA, as *N*-trifluoroacetyl methyl esters, were analyzed by GC as described previously (4).

Determination of radioactivity. The radioactivities of FA and β-AA were determined by liquid scintillation counting in a Tri Carb 2100 instrument (Packard Instruments, Downers Grove, IL).

RESULTS AND DISCUSSION

The influence of isoleucine, leucine and valine on antibiotic production was studied. No significant changes were observed by addition of these branched-chain α-amino acids to the culture medium of *B. subtilis* BD and *B. subtilis* BL. In the case of *B. subtilis* MS, the addition of leucine did not modify antibiotic production while the addition of isoleucine or valine increased it five-fold.

The antibiotics produced in the different media were purified, their β-AA were prepared and analyzed by GC. Lipids were extracted from the different strains of *B. subtilis* grown in various media, and FA were prepared and analyzed by GC. Figures 2-4 show the effects of branched-chain α-amino acids on the percentages of increased β-AA and FA. With the three strains, addition of isoleucine increased both odd *anteiso* β-AA and FA, leucine both odd *iso* β-AA and FA, and valine both even *iso* β-AA and FA.

In the case of *B. subtilis* MS (Fig. 2), the increase induced by each α-amino acid on FA roughly paralleled those of the corresponding β-AA. In the presence of isoleucine (4 g/l), odd *anteiso* β-AA and FA represent about 90% of the total. In the presence of valine or leucine (4 g/l), the increases reached 60% of the total β-AA and FA. Similar results had been previously ob-

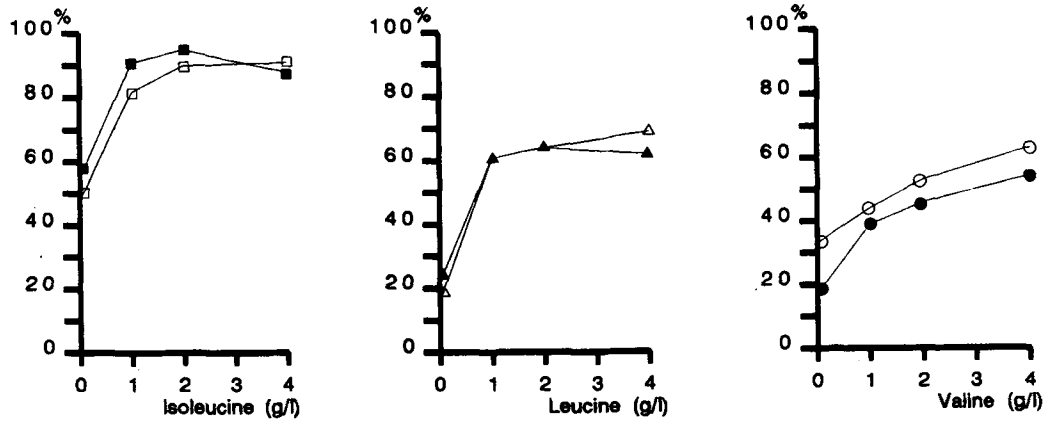

FIG. 2. Increases of FA and β-AA of mycosubtilin induced by the addition of branched-chain α-amino acids to the culture medium. *B. subtilis* MS was grown in the presence of various concentrations of isoleucine, leucine or valine for 100 hr at 35°C. The β-AA of mycosubtilin and cellular FA were prepared and analyzed by GC. The addition of isoleucine modified the percentages of *anteiso* C_{17} β-AA (□) and odd *anteiso* FA (■), the addition of leucine *iso* C_{17} β-AA (△) and odd *iso* FA (▲) and the addition of valine *iso* C_{16} β-AA (○) and even *iso* FA (●).

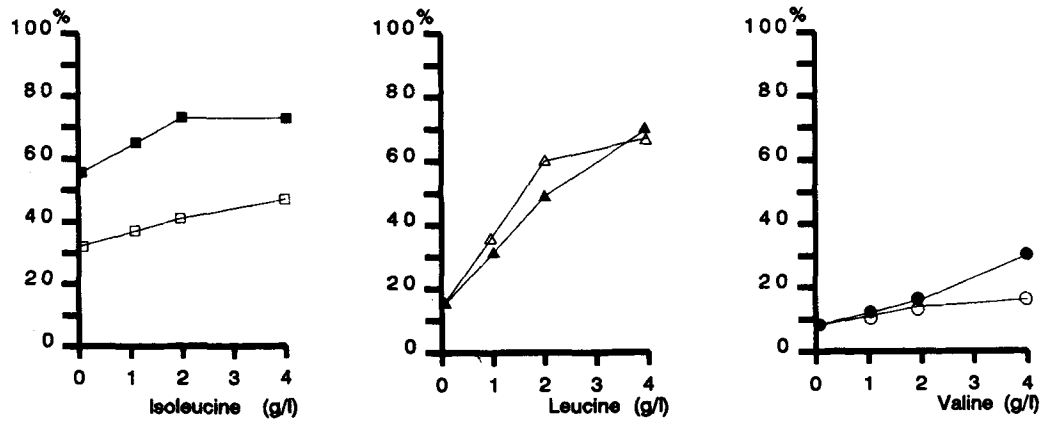

FIG. 3. Increases of FA and β-AA of bacillomycin L induced by the addition of branched-chain α-amino acids to the culture medium. *B. subtilis* BL was grown in the presence of various concentrations of isoleucine, leucine or valine for 100 hr at 35°C. The β-AA of bacillomycin L and cellular FA were prepared and analyzed by GC. The addition of isoleucine modified the percentages of *anteiso* C_{15} β-AA (□) and odd *anteiso* FA (■), the addition of leucine *iso* C_{15} β-AA (△) and odd *iso* FA (▲) and the addition of valine *iso* C_{16} β-AA (○) and even *iso* FA (●).

tained in the case of the bacillomycin F producer (3).

With *B. subtilis* BD and BL (Figs. 3 and 4), the most important effects were obtained with leucine. Both odd *iso* β-AA and FA increased similarly in the presence of leucine, whereas with valine or isoleucine, the effects on FA were 1.5 to 3.5 times more pronounced than those on the corresponding β-AA. Similar results had previously been obtained in the case of the iturin producer (4). Increasing leucine concentrations induced parallel increases of odd *iso* β-AA and FA. In the presence of valine (4 g/l), even *iso* FA was about 1.5 times more abundant than even *iso* β-AA.

Thus, considering the effects of branched-chain α-amino acids on the biosynthesis of carbon chains of β-AA and FA, the antibiotics of the iturin group could be divided into two groups: One with bacillomycin F and mycosubtilin, the other with bacillomycin D, bacillomycin L and iturin. Antibiotics of each group were characterized by the chain length and the nature of the carbon chain of their β-AA (Fig. 5). Bacillomycin F and mycosubtilin contain 3% of *n* β-AA (C_{16}) and 33-49% of *iso* C_{16} β-AA; by contrast, bacillomycin D, bacillomycin L and iturin contain 30-60% of *n* β-AA and only 5-9% of *iso* C_{16} β-AA.

If the β-AA composition of the five antibiotics is compared to the FA composition of the five *B. subtilis* strains (Figs. 5 and 6), it appears that the producers of mycosubtilin or bacillomycin F synthesized two times more *iso* C_{16} FA than the other strains. Conversely, the producers of bacillomycin D, bacillomycin L and iturin synthesized three times more *n* C_{16} FA than the other strains. These differences are similar to those observed in the case of β-AA.

The relationship between the biosynthesis of FA

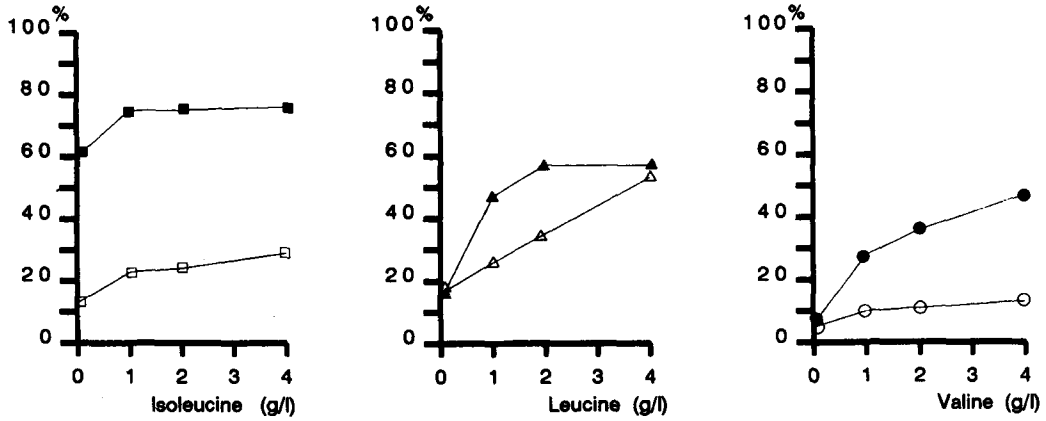

FIG. 4. Increases of FA and β-AA of bacillomycin D induced by the addition of branched-chain α-amino acids to the culture medium. *B. subtilis* BD was grown in the presence of various concentrations of isoleucine, leucine or valine for 100 hr at 35°C. The β-AA of bacillomycin D and cellular FA were prepared and analyzed by GC. The addition of isoleucine modified the percentages of *anteiso* C_{15} β-AA (□) and odd *anteiso* FA (■), the addition of leucine *iso* C_{15} β-AA (△) and odd *iso* FA (▲) and the addition of valine *iso* C_{16} β-AA (○) and even *iso* FA (●).

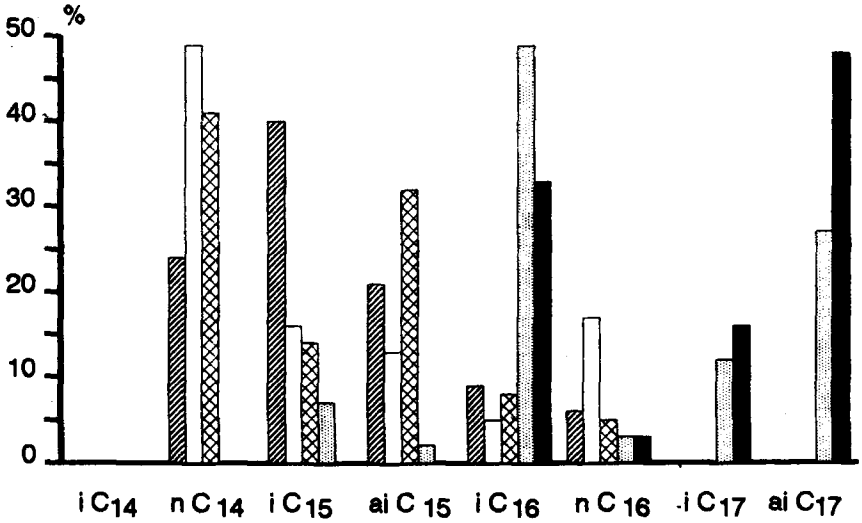

FIG. 5. Nature of the carbon chains of the β-AA of the different iturinic antibiotics. The following compounds were prepared and analyzed by GC: β-AA of iturin (hatched bars), β-AA of bacillomycin D (open bars), β-AA of bacillomycin L (cross-hatched bars), β-AA of bacillomycin F (dotted bars) and β-AA of mycosubtilin (solid bars). The results are expressed in percentages of total β-AA.

and β-AA was studied by incorporating a radioactive precursor of the carbon chains into FA and β-AA. Six cultures of *B. subtilis* producing iturin were chosen at five hr intervals, sodium [1-^{14}C]acetate was added to a culture. When the maximum of antibiotic production was reached, iturin was extracted from the culture media, purified, and its radioactivity was determined. Maximum radioactivity was incorporated into iturin when sodium [1-^{14}C]acetate was added after 10 hr of growth (Fig. 7). Iturin obtained from the six cultures was then hydrolyzed and the β-AA radioactivity was determined (Fig. 7). As in the case of iturin, the maximum of radioactivity was found into β-AA when sodium [1-^{14}C]acetate was added after 10 hr of growth; then, the β-AA represents 58% of the iturin radioactivity.

In addition, FA of the cells from the six cultures were prepared and their radioactivities were determined (Fig. 8). Maximum radioactivity was incorporated into FA when sodium [1-^{14}C]acetate was added at the beginning whereas the maximum incorporation into β-AA was obtained when the radioactive precursor was added after 10 hr of growth. Moreover, when sodium [1-^{14}C]acetate was added after 5 to 25 hr, the level of FA radioactivity was about half that of β-AA.

In conclusion, the five *B. subtilis* strains produc-

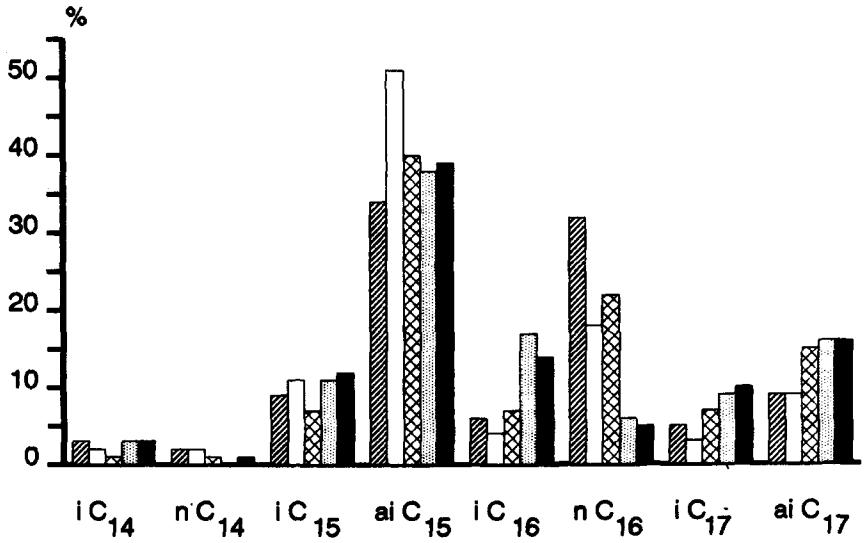

FIG. 6. Nature of the carbon chains of the cellular FA of the different strains producing iturinic antibiotics. The following compounds were prepared and analyzed by GC: FA of *B. subtilis* IT (hatched bars), FA of *B. subtilis* BD (open bars), FA of *B. subtilis* BL (cross-hatched bars), FA of *B. subtilis* BF (dotted bars), FA of *B. subtilis* MS (solid bars). The results are expressed in percentages of total FA.

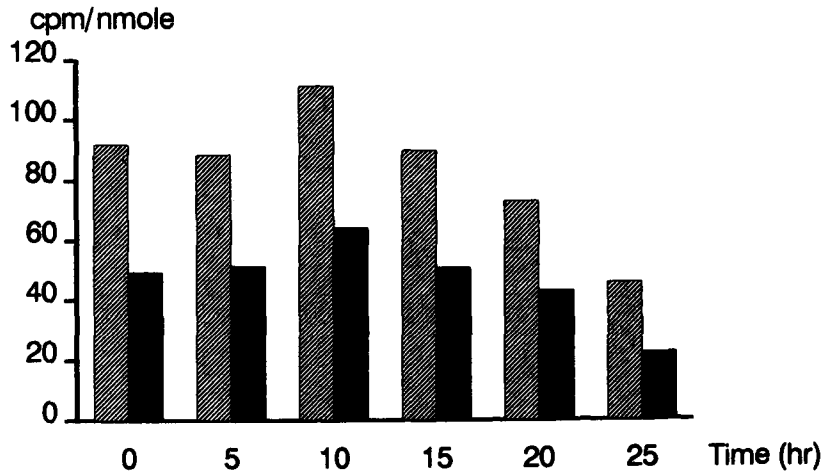

FIG. 7. Incorporation of sodium [1-^{14}C]acetate into iturin and its β-AA. Six cultures of *B. subtilis* IT were realized and, at five hr intervals (corresponding to the indicated times on the Figure), the radioactive precursor was added to one culture. After 100 hr of total growth, the iturin and its β-AA were prepared from each culture. The specific radioactivities of iturin (hatched bars) and β-AA (solid bars) were determined.

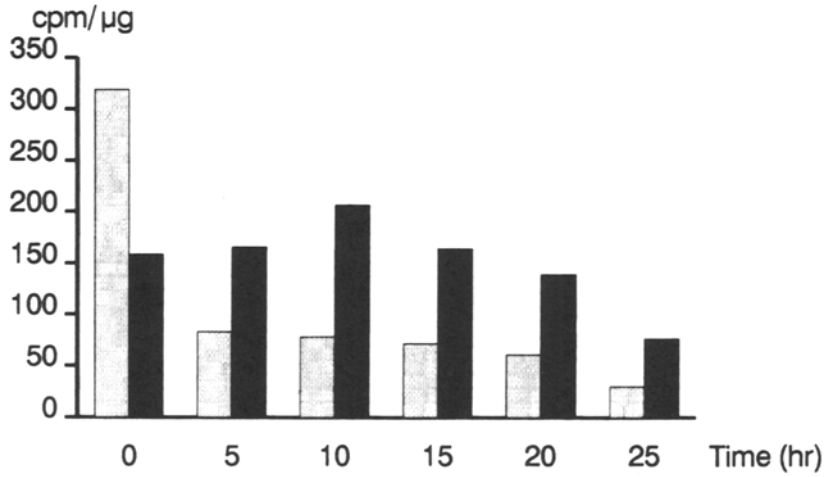

FIG. 8. Incorporation of sodium [1-^{14}C]acetate into the cellular FA of *B. subtilis* IT and into the β-AA of iturin. Six cultures of *B. subtilis* IT were realized and, at five hr intervals (corresponding to the indicated times on the Figure), the radioactive precursor was added to one culture. After 100 hr of total growth, the β-AA of iturin and the cellular FA were prepared. The radioactivities of FA (dotted bars) were determined and compared with those of β-AA (solid bars).

ing iturinic antibiotics can be divided into two groups, one with mycosubtilin and bacillomycin F producers and the other with bacillomycin D, bacillomycin L and iturin producers. The first group is characterized by an active synthesis of *iso* C_{16} β-AA and FA, the second one by the synthesis of *n* β-AA and FA. The higher ability of the first group to synthesize even *iso* carbon chain was also observed when the culture medium was modified. Indeed, the addition of valine to the culture medium of the five *B. subtilis* strains increased even *iso* β-AA and FA but these modifications varied according to the group. In the case of mycosubtilin and bacillomycin F producers, the increase of even *iso* β-AA and FA were similar, while, in the case of bacillomycins D, L and iturin producers, the increases of even *iso* FA were 1.5 to 3.5 times more prominent than those of *iso* β-AA.

On the other hand, the biosynthesis of the carbon chains of FA and β-AA was studied by adding sodium [1-^{14}C]acetate to the culture medium. Maximum radioactivity was incorporated into FA during the first several hours of growth while maximum incorporation into β-AA was observed later. This result can explain the absence of direct correlation in the composition of FA and β-AA although the carbon skeletons of the two molecules resemble each other in some steps.

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Total and Peroxisomal Oxidation of Various Saturated and Unsaturated Fatty Acids in Rat Liver, Heart and M. Quadriceps

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Rates of total and peroxisomal fatty acid oxidation were estimated from the production of ^{14}C-labeled CO_2 and acid-soluble products from differently labeled [^{14}C]fatty acids, in the absence and presence of antimycin-rotenone, in homogenates of liver, heart and m. quadriceps. Total and peroxisomal oxidation rates of palmitic, oleic and linoleic acid were 3-4 times higher than those of arachidonic and adrenic acid which had higher oxidation rates than those of lignoceric and erucic acid. The peroxisomal contribution to the oxidation of the last fatty acids was similar to or higher than that of palmitic acid. For all fatty acids tested in these tissues, the mitochondrial contribution to β-oxidation was higher than the peroxisomal contribution.

Production of $^{14}CO_2$ and ^{14}C-labeled, acid-soluble metabolites from [13-^{14}C]arachidonic acid indicated that polyunsaturated fatty acids can be chain-shortened beyond their double bonds in m. quadriceps and heart as well as in liver. Although 2,4-dienoyl-CoA reductase requires NADPH, addition of this coenzyme did not influence arachidonic acid oxidation. Arachidonic acid oxidation was inhibited by palmitic acid in mitochondria and peroxisomes, but arachidonic acid had only a slight effect on palmitic acid oxidation.
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Saturated and unsaturated fatty acids are chain-shortened by β-oxidation in mitochondria and peroxisomes. Auxiliary enzymes are necessary for the oxidation of the unsaturated fatty acids (1,2). These enzymes, 3-*cis*, 2-*trans*-enoyl-CoA isomerase and 2-*trans*,4-*cis*-dienoyl-CoA reductase, are present in both mitochondria and peroxisomes in rat liver and heart (2-7). 3-Hydroxyl-CoA epimerase described by Stoffel *et al.* (8) was suggested to play a role in a minor peroxisomal pathway (9), but appeared to be a mixture of enoyl-CoA hydratases (10). The availability of NADPH for 2,4-dienoyl-CoA reductase can be rate-limiting for the β-oxidation of polyunsaturated fatty acids (11). The activity of mitochondrial transhydrogenase may restrict oxidation of polyunsaturated fatty acids and lead to accumulation of inhibitory enoyl-CoA β-oxidation intermediates in tissue homogenates (11,12). Therefore the effect of NADPH addition needs investigation.

Studies on the oxidation of (poly)unsaturated fatty acids have mainly been carried out in isolated mitochondria and peroxisomes from rat liver and heart (12-20) and in intact hepatocytes (21,22). The radiochemical assay of fatty acid oxidation in the absence and in the presence of antimycin and rotenone is an accurate and sensitive tool to study peroxisomal oxidation in relation to the total oxidation in homogenates of various tissues (23-25). The validity of this approach is supported by data on peroxisomal fatty acid oxidation in hepatocytes and perfused liver without the use of inhibitors (26,27), which showed the same peroxisomal contribution as the radiochemical assay in liver homogenates (23-25) and hepatocytes (21,22) with inhibitors. The use of antimycin has, however, recently been criticized, since it may inhibit acyl-CoA oxidase (28).

The aim of the present study was to compare the capacity of peroxisomal relative to total β-oxidation of various saturated and (poly)unsaturated fatty acids in liver, heart and m. quadriceps. The experiments were performed under conditions so that the fatty acid concentration was not rate-limiting for both mitochondrial and peroxisomal oxidation. For a closer examination of our assay we first investigated the effects of antimycin on the acyl-CoA oxidase activity under our conditions and of NADPH addition on total and peroxisomal oxidation. Fatty acids with a radioactive label beyond the double bonds were used to determine the degree of chain-shortening and the substrate preference of mitochondria and peroxisomes. The interaction between palmitic and arachidonic acid oxidation was also studied to gain information about the influence of polyunsaturated fatty acids on fatty acid oxidation.

MATERIALS AND METHODS

Materials. [1-^{14}C]Arachidonic acid, [2-^{14}C]adrenic acid, [1-^{14}C]oleic acid, and [1-^{14}C]palmitic acid were obtained from Amersham International, Little Chalfont, U.K.; [1-^{14}C]linoleic acid from New England Nuclear, Dreieichenhain, F.R.G.; [13-^{14}C]arachidonic acid, [1-^{14}C]erucic acid, [14-^{14}C]erucic acid, and [10-^{14}C]oleic acid from CEA, Gif-sur-Yvette, France.

Palmitic and oleic acid were from Merck, Darmstadt, F.R.G.; arachidonic, erucic and linoleic acid from Sigma Chemical Co., St. Louis, MO; adrenic acid from Nu-Chek Prep, Elysian, MN; NADPH from Boehringer Mannheim, Mannheim, F.R.G. Origin of chemicals for the acyl-CoA oxidase assay was given previously (23). All other reagents were of analytical grade.

Animals and tissues. Male random-bred Wistar rats (190 ± 10 g) were starved for 18 hr and killed by cervical dislocation. Liver, heart and m. quadriceps were removed and immediately cooled in ice-cold buffer, consisting of 0.25 M sucrose/2 mM EDTA/10 mM Tris-HCl (pH 7.4). Whole homogenates (5% w/v) were prepared in the same buffer by manual homogenization. Pestles with different diameters were used (0.20, 0.12, 0.05 mm intervening space). Homogenates of liver, heart and m. quadriceps contained 12.0 ± 1.3 (n=26), 8.2 ±

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Abbreviations: CoA, coenzyme A; EDTA, ethylenediamine tetraacetic acid; HCl, hydrochloric acid; NAD, nicotinamide adenine dinucleotide; NADPH, reduced nicotinamide adenine dinucleotide phosphate.

1.2 (n=11) and 7.7 ± 1.2 (n=25) mg protein per ml respectively.

Analytical procedures. Oxidation of fatty acids bound to albumin in a 5:1 molar ratio was assayed under conditions which were optimal with respect to time and tissue material, as described before (24). The reaction medium contained the following cofactors: 5 mM ATP, 1 mM NAD^+, 25 μM cytochrome c, 200 μM coenzyme A, 0.5 mM L-carnitine and 0.5 mM L-malate. In all experiments, 120 μM fatty acid was used, except as otherwise indicated. A fixed concentration was chosen to compare between mitochondria and peroxisomes and between the various fatty acids, because the optimal concentration is different for each organel and each fatty acid (13,15,16,22). The concentration of lignoceric acid, bound to celite (1 nmol/mg), was only 20 μM. Cyclodextrin was added in a concentration of 2 mg/ml (24). Specific activities of [^{14}C]fatty acids were about 2000–5000 dpm/nmol. Peroxisomal oxidation rates were measured in the presence of 72 μM antimycin and 10 μM rotenone.

Acyl-CoA oxidase activity was assayed in liver extracts as H_2O_2-dependent leuko-dichlorofluorescein oxidation (23). Protein concentrations were determined according to the procedure of Lowry *et al.* (29) with bovine serum albumin as standard.

Data were statistically analyzed by Student's t-test. Relative values were used to eliminate differences in the absolute rates for individual animals or experiments.

RESULTS

In the study of Vamecq *et al.* (28), acyl-CoA oxidase was inhibited 7 μM antimycin, but the inhibition was completely alleviated at a protein concentration of 2 mg per ml. Our reaction mixture for the peroxisomal oxidation assay contained at least 2.2 mg protein, but we used 72 μM antimycin, because long-chain fatty acids alleviate the inhibition of low antimycin concentrations (30). Therefore we measured the acyl-CoA oxidase activity in absence and in presence of this high antimycin concentration. In liver extracts, the acyl-CoA oxidase activity was 38.1 ± 1.8 nmol dichlorofluorescein per min per ml without antimycin and 33.1 ± 2.2 with antimycin (n=3), with 30 μM palmitate-albumin as substrate and 0.65 mg total protein per ml. The paired Student's t-test did not show significant differences. These results justify the use of antimycin as inhibitor of mitochondrial oxidation.

Since the availability of NADPH could affect the peroxisomal fatty acid oxidation (11), we studied the influence of NADPH addition. In liver homogenates, NADPH inhibited the peroxisomal oxidation of palmitic and linoleic acid, but not of arachidonic acid (Table 1). Total oxidation was not changed. It also had no effect on the total and peroxisomal oxidation in homogenates of heart and m. quadriceps (data not shown). In further experiments NADPH was not added.

Palmitic, oleic and linoleic acid showed the highest total and peroxisomal oxidation rates in homogenates in all three tissues (Table 2). The total oxidation rate of arachidonic acid was relatively higher (compared to palmitic acid) in muscle homogenates than in liver and heart. Adrenic acid was oxidized much faster than erucic acid and relatively more by the mitochondria. The peroxisomal contribution was less than 50% for all fatty acids tested in all tissue homogenates. Therefore, the mitochondrial oxidation rate is always larger than the peroxisomal one, also for fatty acids, which show a relatively high peroxisomal oxidation rate such as erucic and lignoceric acid.

The production of $^{14}CO_2$ can be used as an indication of citric acid cycle activity in relation to fatty acid oxidation, although the addition of malate traps acetyl units and reduces CO_2 production (25). $^{14}CO_2$ production is negligible in liver, except with arachidonic acid as substrate. It is generally considerable in heart and higher than in skeletal muscle. The low CO_2 contribution to the oxidation of adrenic acid is due to the 2-position of the label. Fatty acids with even-numbered labeled carbon produce [2-^{14}C]acetyl-CoA, which releases $^{14}CO_2$ after 2 revolution of the citric acid cycle. In all cases a higher peroxisomal contribution is matched with a lower CO_2 contribution to the total oxidation.

The total oxidation rates of oleic, arachidonic and erucic acid are not influenced by the position of the label (compare Table 3 with Table 2). The degradation of [13-^{14}C]arachidonic acid to ^{14}C-labeled acid-soluble products and $^{14}CO_2$ (Table 3) indicates that polyunsaturated fatty acids are oxidized beyond the double bonds not only in liver (15), but also in heart and skeletal muscle. The CO_2 contribution to the oxidation of arachidonic acid remains substantial for 7 cycles,

TABLE 1

The Influence of NADPH on [1-^{14}C]Fatty Acid Oxidation in Rat Liver Homogenates

Fatty acid	Concentration NADPH (μM)	Fatty acid oxidation rates (nmol/min per g tissue)	
		Total	Peroxisomal
Palmitic acid	0	538 ± 40	161 ± 47
	100	546 ± 71	102 ± 36[a]
	500	543 ± 84	83 ± 15[b]
Linoleic acid	0	764 ± 90	276 ± 88
	100	895 ± 150	204 ± 68[a]
Arachidonic acid	0	242 ± 31	35 ± 7
	100	214 ± 8	32 ± 11

Values are means ± S.D. for 3–5 experiments.
[a]Significantly different compared to control; $p<0.01$.
[b]Significantly different compared to control; $p<0.001$.

TABLE 2

Total and Peroxisomal Oxidation of 1- or 2-^{14}C-Labeled Fatty Acids with Different Numbers of Double Bonds in Rat Tissue Homogenates[a]

	Fatty acid oxidation rates (nmol/min per g tissue)			Relative contribution (% of total)	
	Total		Peroxisomal	Peroxisomes	CO_2
Liver					
16:0	615 ± 103	(9)	150 ± 42	25 ± 10	3 ± 2
18:1(n-9)	569 ± 87	(8)	101 ± 33	18 ± 5	7 ± 3
18:2(n-6)	664 ± 112	(7)	187 ± 101	28 ± 13	2 ± 1
20:4(n-6)	227 ± 33	(9)	33 ± 10	15 ± 4	16 ± 8
22:1(n-9)	85 ± 37	(4)	19 ± 1	24 ± 7	4 ± 3
22:4(n-6)	156 ± 24	(4)	22 ± 2	15 ± 3	4 ± 2
24:0	10 ± 2	(5)	3 ± 1	31 ± 5	1 ± 6
Heart					
16:0	499 ± 52	(5)	137 ± 47	27 ± 8	34 ± 14
18:1(n-9)	517 ± 109	(8)	142 ± 54	27 ± 5	43 ± 9
18:2(n-6)	571 ± 143	(5)	114 ± 31	20 ± 3	39 ± 5
20:4(n-6)	190 ± 61	(7)	42 ± 28	22 ± 12	46 ± 9
22:1(n-9)	44 ± 6	(4)	20 ± 7	46 ± 12	16 ± 7
22:4(n-6)	146 ± 8	(3)	20 ± 1	14 ± 1	8 ± 5
24:0	8 ± 1	(5)	2.7 ± 0.3	36 ± 3	15 ± 7
M. quadriceps					
16:0	89 ± 27	(7)	25 ± 5	30 ± 8	16 ± 8
18:1(n-9)	146 ± 80	(8)	25 ± 14	18 ± 7	13 ± 5
18:2(n-6)	153 ± 94	(7)	31 ± 9	26 ± 14	13 ± 5
20:4(n-6)	79 ± 23	(6)	14 ± 6	18 ± 5	21 ± 4
22:1(n-9)	8 ± 2	(4)	4 ± 3	38 ± 14	6 ± 2
22:4(n-6)	44 ± 14	(3)	15 ± 4	35 ± 10	5 ± 5
24:0	1.5 ± 0.6	(12)	0.4 ± 0.3	28 ± 12	8 ± 6

[a]Values are means ± S.D. of the number of experiments within parentheses. Fatty acid concentration was 120 μM, except for lignoceric acid (20 μM). All fatty acids were 1-^{14}C-labeled, except adrenic acid, which was labeled on the second carbon. Statistical analysis ($p<0.01$, unpaired Student's t-test; 16:0 = 100%. Total oxidation: liver — 16:0, 18:1, 18:2 > 20:4 > 22:4 > 22:1 > 24:0 — heart — 16:0, 18:1, 18:2 > 20:4, 22:4 > 22:1 >24:0 — m. quadriceps —16:0, 18:1, 18:2, 20:4, 22:4 > 22:1 > 24:0. Peroxisomal oxidation: liver — 16:0, 18:1, 18:2 > 20:4, 22:1 > 22:4 > 24:0 — heart —16:0, 18:1, 18:2 > 20:4, 22:1, 22:4 > 24:0 — m. quadriceps — 16:0, 18:2 > 20:4, 22:1, 22:4 > 22:1 (24:0 significant the lowest rates). Peroxisomal contribution: liver — 24:0 > 18:1, 20:4, 22:4; heart — 24:0, 22:1 > 18:1, 18:2, 22:4; 18:1 > 22:4 — m. quadriceps 16:0 > 18:1, 20:4; 22:1 > 18:1; 22:4 > 20:4.

even in liver. The peroxisomal oxidation rates for the fatty acids labeled beyond the ninth carbon appear to be markedly lower than for the [1-^{14}C]fatty acids. Peroxisomal production rates of ^{14}C-labeled acid-soluble products of [10-^{14}C]oleic reflect those of [14-^{14}C]erucic acid, but are higher due to the more proximal position of the label. The low $^{14}CO_2$ contribution of [10-^{14}C]oleic and [14-^{14}C]erucic acid is due to the position of the label.

The mutual influence of palmitic and arachidonic acid on each other's oxidation was studied at the usual fatty acid/albumin ratio of 5:1 (Table 4). Total palmitic acid oxidation is only slightly reduced upon addition of arachidonic acid, but the inhibitory effect of palmitic acid on arachidonic acid oxidation is considerable. The peroxisomal oxidation of palmitic acid is not significantly affected by addition of arachidonic acid, but that of arachidonic acid is even no longer detectable in the presence of palmitic acid (data not shown).

DISCUSSION

Previously, we have extensively discussed our data on peroxisomal palmitic acid oxidation in comparison to literature data (24). Our results on rat liver agree quite well with those of most other investigators, and for rat heart and muscle with those of other radiochemical assays (31,32). Differences, especially in the peroxisomal oxidation by rat heart and muscle, may be related to the analytical procedures and cell-free systems used. The radiochemical assay measures the number of fatty acid molecules that have passed through one β-oxidation cycle at least. The difference of the oxidation rates of [1-^{14}C]- and [16-^{14}C]palmitic acid, which represents chain-shortening to acid-insoluble compounds, did not markedly change in the presence of antimycin (24) and is similar to the oxidation rate of [1-^{14}C]palmitic acid in the presence of antimycin in both our previous (24) and in this report for liver, heart and skeletal muscle homogenates. The estimations of acyl-CoA-dependent NAD^+ reduction and H_2O_2 production are related to the number of oxidation cycles and have been mostly carried out after cell fractionation. For heart or muscle, an acyl-CoA oxidase assay based on H_2O_2 production did not appear to be a valid measure, even in a peroxisome enriched fraction (23). The cyanide-insensitive NAD^+ reduction is generally used as a measure for the peroxisomal β-oxidation.

TABLE 3

Total and Peroxisomal Oxidation of [10-^{14}C]Oleic Acid, [13-^{14}C]Arachidonic Acid and [14-^{14}C]Erucic Acid in Rat Tissue Homogenates

	Fatty acid oxidation rates (nmol/min per g tissue)		Relative contribution (% of total)	
	Total	Peroxisomal	Peroxisomes	CO_2
Liver				
18:1(n-9)	590 ± 49	19 ± 12[b]	3.3 ± 1.8[b]	0.5 ± 0.1[b]
20:4(n-6)	231 ± 32	3 ± 1[b]	1.4 ± 0.4[b]	4.5 ± 1.9[a]
22:1(n-9)	53 ± 43	0.1 ± 0.1[b]	0.1 ± 0.2[b]	0.3 ± 0.4[b]
Heart				
18:1(n-9)	587 ± 22	122 ± 20[a]	20.7 ± 3.3[a]	4.3 ± 0.5[b]
20:4(n-6)	157 ± 32	10 ± 5[b]	6.3 ± 2.9[a]	32.2 ± 0.9
22:1(n-9)	32 ± 13	3 ± 1[b]	9.7 ± 3.8[a]	5.2 ± 2.6[a]
M. quadriceps				
20:4(n-6)	59 ± 12	2 ± 1[b]	2.8 ± 0.4[b]	16.3 ± 3.5
22:1(n-9)	4 ± 2	0.2 ± 0.2[b]	6.0 ± 9.5[a]	0.7 ± 1.2[a]

Values are means ± S.D. for 3-4 experiments.
[a]Significantly different from data with [1-^{14}C]fatty acid in the same homogenate: $p<0.01$.
[b]Significantly different from data with [1-^{14}C]fatty acid in the same homogenate: $p<0.001$.

TABLE 4

Interaction of Palmitic and Arachidonic Acid Oxidation in Rat Tissue Homogenates

	Relative total oxidation rate (% of control)	
Tissue	[1-^{14}C]palmitic acid and arachidonic acid	[1-^{14}C]arachidonic acid and palmitic acid
Liver	93.6 ± 19.3	48.7 ± 4.0[b]
Heart	89.5 ± 1.3[a]	23.9 ± 3.1[b]
M. quadriceps	94.5 ± 13.0	13.2 ± 4.4[b]

Values are means ± S.D. of 3-4 experiments.
[1-^{14}C]fatty acid and competitive fatty acid were both present in 100 μM concentration together with 40 μM albumin. The control contained 100 μM [1-^{14}C]fatty acid and 20 μM albumin.
[a]Significantly different compared to control: $p<0.01$.
[b]Significantly different compared to control: $p<0.001$.

Cyanide is known to inhibit catalase activity (33), which may lead to accumulation of H_2O_2 in peroxisomes and may consequently suppress the fatty acid oxidation (34) and H_2O_2 may reoxidize NADH (35). Cyanide also binds NAD^+ (36). The use of cyanide and the purification procedure and its associated enzyme leakage (37,38) may bias the assay of NAD^+ reduction in, e.g., heart peroxisomes (39) which gave a much lower peroxisomal oxidation rate than we found.

The present study demonstrates that (poly)unsaturated fatty acids are oxidized beyond the double bonds in skeletal muscle and heart as they are in liver (11,22,40). Auxiliary enzymes, like 2,3-enoyl isomerase and 2,4-dienoyl reductase, must therefore be present in these tissues. On the basis of the total oxidation rates, we conclude than, in our homogenates with intact mitochondria, sufficient NADPH is formed by energy-dependent $NAD(P)^+$ transhydrogenase. Addition of NADPH was even inhibitory to the peroxisomal oxidation of palmitic and linoleic acid in liver homogenate. This was also observed with palmitoyl-CoA oxidation in isolated rat liver peroxisomes (20). NADPH stimulated docosahexaenoic acid oxidation in isolated rat liver peroxisomes, but had no effect on oleic acid oxidation (15).

All fatty acids studied were degraded at higher rates by mitochondrial than by peroxisomal β-oxidation in all three tissues (Table 2). Total and peroxisomal oxidation rates decreased markedly with substrates exceeding eighteen carbons in homogenates, as was previously observed in isolated mitochondria and peroxisomes (13-15,17). In intact hepatocytes, however, the total oxidation rates of oleic, linoleic acid, arachidonic and adrenic acid were in the same range (22). The high peroxisomal oxidation rates of adrenic acid in hepatocytes (21,22) are due to an incomplete inhibition of mitochondrial oxidation with (+)decanoylcarnitine since $^{14}CO_2$ production was still present. The fatty acid oxidation activity in hepatocytes depends also on the fatty acid uptake (41), in contrast to β-oxidation in homogenates and isolated cell organelles.

Erucic acid appeared to be a poor substrate for fatty acid β-oxidation in other studies (13,14,17,27). Based on studies of NAD^+ reduction with isolated peroxisomes and mitochondria, Osmundsen concluded that the peroxisomal capacity to chain-shorten erucic acid exceeded the mitochondrial capacity (19). In our homogenate system the peroxisomal contribution to erucic acid was considerable, but always less than the mitochondrial one. The equal peroxisomal oxidation rates for oleic and linoleic and for erucic and adrenic acid in liver and heart homogenates (Table 2) contrast with the observations that β-oxidation of isolated peroxisomes shows a preference for the more polyunsaturated fatty acids (15,16). These discrepancies are due to the above described differences in the used assays and the fact that our rats were not treated with clofibrate. Clofibrate induces erucic acid oxidation to a greater extent than palmitic acid oxidation in rat hepatocytes (42,43).

On the basis of KCN insensitivity, Singh *et al.* (44) found that the oxidation of lignoceric acid took place

mainly in the peroxisomal fraction of rat liver and human fibroblasts. This does not agree with our results in homogenates of liver, heart and skeletal muscle (Table 2). In homogenates of human fibroblasts the peroxisomal contribution to oxidation of palmitic and lignoceric acid was 21 ± 4% (n=13) and 23 ± 10% (n=8), respectively. It may be possible that in homogenates, lignocerate, activated by peroxisomes and microsomes to lignoceroyl-CoA, is chanelled into mitochondria and oxidized as described (45). The use of cyanide and isolated peroxisomes is discussed above.

The peroxisomal contribution becomes less with a more distal position of the ^{14}C-label, since peroxisomal oxidation rates, based on ^{14}C-labeled acid-soluble products, always appear to depend on the position of the ^{14}C-label in the fatty acid (23,24,46,47) due to incompleteness of peroxisomal oxidation in general (26,48) and to the low number of peroxisomal β-oxidation cycles in homogenates (24) and isolated peroxisomes (18,20).

A marked inhibition of palmitic acid oxidation by arachidonoylcarnitine was observed in uncoupled mitochondria of rat liver (49). This phenomenon was due to limiting NADPH supply and accumulating enoyl-CoA esters and appears to be unlikely during normal hepatic metabolism. The slight inhibition of palmitic acid oxidation by arachidonic acid in our homogenates with intact mitochondria (Table 4) suggests that for oxidation palmitic acid is preferred. However, arachidonic acid oxidation was only partly inhibited by palmitic acid. This cannot be explained by different binding characteristics to albumin of the two fatty acids (41). Probably the observed differences are due to the existence of two acyl-CoA synthetases: nonspecific acyl-CoA and arachidonoyl-CoA synthetase (50,51). Palmitic acid probably inhibits the activity of nonspecific acyl-CoA synthetase for arachidonic acid as was reported for oleic acid (50,51). Tissue-specific ratios of nonspecific acyl-CoA to arachidonoyl-CoA synthetase (50) may explain the differences in inhibition between liver, heart and muscle. The fact that peroxisomal arachidonic acid oxidation is no longer detectable in the presence of palmitic acid may be due to its low oxidation rate or to the absence of arachidonoyl-CoA synthetase on peroxisomes. Recently Schepers *et al.* (52) observed that a metabolite of arachidonic acid, prostaglandin E_2 is activated to its CoA-ester by microsomes but not by peroxisomes.

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Characterization of Porcine Omental Lipids

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Porcine omental lipid extracts were fractionated and the major lipid components characterized. Approximately 97% of the chloroform/methanol extract consisted of triglycerides containing primarily 16:0, 18:0, 18:1, and 18:2 fatty acids. Small quantities of free fatty acids, cholesterol, di- and monoglycerides were also detected. The phospholipid fraction, obtained by solvent partition and Unisil column chromatography and characterized by high performance liquid chromatography (HPLC) and HPLC-mass spectrometry, was found to consist primarily of phosphatidylcholine, sphingomyelin, phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol. The neutral glycolipids, isolated by solvent partition and Unisil column chromatography and identified by HPTLC and HPLC, were found to consist primarily of di-, tri-and tetraosylceramides. The complex glycolipid fraction, obtained from Folch upper phase solvent partition and characterized by HPTLC and immunoblotting, was found to consist primarily of ganglio-, globo-, and neolacto- neutral glycolipids and ganglio-, globo-, neolacto- and fucosylated gangliosides.
Lipids 24, 951–956 (1989).

Surgeons have long used omentum to promote healing of the intestine, and in 1935 Beck (1) sutured omentum to the myocardium and reported the formation of blood vessel anastomoses. Omental tissues have also been used in surgical techniques as a pouch or fold to mechanically hold transplanted tissues and to increase collateral circulation in areas where poor circulation exists (2–5). Pedicled omentum has been helpful in the treatment of human peripheral vascular disease (6). Grafts of omental pedicle are now being used to nourish the bronchus of human lung transplantation (7). The improved healing produced by omentum may depend on new blood vessel formation, i.e., angiogenesis. Omental and other adipose tissues from rabbits have been shown to be angiogenic in the rabbit cornea (8). Angiogenic lipids have been isolated from the culture medium of 3T3-L1 fibroblasts which have differentiated into adipocytes (9,10); some evidence suggests that PGE_1 and PGE_2 may play a role in angiogenesis (11,12). A chloroform/methanol extract from feline omentum has been reported (13) to produce excellent angiogenic activity after a single injection into the central cornea of rabbits. The lipid material has also been injected intramuscularly in the area of a standardized wound and this action resulted in increased perfusion in the wound as measured by *in vivo* nuclear imaging techniques with ^{99m}technetium labeled erythrocytes (14).

While angiogenic peptides have been the subject of intensive study, the angiogenic potential of normal omental tissue and other lipids has received relatively little attention. Recently, Silverman *et al.* (8) reported that rabbit subcutaneous and omental fat tissue induced neovascularization in rabbit corneas and this conclusion suggested that prostaglandins are associated with fat-induced angiogenesis. Thus, normal tissues, such as porcine omentum which is available in large quantities and contains angiogenic activity, provide opportunities for the isolation, structural analysis and extensive animal and clinical studies of active components.

Rabinowitz *et al.* (15) described the nonpolar lipid and phospholipid composition of human omental tissue. We have previously examined the lipids of feline omentum (16) which was available only in limited amounts. The present study is an examination of the nonpolar lipid, phospholipid and glycolipid composition of porcine omentum.

MATERIALS AND METHODS

Materials. HPLC grade methanol and other reagent grade solvents and chemicals were obtained from Fisher Chemical Scientific (Fairlawn, NJ); Iatrobeads 6RS-8060 and 6RS-8010 were from Iatron Industries (Tokyo, Japan); DEAE-Sephadex (A-25) was from Pharmacia Fine Chemicals (Piscataway, NJ); Unisil was from Clarkson Chemical Company (Williamsport, PA); and HPTLC plates were obtained from E. Merck (Darmstadt, Germany). Ganglioside and glycolipid standards were prepared as previously described (16). *V. cholerae* neuraminidase was obtained from Sigma Chemical Co. (St. Louis, MO). BondElut C18 cartridges were obtained from Analytichem International (Harbor City, CA).

GC analysis of fatty acid methyl esters. Ester lipids were subjected to alkaline methanolysis (17) to prepare fatty acid methyl esters. The esters were analyzed by gas liquid chromatography as previously described (16) to obtain quantitative data on the percent distribution of the different fatty acids.

HPTLC and HPLC analysis of neutral glycolipids. HPTLC plates were developed with chloroform/methanol/water (60:35:8, v/v/v) and the spots were visualized with the orcinol spray reagent (17). For HPLC, the glycolipid fractions were perbenzoylated with benzoyl chloride in pyridine and the benzoylated glycosphingolipids were separated and measured by HPLC as previously described (18,19). This procedure provides quantitative data on the percent distribution of the different glycolipids.

HPTLC analysis of phospholipids. Whatman HP-KF 10 cm × 10 cm silica gel HPTLC plates were utilized for two-dimensional HPTLC developed with

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Abbreviations: GlcCer, glucosylceramide; GalCer, galactosylceramide; LacCer, lactosylceramide; $GaOse_2Cer$, galabiglycosylceramide, Gal(α1-4)Gal(β1-1)Cer; $GbOse_3Cer$, globotriglycosylceramide, Gal(α1-4)Gal(β1-4)Glc(β1-1)Cer; $GbOse_4Cer$, globotetraglycosylceramide, GalNAc(β1-3)Gal(α1-4)Gal(β1-4)Glc(β1-1)Cer; $GalNAcGbOse_4Cer$, Forssman glycolipid.

chloroform/methanol/water (65:25:4, v/v/v) in the first dimension and with n-butanol/acetic acid/water (60:20:4, v/v/v) in the second dimension. The phospholipids were visualized with ninhydrin spray reagent, Dittmer-Lester phosphate spray reagent, iodine vapors, and by charring with 20% sulfuric acid in methanol (17).

Mass spectrometry. Mono- and diglyceride fractions were analyzed as their trimethylsilyl ether derivatives by direct probe mass spectrometry. The direct probe conditions utilized were the same as previously reported (16) for the analysis of trimethylsilylated glycolipid samples. Liquid chromatography-mass spectrometry (LC-MS) of phospholipids was performed by methods previously reported by this laboratory (16,20) except that the HPLC column was eluted with a linear gradient consisting of solvent A, isooctane/2-propanol/chloroform/acetic acid (79:16:4:1, by vol) to solvent B, isooctane/2-propanol/chloroform/water/acetic acid (44:41:9:5:1, by vol) in 10 min at a flow rate of 0.75 ml/min. The sample to be injected was dissolved in isooctane/2-propanol (1:1, v/v) at a concentration of about 1 μg phospholipid per μl.

Immunoblot HPTLC. Glycolipids were chromatographed on aluminum-backed HPTLC plates (E. Merck) with chloroform/methanol/water (60:35:8, v/v/v), dried, and then dipped in polyisobutyl methacrylate in hexane as described by Brockhaus *et al.* (21). The plates were then soaked in phosphate-buffered saline containing bovine serum albumin before exposure to the antibody. The TLC plates of upper phase neutral glycolipids, gangliosides, and desialylated gangliosides were treated with monoclonal antibodies purchased from American Type Culture Collection T1B 121 (Rockville, MD) and with antibodies prepared at the E.K. Shriver Center by Drs. G. Schwarting and M. Yamamoto. The source and specificity of the antibodies used are shown in Table 1. The plates were washed in PBS and exposed to goat anti-mouse IgG and IgM conjugated to horseradish peroxidase. The plates were again washed with PBS and color developed with 33 mM 4-chloronaphthol and H_2O_2.

Extraction of total omentum lipids. Fresh porcine omenta obtained from the slaughter house were minced and then cryoground in liquid nitrogen. The omentum cryopowder was allowed to warm to 4°C and then was extracted with 10 volumes of chloroform/methanol (2:1, v/v). The extract was centrifuged (or vacuum-filtered) to remove particulate matter. The clear supernatant was evaporated initially at 37°C and finally at 55°C to dryness *in vacuo* to obtain the total omental lipids

TABLE 1

Specificity of Antibodies Used for Immunoblotting

Antibodies (+)	Source (*)	Specificity
5A (SSEA-3)	GS	Galβ1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-R
7A (SSEA-1)	MY	Galβ1-4GlcNAcβ1-R 3 | 1αFuc
AGM_2 (Asialo GM_2)	ATCC	GalNAcβ1-4Galβ1-4Glcβ1-R
AGM_1 (Asialo GM_1)	GS	Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-R
CA3	ATCC	Galβ1-3GlcNAcβ1-3Galβ1-R 4 | 1αFuc
WAF	GS	αGal(αFuc)-R (acidic and neutral compounds)
WEB	GS	αGal(αFuc)-R (only neutral compounds)
WCC4	GS	αGal(αFuc)GM_1-R (also reacts with neutrals — probably asialo)
IB2	GS	GAlβ1-4GlcNAcβ1-3Galβ1-4Glcβl-R
Forssman	ATCC	GalNAcα1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-R
Globoside	GS	GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-R
7C7 (GD_3)	GS	Galβ1-4Glcβ1-R 3 | 2αNeuAc8-2αNeuAc
4F4 (HNK-1)	GS	Sulfate-3-GluAβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-R
Cholera Toxin (B-Subunit)	LB	Primarily GM_1, but it also reacts with AGM_1, AGM_2, and GD1b

(*) GS, Dr. Gerald Schwarting E.K. Shriver Center; MY, Dr. Miyuki Yamamoto E.K. Shriver Center; ATCC, American Type Cell Culture, Rockville, MD; LB, List Biomedicals, Campbell, CA.

(+) SSEA, Stage Specific Embryonic Antigen; HNK, Human Natural Killer; WEB, WAF, WCC4, 7C7, 4F4, arbitrary laboratory designations.

or the chloroform/methanol fraction (CMFr). A total of 11 omenta were extracted and the extracts pooled for further analysis.

Fractionation of CMFr. CMFr was fractionated by a slight modification of the previously described procedure (16) as shown in Figure 1. For separation of the neutral lipids from the polar lipids, the CMFr was dissolved in hexane (approximately 60 ml/10 g of extract) and 0.66 volumes of 87% ethanol added. The phases were thoroughly mixed and allowed to separate. The lower phase was removed and the upper phase (hexane layer) re-extracted with 87% ethanol. The lower phases were combined and re-extracted with a fresh volume of hexane and the hexane phases combined. The phases were then taken to dryness *in vacuo* to obtain hexane upper phase material (hexane-UP) and ethanol lower phase material (ethanol-LP).

To further fractionate the hexane-UP material, 100 mg was dissolved in hexane and applied to an SRS-8010 4 mm × 50 cm Iatrobead column. The column was developed with a gradient of 100% benzene to 100% benzene/ethyl acetate/acetic acid (95:5:1) and fractions were collected and analyzed by HPTLC with benzene/ethyl acetate/acetic acid (90:10:0.2). The bulk of the material was eluted first and consisted only of triglycerides. Subsequently, diglyceride, cholesterol and free fatty acid fractions were eluted. The fractions containing spots migrating as the 1,2-diglycerides and those migrating as 1,3-diglycerides were collected separately. The fatty acid compositions, as determined by GC and direct probe MS analysis of the trimethylsilylated diglycerides, allowed the assignment of diglyceride molecular species.

The ethanol-LP fraction was subjected to solvent partition as described by Folch *et al.* (22). The material was dissolved in chloroform/methanol (2:1, v/v) (20 ml/g), and 0.2 volumes of water were added. Phases were thoroughly mixed and allowed to separate. The upper phase was removed and the lower phase was washed with 0.4 volumes of methanol/water (1:1, v/v). The upper phases were combined to obtain the Folch-UP fraction, and the lower phase was taken to dryness *in vacuo* to obtain Folch-LP material.

The Folch-LP material was dissolved in chloroform and subjected to chromatography on a silicic acid (Unisil) column (23). The Unisil column was eluted successively with 20 column volumes of chloroform, acetone/methanol (9:1, v/v), and methanol to obtain the neutral lipid, glycolipid and phospholipid fractions, respectively. Each fraction was examined by HPTLC.

The Folch-UP fraction (containing gangliosides and complex neutral glycolipids) was made 0.1 M with respect to KCl and applied to a C18 reversed-phase cartridge (BondElut) (24) which was then washed with 4 volumes of methanol/water (1:1, v/v) followed by elution with 4 volumes of methanol and then chloroform/methanol (2:1, v/v). The two washes were separately collected and evaporated to dryness *in vacuo* to obtain nonlipid upper phase material (nonlipid-UP) and lipid upper phase material (lipid-UP), respectively. The lipid-UP was dissolved in methanol and applied to a DEAE-Sephadex [acetate] column (25). The column was eluted with 10 volumes of methanol to obtain the neutral lipid

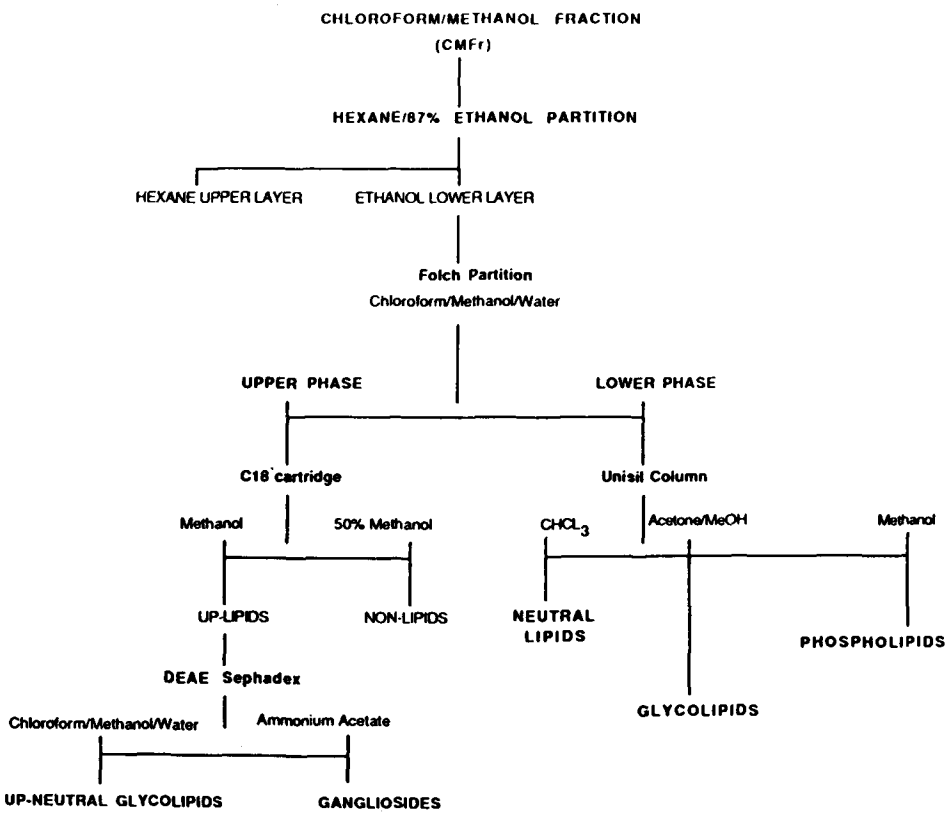

FIG. 1. Flow diagram for the fractionation of porcine omentum chloroform/methanol fraction (CMFr).

upper phase fraction (neutral lipid-UP) and then with 0.5 M ammonium acetate in methanol to obtain the ganglioside fraction. The fractions were evaporated to dryness *in vacuo*. The ganglioside fraction was desalted with a C18 reversed-phase cartridge as described above.

RESULTS

Table 2 lists the weight percent distribution of the various lipids subfractions described below.

The hexane-UP fraction (nonpolar lipids). This fraction contained approximately 99% of the material in the CMFr and was shown to consist primarily of triglycerides as determined by HPTLC. Small quantities of material migrating on HPTLC as free fatty acids, cholesterol, cholesterol ester, diglycerides, and monoglycerides were also present. Alkaline methanolysis of this fraction followed by GC and GC/MS analysis of the resulting fatty acid methyl esters revealed that 16:0 (28%), 18:0 (20%), 18:1 (39%) and 18:2 (12%) were the major fatty acids present.

The fatty acid composition of the isolated 1,2- and the 1,3- diglycerides was nearly identical and gave the following percent compositions: 14:0 (2.5%); 16:0 (34.5%); 16:1 (2.5%) 18:0 (20.6%); 18:1 (30.7%); 18:2 (9.2%). In both fractions the decreasing order of concentration of the molecular species as determined from the relative intensities of the diglyceride ions in the MS spectra was: 16:0-18:1>18:1-18:1>16:0-18:0>16:0-16:0>18:1-18:0>18:2-18:1. No differences between the two isomers were found or expected because considerable acyl migration is predicted for the sample preparation procedures utilized.

Folch lower phase glycolipids. The glycolipids were analyzed by HPTLC and visualized with the orcinol spray reagent (Fig. 2). Components migrating as mono-, di-, tri- and tetraosylceramides were present, the triaosylceramides being the major constituents. Each glycolipid band appeared as a doublet or triplet probably indicating differences in the fatty acid composition of these glycolipids. The glycolipid fraction was also analyzed by HPLC and the results are shown in Figure 3. These data confirmed the HPTLC results and provided quantitative information on the relative percent distribution of the major components as given in Figure 3's legend.

Phospholipids. The phospholipid fraction was examined by two-dimensional HPTLC and by LC-MS. The HPTLC data revealed components (as visualized with phosphate spray reagent, ninhydrin reagent and charring) that migrated as phosphatidylcholine, sphingomyelin, phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol, listed in order of decreasing concentration as judged by the intensity of charring. The LC-MS data confirmed the identification of these phospholipids and provided data on the molecular species composition of each phospholipid class. The carbon number and number of double bonds for each molecular species were identified from the mass spectra and the probable diacyl or alkylacylglycerol structures assigned and listed in order of decreasing ion intensity as follows: phosphatidylcholine (18:0-18:2, 16:0-18:1 and 16:0-16:0); phosphatidylethanolamine (18:0-18:2, alkyl 18:1-18:2, 18:0-20:4, alkyl 18:1-20:4, alkyl 16:0-20:4, alkyl 16:1-20:4, 18:0-18:3, 16:0-18:1 and alkyl 16:0-18:2); phosphatidylserine (18:0-18:1, 18:0-18:2); phosphatidylinositol (18:0-20:4). The ceramide molecular species present in the sphingomyelin peak were identified as 18:0, 22:0, 24:1, 16:0, 20:0 and 24:0 fatty acids all coupled to C18-sphingosine (sphingenine).

Complex neutral glycolipids. The neutral lipid-UP material contained several components as shown by HPTLC (Fig. 2) but lacked the Forssman glycolipid which was the major constituent of this fraction in the feline preparation (16). The major porcine component migrated as globoside, and several orcinol positive components migrating below globoside were noted. Im-

TABLE 2

Weight Percent Distribution of Fractions from Porcine Omentum Chloroform/Methanol Extracts*

Fraction	Percent of starting wt.	Percent of ethanol phase
CMFr	100	
Hexane-UP	99	
Ethanol-LP	0.4	100
Folch Lower Phase	0.3	71
**Unisil Column		
Neutral lipids	0.173	43
Glycolipids	0.015	3.7
Phospholipids	0.080	19.1
Folch Upper Phase	0.1	28
Nonlipid	0.1	23
Lipid	0.02	4.5
Neutral glycolipids	0.0005	0.1
Gangliosides	0.01	2.5

*The amount of each fraction was determined gravimetrically.
**Average of two preparations.

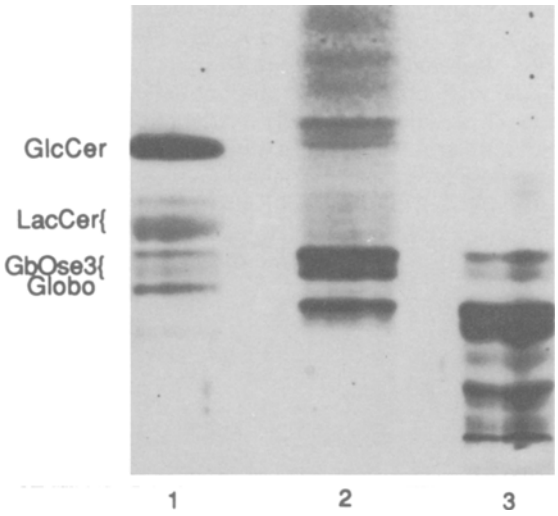

FIG. 2. HPTLC of Folch upper and lower phase neutral glycolipids (1, glycolipid standards; 2, lower phase neutral glycolipids; 3, upper phase neutral glycolipids). The plate was developed with chloroform/methanol/water (60:35:8, v/v/v) and the spots visualized with the orcinol spray reagent. Standard glycolipids are glucosylceramides, galabiosylceramides, globotriaosylceramides and globotetraosylceramides.

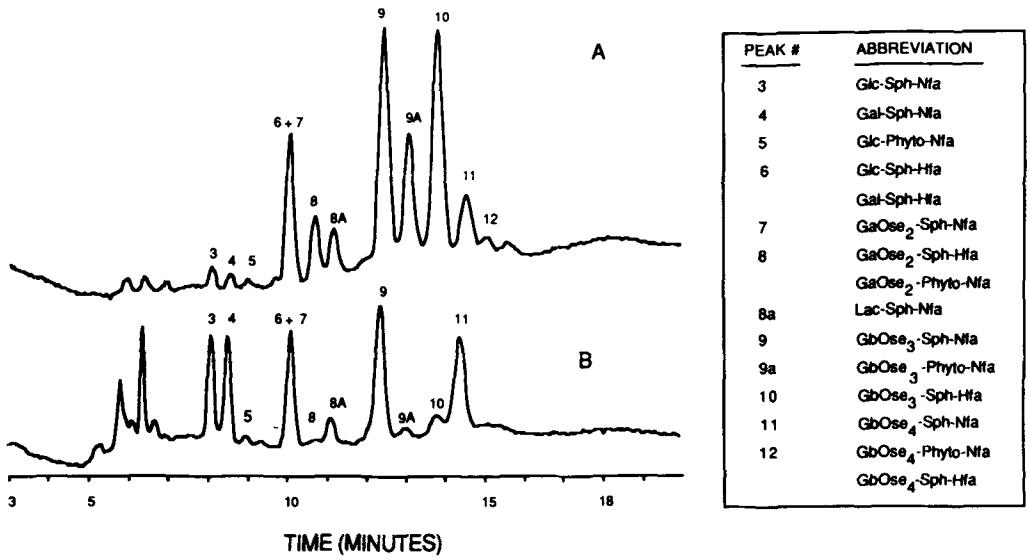

FIG. 3. HPLC of Folch lower phase glycolipids. The glycolipid fraction was perbenzoylated and examined by gradient elution HPLC on a Zipax column with 230 nm detection as previously described (18,19). A, standard male mouse (C57BL/6J) kidney glycolipids; B, porcine omentum upper phase glycolipids. The percent distribution of glycolipid components, listed as peak numbers, was found to be: 3, 12.0%; 4, 12.5% 5, 0.5%; 6+7, 17.2%; 8a, 4.5%; 9, 26.7%; 9a, 2.3%; 10, 3.4%; 11, 20%.

munoblot-HPTLC with several monoclonal antibodies of known specificity (Table 2) indicated the presence of ganglio-, globo- and neolactoglycolipids in four major areas. A doublet band, which migrated like globoside standard, reacted positively with the globoside and 5A antibodies, suggesting that both bands contain the globoside structure and that the doublet is most likely due to fatty acid differences. Just below globoside, several orcinol positive bands were present which reacted with WAF, WCC4, and WEB monoclonal antibodies indicating that they contain terminal α-galactose and α-fucose residues on ganglio structures. Antibody 5A had a different pattern of reactivity with components in this area of the HPTLC plate than did WAF, WCC4 and WEB, indicating that components containing a globo structure were also present in this area. The third group of glycolipids migrated just ahead of the SSEA-1 standard and reacted with both WCC4 and WEB but not WAF which indicated that these bands also consist of ganglio structures containing terminal β-galactose and fucose residues. Even more polar components are present which migrate just above the origin and also react with WEB and WCC4. The major components of this complex neutral glycolipid fraction are being isolated for further structural analyses.

Gangliosides. The ganglioside fraction was examined by HPTLC and at least six major resorcinol positive components were seen (Fig. 4). These corresponded in mobility to GM3, GM1, GD3, GD1a, GD1b, and GT1b. Each ganglioside was present as a doublet band which probably reflected the presence of short- and long-chain fatty acids. The relative amounts of the gangliosides (tentatively identified) were visually estimated to be: GD1a>GM3>GM1>GD3>GD1b>GT1b. The ganglioside fraction contained a yellow pigment

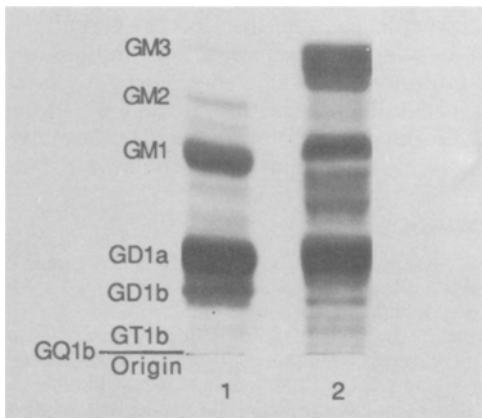

FIG. 4. HPTLC of porcine omentum gangliosides. The plates were developed with chloroform/methanol/0.25% calcium chloride (55:45:10, v/v/v) and visualized with resorcinol spray reagent. 1, bovine brain ganglioside standard; 2, porcine omentum gangliosides.

which streaked during HPTLC and interfered with the visualization of some components. Prior to further analysis, the ganglioside fraction was treated with 0.25 N sodium hydroxide in methanol for 2 hrs at 37°C, neutralized with glacial acetic acid and desalted with a C18 reversed-phase cartridge. Immunoblot-TLC of this preparation indicated the presence of ganglio and neolacto series gangliosides. The ganglioside fraction contained components that reacted with Cholera toxin, WCC4 and 7C7. A portion of the gangliosides was desialylated with 1% acetic acid for 2 hours at 100°C (26), and the asialo derivatives were examined by immunochromatography. Components reacting with AGM1, AGM2, WCC4, WEB, 7A, HNK and IB2 were detected indicating the presence of fucosylated globo-

and neolacto-type gangliosides as well as small amounts of sulfated glucuronic acid containing glycolipids. The isolation and structural analysis of the major components of this complex ganglioside fraction are in progress and the results will be published elsewhere.

DISCUSSION

The data reported here are the first on porcine omental lipids. The triglyceride fatty acid composition of porcine omentum resembled that reported by Rabinowitz *et al* (15) for their control human omental tissue except for the higher content of stearic acid. The identity and relative concentration of phospholipid species in the porcine tissue also closely paralleled that reported for human tissue.

Although some glycolipids may be characteristic of omentum, it is clear from the studies of pooled omentum extracts from feline (16), porcine and bovine (data not shown) that the glycolipid patterns are species specific. It is anticipated that the identification of the lipid species reported here will be useful in characterizing any biologically active lipid components of this tissue. The presence of mono- and diglycerides, mono-, di-, tri- and tetraglycosyl ceramides of the globoseries glycolipids, as well as gangliosides, could possibly relate to the angiogenic activity observed for the lipid extracts. Recently, Niinikoski *et al.* (27) reported that a hexosylceramide fraction from calf blood exerted an accelerating effect on wound-healing angiogenesis. Gullino (28) reviewed reports on factors that can trigger angiogenesis and provided evidence that heparin, fibronectin and gangliosides are involved in the mobilization of capillary endothelium.

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The ganglioside nomenclature used is according to the system of Svennerholm (*J. Neurochem. 10*, 613-623, 1963). The abbreviations and nomenclature used for glycosphingolipids are those recommended by IUPAC (1976) *Lipids 12*, 455-468.

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The Processing of Exogenous Cholesterol in the Alveolar Compartment of the Rat Lung

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We have examined the esterification of [^{3}H]cholesterol following the intratracheal instillation of a tracer amount into the isolated rat lung perfused with Krebs bicarbonate containing 4.5% albumin. At 5, 30 and 60 min after instillation, lungs were lavaged at 2°C with 3 x 10 ml of 0.15 M NaCl, each volume instilled and withdrawn three times. Each lung was lavaged at only one time point. The saline recovered was centrifuged at 150 g (5 min) to sediment the macrophage-rich fraction, leaving the surfactant in the supernatant. The amounts and specific activity of cholesterol and cholesteryl ester were measured following isolation by high performance liquid chromatography of the free cholesterol and the hydrolyzed ester-derived cholesterol. There was a rapid fall in [^{3}H]cholesterol in the surfactant fraction, accompanied by a reciprocal increase in [^{3}H]cholesteryl ester. Likewise, there was a rapid increase in [^{3}H]cholesteryl ester in the macrophage-rich fraction, while the level of free [^{3}H]cholesterol in that fraction remained very low. These data are consistent with exogenous cholesterol being rapidly esterified in the alveolus, and the ester then being cleared by the macrophages. We were unable to locate the actual site of esterification. Lipids *Lipids* 24, 957–961 (1989).

Cholesterol appears to be an integral component of pulmonary surfactant, being found in about equal amounts in both the lamellar bodies of the alveolar type II cell (1) and in the alveolar compartment of the lung. It is second only to dipalmitoyl phosphatidylcholine (DPPC) as the major component of surfactant, comprising some 10% by mass and 20% mol (2,3). Surprisingly little attention has been paid to cholesterol in surfactant, most of the attention being focused on the phospholipids, and, more recently, on the protein component. Cholesterol may aid the disperison of surfactant in the alveolar hypophase (4), enhance the stability of surfactant (5), and control the fluidity of the gas/liquid monomolecular layer. It may also influence the rate of synthesis of the surfactant phospholipids. Although much has been published recently on the processing of the phospholipids once released into the hypophase (6), nothing is known of the processing of cholesterol.

In the present study, we have investigated the possible esterification of cholesterol in the alveolar compartment and the involvement of macrophages in the fate of alveolar cholesterol.

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Abbreviations: BSTFA, bis(trimethylsilyl)trifluoracetamide; DPPC, dipalmitoyl phosphatidylcholine; GC-MS, gas chromatography-mass spectroscopy; HPLC, high performance liquid chromatography.

METHODS

The isolated perfused lung preparation. Perfusion methods were similar to those previously described (7). Briefly, male Porton rats (230-280 g) were heavily anesthetized with intraperitoneal methohexital sodium (160 mg/kg, Eli Lilly, Sydney, Australia), a tracheal catheter inserted, and the lungs ventilated with positive pressure at 60 breaths/min with 5% CO_2/95% O_2 (tidal volume 2.5 ml, end expired pressure 2 cm H_2O) via a Harvard Rodent Respirator (model 680, Harvard Instruments, MA). The thorax was then opened and catheters placed in the pulmonary artery via the right ventricle and in the left atrium via the left ventricle. Without interrupting the circulation, the lungs were perfused at 10 ml/min with the plasma substitute Hemaccel (Behring Institute, Federal Republic of Germany) for about three min while the lungs were removed. They were then placed in a closed chamber saturated with water vapor at 37°C and the positive pressure ventilation continued. The Hemaccel perfusate was then replaced by Krebs bicarbonate containing 4.5% bovine serum albumin (Cohn fraction V), recirculating through a 25 ml reservoir. The lungs were allowed to equilibrate for 10 min prior to instillation of radiolabeled cholesterol.

The instillation of radiolabeled cholesterol into the isolated perfused lung. [1,2(n)-^{3}H]Cholesterol (33 Ci/mmol; Amersham, Sydney, Australia) was instilled as a 150 μl bolus down the tracheal catheter. This amounted to ca 33,000 dpm of [^{3}H]cholesterol, or 0.17 ng, sonicated in 0.15 M saline. At 5, 30 and 60 min after instillation, the lungs were removed from the chamber, degassed, and lavaged at 2°C with three 10 ml volumes of 0.15 M NaCl, each volume instilled and withdrawn three times. Each lung was lavaged at only one time point. The recovered fluid (ca 95% of that instilled) was centrifuged at 150 g for five min at 2°C to sediment the macrophage-rich fraction, leaving the supernatant containing surfactant in tubular myelin-rich and poor forms. Each sample was analyzed for cholesterol and cholesteryl ester as described below.

Estimation of cholesterol esterification in vitro. The lungs from 12 rats were ventilated, perfused and removed as described above. Each lung was lavaged with a single 10 ml volume of Krebs bicarbonate (without albumin), instilled and withdrawn three times at 2°C. The lavage was centrifuged at 150 g for five min at 2°C to sediment the macrophage-rich fraction, leaving the supernatant containing surfactant. The macrophage-rich fractions from the different rats were combined and washed four times with Krebs bicarbonate. The fraction was pelleted again and then taken up in 60 ml of Krebs bicarbonate, gently mixed and divided in 12 x 5 ml aliquots. While six aliquots were maintained at 2°C, the remaining six were heated to 37°C in an oscillating water bath (30 oscillations per min). One-ml samples were removed from each tube immediately after the addition of 22,000 dpm of [^{3}H]cholesterol (33 Ci/mmol) (as a 50 μl bolus in Krebs

bicarbonate), and then at 30 and 60 min. Each sample was analyzed for cholesterol and cholesteryl ester according to the methods described below.

The surfactant-containing supernatants from the 12 lavages were also combined and then made up to a total volume of 120 ml with Krebs bicarbonate. This was subsequently divided into 24 x 5 ml aliquots. Twelve samples were maintained at 2°C, and the remaining were heated to 37°C. Aliquots of 500 μl were removed for analysis at the identical time described for the macrophage-rich fraction.

Analysis of cholesterol and cholesteryl ester. The supernatant fraction was lyophilized and lipids were extracted from both this and the macrophage-rich fraction by the method of Bligh and Dyer (8). The extracts were stored in chloroform at -20°C prior to analysis, when they were dried under nitrogen at room temperature and reconstituted with 500 μl of hexane, of this, 200 μl was assayed directly for free cholesterol, the remainder was dried under nitrogen, saponified and extracted according to the method of Abell-Kendall (9). Briefly, 5 ml of alcoholic KOH (6 ml of 33% KOH in water, diluted to 100 ml with absolute ethanol) was added to the dried lipid extract in a 20 × 150 mm glass culture tube with a PTFE-lined screw cap. The tubes were incubated at 45°C for 60 min and, after the addition of 5 ml of water to each, were allowed to cool to room temperature. Ten ml of hexane were added to each tube, which was then shaken mechanically for 10 min. The hexane layer was separated and evaporated to dryness under nitrogen at room temperature. The residue was dissolved in 300 μl of fresh hexane and assayed for total cholesterol. The amount of cholesteryl ester was determined as the difference between total and free cholesterol levels.The standards for determining free cholesterol were prepared as varying injected volumes (25, 50, 100, 150 and 200 μl) of a 200 μg/ml solution of cholesterol in hexane. The standards for determining total cholesterol were prepared by adding 5 ml of alcoholic KOH to known volumes of the 200 μg/ml cholesterol standard solution and then incubating, extracting, evaporating and dissolving the residue as described above. The mean coefficients of variation derived from the 12 calibration curves run concurrently with the analyses of free and total cholesterol were 4.5% and 5.8%, respectively.

The high performance liquid chromatography (HPLC). This system comprised a solvent delivery pump (model 45, Waters Associates, Milford, MA), a Wisp® autoinjector (Waters Associates), and a variable wavelength detector (model 481, Waters Associates) operated at 206 nm. Prepacked μPorasil columns (15 cm = 3.9 mm) were obtained from Waters Associates, as were the HPLC-grade solvents. The mobile phase consisting of hexane/isopropyl alcohol/acetic acid (750:10:0.1, v/v/v) was pumped at 1 ml/min, and the system was maintained at 21°C.

Our HPLC system for free cholesterol was based on a method of Hamilton and Comai (10) for the isolation and quantitation of cholesterol, cholesteryl ester, free fatty acids and triacylglycerides in tissue extracts. The elution time for free cholesterol was 6.0 min. In our experiment, where we identified the acyl moieties for esterified cholesterol, we first separated the lipid classes with silica Sep-Paks® (Waters Associates), and then used the HPLC system to resolve esterified cholesterol from free cholesterol and other endogenous pulmonary lipids. The cholesteryl esters were eluted as a single peak at 2.5 min and were free of any interfering peaks.

We verified the absolute structure of both the unesterified (eluted at 6.0 min) and the esterified sterol (eluted at 2.5 min) by gas chromatograpic-mass spectroscopy (GC-MS). Briefly, the HPLC eluted peaks from both surfactant and macrophage-rich fractions were collected separately as free and esterified "cholesterol". The esterified cholesterol eluted was saponified and the cleaved sterol extracted with hexane.

Both free and saponified sterol were evaporated to dryness with nitrogen and derivatized as the trimethylsilyl derivatives with *bis*(trimethylsilyl)trifluoroacetamide (BSTFA) in pyridine (Pierce, Rockford, IL). They were injected into a gas chromatography (model 5890 Hewlett-Packard) electron impact mass spectrometry system (Jeol Jms Dx-303 single-beam, double-focusing) (GC-MS). A Jeol Jma-Da5000 data system was used. Chromatographic separation was achieved with a splitless injector coupled to a BP I (SGE, Melbourne, Australia) bonded phase fused silica column (Scientific Glass Engineering, Melbourne, Australia), of 12 m x 0.22 mm internal diameter and 0.25 μm phase thickness, directly inserted into the mass spectrometer source. The injector temperature was 250°C, the helium flow rate was 1.0 ml/min and the column was temperature programmed from 160 to 320°C at 4°C/min. The mass spectrometry fragmentation pattern of the single eluted gas chromatographic peak—obtained from both macrophage-rich and macrophage-free lavage samples—was identical to that obtained when a pure cholesterol standard (BDH, Poole, England) was passed through the GLC-MA system.

Radiopurity of the instilled [^{3}H]cholesterol was verified by counting the eluted cholesterol peak (cold cholesterol carrier was combined with radiolabeled cholesterol prior to HPLC injection). Eluted cholesterol peaks from each sample injected were collected in borosilicate glass tubes and the mobile phase evaporated to dryness in order to determine radioactivity of free and saponified cholesterol eluates. Samples were counted in a Searle Nuclear Chicage Mark III β scintillation counter (model 6880, Searle Analytical Inc., Des Plaines, IL) using a scintillation cocktail consisting of 7g of 2, 5-diphenyloxazole, 300 ml ethoxyethanol, and made up to 1 l with xylene.

The identity of the acyl moieties of the cholesteryl ester isolated from the surfactant and macrophage-rich fractions was determined by transmethylating in 1% (vol/vol) H_2SO_4 in methanol, and then analyzing the methyl esters using twin columns of SP2310 (Supelco Inc., Bellefonte, PA) in a Hewlett-Packard 5840A gas chromatograph programmed for 125-225°C at 4°C/min.

RESULTS

Esterification of cholesterol in the isolated perfused lung. The mass of free cholesterol, the percentage of dpm of free cholesterol normalized to the total recovered dpm (free plus esterified), and the specific activity of free cholesterol in both surfactant and macrophage-rich fractions are presented in Table 1. The corresponding values for cholesteryl ester are also presented. There was no

TABLE 1

The Mass and Specific Activity of Free Cholesterol and Cholesteryl Ester in the Alveolus Following the Intratracheal Instillation of [^{3}H] Cholesterol.

	μg/GDL	Percent dpm recovered	dpm/μg cholesterol	μg/GDL	Percent dpm recovered	dpm/μg cholesterol
	Free cholesterol in surfactant			Free cholesterol in macrophages		
5 min	560 ± 24	76.5 ± 3.1	250.0 ± 9.9	124 ± 10	2.7 ± 0.7	41.1 ± 11.3
30 min	535 ± 27	19.7 ± 2.3[a]	62.8 ± 6.8[a]	120 ± 7	2.9 ± 0.9	40.0 ± 10.2
60 min	581 ± 35	1.8 ± 0.5[a]	4.2 ± 1.2[a]	122 ± 6	2.5 ± 0.5	39.3 ± 3.3
	Cholesteryl ester in surfactant			Cholesteryl ester in macrophages		
5 min	130 ± 10	14.4 ± 2.1	209.0 ± 34.0	69 ± 7	5.8 ± 0.9	167.0 ± 39.2
30 min	129 ± 7	41.4 ± 4.1[a]	547.8 ± 41.2[a]	71 ± 6	39.5 ± 2.0[a]	1003.0 ± 132.6[a]
60 min	137 ± 6	45.9 ± 4.3	567.0 ± 38.0	71 ± 6	50.2 ± 4.3[a]	1390.8 ± 122.5[a]

[a]Significantly different from value immediately above, $P < 0.001$.

Values are expressed as mean ± SEM; six lungs/group. GDL = gram dry lung. At time zero, 33,000 dpm of [^{3}H]cholesterol in 150 μl NaCl was instilled down the trachea of the isolated perfused rat lung. At 5, 30 and 60 min, the lungs were lavaged and the lavage fluid centrifuged at 150 g (5 min) to sediment the macrophage-enriched fraction.

change in the absolute mass of either cholesterol or cholesteryl ester at any time point. In the surfactant fraction the specific activity of free cholesterol fell rapidly, while there was a concomitant increase in the specific activity of cholesteryl ester. This increase paralled an increase in the specific activity of cholesteryl ester in the macrophage-rich fraction. When we expressed the recovery of dpm of cholesterol and ester as a percentage of the total harvested from surfactant plus macrophage-enriched fractions, we found no significant decrease in recovery up to 60 min of perfusion (5 min, 99.2 ± 2.8%; 30 min, 98.2 ± 2.7%, 95.5 ± 1.4%; mean ± SEM of six lungs/group).

The percentage distribution of the major fatty acid moieties of the cholesteryl esters recovered in the surfactant and macrophage-enriched fractions is presented in Table 2. The distribution of esters did not differ between the two fractions. The monoenoic fatty acid oleic acid accounted for 33%, while the saturated fatty acid palmitate comprised approximately 20% in both fractions.

TABLE 2

The Distribution of the Major Fatty Acids Derived from Cholesteryl Esters in Surfactant and Macrophage-Rich Fraction

Acyl moiety	Surfactant	Macrophage
14:0	6.7 ± 0.05	6.6 ± 0.59
16:0	20.1 ± 1.1	21.8 ± 4.5
18:0	7.0 ± 0.64	6.1 ± 0.70
18:1	33.7 ± 1.7	32.6 ± 3.3
18:2	12.4 ± 0.73	12.1 ± 1.0

Results expressed as mean ± SEM in percentages, four lungs/groups. There were no significant differences between groups.

Esterification of cholesterol in vitro. When the macrophage-rich fraction was incubated with radiolabeled cholesterol at either 2 or 37°C there was no change in the specific activity of free cholesterol with time (Table 3). We could not detect radiolabeled esterified cholesterol in the macrophage, even after 60 min of incubation. Likewise, when we incubated the surfactant-rich supernatant with [^{3}H]cholesterol, we found no evidence of esterification, even after 60 min at 37°C (Table 3).

TABLE 3

The Specific Activity of Free Cholesterol and Cholesteryl Ester in the Alveolar Surfactant and Macrophage-Rich Fractions Lavaged from Rat Lungs Following Incubation of These Fractions with [^{3}H]cholesterol

Temp	Free cholesterol			Cholesteryl ester		
°C	0 min	30 min	60 min	0 min	30 min	60 min
Macrophage-rich fraction						
2	18.3 ± 4.9	20.1 ± 3.9	19.8 ± 1.0	N.D.	N.D.	N.D.
37	19.7 ± 3.2	21.2 ± 4.7	18.5 ± 3.6	N.D.	N.D.	N.D.
Supernatant fraction						
2	21.7 ± 1.9	20.4 ± 2.0	20.9 ± 2.6	N.D.	N.D.	N.D.
37	18.8 ± 4.2	21.6 ± 3.5	19.8 ± 1.7	N.D.	N.D.	N.D.

Results are expressed as mean ± SEM in dpm × 10^3 per original 5 ml volume of incubate, six incubations/group in the macrophage-rich fraction, 12 incubations/group in the supernatant fraction. N.D. = dpm not detected above background. There were no significant differences between the different periods of incubation, or between the two different temperatures.

DISCUSSION

We have found that a tracer amount of free cholesterol, instilled down the trachea of an isolated perfused rat lung, is rapidly esterified and that the esters appear in the alveolar macrophages. After 60 min over 95% of the total amount of radioactivity instilled can be lavaged from the alveolar compartment. If this also reflects the fate of endogenous cholesterol, then this sterol is being handled very differently from the phospholipid component of alveolar surfactant. The latter is taken up into the alveolar type II cell as intact molecules (11) and macrophages play little part in their fate (12).

Validation of methods. We chose to use the isolated perfused rat lung preparation as it enabled us to control respiration, end expired pressure, and perfusion rate.

Furthermore, it was much easier to instill a solution down the accessible trachea of a vertically orientated isolated lung than it is *in vivo*.

It is difficult to determine whether the radiolabeled cholesterol we instilled was treated as endogenous sterol. We cannot discount the possibility that under physiological conditions the alveolar cholesterol is protected by its association with the other components of surfactant. One way to circumvent this problem would be to instill isolated lamellar bodies containing radiolabeled cholesterol. The amount of high specific activity [^{3}H]cholesterol instilled represented a true tracer amount, being less than 0.0001% of the total endogenous cholesterol in the alveolar compartment. There was no measurable change in the total amount of unlabeled cholesterol or cholesteryl ester in either the alveolar surfactant or macrophage-rich fractions during the total 60 min period, suggesting a steady-state condition. This is also consistent with a constant number of lavagable macrophages present up to 60 min. We did not count the number of macrophages. From previous studies we know that when this volume of saline containing lamellar bodies was instilled, a significant proportion reached a marked increase in the numbers of whole and unravelling lamellar bodies in the alveolar compartment (unpublished data). Lamellar bodies are usually not seen in the alveolar compartment of adult rat lungs. Hence, we are confident that some cholesterol was reaching the alveolar compartment, although the proportion is unknown. The possibility remains that the rate- limiting step in the esterification of cholesterol is the time taken for the cholesterol is the time taken for the cholesterol to reach the alveolar compartment. However, the same criticism can be levelled at all the recent reports where investigators have instilled different forms of surfactant and measured the apparent rate of disappearance from the alveolar compartment. Furthermore, the mucociliary-escalator would be moving material from the distal to proximal lung, i.e., in the opposite direction.

We are confident that our HPLC method accurately identifies and quantitates cholesterol. The mass spectrum and retention time of the sterol obtained from our surfactant and macrophage-rich fractions were identical to those obtained after a pure standard of cholesterol was derivatized and chromatographed under indentical conditions.

Metabolism of cholesterol in the surfactant and macrophage fractions in vitro. Although we could show no esterification when we incubated either the macrophage-rich or the surfactant fraction with [^{3}H]cholesterol, a major reservation exists regarding the incubating medium. Whereas we used Krebs bicarbonate, the alveolar hypophase is almost certainly of very different composition and pH. However, in the absence of accurate knowledge of its composition, there was no alternative. Therefore, it is possible that the conditions were not optimal for the action of a cholesterol esterifiying enzyme. It is also possible that the cholesterol esterifying enzymes were inactivated during the processing.

Metabolism of cholesterol in the isolated perfused lung. We found 81% of free unlabeled cholesterol in the surfactant fraction and 18% in the macrophage-rich fraction over the 60 min duration of these experiments. In contrast, the same relative distribution of cholesteryl ester was 65 and 35%, respectively. These distributions remained constant over 60 min of perfusion. Although after 60 min we could still harvest over 95% of the amount of instilled [^{3}H]cholesterol, the distribution of radiolabel had changed markedly. At that time the amount of free radiolabeled cholesterol in the surfactant fraction had fallen to near zero, and there was a concomitant increase in radiolabeled ester which appeared to plateau after 30 min. In contrast, the percent of radiolabeled free cholesterol in the macrophage fraction remained very low and constant. After 60 min 50% of the total amount of radiolabel lavaged was accounted for by the esterified cholesterol in the macrophage fraction. These results are consistent with cholesterol being esterified in the surfactant fraction and the esters taken up by the macrophages. The fact that these two fractions had identical acyl moieties is also consistent with a precursor-product relationship between the two. Alternatively, the esterification may have occurred either on the surface of the macrophages or immediately upon the sterol reaching the cytoplasm. If, despite our reservations with the incubation medium, our negative results with the *in vitro* incubation of macrophages and surfactant fraction do reflect the situation *in vivo*, the possibility remains that the esterification occurs at the epithelial cells.

The fact that even after 60 min we could still recover over 95% of instilled cholesterol, strongly suggests that the sterol is remaining in the alveolar compartment, and also that the same population of macrophages remain. From our present data it is very difficult to attribute precursor-product relationships. We suggest that the precipitous fall in the specific activity of free cholesterol in surfactant, when viewed in light of the simultaneously high specific activities in the cholesteryl ester in surfactant and, in particular, in the macrophage fraction, reflects the existence of more than one pool within our surfactant fraction. There must be a small pool with a very high specific activity, possibly containing only the labeled exogenous cholesterol, and a large pool with a low specific activity. Certainly we can conclude that exogenous cholesterol is very rapidly and completely esterified in the airspaces of the lung, with a half life of about 15 min. This may well be the fate of cholesterol instilled as part of an exogenous or artificial surfactant used in therapy for neonatal respiratory distress syndrome.

Cholesterol is known to have profound effects on fluidity of membranes. It intercalates lipid bilayers, with its β-OH group at the aqueous interface and the rings and hydrocarbon tail towards the inside (13). At temperatures above the phase transition of the bilayer, cholesterol depresses fluidity, whereas if the temperature is below, it increase fluidity. Cholesterol appears to act by either enhancing or inhibiting the van der Waals interactions between apposed fatty acyl chains. The β-OH group favors the oxygen on the 2-acyl group, and cholesterol aligns its planar surface to this acyl group. It extends beyond the C-9 position, which is the usual site of the first double bond, and hence would tend to retard flexing (14-16). The actual effect of cholesterol will depend on the phospholipid composition of the membrane. Esterification of cholesterol at the β-OH group would tend to make the molecule drop out of the bilayer. Whether this is the case with a surfactant monolayer is unknown, but if so, the esterification of cholesterol could affect the surface chemistry of alveolar surfactant. In contrast to cholesterol, cholesteryl ester has little effect on the minimum

surface tension attained by DPPC (16), at least at 25°C. Hence the cholesterol:cholesteryl ester ratio may greatly affect the surface tension of the monolayer. By rapidly esterifying free alveolar cholesterol, the lung inactivates an agent which can profoundly affect fluidity of surfactant.

We conclude that exogenous cholesterol is rapidly esterified in the alveolar compartment and suggest that it is handled very differently from endogenous surfactant-associated cholesterol. We speculate that the cholesterol:cholesteryl ester ratio may influence the surface tension at the gas-liquid interface.

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A Tetrameric Dialdehyde Formed in the Reaction of Butyraldehyde and Benzylamine: A Possible Intermediary Component for Protein Cross-Linking Induced by Lipid Oxidation

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We investigated the reaction of butyraldehyde and benzylamine and analyzed the products to identify the components that produce protein cross-linking in the reaction of butyraldehyde and proteins. When the mixtures of butyraldehyde and benzylamine were incubated at pH 7 and 37°C for 48 hr, many reaction products other than 2-ethyl-2-hexenal and Schiff bases of butyraldehyde and 2-ethyl-2-hexenal were produced. Fluorescent substance(s) were formed only in the presence of dissolved oxygen in the reaction mixture. Three new nonflourescent products—*d*,*e* and *f*—were isolated, and their structures are suggested to be 2,9-dibenzyl-4,6,8-triethyl-7-propyl-2,9-diazabicyclo[3,3,1]nona-3-ene (*d*), 1-phenyl-2-benzyl-4,5,7-triethyl-6-propyl-1H,2H,3H,5H,6H,7H,8H-pyrido[1,2-c]pyrimidine (*e*) and 1-phenyl-2-benzyl-4,5,7-triethyl-6-propyl-1H,2H,4aH,5H,6H,7H,8H-pyrido[1,2-*c*]pyrimidine (*f*). Formation of these compounds suggested that the protein cross-linking with butyraldehyde is caused by the tetrameric dialdehyde formed by repeated aldol condensation and Michael reaction of butyraldehyde.
Lipids 24, 962-969 (1989).

It is well-known that a variety of aldehydes such as malonaldehyde, alkanals, alkenals, alkadienals and 4-hydroxyalkenals is generated as a result of lipid oxidation (1,2). Induction of lipid oxidation in erythrocyte membrane results in the formation of cross-links (3-9) together with fluorophores (8) and borohydride-reducible functions (9) in the membrane proteins. Malonaldehyde can produce cross-links, fluorescence and borohydride-reducible functions in proteins (10-12). However, because of its extremely low content in oxidized lipids (13,14), this aldehyde is an unlikely compound for induction of protein damage during lipid oxidation. On the other hand, various aldehydes other than malonaldehyde are considered to be more important lipid oxidation products to induce protein damage (11,12). Fluorescence spectra of the membrane proteins modified during lipid oxidation are more similar to those of the alkanal-, alkenal- and alkadienal-modified proteins than to those of the malonaldehyde-modified proteins (8,15-17). Alkanals, alkenals and alkadienals can also produce cross-links (8,15,16,18) and borohydride-reducible functions (9). However, the structures of the products and the reaction mechanisms of these aldehydes have not yet been elucidated.

Hexanal is one of the major aldehydes produced by lipid oxidation (1). Butyraldehyde is a closely related aldehyde with a shorter alkyl chain and may undergo the same reactions as hexanal. We have previously demonstrated that butyraldehyde is dimerized into 2-ethyl-2-hexenal as a consequence of aldol condensation by catalysis of methylamine, which is, in turn, transformed into fluorescent products by reaction with methylamine in the presence of molecular oxygen (19). Furthermore, it has been shown that the reaction of butyraldehyde with proteins gives rise to cross-links between the amino groups in the proteins (16). We have now investigated the reaction of butyraldehyde and benzylamine, and analyzed the reaction products to identify the components that produce cross-links in proteins. Benzylamine bears no reactive functions except for an amino group, and its reaction products may be surveyed by its phenyl chromophore.

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Abbreviations used: NMR, nuclear magnetic resonance; GC, gas chromatography; GC-MS, gas chromatography-mass spectrometry; HPLC, high pressure liquid chromatography.

MATERIALS AND METHODS

Materials. Butyraldehyde and 2-ethyl-2-hexenal were obtained from Wako Pure Chemical Industries, Osaka and Tokyo Kasei Kogyo Company, Tokyo, respectively.

Analysis. Ultraviolet absorption spectra were taken with a Hitachi 557 double wavelength, double beam spectrophotometer. Fluorescence spectra were measured with a Hitachi 650-40 fluorescence spectrophotometer. ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were taken on a Bruker AM400 FT-NMR spectrometer using $CDCl_3$ as a solvent. Signals in ^{13}C NMR spectra were assigned by the *complete decoupling technique* and the *distortionless enhancement by polarization transfer* (DEPT) technique. Mass spectra were obtained on a Hitachi M-80 double focusing mass spectrometer, and measured by an electron impact (EI) ionization technique using an ionization energy of 70 eV and ion source temperature of 200°C.

Gas chromatography-mass spectrometry (GC-MS) was performed on a Hitachi M-80 GC-MS attached with a Silicone OV-101 (1.5% on Chromosorb W) column (0.3 x 20 cm). GC conditions were: injection port temperature was 150°C; GC column oven temperature was programmed from 120°C at 5°C/min to 240°C; carrier gas (helium) flow rate was set to 40 ml/min. GC-MS elution profiles were recorded with a total ion current.

High pressure liquid chromatography (HPLC) was carried out with a Hitachi 655 liquid chromatograph. For analytical purposes, a column of Inertsil ODS (4.6 x 250 mm) (Gaskuro Kogyo Company, Ltd., Tokyo) was used. Elution was performed with acetonitrile or acetonitrile/water (8:2, v/v) at a flow rate of 0.5 ml/min. For preparative purposes, a column of Inertsil ODS (20 x 250 mm) was used and eluted with the same solvent at a flow rate of 5.0 ml/min. The peaks were detected by a Shimadzu RF-530 fluorescence spectromonitor and a Hitachi 638-41 variable wavelength UV monitor.

Formation of fluorescent and nonfluorescent compounds in the reaction of butyraldehyde and benzylamine. Into a test tube (13-ml content) with a screw cap was

placed a 10-ml mixture of butyraldehyde (or 2-ethyl-2-hexenal) and benzylamine with the indicated reactant concentrations in 90% methanol/0.1 M phosphate buffer (pH 7.0). The mixture was incubated at 37°C for 48 hr. In order to know the influence of oxygen on the reaction, dissolved oxygen was freed from the mixture by bubbling nitrogen gas prior to the reaction. In order to know the effect of borohydride on the reaction products, the reaction mixture was treated with 5 mmol sodium borohydride at 0°C for 1 hr after the reaction. Fluorescence spectrum of the reaction mixture was directly measured after dilution into methanol, and fluorescence intensity relative to that of 0.1 μM quinine sulfate in 0.1 N sulfuric acid was obtained. The reaction mixture was extracted with 4 volumes of chloroform and 3 volumes of water, and the chloroform extract was subjected to GC-MS. The chloroform extract was evaporated to dryness, dissolved into 2.0 ml of methanol and subjected to analytical HPLC.

Attempts to isolate the fluorescent substance(s). A 300-ml mixture of 200 mM butyraldehyde and 100 mM benzylamine in 90% methanol/0.1 M phosphate buffer (pH 7.0) was incubated at 37°C for 48 hr. To the mixture were added 4 volumes of chloroform and 3 volumes of water, and the chloroform extract was evaporated to dryness (7 g). The residue was subjected to preparative HPLC and the column was eluted with acetonitrile/water. The fluorescent peak detected at 360 nm (excitation) and 440 nm (emission) was collected. The peak fraction was purified by analytical HPLC by elution with the same solvent. The fluorescent peak fraction was evaporated to dryness to yield 0.9 mg of a colorless oil. The oil showed a single peak with a retention time of 10.2 min in GC. The oil showed absorption maxima at 291 and 364 nm (methanol) and fluorescence with an excitation maximum at 369 nm and an emission maximum at 439 nm (methanol). The following data of the oil indicated that the major component was N-benzybutyramide, and the fluorescent substance(s) were the minor components in the oil—EI mass spectrum: *m/z* (rel intensity), 177 [M^+] (100), 162 [M^+-15] (20), 149 (30), 135 (10), 106 (95) and 91 [benzyl] (100); ^{13}C NMR spectrum ($CDCl_3$): ppm, 172.9 (CO), 138.4 (phenyl C), 127.5, 127.8, 128.7 (phenyl CH), 43.6 (benzyl CH_2), 38.7 (alkyl CH_2), 19.2 (alkyl CH_2) and 13.8 (CH_3); ^1H NMR spectrum ($CDCl_3$): ppm, 7.38-7.26 (5H, *m*, phenyl), 5.84 (1H, *bs*, NH), 4.45 (2H, *d*, benzyl CH_2, J = 5.7 Hz), 2.20 (2H, *t*, CH_2), 1.65 (2H, *m*, CH_2) and 0.95 (3H, *t*, CH_3).

Isolation of nonfluorescent compounds d, e and f. A 1000-ml mixture of 200 mM butyraldehyde (or 100 mM 2-ethyl-2-hexenal) and 100 mM benzylamine in 90% methanol/0.1 M phosphate buffer (pH 7.0) was incubated at 37°C for 48 hr. After treatment of the reaction mixture with 500 mmol sodium borohydride, the mixture was extracted with chloroform. The extract was washed with water and evaporated to dryness to yield oily residues: 15.3 g for butyraldehyde and 16.2 g for 2-ethyl-2-hexenal. The oily residues were subjected to preparative HPLC and eluted with acetonitrile. The peaks were detected at 250 nm. The peak fractions corresponding to compounds *d* and *e* were obtained from the reaction of butyraldehyde. The peak fraction corresponding to compound *f* was obtained from the reaction of 2-ethyl-2-hexenal. These peak fractions were evaporated to dryness and rechromatographed on analytical HPLC by elution with acetonitrile. The purified peak fractions showed single ultraviolet absorbing peaks with retention times of 42 min for *d*, 47 min for *e* and 42.5 min for *f*. Each peak fraction was evaporated to dryness to yield 4.7 mg of *d*, 10.2 mg of *e* and 8.4 mg of *f* as colorless oils. Compounds *d*, *e* and *f* showed the same ultraviolet absorption spectrum with an end absorption and a broad shoulder at 240 nm.

RESULTS

The mixtures of butyraldehyde (or 2-ethyl-2-hexenal) and benzylamine with different reactant concentrations in methanol/phosphate buffer (pH 7) were incubated at 37°C for 48 hr (Table 1). Reactions **1-4**, in which the ratios of the reactants were different, produced similar fluorescence with excitation maxima at 350-370 nm and emission maxima at 410-440 nm. Reaction **3**, in which the ratio of butyraldehyde and benzylamine was 2:1, yielded the highest fluorescence. When the mixture of reaction **3** was freed from dissolved oxygen prior to the reaction, formation of fluorescence was completely suppressed. When the mixture of reaction **3** was treated with borohydride after the reaction, produced fluorescence was com-

TABLE 1

Fluorescence Spectra and Intensities of the Reaction Mixtures of Butyraldehyde (or 2-Ethyl-2-Hexenal) and Benzylamine Incubated at 37°C for 48 hr

	Reaction mixture					Fluorescence		
Reaction	Butyraldehyde (mM)	2-Ethyl-2-hexenal (mM)	Benzylamine (mM)	O_2[a]	Treatment with borohydride[b]	Excitation max (nm)	Emission max (nm)	Relative intensity
1	25		275	+	-	363	414	9
2	100		200	+	-	364	440	32
3	200		100	+	-	364	441	371
				-	-			2
				+	+			4
4	275		25	+	-	351	422	104
5		100	100	+	-	360	433	42
				-	-	366	426	21

[a]Dissolved oxygen in the reaction mixture.
[b]Treatment after the reaction.

pletely destroyed. The reaction of 2-ethyl-2-hexenal with benzylamine in a 1:1 ratio (reaction **5**) yielded a similar fluorescence but the intensity was lower than that of reaction **3**.

HPLC of the chloroform extract of the reaction mixture of reaction **3** (Fig. 1A) revealed a flourescent peak at a retention time of 8 min and a large number of ultraviolet absorbing peaks. The reaction conducted under anaerobic conditions produced the same ultraviolet absorbing peaks but no fluorescent peaks. HPLC of the reaction mixture after borohydride treatment (Fig. 1B) indicated that the fluorescent peak completely disappeared, but most of the ultraviolet absorbing peaks remained unchanged. HPLC of the chloroform extract of the reaction mixture of reaction **5** revealed fluorescent peaks and ultraviolet absorbing peaks (Fig. 1C), and that after treatment with borohydride revealed no fluorescent peaks but did reveal ultraviolet absorbing peaks (Fig. 1D). The reaction conducted under anaerobic conditions produced the same ultraviolet absorbing peaks.

The fluorescent substance(s) produced in reaction **3** was purified by preparative HPLC and obtained as a colorless oil. The oil showed a fluorescence spectrum with an excitation maximum at 369 nm and an emission maximum at 439 nm, and ultraviolet absorption maxima at 291 and 364 nm. However, the major component in the oil was identified as nonfluorescent *N*-benzylbutyramide by mass and NMR spectra (see Materials and Methods section). An attempt to isolate the fluorescent substance(s) was unsuccessful.

Total ion monitoring in GC-MS analysis of the chloroform extract of the borohydride-treated reaction mixture of reaction **3** indicated the presence of various products (Fig. 2A). The ion peak at a retention time of 1.1 min showed the mass spectrum with *m/z* 128 [M^+] (relative intensity 53), 99 [M^+-29 ($C_2H_5\cdot$)] (50) and 85 [M^+-43 ($C_3H_7\cdot$)] (100), indicating that the product (product *a*) was 2-ethyl-2-hexenol. The ion peak at a retention time of 3.3 min showed the spectrum with *m/z* 163 [M^+] (5), 120 [M^+-43 ($C_3H_7\cdot$)] (90) and 91 [benzyl] (100), indicating the product (product *b*) was *N*-butylbenzylamine. The ion peak at a retention time of 9.1 min showed the spectrum with *m/z* 217 [M^+] (5), 188 [M^+-29 ($C_2H_5\cdot$)] (40),174 [M^+-43 ($C_3H_7\cdot$)] (40), 120 [M^+-97] (40) and 91 [benzyl] (100),indicating the product (product *c*) was *N*-(2-ethyl-2-hexenyl) benzylamine. Many ion peaks including those corresponding to compounds *d* and *e* were observed at the longer retention times. They showed mass spectra with molecular or fragment ion peaks of more than 210 *m/z* and a fragment ion peak of benzyl. When ultraviolet absorbing HPLC peak fractions at retention times of 40 and 45 min from the same reaction mixture (Fig. 1B) were subjected to GC-MS analysis, they showed sharp ion peaks at GC-MS retention times corresponding to compounds *d* and *e*, respectively. Furthermore, ultraviolet absorbing HPLC peak fractions at the same retention times from the non-reduced reaction mixture of reaction **3** (Fig. 1A) also revealed sharp ion peaks corresponding to compounds *d* and *e*, respectively. Thus, compounds *d* and *e* were the products of butyraldehyde and benzaylamine and not the products of borohydride treatment.

Total ion monitoring of the chloroform extract of the borohydride-treated reaction mixture of reaction **5** (Fig. 2B) revealed several ion peaks including that corresponding to compound *f*, besides the ion peaks corresponding

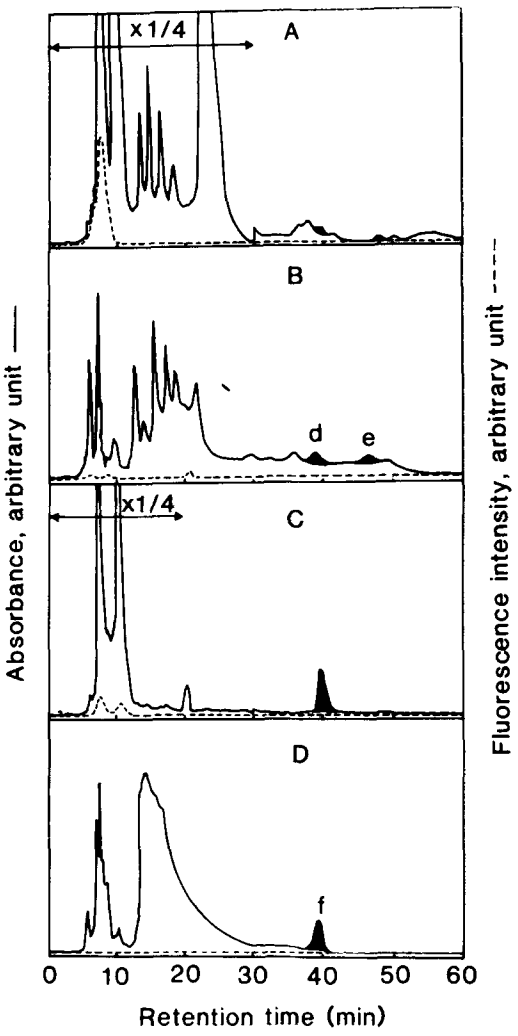


FIG. 1. HPLC of the chloroform extract of the reaction mixture of butyraldehyde (A, B) or 2-ethyl-2-hexenal (C, D) with benzylamine.

The reaction mixtures from reaction 3 (A) and 5 (C) in Table 1 and those treated with borohydride (B and D, respectively) were extracted with chloroform. The chloroform extracts were evaporated to dryness, dissolved into methanol and subjected to analytical HPLC with an Inertsil ODS column and eluted with acetonitrile. Absorbance at 250 nm (———) and fluorescence with excitation at 360 nm and emission at 440 nm (- - - - -) were monitored.

to *a* and *c*. They appeared at the longer retention times and showed mass spectra with molecular or fragment ion peaks of more than 230 *m/z* and a benzyl fragment ion peak. When the ultraviolet absorbing HPLC peak fraction at a retention time of 40 min from the same reaction mixture (Fig. 1D) was subjected to GC-MS analysis, it showed a sharp ion peak corresponding to compound *f*. Similarly the ultraviolet absorbing HPLC peak fraction at the same retention time from the nonreduced reaction mixture (Fig. 1C) showed the ion peak corresponding to compound *f*. Compound *f* was the product of 2-ethyl-2-hexenal and benzylamine.

It was found that the reaction of butyraldehyde and benzlamine gave many products other than 2-ethyl-2-hexenal and the Schiff bases of butyraldehyde and 2-ethyl-2-hexenal. Among the many products, compounds *d* and *e* were purified by preparative HPLC of the

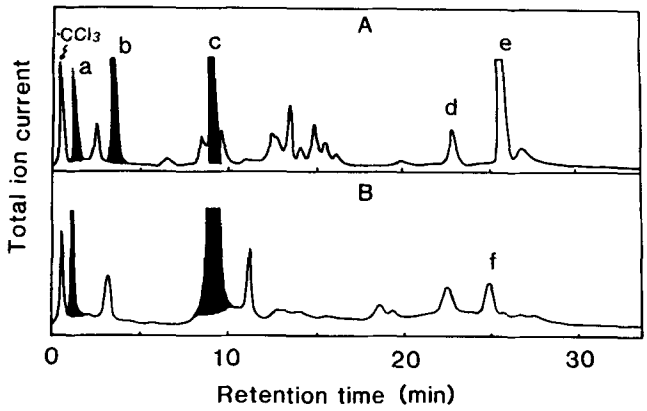


FIG. 2. GC-MS of the chloroform extract of the borohydride-treated reaction mixture of butyraldehyde (A) or 2-ethyl-2-hexenal (B) with benzylamine.

The reaction mixtures from reaction 3 (A) and 5 (B) in Table 1 were treated with borohydride and extracted with chloroform. The chloroform extract was subjected to GC-MS with a Silicone OV-101 column and helium as a carrier gas.

borohydride-treated reaction mixture of reaction **3**, and were obtained as colorless oils. Compound *f* was similarly purified from both the nonreduced and the reduced reaction mixtures of reaction **5** and obtained as a colorless oil. When compound *f* from the nonreduced reaction mixture was treated with borohydride, its retention time remained unchanged. Mass spectra of *f* from the nonreduced and the reduced reaction mixtures were identical.

Mass spectra of compounds *d*, *e* and *f* showed the same molecular ion peak at 430 *m/z*. The fragment ion peaks showed loss of ethyl, propyl and benzyl radicals and the base ion peak due to benzyl radical was at 91 *m/z* (Table 2). High resolution mass spectra indicated the formulae of *d*, *e* and *f* were identical and found to be $C_{30}H_{42}N_2$. The contents of carbon and nitrogen atoms suggested that these compounds were derived from 4 molecules of butyraldehyde (or 2 molecules of 2-ethyl-2-hexenal) and 2 molecules of benzylamine. The reaction may proceed as illustrated in Scheme 1: two molecules of butyraldehyde were condensed by aldol condensation and dehydrated to produce 2-ethyl-2-hexenal, and the dimer aldehyde may undergo Michael addition with one molecule of butyraldehyde and subsequently aldol condensation with one molecule of butyraldehyde forms the tetrameric dialdehyde. Alternatively, 2-ethyl-2-hexenal may be converted into the tetrameric dialdehyde by Michael addition to itself. These reaction sequences may be supported by the earlier observation that 2-ethyl-2-hexenal is transformed via the intermediary tetrameric dialdehyde under rather caustic alkaline conditions (20). The formulae of compounds *d*, *e* and *f* were consistent with the Schiff base structure of the tetrameric dialdehyde and benzylamine as shown in Scheme 1. However, NMR spectra of these compounds did not support the Schiff base structure. It is tentatively suggested that the unstable Schiff base of the tetrameric dialdehyde was cyclized to produce its stable isomeric compounds *d*, *e* and *f* that were resistant to the borohydride treatment.

TABLE 2

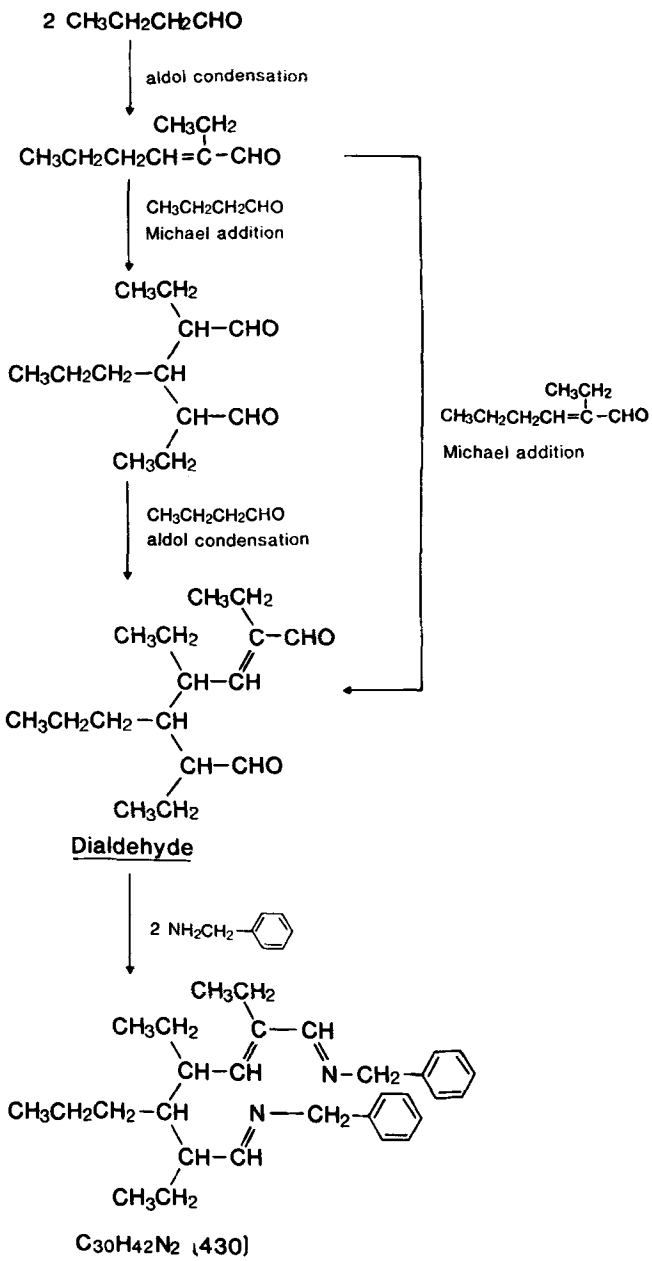
EI Mass Spectra of Compounds *d*, *e* and *f*

Compound *d*	Compound *e*	Compund *f*
	m/z (rel intensity)	
430 [M⁺] (22)	430 [M⁺] (16)	430 [M⁺] (20)
401 [M⁺-29] (7)	401 [M⁺-29] (3)	401 [M⁺-29] (12)
	387 [M⁺-43] (5)	387 [M⁺-43] (1)
339 [M⁺-91] (6)	339 [M⁺-91] (10)	339 [M⁺-91] (10)
301 (7)	301 (35)	301 (2)
		270 (8)
	268 (21)	
	240 (15)	
		230 (20)
226 (78)	226 (12)	
216 (30)		216 (23)
		180 (22)
	174 (14)	
		138 (17)
120 (13)	120 (10)	120 (10)
		106 (50)
91 [benzyl] (100)	91 [benzyl] (100)	91 [benzyl] (100)
High resolution ion mass (Calc'd for $C_{30}H_{42}N_2$: 430.3346)		
430.3353	430.3337	430.3346

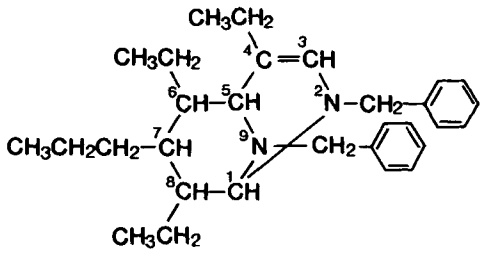
Compound *d* was suggested to be 2,9-dibenzyl-4,6,8-triethyl-7-propyl-2,9-diazabicyclo[3,3,1]nona-3-ene cyclized from the Schiff base (Scheme 2) by analysis of its ^{13}C and ^{1}H NMR spectra (Tables 3 and 4). Four methyl carbon signals at 14.3-11.5 ppm and five secondary carbon signals at 34.7-21.2 ppm were assignable to three ethyl and one propyl carbons. Two quaternary carbon signals at around 140 ppm were assigned to the carbons of two phenyls. Tertiary carbon signals at 128.5-126.6 ppm were assignable to the carbons of two phenyl groups and that at the 3-position of the six-membered ring. Two secondary carbon signals at 58.4 and 57.3 ppm were assigned to two benzyl methylene carbons. Five tertiary carbon signals at 71.4, 59.5, 42.8, 37.1 and 34.2 ppm were assignable to the six-membered ring carbons at 1-, 5-, 6-, 7- and 8-positions, respectively. One quaternary carbon signal at 110.4 ppm can be assigned to the C= carbon at the 4-position of the six-membered ring.

A proton signal appearing as a singlet at 5.59 ppm could be assigned to the CH= proton at the 3-position of the six-membered ring. Two doublets at 4.22 and 4.06 ppm were assigned to the geminal protons of one of the two benzyl methylenes, and a singlet at 3.90 ppm was assigned to two protons of another benzyl methylene. A singlet at 3.58 ppm was assignable to the fixed proton at the 1-position. A quartet at 1.98 ppm was assigned to the methylene protons of the ethyl group attached to the 4-position.

Compound *e* was suggested to be 1-phenyl-2-benzyl-4,5,7-triethyl-6-propyl-1H,2H,3H,5H,6H,7H,8H-pyrido[1,2-*c*]pyrimidine cyclized from the Schiff base (Scheme 3) by analysis of its ^{13}C and ^{1}H NMR spectra (Tables 3 and 4). A quaternary carbon signal other than those due to the phenyl carbons appeared at 141.6-139.5 ppm, which was assignable to the carbon at the 4a-position. Another quaternary carbon signal at 103.1 ppm was assignable to the C= carbon at the 4-position. The benzyl carbon signal appeared at 57.8 ppm as a secondary carbon. A tertiary carbon signal at 73.0 ppm was assignable to the carbon at the 1-position. Two secondary carbon signals at 51.5 and 48.8 ppm were assignable to the methylene carbons at the 3- and the 8-position, respec-

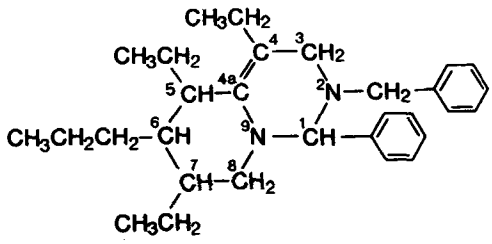


SCHEME 1



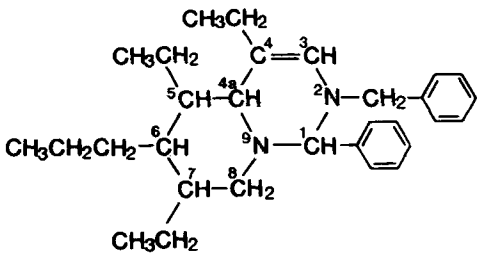
Compound d

SCHEME 2



Compound e

SCHEME 3



Compound f

SCHEME 4

tively, in the six-membered ring systems. Proton signals appearing as doublets at 4.48 and 4.22 ppm were assignable to the geminal protons of the benzyl group. Two doublets at 3.73 and 3.57 ppm were assignable to the geminal protons at the 3-position. A singlet at 3.55 ppm was reasonably assigned to the CH proton at the 1-position, since the chemical shift occurring at high field may be due to the shielding effect of $^{4}C=^{4a}C$ double bond (21).

Compound *f* was suggested to be 1-phenyl-2-benzyl-4,5,7-triethyl-6-propyl-1H,2H,4aH,5H,6H,7H,8H-pyrido[1,2-*c*]pyrimidine cyclized from the Schiff base (Scheme 4) by the analysis of its ^{13}C and ^{1}H NMR spectra (Tables 3 and 4). A quaternary carbon signal at 108.4 ppm was assignable to the C= carbon at the 4-position. The benzyl carbon signal appeared as a secondary carbon at 60.1 ppm. A tertiary carbon signal at 74.3 ppm was assignable to the carbon at the 1-position. A secondary carbon signal at 57.6 ppm was assignable to the carbon at the 8-position, and a tertiary carbon signal at 55.2 ppm was assigned to the carbon at the 4a-position. A proton signal appearing as a singlet due to two protons at 4.09 ppm was assignable to the benzyl protons, and a singlet at 3.83 ppm was assigned to the CH proton at the 1-position. A singlet at 6.08 ppm could be assigned to the CH= proton at the 3-position, and two doublets at 3.16 and 3.09 ppm were assigned to the geminal protons at the 8-position. A singlet at 2.80 ppm may be due to the fixed 4a-proton, and a quartet at 1.83 ppm may be due to the methylene protons of the ethyl group attached to the 4-position.

TABLE 3

^{13}C NMR Spectra of Compounds *d*, *e* and *f* ($CDCl_3$)

Compound *d*			Compound *e*			Compound *f*		
ppm	DEPT	Assignment	ppm	DEPT	Assignment	ppm	DEPT	Assignment
141.2	C	phenyl	141.6			140.5	C	phenyl
140.5			140.6	C	phenyl,	139.7		
128.5			139.5		4a	128.9		
128.4			128.4			128.7	CH	phenyl
128.2			128.1			128.5		
128.1	CH	phenyl,	128.0	CH	phenyl	128.4		
128.0		3	127.7			127.9		
127.3			126.5			127.2	CH	phenyl, 3
126.8			103.1	C	4	126.4		
126.6			73.0	CH	1	108.4	C	4
110.0	C	4	57.8	CH_2	benzyl	74.3	CH	1
71.4	CH	1	51.5	CH_2	3	60.1	CH_2	benzyl
59.5	CH	5	48.8	CH_2	8	57.6	CH_2	8
58.4	CH_2	benzyl	42.9			55.2	CH	4a
57.3			38.9	CH	5,6,7	48.2		
42.8	CH	6,7 or 8	38.5			42.1	CH	5,6,7
37.1			30.2			35.0		
34.7	CH_2	methylene at 4	26.4			31.4		
34.2	CH	6,7 or 8	25.2	CH_2	methylene	25.6		
28.2			24.1			21.2	CH_2	methylene
26.6	CH_2	methylene	23.5			20.0		
23.3			14.8			16.3		
21.2			14.4	CH_3	methyl	14.4		
14.3			12.2			14.1	CH_3	methyl
14.0	CH_3	methyl	11.6			12.9		
13.1						12.8		
11.5								

Formation of compounds *d*, *e* and *f* with the above structures from the reaction of butyraldehyde and benzylamine strongly supported the idea that the reaction of butyraldehyde and benzylamine proceeded via the formation of the tetrameric dialdehyde shown in Scheme 1.

DISCUSSION

Lipid oxidation induced in the cell membrane results in various types of damage to membrane proteins: formation of fluorescence, cross-links and borohydride-reducible functions are such events (3-12). It has long been considered that malonaldehyde generated during lipid oxidation is the major component that causes damage to the proteins (10). This concept is based on the findings that malonaldehyde produces fluorescent conjugated Schiff bases between two amino groups in proteins (10), and a large amount of malonaldehyde is detected in oxidized lipids by the thiobarbituric acid assay (22). However, we have demonstrated recently that malonaldehyde produces fluorescent 1,4-dihydropyridine-3,5-dicarbaldehyde moieties and cross-links due to the conjugated Schiff bases in proteins, but none of these characteristic fluorophores are found in the proteins modified with oxidized lipids (11,12). Furthermore, determination of malonaldehyde contents by alternative methods showed that they are much lower that those estimated by the thiobarbituric acid assay (13,14). More recently it has been demonstrated that the thiobarbituric acid-reactive substances in oxidized lipids are aldehyde species other than malonaldehyde (23,24). From the results obtained so far, it appears likely that the role of malonaldehyde in the oxidized lipid-induced damage of proteins has been exaggerated.

Alternative candidates in oxidized lipids that give rise to damage to proteins are alkanals, alkenals, alkadienals and 4-hydroxyalkenals (8,15-19). These aldehydes also produce fluorescence, cross-links and borohydride-reducible functions in proteins (16). However, no mechanistic consideration has been offered for the formation of fluorescence and cross-links. It has been shown that acetaldehyde forms cross-links or stable adducts in proteins such as erythrocyte membrane proteins, spectrin, bovine serum albumin and hemoglobin (25-30). Tuma and Sorrell proposed the mechanisms that the formation of the stable adducts may be due to the reaction of sulfhydryl groups with the unstable Schiff bases initially produced (28). But there were no evidences for the structures of the stable adducts. The cross-linking by acetaldehyde requires both amino and sulfhydryl groups, but the cross-

TABLE 4

^{1}H NMR Spectra of Compounds *d*, *e* and *f* ($CDCl_3$)

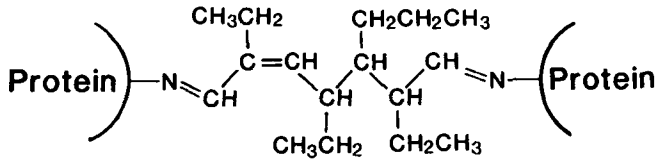
ppm	Multiplicity	Coupling constant (Hz)	No. of protons	Assignment
Compound *d*				
7.20-7.34	*m*		10	phenyl
5.59	*s*		1	3
4.22	*d*	14.8	1	benzyl methylene
4.06	*d*	14.8	1	
3.90	*s*		2	benzyl methylene
3.58	*s*		1	1
2.72	*m*		1	5
2.37	*m*		1	8
1.98	*q*		2	methylene at 4
1.65-1.07	*m*		10	6,7, methylene
0.97	*t*		6	methyl
0.89	*t*		3	
0.72	*t*		3	
Compound *e*				
7.12-7.31	*m*		10	phenyl
4.48	*d*	16.2	1	benzyl methylene
4.22	*d*	16.2	1	
3.73	*d*	14.4	1	3
3.57	*d*	14.4	1	
3.55	*s*		1	1
2.14-2.38	*m*		5	5,8, methylene at 4
1.35-1.70	*m*		10	6,7, methylene
0.96	*t*		6	methyl
0.87	*t*		3	
0.67	*t*		3	
Compound *f*				
7.16-7.32	*m*		10	phenyl
6.08	*s*		1	3
4.09	*s*		2	benzyl
3.83	*s*		1	1
3.16	*d*	13.7	1	8
3.09	*d*	13.7	1	
2.80	*s*		1	4a
1.83	*q*		2	methylene at 4
1.03-1.70	*m*		11	5,6,7, methylene
0.91	*t*		6	methyl
0.86	*t*		3	
0.62	*t*		3	

Multiplicity: *s* = singlet, *d* = doublet, *t*= triplet, *q* = quartet and *m* = multiplet.

linking by butyraldehyde requires amino groups alone. Polylysine without sulfhydryl groups can be effectively cross-linked by butyraldehyde (16).

The present investigation demonstrated that the reaction of butyraldehyde with benzylamine under mild conditions gave fluorescent compound(s) and nonfluorescent compounds *d*, *e* and *f*, besides 2-ethyl-2-hexenal, as a consequence of aldol condensation of butyraldehyde, and the Schiff bases of butyraldehyde and 2-ethyl-2-hexenal. The structures of compounds *d*, *e* and *f* were tentatively suggested as shown in Scheme 2-4. They were isomers of the Schiff base of the tetrameric dialdehyde shown in Scheme 1 and were composed of 4 molecules of butyraldehyde and 2 molecules of benzylamine. Although these compounds do not have an important significance by themselves, the formation of these compounds strongly suggested the transformation of butyraldehyde into the tetrameric dialdehyde prior to the reaction with benzylamine. This transformation of butyraldehyde involves repeated aldol condensation and Michael addition of butyraldehyde catalyzed by benzylamine.

Formation of the tetrameric dialdehyde from butyraldehyde under mild conditions has an important significance in the protein cross-linking by butyraldehyde and its related aldehydes. The formation of the tetrameric dialdehyde may be induced by primary amines, amino acids and proteins. In the reaction of proteins with butyraldehyde, the tetrameric dialdehyde may be initially produced by the catalytic action of proteins, and the tetrameric dialdehyde may produce unstable Schiff bases between ε-amino groups of the protein as illustrated in Scheme 5. The Schiff bases may be transformed into stable adducts similar to those found in compounds *d*, *e* and *f*. Thus, the stable cross-links can be formed in the protein molecules.



SCHEME 5

In conclusion, it is suggested that the mechanisms of the cross-linking of proteins by butyraldehyde involve the formation of the tetrameric dialdehyde by repeated aldol condensation and Michael addition of butyraldehyde. The tetrameric dialdehyde may produce unstable Schiff bases which are, in turn, transformed into the stable cross-links.

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Specific Susceptibility of Docosahexaenoic Acid and Eicosapentaenoic Acid to Peroxidation in Aqueous Solution

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The peroxidation of different polyunsaturated fatty acids (PUFA) after photoirradiation in aqueous solution was evaluated by measuring fatty acid loss and malonaldehyde production in the medium. The oxidation rates of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), two highly unsaturated fatty acids of the n-3 series, were surprisingly lower (14 and 22%, respectively) than the oxidation rates of linoleic, α-linolenic, γ-linolenic, dihomo γ-linolenic, and arachidonic acids (62-90%). The quantities of malonaldehyde (MA) produced were assayed simultaneously by gas chromatography (GC) and high performance liquid chromatography (HPLC). MA production was found to be related to both the degree of unsaturation and the metabolic series of the fatty acid. The maximum value was observed with arachidonic acid (MA production from 2 mM arachidonic acid in aqueous solution was estimated at 44.9 ± 6.0 μM by GC and 46.8 ± 4.0 μM by HPLC). Eicosapentaenoic acid and docosahexaenoic acid produced lower MA quantities compared to arachidonic acid (MA production from 2 mM EPA and 2 mM DHA was estimated at 17.9 ± 1.5 μM and 37.9 ± 0.7 μM, respectively, by GC, and 26.3 ± 4.9 μM and 37.3 ± 4.2 μM, respectively, by HPLC). The MA yield, defined as the amount of MA (nmols) produced per 100 nanomoles of oxidized fatty acid, was used to express the susceptibility of individual PUFA to peroxidation. The MA yield correlated well with the degree of unsaturation, but was independent of carbon chain length and metabolic series. The study suggests that adequate assessment of lipid peroxidation cannot be achieved by measuring MA formation alone, but it also requires knowledge of the fatty acid composition of the system studied.

Lipids 24, 970-975 (1989).

Lipid peroxidation has been defined as the oxidative deterioration of polyunsaturated lipids, i.e., lipids that contain more than two covalent carbon-carbon double bonds (1). Cholesterol (2) and polyunsaturated fatty acids (PUFA) (3,4) are the basic lipid structures that are most susceptible to peroxidation. PUFA are readily oxidized during storage, and their oxygen-dependent deterioration leads to rancidity of fats and oils. The physiological importance of PUFA has been emphasized in a recent report (5), where it was found that their presence in cell membranes exposes cells to the risk of lipid peroxidation. Oxidative degradation of PUFA also occurs in vitro in tissue preparations, however, little is known about the susceptibility of individual fatty acids to peroxidation.

In order to evaluate the peroxidation process, we have measured both fatty acid loss in the medium and the formation of malonaldehyde (MA). MA is a secondary end product of the peroxidative degradation process and is of considerable biological significance (6). Most of the methods involving an MA derivatization procedure before chromatography require a heat and/or acid treatment (7-11), which can lead to artifactual production of MA, and thus to an overestimation of the MA levels that were initially present in the sample. Some high performance liquid chromatographic (HPLC) methods (12-14) do not require derivatization and permit direct analysis of MA, but they are less specific and less sensitive (15). In the present study, we combined a gas chromatographic (GC) method (8), using a derivatization procedure with a direct HPLC method (12) in order to ensure accurate measurement of MA production in peroxidized fatty acid solutions, as well as to identify those fatty acids which are most susceptible to MA formation during peroxidation. The peroxidation process was initiated by UV photoirradiation under aerobic conditions at room temperature. It has recently been demonstrated by electron paramagnetic resonance studies that photoirradiation produces superoxide radicals ($O_2^{\cdot-}$) under aerobic conditions (16), which may (via Haber-Weiss type reactions) yield highly reactive oxygen species ($OH^{\cdot}$), and hence contribute to initiation processes leading to the autoxidation of PUFA.

The present work was intended to study the peroxidation of fatty acids as a function of degree of unsaturation, number of carbon atoms and metabolic series (n-3, n-6). Particular attention was paid to docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) because of their assumed beneficial effects on platelet aggregability and in atherosclerosis (5,17).

MATERIALS AND METHODS

Reagents. Stearic (18:0), linoleic (18:2n-6), α-linolenic (18:3n-3), γ-linolenic (18:3n-6), dihomo-γ-linolenic (20:3 n-6), arachidonic (20:4n-6), eicosapentaenoic (20:5n-3), and docosahexaenoic (22:6n-3) acids were purchased from Sigma France, Ltd. Ammonium iron (II) sulfate hexahydrate was obtained from Prolabo, France. 2-Hydrazinobenzothiazole (HBT) was obtained from Eastman Kodak, and 1,1,3,3-tetramethoxypropane (TMP) from Merck (West Germany). 2-(Pyrazol-1'-yl)benzothiazole (HBT-MA) and 2-(3',5'-dimethylpyrazol-1'-yl)benzothiazole (HBT-AA) were prepared by the reactions of HBT with malonaldehyde (MA), and acetylacetone (AA), respectively, as previously described (8). All other reagents and solvents were of analytical grade.

Preparation of photoirradiated fatty acids in aqueous solution. Fatty acids were dispersed with 0.5% Tween 20, saponified by 1N NaOH, and dissolved at a concentration

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Abbreviations used: AA, acetylacetone; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FID, flame ionization detector; GC, gas chromatography; HBT, 2-hydrazinobenzothiazole; HBT-AA, 2-(3',5'-dimethylpyrazol-1'-yl)benzothiazole; HBT-MA, 2-(pyrazol-1'- yl)benzothiazole; HPLC, high performance liquid chromatography; MA, malonaldehyde; NPD, nitrogen phosphorus detector; PUFA, polyunsaturated fatty acids; TBA, thiobarbituric acid; TMP, 1,1,3,3-tetramethyoxypropane.

of 2 mM in a 25 mM borate buffer, pH 6.9, under nitrogen to minimize autoxidation, following a modification of the method described by Cillard and Cillard (18). Five ml of aqueous fatty acid solution was placed in an open polystyrene flask (3 cm i.d.), and then irradiated under aerobic conditions at a distance of 40 cm from a 30 W-254 nm UV lamp (Bioblock Scientific, intensity of 1780 $\mu W/cm^2$ at 15 cm from the filter), at 23°C, in a closed reaction chamber (40 cm x 60 cm x 70 cm) for various time periods. The peroxidation process was stopped by cooling the samples on ice in the dark.

Fatty acid analysis. Fatty acids were analyzed as methyl esters on a Varian model 3300 GC equipped with a flame ionization detector (FID), using a spirawax capillary column (25 m x 0.2 mm i.d.) and temperature programming (150-210°C; 1.5°C/min). GC peak areas were measured with a Merck model D 2000 integrator. After extraction of free fatty acids according to the method of Folch *et al.* (19), fatty acid methyl esters were prepared according to Hagenfeldt (20). Before methylation, nonadecanoic acid was added to the mixture as internal standard. Fatty acid loss after irradiation was estimated by calculating the difference between the initial fatty acid level (T_0) and the level after 24 hr exposure to UV light (T_{24}).

MA analysis by gas chromatography. Malonaldehyde (MA) was measured on a Packard model 437 GC equipped with a nitrogen phosphorus detector (NPD) using a glass column (3 m x 3 mm i.d.) packed with 3% OV 17 on 80/100 mesh chromosorb, which was maintained at 215°C. GC data were recorded with a Packard model 641 recorder. MA quantities were determined according to the procedure of Beljean-Leymarie and Bruna (8). 0.6 ML of 0.1 M citrate buffer, pH 2.5, and 0.3 ml of 5 mM HBT solution were added to 0.1 ml of the fatty acid solution. The mixtures were placed in capped glass tubes and were heated in a water bath for 30 min at 70°C. The HBT-MA derivative was extracted with 1 ml of hexane containing 2.5 μM HBT-AA as internal standard.

MA analysis by high pressure liquid chromatography. MA was analyzed using Varian model 500 liquid chromatograph equipped with a TSK G 2000 PQ column (70 mm x 7.5 mm i.d.) which was perfused with a mobile phase of 0.1 M sodium phosphate buffer, pH 8.0, at a flow rate of 0.3 ml/min. Free MA in the PUFA aqueous solutions was separated and quantified within 10 min. Absorbance was monitored at 267 nm and HPLC peaks were measured with a Merck model D 2000 integrator. MA levels were determined according to the procedure by Csallany *et al.* (12). Fifty μl of fatty acid solution was directly injected onto the HPLC column. To prepare the same calibration curves for MA analysis by GC and by HPLC, aliquots of a 6.3 mM stock solution of an acid hydrolyzed TMP solution [prepared as previously described (8)] were diluted with 0.01 M phosphate saline buffer solution, pH 7.0, to give final concentrations of standards ranging from 0.5-60 μM.

Analysis of metal catalysts. Iron assays were performed using a Perkin Elmer 300 atomic absorption spectrophotometer equipped with an acetylen-oxygen burner. The detection limit of the assay was 1 μM. Calibration curves for iron assays were prepared by successive dilution of a 180 μM iron solution. Copper was assayed using a Perkin Elmer 380 atomic absorption spectrophotometer equipped with an HGA 500 electrothermal atomizer. The detection limit was 0.02 μM.

RESULTS

Analytical features of MA determination in fatty acid solutions. In order to achieve precise measurement of MA formation, we have compared two analytical methods. Arachidonic acid, a well known source of MA (21), was the PUFA used for these comparative studies. MA that was produced from 2 mM arachidonic acid solutions after six hr irradiation was simultaneously estimated by GC and HPLC and expressed as the difference between MA concentrations produced at six hr and MA concentrations at time 0 (prior to exposure). The results were 9.9 ± 1.1 μM (mean ± SD, n = 17) by GC, and 10.3 ± 1.5 μM (mean ± SD, n = 17) by HPLC. Reproducibility was tested using samples of 5 μM MA in aqueous solution (prepared by successive dilution of a 6.3 mM MA stock solution with 25 mM borate buffer, pH 6.9) and of 2 mM arachidonic acid in aqueous solution, prepared according to the experimental procedure described in Materials and Methods and photo-irradiated by UV light for 24 hr. The coefficients of variation for the GC method were 1.9% (n = 17) and 6.0% (n = 17), respectively, and for HPLC, 6.9% (n = 17) and 11.0% (n = 17), respectively. The statistical comparison of the relative accuracy of the two methods was performed with Student's paired *t*-test (22)—a series of 16 different samples of 2 mM arachidonic acid was analyzed for MA production after 24 hr UV-induced peroxidation, and the difference between each pair of results was calculated and compared. There was no significant

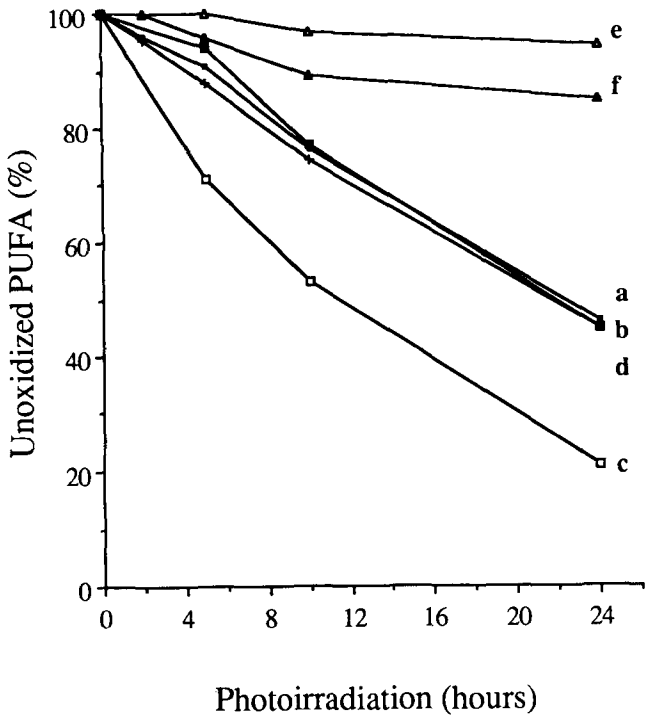

FIG. 1. Kinetics of PUFA oxidation. PUFA in aqueous solution were exposed to UV light for different periods of time (λ = 254 nm at 23°C). Unoxidized PUFA (%) were quantified by GC. a- 18:3n-3; b- 18:3n-6; c- 20:3n-6; d-20:4n-6; e- 20:5n-3; f- 22:6n-3. Data shown are the results of one experiment.

difference between the accuracy of the two methods (Student's paired *t*-test: n = 16, t = 0.795).

Fatty acid autoxidation. Eight fatty acids selected for their different double bonds, number of carbon atoms, and metabolic series (n-3 and n-6) were incubated under the oxidation conditions described in Materials and Methods. Autoxidation of these PUFA was assessed simultaneously by GC of the unoxidized PUFA, and by GC and HPLC of MA produced. Figure 1 shows the PUFA oxidation rates, and Figure 2 shows the concomitant MA production as a function fo UV irradiation time. Under our experimental conditions, optimal differences were observed after 24 hr photoirradiation. The oxidation and MA production kinetics of the fatty acids were specific for these chemical structures.

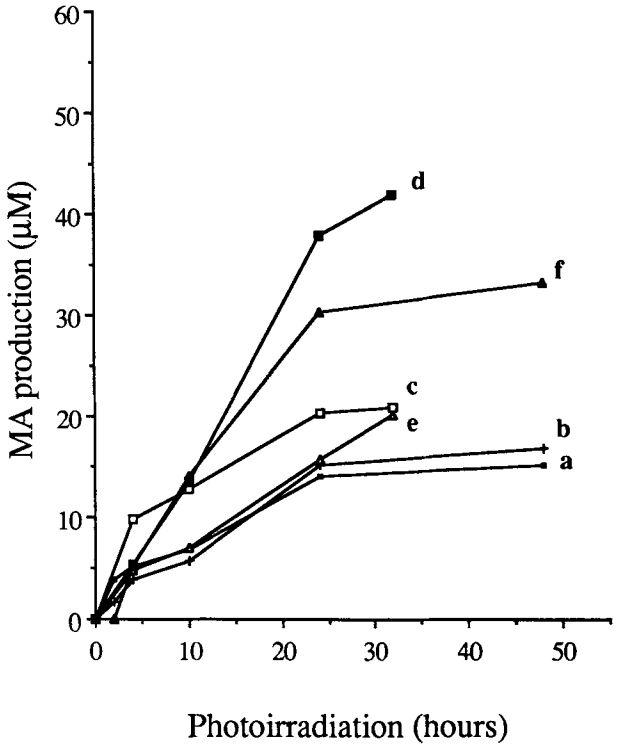

FIG. 2. Kinetics of MA production. MA produced from aqueous solutions of different PUFA after varying periods of UV irradiation was quantified by GC as described in Materials and Methods. a- 18:3n-3; b- 18:3n-6; c- 20.3n-6; d- 20:4n-6; e- 20:5n-3; f- 22:6n-3. Data shown are the results of one experiment.

Fatty acid loss. The results obtained for each of the fatty acids are presented in Figure 3 and show that only PUFA with at least two double bonds were oxidized, whereas saturated and monounsaturated fatty acids were not oxidized under our experimental conditions. Our data reveal that the peroxidation rates of the highly unsaturated acids 20:5n-3 (EPA) and 22:6n- 3 (DHA) (14 and 22%, respectively) were lower than those of the other PUFA studied (62-90%). In order to verify that the relative stability of DHA and EPA was not dependent on a lower level of contamination by copper or iron, the peroxidation system was analyzed with regard to the presence of these metal catalysts. Measurement of the ions by atomic absorption spectrophotometry revealed that these

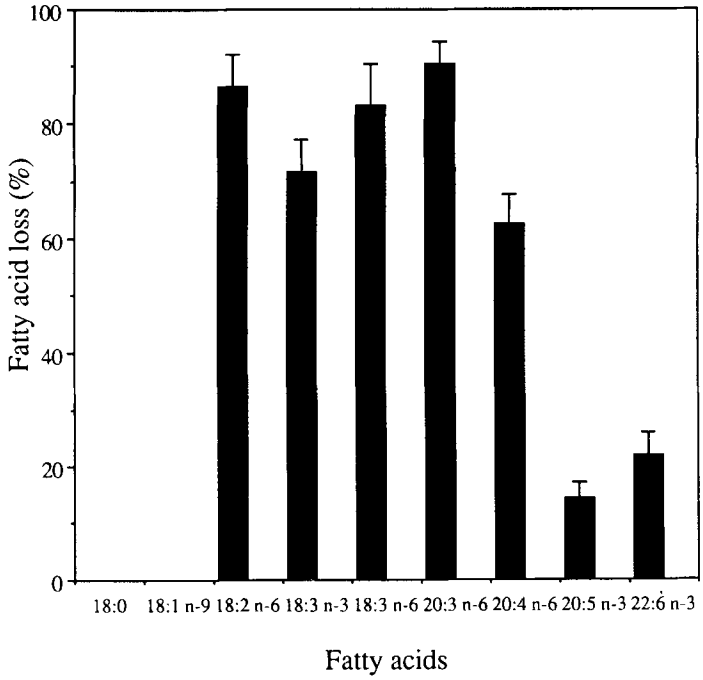

FIG. 3. PUFA oxidation as a function of degree of unsaturation. PUFA oxidation, expressed by the PUFA loss in percent, was estimated by calculating the difference between the initial concentration of PUFA and the concentration after 24 hr of UV irradiation. Peroxidation conditions: Five ml each of different PUFA in aqueous solution (2 mM) was exposed to UV light (254 nm) at 23°C for 24 hr. Each sample was assayed in duplicate. Evaporation of solvent during the exposition was negligible. Results are the mean values (± sem) of six determinations.

metals were not present in detectable quantities in the reaction medium. Moreover, while the addition of known amounts of ferrous ions (1.25 and 2.5 μM) to the reaction medium resulted in increased rates of oxidation, the relative differences between the oxidation rates of DHA, EPA and arachidonic acid remained unchanged (Fig. 4).

MA production. The susceptibility of each fatty acid to peroxidation was expressed as the MA concentration produced after 24 hr irradiation. MA was determined in each fatty acid solution before UV exposure (T_0) and after 24 hr irradiation (T_{24}), and MA production was calculated from the difference between these two values (Table 1). The initial MA (T_0) recorded for each PUFA depended on the chemical nature of the PUFA. The results reported in Figure 5 confirm that all the PUFA studied, even linoleic acid (18:2n-6), can produce MA. However, only PUFA with at least three double bonds generated significant MA concentrations. MA production was found to be both a function of the degree of unsaturation and characteristic for each metabolic series. Fatty acids of the n-6 series seemed to produce more MA than did fatty acids of n-3 series. A maximum value was obtained for arachidonic acid (MA production from 2 mM arachidonic acid in aqueous solution was estimated at 44.9 ± 6.0 μM by GC and 46.8 ± 4.0 μM by HPLC). Eicosapentaenoic acid and docosahexaenoic acid, although highly unsaturated, produced lower MA quantities as compared to arachidonic acid (Table 1).

MA yield. The MA yield expresses the susceptibility to peroxidation of each PUFA. MA yield was defined in this

TABLE 1

MA Concentrations (μM) Produced by 2 mM PUFA Aqueous Solutions[a]

	T_0[b]		T_{24}		$T_0 - T_{24}$	
	GC	HPLC	GC	HPLC	GC	HPLC
20:4 n-6	8.6 ± 0.5[c]	1.2 ± 0.3	57.0 ± 6.1	47.6 ± 3.9	44.9 ± 6.0	46.8 ± 4.0
20:5 n-3	9.8 ± 2.2	1.7 ± 1.4	27.6 ± 3.7	29.7 ± 5.8	17.9 ± 1.5	26.3 ± 4.9
22:6 n-3	97.2 ± 0.4	6.8 ± 3.4	45.1 ± 0.8	40.7 ± 2.3	37.9 ± 0.7	37.3 ± 4.2

[a]The reaction was carried out as described in Materials and Methods.
[b]T_0: before UV exposition, T_{24}: after 24 hours UV-induced peroxidation.
[c]Means values ± sem of seven determinations.

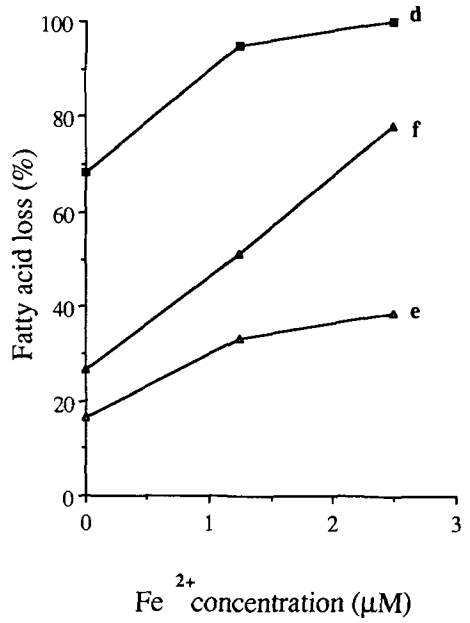

FIG. 4. Influence of ferrous ions on long PUFA autoxidation. Increasing quantities of ferrous ions were added to the PUFA aqueous solutions. PUFA loss (%) was estimated as in Figure 3. d- 20:4n-6; e- 20:5n-3; f- 22:6n-3. Results are the mean values of two determinations.

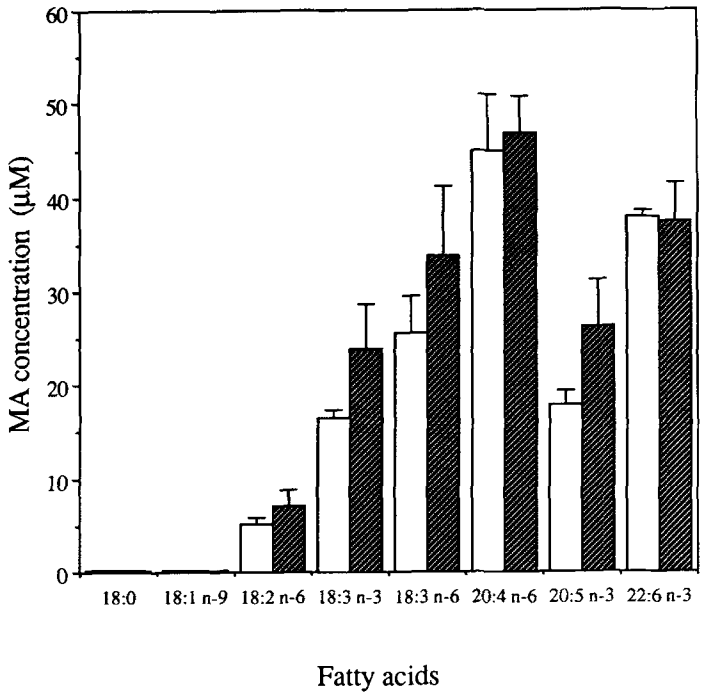

FIG. 5. MA concentrations generated by peroxidation of aqueous solutions (2 mM) of PUFA containing increasing numbers of double bonds. Peroxidative conditions were the same as in Figure 2. MA production was simultaneously evaluated by GC (open bars) and HPLC (hatched bars), as described in Materials and Methods, and was calculated as the difference between MA concentration after 24 hr photoirradiation and initial MA concentration in the medium. Results are the mean values (± sem) of six determinations. For 20:4 n-6 , 20:5 n-3 , and 22:6 n-3, the results are the mean values (± sem) of seven determinations.

study as the amount of MA (nmols) produced during 24 hr UV irradiation per 100 nanomoles of oxidized PUFA (e.g., 100 nmols of oxidized arachidonic acid produced 4 nmols of MA, the MA yield of arachidonic acid was 4%). This criterion allowed us to evaluate the peroxidation of each PUFA in relation to its metabolic series, carbon chain length, and degree of unsaturation. Figure 6 shows that the MA yield calculated for three different PUFA with three double bonds was independent of the carbon chain lengths (18 and 20 carbon atoms) and metabolic series (n-3 and n-6). In contrast, the results in Figure 7 show a close relationship of MA yield to the degree of unsaturation of each fatty acid. Moreover, when the MA yield was divided by the number of double bonds, we found that the resultant value was not constant, but appeared to be an increasing function of the degree of unsaturation of the fatty acid (Fig. 8).

DISCUSSION

The aim of this study was to evaluate the peroxidation of different PUFA as a function of their degrees of unsaturation, carbon chain lengths, and metabolic series. The UV-induced autoxidation process can be quantitatively assessed by determining the PUFA loss and MA production in the medium. A comparison of the two analytical methods used permits precise quantification of the actual MA production resulting from peroxidation. When MA levels are calculated as the difference between levels after 24 hr irradiation and those at time 0, it is found that the corresponding values obtained by GC and HPLC are

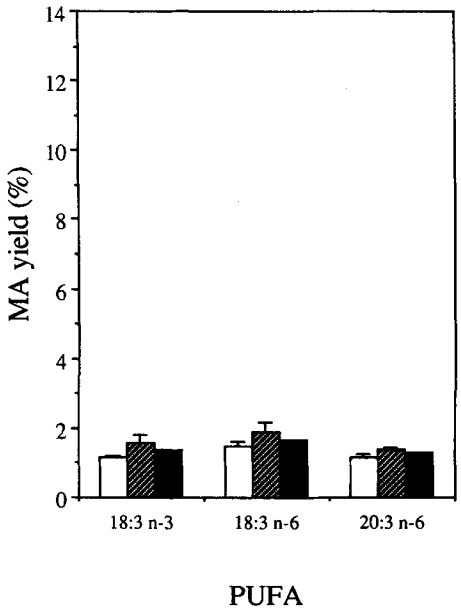

FIG. 6. MA yield of different photoirradiated PUFA with the same degree of unsaturation. The yield was calculated as the amount of MA (nmols) per 100 nmols of oxidized PUFA after UV irradiation (254 nm, 24 hr 23°C). MA yield was calculated using the MA production estimated a) by GC (open bars), b) by HPLC (hatched bars), and c) the mean of GC + HPLC results (dark bars). Results are the mean values (± sem) of five determinations.

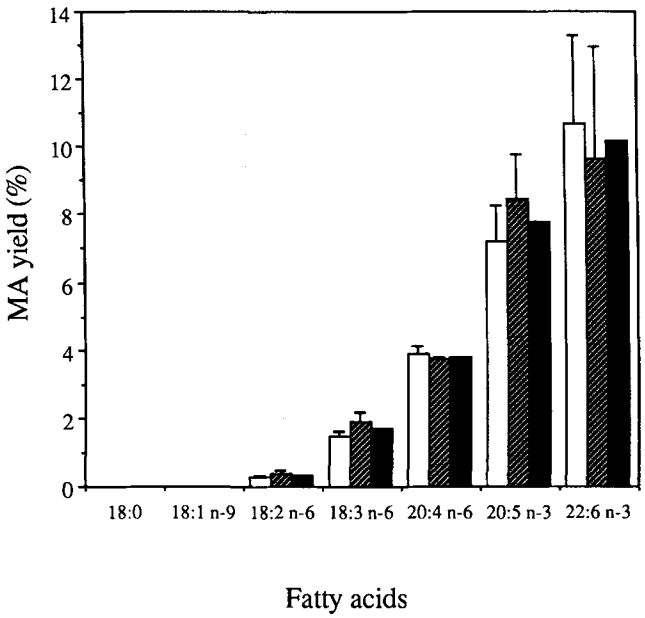

FIG. 7. MA yield as a function of degree of unsaturation of photoirradiated PUFA. MA yield was calculated and expressed as described in the Figure 6. Results are the mean values (± sem) of five determinations.

quite similar. The procedure permits the differentiation of MA initially present in the reaction medium and/or produced by the decomposition of intermediate molecules during the derivatization procedure (7,9) and MA actually produced during photoirradiation.

Our results indicate that the amounts of MA produced from UV- irradiated PUFA are essentially correlated to

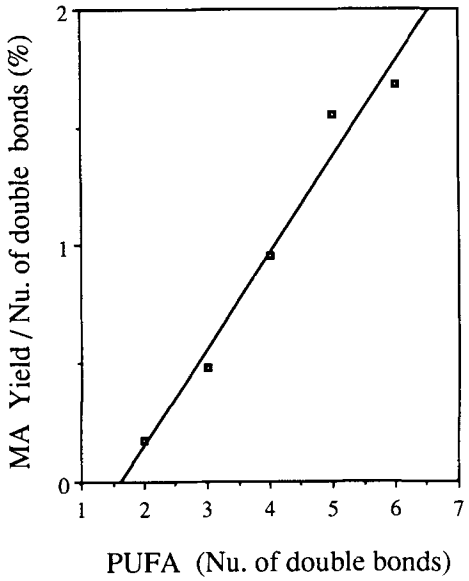

FIG. 8. MA yield divided by the number of double bonds as a function of PUFA double bond content. Yields were calculated using the mean of MA production measured by GC and HPLC. Results are the mean values of ten determinations.

the double bond content of the fatty acid. Paradoxically, DHA, and particularly EPA, both highly unsaturated fatty acids, are weakly oxidizable and produce less MA than does arachidonic acid in aqueous solution. The marked oxidative degradation of arachidonic acid relative to EPA and DHA was not related to detectable contamination by metal catalysts (Fe and Cu), but only to the structure of the fatty acid; the addition of known amounts of Fe^{2+} showed that at identical Fe concentrations, arachidonic acid continued to exhibit greater susceptibility to oxidation than DHA and EPA. Our results are in accordance with those of Gutteridge (7), who has shown that after three days at room temperature and under normal fluorescent laboratory lighting, DHA in aqueous dispersion was approximately 33% less TBA (thiobarbituric acid) reactive than arachidonic acid. In contrast, the results of Cosgrove *et al.* (23) show that in nonaqueous medium, the oxidizability of DHA is greater that that of arachidonic acid independent of the chemical initiator employed. We found that the peroxidative breakdown of a PUFA was also dependent on its metabolic origin. This observation is in agreement with other recent reports (5) which indicated that the production of MA by human platelet membrane liquid enriched in EPA and DHA was reduced significantly. Furthermore, our findings reinforce the previous results by Smith *et al.* (21), who postulated that arachidonic acid was a primary precursor of MA in biological systems, as well as those of Umano *et al.* (9), who recently showed that only PUFA with more than one double bond can produce detectable amounts of MA upon photoirradiation. Our data demonstrate that although linoleic acid (18:2n-6) produced very small but detectable amounts of MA, it was highly autoxidizable. A biological system containing substantial quantities of linoleic acid can be oxidized without producing substantial amounts of MA. These data suggest that adequate assessment of lipid peroxidation cannot be achieved by

measurement of MA formation alone, but it also requires knowledge of the fatty acid composition and the secondary degradation products of PUFA (24).

It is of interest to note that the MA yield of a PUFA, defined as the amount of MA (nmols) produced per 100 nanomoles of oxidized fatty acid, was directly related to the degree of unsaturation, but was independent of carbon chain length and metabolic series. Moreover, the probability of a peroxidative breakdown in MA increases with the degree of unsaturation since the MA yield divided by the number of double bonds is not a constant, but an increasing, function of the degree of unsaturation.

In conclusion, of the PUFA tested, EPA and DHA were the two most highly unsaturated fatty acids and, paradoxically, the least sensitive to lipid peroxidation. Formation of MA by peroxidation of cell membrane lipids may be a phenomenon of considerable pathophysiological importance (6,25). The present study shows that the type of PUFA present in the cell membrane is a significant factor in assessing *in vivo* lipid peroxidation processes.

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METHODS

Rapid Headspace Gas Chromatography of Hexanal as a Measure of Lipid Peroxidation in Biological Samples[1]

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A rapid, sensitive and convenient capillary gas chromatographic-headspace method was developed to determine hexanal as an important volatile decomposition product of hydroperoxides formed from n-6 polyunsaturated fatty acids in rat liver samples. Total volatiles were also determined as a measure of overall lipid peroxidation. Samples of headspace taken from sealed serum bottles incubated at 37°C were injected into a gas chromatograph. It was possible to make 15 determinations per hour. This method is convenient because no special sample manipulations are necessary. The addition of 0.5 mM ascorbic acid prior to gas chromatographic analysis significantly increased hexanal production. The applicability of the method was demonstrated in studies of the effect of iron in the presence or absence of hydroperoxides of methyl linoleate and methyl linolenate and *tert*-butyl hydroperoxide on rat liver homogenates, slices and microsomes. A rapid silica cartridge chromatographic procedure was used to purify hydroperoxides from autoxidized methyl linoleate and methyl linolenate, and hydroperoxy epidioxides (cyclic peroxides) from autoxidized methyl linolenate in 20–40 mg quantities. The hydroperoxides and hydroperoxy epidioxides of methyl linolenate were effective inducers of n-6 polyunsaturated fatty acid peroxidation in liver homogenates. Hexanal and thiobarbituric acid-reacting substances were significantly correlated in liver homogenates and microsomes but not in slices. This specific method for hexanal, a known product of peroxidation of n-6 polyunsaturated fatty acids, can be used as a good measure of lipid peroxidation.
Lipids 24, **976–981 (1989).**

The literature evaluating different methods to measure lipid peroxidation in foods and biological systems has been reviewed recently (1–4). Different measures of lipid peroxidation include lipid hydroperoxides, fluorescence, loss of polyunsaturated fatty acids (PUFA), oxygen absorption, diene conjugation, thiobarbituric acid-reacting substances (TBARS), alkanes, carbonyls and chemiluminescence. The thiobarbituric acid (TBA) reaction has been extensively used in food and biological systems to determine lipid peroxidation. However, it is generally agreed that more useful and valid biochemical information may be obtained by use of more than one assay for lipid peroxidation (1,5).

Biological damage is induced by active-oxygen species formed by hyperbaric oxygen, radiation, xenobiotics, inhibitors of antioxidant enzymes, metals, hydrogen peroxide and organic peroxides or hydroperoxides (6,7). Many commercially available peroxides and hydroperoxides have been used as prooxidant models in studies of tumor formation (8,9) and biological peroxidation (10,11). Very little work has been reported on the biological effects of pure fatty acid hydroperoxides, even though they are more valid models of primary lipid peroxidation products than are synthetic and markedly more stable organic hydroperoxides. In this study pure hydroperoxides from autoxidized methyl linoleate and methyl linolenate, and hydroperoxy epidioxides (cyclic peroxides) from methyl linolenate were prepared by a rapid and convenient chromatographic method and compared with *tert*-butyl hydroperoxide as *in vitro* biological oxidants.

Several gas chromatographic (GC) methods have been reported for the analysis of volatile flavor products in oils and oil-based foods (12). Headspace GC analysis is a simple technique that measures volatile compounds equilibrated with liquid or solid samples in a closed system (13,14). This method has been used to analyze hexanal as a lipid peroxidation product in cereal foods (15). Capillary gas chromatography has recently provided a significant improvement in methodology to investigate volatile decomposition products in oxidized vegetable oils (16–19), and to follow oxidative deterioration in soybean seeds during storage under adverse conditions (20). This paper describes a rapid headspace GC method to determine hexanal, a major volatile decomposition product of n-6 PUFA, and total volatiles produced by rat liver preparations. This method is convenient and appears suitable as a biological assay for lipid peroxidation, and it permits the analyses of a large number of samples within a short period of time after tissue preparation.

EXPERIMENTAL PROCEDURES

Materials. All solvents were reagent or spectral quality. To avoid interference in peroxidation studies, peroxide contaminants and antioxidant preservatives were removed from the diethyl ether by chromatographing 10 ml through a Sep-Pak silica cartridge (Waters Associates, Milford, MA) immediately prior to use. *tert*-Butyl hydroperoxide was obtained commercially (Polysciences, Warrington, PA) and used without purification.

Hexanal used as standard for the headspace GC analyses was purified by passing a sample of about 0.5 ml (Aldrich Chemical Co., Milwaukee, WI) through a Waters Sep-Pak silica cartridge and sealing it in a serum bottle under nitrogen.

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Abbreviations: GC, gas chromatography; MeLnCyclic, methyl linolenate hydroperoxy epidioxides; MeLnOOH, methyl linolenate hydroperoxides; MeLOOH, methyl linoleate hydroperoxides; PUFA, polyunsaturated fatty acids; TBA, thiobarbituric acid; TBARS, thiobarbituric acid-reacting substances; *t*-BOOH, *tert*-butyl hydroperoxides.

METHODS

Hydroperoxides of methyl linoleate, methyl linolenate and hydroperoxy epidioxides of methyl linolenate were prepared by a rapid chromatographic procedure using a Waters Sep-Pak silica cartridge. A sample of methyl linoleate (NuChek Prep, Elysian, MN) was autoxidized with pure oxygen at 40°C. The absorptivity at 231 nm was 5738, which corresponded to 21% oxidation. The chromatographic procedure consisted of passing a 200 mg sample of autoxidized methyl linoleate dissolved in about 0.5 ml of 5% ether in hexane (v/v) through a Sep-Pak silica cartridge. The first fraction, which contained unoxidized methyl linoleate (140 mg), was eluted with 4 ml of 5% ether in hexane. The second fraction, which contained the purified hydroperoxides (37 mg), was eluted with 4 ml of 25% ether in hexane. The third and final fraction, which contained secondary oxidation products (4 mg), was eluted with 4 ml of 25% ether in hexane. These fractions were characterized by qualitative thin layer chromatography (21). The samples were dried under nitrogen at room temperature. The absorptivity of the hydroperoxide fraction at 231 nm was 28,640 (literature values, 26,000 for pure *cis,trans* isomers of methyl linoleate 9- and 13-hydroperoxides, and 28,600 for pure *trans,trans* isomers of methyl linoleate 9- and 13-hydroperoxides [22]). The time required for purification was about one hour.

To purify methyl linolenate hydroperoxides and the hydroperoxy epidioxides, a 200 mg sample of autoxidized methyl linolenate (absorptivity at 235 nm was 6683, corresponding to 25% oxidation) dissolved in 0.5 ml of 6% ether in hexane (v/v) was passed through a Sep-Pak silica cartridge. The first fraction, which contained unoxidized methyl linolenate, was eluted with 4.5 ml of 6% ether in hexane. The second fraction, which contained the purified methyl linolenate hydroperoxides, was eluted with 3.5 ml of 12% ether in hexane. The third fraction, which contained a mixture of methyl linolenate hydroperoxides and hydroperoxy epidioxides, was eluted with 4 ml of 12% ether in hexane. The fourth and final fraction, which contained hydroperoxy epidioxides, was eluted with 4 ml of 25% ether in hexane. These fractions were characterized by qualitative thin layer chromatography (21). The hydroperoxide fraction had an absorptivity of 23,772 at 235 nm (literature value, 24,600 for an isomeric mixture of *cis,trans* and *trans,trans* methyl linolenate hydroperoxides [23]). The hydroperoxy epidioxide fraction had an absorptivity of 24,975 at 235 nm (literature values, 24,200 for pure *cis,trans* isomers of methyl linolenate hydroperoxy epidioxides, and 28,700 for pure *trans,trans* isomers of methyl linolenate hydroperoxy epidioxides [21]).

Liver homogenates, liver slices and microsomes were prepared by the procedures described by Fraga *et al.* (11), except that a 1.15% KCl/0.01 M phosphate buffer (pH 7.4) was used to prepare the homogenates. Slices of liver (0.1 g in 4 ml buffer) and 4-ml aliquots of homogenates or microsomes (11) were placed in a 10-ml serum bottle, which was sealed with a rubber septum, and incubated at 37°C for 60 min unless otherwise stated. Fatty ester hydroperoxides in methanol (20 μl) and *tert*-butyl hydroperoxide in water (20 μl) were added to liver samples as inducers of peroxidation.

Headspace capillary GC method for hexanal and total volatiles. For testing of a large number of oxidatively-damaged liver samples, a method was developed to determine hexanal within 3 min using a short capillary DB-1 column (10 meter × 0.25 mm, J & W, Folsom, CA) isothermally at 37°C. For determination of both hexanal and total volatiles, a longer DB-5 column (30 meter × 0.329 mm) was used isothermally at 40°C.

Solutions of hexanal standards were allowed to reach equilibrium in the headspace of sealed 10-ml serum bottles by incubating at 37°C for varying times. GC peak areas reached a maximum between 6 and 10 min, and an incubation period of 10 min prior to GC analysis was adopted as the equilibrium time. Although pentane and propanal were identified in the gas chromatograms of headspace from incubated liver homogenates, a method was standardized in which hexanal, with a retention time ranging between 1.5 and 2.2 min, was the only component separated from volatile components that eluted earlier. Total volatiles were based on total integrated peak areas for all volatiles eluted through hexanal. Samples of 1 ml headspace were injected with a gas-tight syringe (Precision Sampling, Baton Rouge, LA) into a gas chromatograph (Varian, model 3400, Palo Alto, CA) every 4 min. The GC conditions were: helium carrier gas flow, 7–9 ml/min; make-up nitrogen gas, 26 ml/min; split, 24 ml/min; injector temperature, 200°C; and detector temperature, 150°C.

After 60 min of incubation, the serum bottles were immediately placed in dry ice/ethanol. The GC measurement consisted of injecting 1 ml of headspace from the sample bottles following 10 min of incubation with vigorous shaking at 37°C. To release more hexanal, ascorbic acid was added in a small volume to a final concentration of 0.5 mM to all samples of liver homogenates before the 10 min incubation period. Standards of 2.5, 5.0 and 7.5 μM hexanal were prepared daily in phosphate-KCl buffer, and 4-ml aliquots were analyzed before and after each group of experimental samples. A standard curve was obtained with 4-ml aliquots of 0.625–3.125 μM hexanal in distilled water in serum bottles. Linear regression analysis gave correlation coefficients of 0.98 and 0.99, $P < 0.005$, for the increase in GC peak areas with hexanal concentration. The hexanal method tested with six replicates of 4 ml phosphate buffer containing 2.5, 5.0 and 7.5 nmol hexanal gave peak areas of 86.5, 214 and 378 integration counts with respective standard errors of means of 5.6, 6.8 and 12.

For the determination of total volatiles, blanks were analyzed in triplicate with 1 ml of laboratory air at the start and end of the day. The mean blank value was subtracted from each analytical value of total volatiles based on total peak areas. Although total volatiles consisted mainly of pentane, propanal and hexanal, for convenience total peak areas were expressed as hexanal.

TBA method. Thiobarbituric acid-reacting substances (TBARS) were determined in homogenates by the method of Tappel and Zalkin (24), using as a reference standard malonaldehyde generated by acid hydrolysis of 1,1,3,3-tetraethoxypropane, and in liver slices and microsomes by a modified fluorometric method (11).

Conjugated dienes. Conjugated dienes were determined in lipid extracts from liver homogenates by the method of Recknagel and Glende (25).

RESULTS AND DISCUSSION

The applicability of the GC method to *in vitro* lipid peroxidation was investigated by determining hexanal in the

METHODS

TABLE 1

Effect of Fe^{++} and Hydroperoxides on Lipid Peroxidation in Rat Liver Homogenates

Conditions	Time (min)	Hexanal (nmol/g)[b] ascorbic acid: Without	Hexanal (nmol/g)[b] ascorbic acid: With[c]	TBARS (nmol/g)[b]	Conjugated dienes (μmol/g)[b]	TBARS/ hexanal[c]
Experiment I[d]						
Control	60	0	0	15	0.00	—
	120	0	0	108	—	—
Fe^{++}	60	—	78	422	0.63	5.38
	120	0	86	616	—	7.15
MeLOOH	60	—	175	193	3.73	1.10
	120	35	178	444	—	2.49
MeLOOH + Fe^{++}	60	—	194	591	4.68	3.05
	120	48	195	699	—	3.58
t-BOOH	60	—	21	238	0.00	11.12
	120	19	57	407	—	7.14
t-BOOH + Fe^{++}	60	—	83	533	0.72	6.42
	120	0	69	511	—	7.33
Experiment II[e]						
Control	60	0	0	13	0.00	—
Fe^{++}	60	51	184	381	0.00	2.07
MeLOOH	60	90	290	328	1.69	1.13
MeLOOH + Fe^{++}	60	105	305	522	2.43	1.71
t-BOOH	60	47	172	290	0.00	1.69
t-BOOH + Fe^{++}	60	55	207	463	0.63	2.23

Regression analysis		Experiment I	Experiment II
Hexanal (with ascorbic acid) vs TBARS	*r*	0.61	0.87
	P <	0.05	0.02
Hexanal (with and without ascorbic acid) vs conjugated dienes	*r*	0.87	0.78
	P <	0.02	0.05

[a] Liver was homogenized in 19 volumes of KCl-phosphate buffer (pH 7.4). A 4-ml aliquot of homogenate was placed in a 10-ml serum bottle, which was sealed with a rubber septum, and incubated at 37°C with the inducers listed or without inducer (control).

[b] Values for hexanal, TBARS, and conjugated dienes are averages of duplicate determinations.

[c] Hexanal was determined in the presence of 0.5 mM ascorbic acid.

[d] Concentrations of added inducers were Fe^{++}, 50 μM and hydroperoxides, 0.5 mM.

[e] Concentrations of added inducers were Fe^{++}, 100 μM and hydroperoxides, 0.5 mM.

headspace of liver homogenates, slices and microsomes incubated in 10-ml serum bottles for 60 or 120 min. Liver preparations were incubated with ferrous iron, methyl linoleate hydroperoxide, methyl linolenate hydroperoxide, *tert*-butyl hydroperoxide and combinations of each hydroperoxide with ferrous iron. The amounts of headspace hexanal were compared with amounts of TBARS and with amounts of conjugated dienes in lipid extracts.

Tables 1-3 summarize data from several experiments carried out with preparations of livers from different rats. The addition of 0.5 mM ascorbic acid to each serum bottle prior to GC analysis significantly increased the amount of hexanal detected. Lipid hydroperoxides are known to be readily decomposed by catalytic reactions of metals, especially in the presence of reducing agents such as ascorbic acid (26,27). Ascorbic acid is involved in the redox process to convert Fe^{+++} to Fe^{++}, which is the form required for the decomposition of hydroperoxides and the formation of hexanal as a major volatile product (28). With one exception (Table 3, Experiment V), hexanal was not detected in control samples. On the other hand, TBARS in control samples ranged from 8-108 nmol/g liver. In 11 out of 12 samples, TBARS increased between 60 and 120 min of incubation, whereas hexanal released in the presence of ascorbic acid during the final 10 min incubation was not time dependent (Table 1, Experiment I). The ratio of TBARS to hexanal (with added ascorbic acid) varied from 1.1 to 11 in liver homogenates (Table 1), from 0.33 to 13 in liver slices (Table 2) and from 0.40 to 1.8 in microsomes (Table 3).

The values for hexanal, produced in the presence of ascorbic acid, were lower than for TBARS in liver slices incubated with iron or with *tert*-butyl hydroperoxide, and higher in liver slices incubated with methyl linoleate hydroperoxides (Table 2). The headspace hexanal measured in the latter liver slices reflects both peroxidation of liver n-6 PUFA and decomposition of methyl linoleate hydroperoxides, which are direct precursors of hexanal (28). In liver microsomes, hexanal produced in the presence of ascorbic acid was higher than TBARS in 6

TABLE 2

Effect of Fe^{++} and Hydroperoxides on Lipid Peroxidation in Rat Liver Tissue Slices

Conditions[a]	Hexanal (nmol/g)[b] ascorbic acid: Without	With[c]	TBARS (nmol/g)[b]	TBARS/ hexanal[c]
Experiment III[d]				
Control	0	0	10	—
Fe^{++}	0	0	59	—
MeLOOH	221	308	101	0.33
MeLOOH + Fe^{++}	330	367	123	0.34
t-BOOH[e]	100	40	123	3.08
t-BOOH + Fe^{++}[e]	0	47	116	2.47
Experiment IV[f]				
Control	0	0	8	—
Fe^{++}	6	7	98	13.24
MeLOOH	187	246	106	0.43
MeLOOH + Fe^{++}	227	289	121	0.42
t-BOOH[e]	42	37	179	4.86
t-BOOH + Fe^{++}[e]	34	37	177	4.85

Regression analysis		Experiment III	Experiment IV
Hexanal (with ascorbic acid) vs TBARS	*r*	0.51	0.10
	P	N.S.	N.S.

[a] Conditions are the same as in Table 1, and the slices were incubated for 120 min at 37°C.

[b] Values for hexanal and TBARS are averages of duplicate determinations.

[c] Hexanal was determined in the presence of 0.5 mM ascorbic acid.

[d] Concentrations of added inducers were Fe^{++}, 50 μM and hydroperoxides, 0.5 mM.

[e] Headspace sample showed peaks that corresponded to unreacted *t*-BOOH.

[f] Concentrations of added inducers were Fe^{++}, 100 μM and hydroperoxides, 0.5 mM.

TABLE 3

Effect of Fe^{++} and Hydroperoxides on Lipid Peroxidation in Rat Liver Microsomes

Conditions[a]	Hexanal (nmol/g)[b] ascorbic acid: Without	With[c]	TBARS (nmol/g)[b]	Conjugated dienes (μmol/g)[b]	TBARS/ hexanal[c]
Experiment IV[d]					
Control	0	0	14	0.29	—
Fe^{++}	137	184	240	0.36	1.30
MeLOOH	404	483	315	3.26	0.65
MeLOOH + Fe^{++}	429	516	403	4.00	0.78
t-BOOH[e]	120	105	191	0.15	1.82
t-BOOH + Fe^{++}[e]	140	173	231	0.38	1.33
Experiment V[d]					
Control	0	53	22	0.00	0.40
Fe^{++}	231	330	317	0.19	0.96
MeLOOH	408	651	339	3.29	0.52
MeLOOH + Fe^{++}	452	459	412	3.34	0.90
t-BOOH[e]	166	172	214	0.11	1.25
t-BOOH + Fe^{++}[e]	115	230	241	0.34	1.05

Regression analysis		Experiment IV	Experiment V
Hexanal (with ascorbic acid) vs TBARS	*r*	0.91	0.83
	P <	0.01	0.05
Hexanal (with ascorbic acid) vs conjugated dienes	*r*	0.91	0.88
	P <	0.01	0.02

[a] Conditions are the same as in Table 1, and the samples were incubated for 120 min at 37°C.

[b] Values for hexanal, TBARS and conjugated dienes are averages of duplicate determinations.

[c] Hexanal was determined in the presence of 0.5 mM ascorbic acid.

[d] Concentrations of added inducers were Fe^{++}, 100 μM, and hydroperoxides, 0.5 mM.

[e] Headspace sample showed peaks that corresponded to unreacted *t*-BOOH.

out of 11 samples (Table 3). A GC peak corresponding to unreacted *tert*-butyl hydroperoxide was detected in headspace from all liver slices and microsomes (Tables 2 and 3) incubated with the hydroperoxide, but not from homogenates incubated with the hydroperoxide (Table 1). This hydroperoxide is apparently more readily metabolized by enzyme systems in liver homogenates than in liver slices or microsomes.

There were significant correlations between data for hexanal, produced in the presence of ascorbic acid, and TBARS in homogenates and microsomes but not in liver slices (Tables 1-3). In the absence of ascorbic acid, the correlation between hexanal and TBARS was significant in microsomes but not in homogenates and slices. There were also significant correlations between all hexanal values, with and without ascorbic acid, and conjugated dienes in homogenates and microsomes (Tables 1 and 3). Hexanal formed by decomposition of methyl linoleate hydroperoxides is apparently better metabolized in homogenates than are TBARS, which include compounds other than aldehydes (1-3). The mean of all hexanal values (plus ascorbic acid) decreased in the order: microsomes > homogenates > slices; the mean of values for TBARS decreased in the order: homogenates > microsomes > liver slices. When cells are disrupted, as in homogenates, the effects of oxidants on lipid peroxidation are apparently reflected more by TBARS than by hexanal.

Methyl linolenate hydroperoxides and hydroperoxy epidioxides, methyl linoleate hydroperoxides and *tert*-butyl hydroperoxide were compared as inducers of lipid peroxidation in liver homogenates in the presence and absence of Fe^{++}. By the hexanal and total volatile determinations, the hydroperoxides ranked as inducers of lipid peroxidation in the decreasing order: methyl linolenate hydroperoxy epidioxides > methyl linolenate hydroperoxides > methyl linoleate hydroperoxides > *tert*-butyl hydroperoxide, in the absence of Fe^{++}. In the presence of Fe^{++}, hexanal formation decreased in the order: methyl linolenate hydroperoxy epidioxides > methyl linoleate hydroperoxides > methyl linolenate hydroperoxides (Table 4). However, the difference between methyl linoleate and methyl linolenate hydroperoxides as inducers of hexanal formation was too small (14 nmol/g) to be significant. The relative amount of hexanal formed directly from decomposition of methyl linoleate hydroperoxides compared to that produced by their activity as peroxide inducers of biological peroxidation in these experiments can be large (Tables 1-3), but this was not separately measured.

Methyl linolenate hydroperoxides were the most potent inducers of TBARS in the absence of Fe^{++}, and methyl linolenate hydroperoxy epidioxides were the most potent inducers in the presence of Fe^{++}. The order of lipid peroxidation inducer potency, as measured by TBARS, agreed better with that obtained by hexanal determinations in samples incubated with Fe^{++} than in samples incubated under any of the other conditions. The hydroperoxides and hydroperoxy epidioxides of methyl linolenate were more effective inducers of lipid peroxidation

TABLE 4

Effect of Fe^{++} and Hydroperoxides on Lipid Peroxidation in Rat Liver Homogenates

Conditions[a]	Hexanal (nmol/g)[b,c]	Total volatiles[d] $\times 10^{-3}$	TBARS (nmol/g)	TBARS/ Hexanal[c]
Control	0	0.001	10	—
Fe^{++}	244	1.87	410	1.7
MeLOOH	156	12.1	358	2.3
MeLOOH + Fe^{++}	239	15.4	642	2.7
MeLnOOH	194	36.5	505	2.6
MeLnOOH + Fe^{++}	225	51.0	591	2.6
MeLnCyclic	206	38.4	481	2.3
MeLnCyclic + Fe^{++}	280	53.9	664	2.4
t-BOOH	151	4.01	284	1.9
t-BOOH + Fe^{++}	171	14.1	515	3.0
Regression analysis				
Hexanal vs TBARS	r 0.91	$P < 0.01$		
Total volatiles vs TBARS	r 0.65	$P < 0.02$		

[a]Conditions are the same as in Table 1, and the samples wre incubated for 60 min at 37°C. Concentrations of added inducers were Fe^{++}, 100 μM; and hydroperoxides, 0.5 mM.

[b]Values for hexanal and TBARS are averages of duplicate determinations.

[c]Hexanal was determined in the presence of 0.5 mM ascorbic acid.

[d]Total volatiles are based on total peak areas and are expressed as nmol hexanal/g liver.

in liver homogenates than was *tert*-butyl hydroperoxide as determined by GC headspace hexanal and total volatiles and by TBARS. For the data in Table 4, the values for hexanal were significantly correlated with those of TBARS. The correlation between the values for total volatiles and TBARS was less significant.

The hydroperoxides and hydroperoxy epidioxides of methyl linolenate were effective inducers of n-6 PUFA peroxidation in liver homogenates. Because they do not produce hexanal upon decomposition, they are more suitable than methyl linoleate hydroperoxides as inducers to measure lipid peroxidation in biological samples by headspace GC analysis of hexanal. In liver homogenates, the hydroperoxides of methyl linolenate and methyl linoleate produced more TBARS than did methyl linolenate hydroperoxy epidioxides. Therefore, the TBARS appeared to be better indices of total decomposition products of hydroperoxides of methyl linoleate and methyl linolenate than of cyclic peroxides that have been suggested as important precursors of malonaldehyde in oxidized lipids containing three or more double bonds (29-31).

In conclusion, a rapid headspace GC method was developed for the determination of hexanal and total volatiles produced by peroxidizing liver samples. Compared to other GC methods used for volatile analyses in foods and lipid systems, including direct injection and dynamic headspace (12), the static headspace technique used in this study has the advantages of being rapid and of requiring no cleaning of injector and trapping systems between analyses of samples (17). These time saving advantages are particularly important in carrying out multiple analyses of unstable and rapidly peroxidizing biological tissues. However, the static headspace method is limited to the analysis of a small number of low molecular weight volatile oxidation products of short elution times (17). Important applications of this method were demonstrated for *in vitro* lipid peroxidation. After homogenization of tissue samples and incubation, about 15 determinations per hour were possible.

Methods development can involve a number of studies; for example, the validity of TBARS determination as an index of lipid peroxidation in biological samples has been a matter of considerable debate in the literature, and many studies have been aimed at improving the method and comparing it with other tests (4,31-36). This headspace GC method has the advantage of being specific for hexanal, an important product of n-6 PUFA peroxidation. Other advantages of the method are that it does not require special sample preparation or procedural modifications for different types of biological samples. A longer time period would be required for GC separation of propanal and pentane (17,37). The analysis of propanal would provide a valuable assay for n-3 PUFA peroxidation in biological samples. Improved precision in this headspace GC method may be expected with an automated headspace gas chromatograph (38,39).

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COMMUNICATIONS

Quantitative Determination of 1,2-Diacylglycerol in Thoracic Aorta of the Rat Using Iatroscan TLC/FID: Effect of Norepinephrine

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This study was undertaken to evaluate the use of the Iatroscan TLC/FID system for quantitating 1,2-diacylglycerol (DG) in the aorta. Cholesteryl acetate was chosen as an internal standard. In order to avoid interference of triglyceride and phospholipids with the separation of the internal standard and 1,2-DG, a stepwise elution of lipids from the silicic acid column was used. The development of Chromarods was done using two solvent systems and a three-step developing technique. Assay and recovery of both 1,2-DG and cholesterol (as compared to cholesteryl acetate) were sufficient to measure changes in the 1,2-DG content in blood vessels. After exposing the thoracic aorta to 10^{-5} M norepinephrine for 10 min, the 1,2-DG content increased nearly two-fold without significant change in cholesterol content.

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Increased formation of 1,2-diacylglycerol (DG) resulting from phosphoinositide hydrolysis by action of a phosphoinositide-specific phospholipase C activates protein kinase C (1). 1,2-DG is widely recognized as an intracellular secondary messenger, and therefore changes in the 1,2-DG content in tissues are thought to be important (2). Also, 1,2-DG is a potential source of arachidonic acid (3,4). Estimates of 1,2-DG production have mostly been based on labeling experiments with radioactive fatty acids (usually arachidonic acid) (5). However, this method does not give information on the mass of 1,2-DG produced. We previously reported a method for the quantitative determination of 1,2-diacylglycerol in rat heart using an Iatroscan TLC/FID (thin-layer chromatography/flame ionization detection) system and a silicic acid column (6). However, with this method, an accurate determination of 1,2-DG in lipid extracts from blood vessels could not be achieved because of difficulties in separating the large amount of triglyceride from the internal standard. In the present study we report on a modified procedure for the mass measurement of 1,2-DG in the aorta by Iatroscan TLC/FID using a stepwise elution procedure. In addition, we describe the effect of norepinephrine on 1,2-DG production in rat aorta.

MATERIALS AND METHODS

Materials. Adult male Wistar rats weighing 300–350 g were used in this study. Following decapitation, the thoracic aorta was excised and washed with Krebs-Henseleit buffer containing (in mM): NaCl 120, $NaHCO_3$ 25, KCl 4.8, KH_2PO_4 1.2, $MgSO_4$ 1.25, $CaCl_2$ 1.25 and glucose 8.6 (pH 7.4). Adipose tissue and blood were carefully removed and the sample was frozen in liquid N_2.

Extraction and stepwise elution of lipids. Following the extraction of crude lipids containing butylated hydroxytoluene as antioxidant and cholesteryl acetate as internal standard, four sequential fractions were obtained by stepwise elution of the silicic acid (minus 325 mesh) column (20 mm × 5.5 mm) which had been equilibrated with *n*-hexane instead of chloroform (6). The first fraction was eluted with 5.5 ml of *n*-hexane/diethyl ether (98:2, v/v) and the second fraction was eluted with 3 ml of *n*-hexane/diethyl ether (95:5, v/v). Fractions three and four were eluted with 3.5 ml of diethyl ether and 3 ml of methanol, respectively. The eluted solutions were concentrated under a stream of N_2 at 40°C, and dissolved in 20 μl chloroform.

Chromarod development and scanning. Two solvent systems were used as previously described (6), with a slight modification. The first and second developments were carried out in a solvent system of 1,2-dichloroethane/chloroform/acetic acid (46:6:0.05, v/v/v) until the solvent front had migrated ca 8 cm. The third development was carried out in a solvent system of *n*-hexane/diethyl ether/acetic acid (98:1:1, v/v/v) in the same direction until the solvent front had migrated ca 11 cm. Lipid analyses were carried out with the Iatroscan TH-10 TLC/FID analyzer (Iatron Inc., Tokyo, Japan), under the conditions reported previously (6). Assays were averaged from the results of three Chromarods.

Effect of norepinephrine on 1,2-DG accumulation in the aorta. The thoracic aorta from six male Wistar rats was cut into 24 pieces and was preincubated in Krebs-Henseleit buffer that was gassed continuously with 95% O_2-5% CO_2, and maintained at 37°C. Following preincubation for a 20 min stabilization period, the pieces of the aorta were divided into two groups—half served as controls, the others were used as the norepinephrine group, which was exposed to 10^{-5} M norepinephrine in Krebs-Henseleit solution for 10 min. Then a pair of pieces, as a single sample, were frozen in liquid N_2 in order to avoid differences between portions of the aorta.

Statistics. Comparisons between the two groups were assessed with the unpaired Student's *t*-test, and a P value of <0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

The lipids in the first fraction obtained by stepwise elution from the silicic acid column were cholesteryl

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Abbreviations used: DG, 1,2-diacylglycerol; FID, flame ionization detector; TLC, thin-layer chromatography.

acetate and a small amount of triglyceride. In the second fraction, a large amount of triglyceride was obtained, and there was apparently no cholesteryl acetate, cholesterol or 1,2-DG in this fraction. The third fraction contained triglyceride, cholesterol and 1,2-DG. The more polar lipids remained at the origin, which mostly consisted of phospholipids. The fourth fraction did not contain cholesterol or 1,2-DG, and was discarded. The second fraction was also discarded. The first and third fractions were combined for the quantitative determinations of cholesterol and 1,2-DG. As shown in Figure 1, good separation of cholesteryl acetate, cholesterol, and 1,2-DG was obtained without interference from triglyceride or phospholipids. The level of 1,3-DG in the tissue was relatively small and

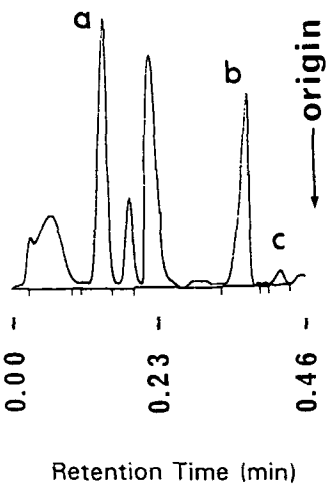

FIG. 1. TLC/FID separation of lipids for the quantitative determination of 1,2-DG and cholesterol from the first and third fractions, according to the stepwise elution described in Materials and Methods. a, cholesteryl acetate; b, cholesterol; c, 1,2-diacylglycerol.

was clearly separated from the 1,2-DG peak (data not shown).

The standard mixture solution of cholesteryl acetate, cholesterol, and 1,2-diolein was applied to a silicic acid column, as mentioned above. As a result, the ratios of 1,2-diolein and cholesterol to cholesteryl acetate were slightly reduced to 84.3 ± 12.3% and 94.9 ± 5.3% (Mean ± SD, n = 5), respectively. However, the recovered 1,2-diolein (one-tenth that of cholesteryl acetate by weight) added to the aorta homogenate was 101.7% (n = 2). Moreover, there was no appreciable conversion of 1,2-DG to 1,3-DG during the extraction (data not shown). The amounts of 1,2-DG and cholesterol in the thoracic aorta of rats obtained from the independent extraction and assay of the same homogenate were 89.1 ± 7.7 ng/mg wet wt and 1.37 ± 0.08 μg/mg wet wt (Mean ± SD, n = 5), respectively, indicating that the intraassay coefficients of variation for 1,2-DG and cholesterol were 8.6% and 5.5%, respectively. The relationships between weight ratio and peak area ratio through the entire extraction and assay procedure of 1,2-DG and cholesterol is compared to cholesteryl acetate, were linear throughout the ratio range tested (0.01 to 0.08 and 0.6 to 4.0 as ratios by weight, respectively).

The aorta incubated for 30 min in Krebs-Henseleit buffer indicated a lower level of 1,2-DG (33.7 ± 6.0 ng/mg wet wt, mean ± SE, n = 6) is compared to that of a sample that was not incubated, suggesting that the aorta excised from rats following decapitation may show stimulated 1,2-DG production. On the other hand, the 1,2-DG content in the aorta incubated for 20 min and followed by exposure to 10^{-5} M norepinephrine for 10 min significantly increased by 96.8% to 66.3 ± 6.7 ng/mg wet wt (Mean ± SE, n = 6, $P<0.01$) without a significant change in cholesterol content (Fig. 2).

In the previous paper, we described a method for quantitative determination of 1,2-DG in rat heart by TLC/FID system (6). We then tried to determine the

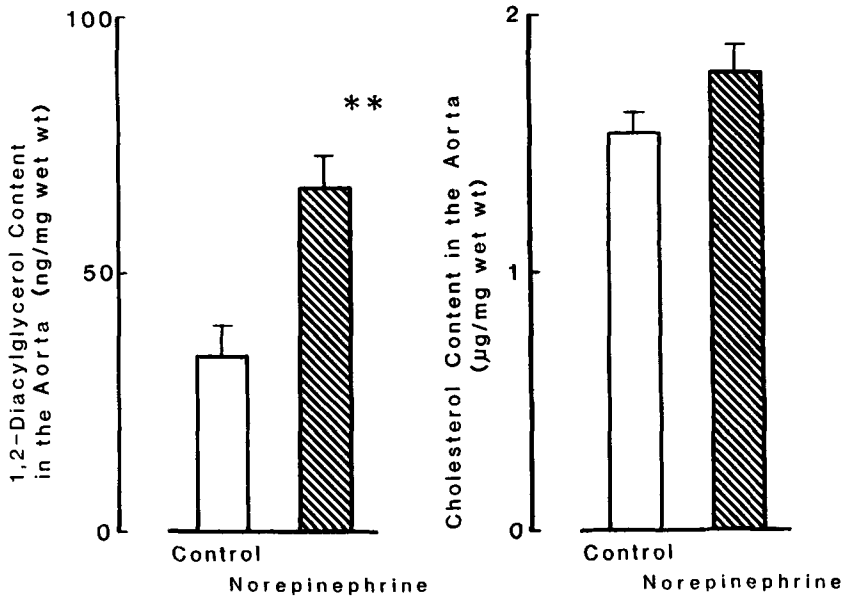

FIG. 2. Effect of 10^{-5} M norepinephrine on 1,2-diacylglycerol and cholesterol content in aorta. Each column represents the Mean ± SE. ** Indicates $P<0.01$ compared to control.

levels of 1,2-DG in blood vessels, but because of the large amounts of triglyceride present in vessel tissue, an alternate approach was chosen. In the present study, the quantitative determination of 1,2-DG in the aorta was accomplished using a stepwise elution procedure. The increase in 1,2-DG in response to norepinephrine is consistent with enhanced phosphatidylinositol turnover induced by norepinephrine in rat aorta (7). Although it is likely that 1,2-DG is produced chiefly via phosphoinositide hydrolysis, several reports suggests that 1,2-DG is also generated from compounds other than phosphoinositides, such as phosphatidylcholine (8), phosphatidic acid (9) and glycolipids (10). The mechanism of 1,2-DG accumulation will be studied using the method described for 1,2-DG determination.

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Lipids and Lipogenic Enzymes in Adipose Tissue of Castrated Male Goats

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Male goats ("Criolla Argentina" breed), castrated at 45 days of age, showed altered lipid metabolism 180 days after castration as compared to control goats. Subcutaneous, perirenal and omental adipose tissues of castrated goats showed increases in fatty acid synthetase, glucose-6-phosphate dehydrogenase and NADP-isocitrate dehydrogenase activities. Castration increased the amount of total lipids and triglycerides, but did not modify the amount of cholesterol, phospholipid and protein in the three types of adipose tissue. The incorporation of [1-^{14}C]acetate into fatty acids of subcutaneous and perirenal adipose tissue was increased in castrated goats in relation to noncastrated goats. Our results suggest that removal of gonadal steroids increases significantly the rate of lipogenesis in adipose tissue of male goats.
***Lipids 24,* 985-987 (1989).**

Previous work has indicated that castration affects fat metabolism in ruminants. Castration of sheep increases fat cell size (1) while treatment of castrated sheep with anabolic steroids decreases the activity of key lipogenic enzymes in adipose tissue (2). Castrated sheep had a greater weight of dissected carcass fat than noncastrated sheep (3). The increment observed was due to an increased weight of dissected subcutaneous fat (4). In order to elucidate whether gonadal steroids regulate fatty acid synthesis in adipose tissue of male goats, we have investigated subcutaneous, perirenal and omental adipose tissues for the effect of castration on: (a) the activities of fatty acid synthetase, glucose-6-phosphate dehydrogenase (EC.1.1.1.49) and cytosolic NADP-isocitrate dehydrogenase (EC.1.1.1.42), (b) the content of total lipids, triglycerides and cholesterol; and (c) the incorporation of [1-^{14}C]acetate into fatty acids. Simultaneously we measured total lipids, triglycerides and cholesterol in serum.

MATERIALS AND METHODS

Chemicals. The substrates for the enzymatic reactions were purchased from Sigma Chemical Co. (St. Louis, MO). Serum lipids were determined using the assay kit from Wiener Laboratory, Rosario, Argentina. [1-^{14}C]Acetic acid sodium salt (2.3 mCi/mmol) was purchased from New England Nuclear (Boston, MA).

Animals and feeding procedures. Male goats ("Criolla Argentina" breed), 45 days old, were separated into two groups. One group was used as a noncastrated control and the other underwent castration. Each group was maintained in separate yards and fed with high quality alfalfa hay with access to water supplemented with minerals and vitamins (5). The daily food intake was about 3 Kg diet per goat. Before slaughtering, 10 ml of blood was extracted from each goat by a puncture of the jugular vein. The animals were sacrified 180 days after castration.

Enzymatic determinations. After animals were bled, the liver and samples of subcutaneous, perirenal and omental adipose tissues were extracted. The liver was washed with cold saline solution (NaCl, 0.9%) dried with filter paper and weighed. Four g of liver was homogenized with 9 ml of potassium phosphate buffer (0.5 M, pH 7) with dithiothreitol (1 mM). The adipose tissue was kept at 37°C in 0.9% NaCl then dried with filter paper and weighed. Four g of each type of adipose tissue was homogenized with 9 ml of potassium phosphate buffer (0.1 M, pH 7) with dithiothreitol (1 mM) and EDTA (1 mM). The temperature was always maintained at 37°C. Afterwards the liver and adipose tissue homogenates were centrifuged at 100,000 × *g* for 1 hr in a Beckman LS-65B ultracentrifuge. The supernatant was used for enzyme and protein determinations. The activities of fatty acid synthetase (FAS) (6), citosolic NADP-isocitrate dehydrogenase (ICD) (7) and glucose-6-phosphate dehydrogenase (G6PD) (8) were determined as described.

Protein assay. Protein was measured by the Biuret reaction (9) using the fraction V of bovine serum albumin as the standard.

Lipid analysis. Lipids were extracted from adipose tissue by the method of Folch *et al.* (10). Total lipids were determined gravimetrically. The extract was resuspended in chloroform methanol (2:1, v/v). Aliquots were taken to determine lipid phosphorus and total cholesterol. Another part of the extract was used to separate the different lipids on TLC plates of silica gel G (Merck, Darmstadt, West Germany) with hexane diethyl ether/acetic acid (80:20:1, by vol) as solvent for development. The lipids were detected by exposing the plate to iodine vapors. They were scraped off and used directly for determination of phospholipid (11), esterified (12) and free cholesterol (13), and triglyceride (14).

Incorporation of [1-^{14}C]acetate into fatty acids. Slices of 200 mg of subcutaneous and perirenal adipose tissue from castrated and control goats were incubated in triplicate in 2 ml of Krebs-Ringer bicarbonate containing 4% bovine serum albumin, 1.27 mM calcium chloride and 6 mM glucose. [1-^{14}C]acetic acid sodium salt (0.1 μCi/ml) was added. The mixture, contained in a vial, was incubated in a shaking water bath at 37°C with O_2/CO_2 (95:5) as gas phase. Under these conditions the incorporation of radioactive substrate into

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Abbreviations: FAS, fatty acid synthetase; ICD, NADP-isocitrate dehydrogenase; G6PD, glucose-6-phosphate dehydrogenase; GH, growth hormone.

lipids was linear with time to over 3 hr. In the standard assay, the incubation continued for 2 hr. At the end of the incubation, the incubation medium was removed by aspiration. The tissue was washed three times with NaCl (0.9%). The lipids were extracted with chloroform methanol (2:1, v/v) and purified (10). An aliquot of the lipid extract was taken to measure the radioactivity incorporated into total lipids. Another aliquot was subjected to saponification with 0.5 N NaOH in methanol at 80°C for 3 hr under reflux. The nonsaponifiable lipids were extracted by using 3 × 5 ml of petroleum ether (bp 30-60°C). Afterwards, the lower phase was acidified by addition of 1 ml of 12 N HCl, and fatty acids were extracted with 3 × 5 ml of petroleum ether (bp 30-60°C). The ether extracts were combined, dried in a stream of N_2, and radioactivity was counted in a Beckman Liquid Scintillation Counter.

Statistical analysis. Values are expressed as mean ± standard deviation. Student's t-test was used.

RESULTS AND DISCUSSION

Figure 1 shows that in the three types of adipose tissue analyzed (subcutaneous, perirenal and omental), the activities of FAS, ICD and G6PD were higher in castrated goats than in the control animals. The increase of G6PD was higher than that of FAS and this was greater than that for ICD in castrated goats. Castration increased the ICD activities in liver, but this increase was less than that observed in adipose tissue. Protein synthesis was not affected by castration in the two tissues studied. ICD activity was higher than G6PD in subcutaneous adipose tissue of control goats. In this tissue ICD provides 33-44% of the reducing equivalent required for lipogenesis from acetate (15). Figure 2 shows that in all types of adipose tissue, castration increases the accumulation of lipids. Under this condition the concentration of triglycerides increases while the amount of phospholipids and cholesterol remains constant. The triglycerides represent more than 90% of the total lipids of adipose tissue and this was consistent with the results reported by others (16).

Table 1 shows that the incorporation of [1-^{14}C]acetate into the lipids of subcutaneous and perirenal adipose tissue of castrated goats increased in relation to noncastrated goats. The rate of fatty acid synthesis was similar in subcutaneous and perirenal adipose tissue in control and castrated goats at 220 days of age. Cheviot lambs of about 250 days of age showed a similar pattern (17). The increased lipogenesis in castrated goats in relation to noncastrated animals shows that the concentration of testosterone in serum could be important in the regulation of lipogenesis in adipose

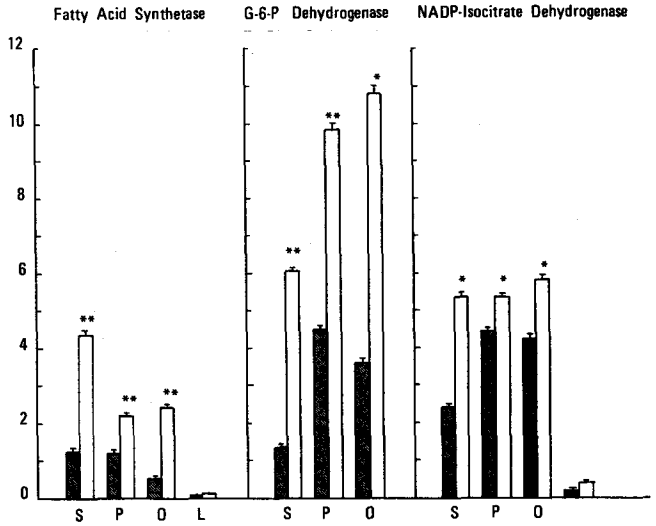

FIG. 1. The activity of FAS, G6PD and ICD in control and castrated male goats. The activity of FAS is expressed as μmol NADP oxidized/min/mg protein. The activities of G6PD and ICD are expressed as μmol NADP reduced/min/mg protein. The enzymatic determinations were performed in the 100,000 × g supernantants of adipose tissues:Subcutaneous, S; Perirenal, P; Omental, O; and Liver, L. Each value represents the mean ± S.D. from eighteen animals analyzed in triplicate. Control (open bars) and castrated (hatched bars). For details see Material and Methods. *P<0.05 —**P<0.001.

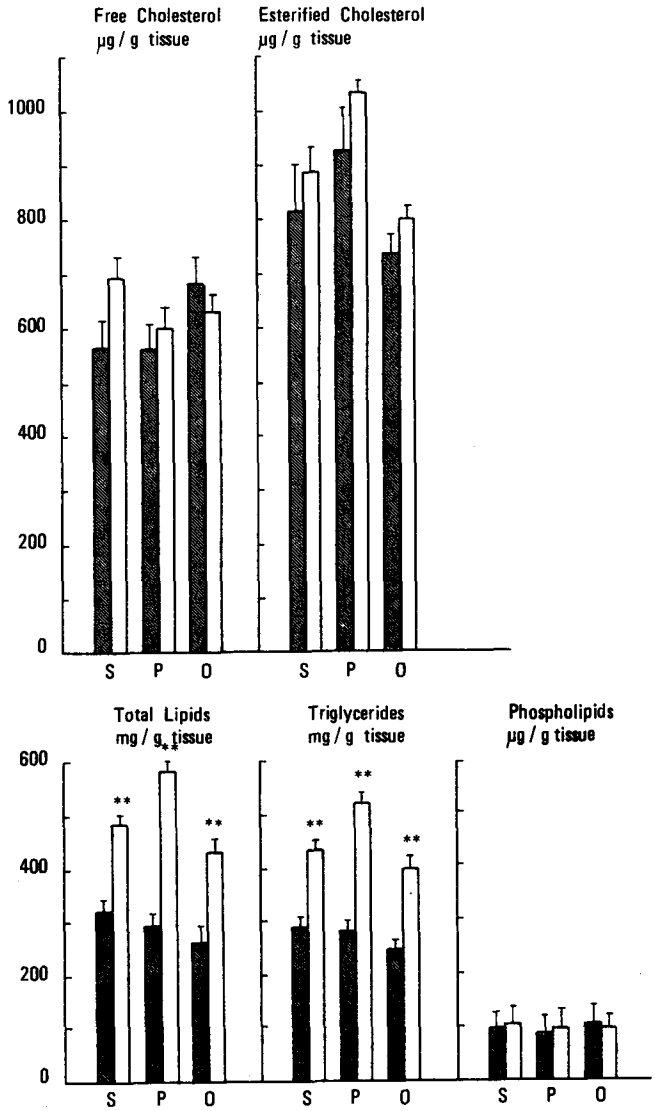

FIG. 2. Lipid content of three types of adipose tissue: Subcutaneous, S; Perirenal, P; and Omental, O; in control and castrated male goats. Each bar represents the mean ± SEM of data from eighteen animals analyzed in triplicated.
**Significantly different P<0.01 compared to the control. See Material and Methods for technical details.

TABLE 1

[1-^{14}C]Acetate Incorporation into Fatty Acids of Adipose Tissue

	[1-^{14}C]Acetate μmol/h/g tissue[a]	
Adipose tissue	Control	Castrated
Subcutaneous	15 ± 5	35 ± 3*
Perirenal	18 ± 6	40 ± 6*

[a]Results are averages of triplicate measurements on six animals ± S.D.
*P<0.01 compared to the control.

tissue of the goat. The testosterone action could be direct or through another hormone the concentration of which is modified when the level of testosterone decreases. Growth hormone (GH) has been implicated as a mediator of the action of testosterone. Plasma concentrations of GH were greater in intact male sheep than in castrated sheep, and the mean concentrations of GH were doubled by the administration of large amounts of testosterone in these animals (18). However, when physiological doses of testosterone were administrated there was no consistent alteration in the amount of GH in the plasma of castrated sheep. The increase of lipogenesis in adipose tissue after 180 days of castration did not modify the body weight and lipid content in serum as compared to control goats. This could be due to a simultaneous increase in the breakdown of protein in skeletal muscle. In castrated male lamb, administration of testosterone reduces the breakdown of protein (19). It is known that the removal of testosterone by castration decreases the lipolytic activity of subcutaneous bovine adipose tissue (20). Lipogenesis and lipolysis are two important processes that control the fat mass in adipose tissue. We conclude that increased lipogenesis is a factor in causing increased storage of lipids in tissues of castrated male goats.

ACKNOWLEDGMENTS

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Interactive Effects of Prenatal Ethanol and N-3 Fatty Acid Supplementation on Brain Development in Mice

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This study assesses the combined effects on brain and behavioral development of ethanol administration and supplementation of the maternal diet with long chain n-3 polyunsaturated fatty acids. From day 7 to 17 of gestation, pregnant mice were fed equivalent daily amounts of isocaloric liquid diets; 20% of the energy was provided by either ethanol or maltose-dextrin, and a further 20% by either safflower oil (rich in linoleic acid, 18:2n-6), or a combination of safflower oil with a fish oil concentrate (rich in eicosapentaenoic acid, 20:5n-3, and docosahexaenoic acid, 22:6n-3). On day 18 the liquid diets were replaced by lab chow; a fifth group was maintained on lab chow throughout the experiment. Measures on the pups included brain weight and the fatty acid composition of the brain phospholipids on days 22 and 32 post-conception (birth ≈ day 19), as well as behavioral development. Maternal weight gain during gestation was decreased by ethanol relative to maltose-dextrin, and increased by fish relative to safflower oil. On day 32, the brain weight of ethanol-treated animals fed fish oil was greater than their safflower oil controls, whereas the reverse was true in the two maltose-dextrin groups; a similar trend was apparent on day 22. The brain phospholipid content of the longer chain fatty acids (20:4n-6, 22:4n-6, 22:5n-6, 20:5n-3, 22:5n-3, 22:6n-3) on day 22 reflected that of the prenatal diet, with the proportion of n-3 compounds being higher and that of n-6 lower in the fish oil than safflower oil groups. Prenatal dietary effects were absent by day 32, with the exception of lower 22:5n-6 in fish oil groups. Dietary supplementation with n-3 fatty acids increased the ratio of 20:3n-6 to 20:4n-6, which is consistent with a blockade of the activity of Δ-5 desaturase. On day 22 the incorporation of dietary long chain n-3 fatty acids into the brain phosphatidylcholine fraction was enhanced in the ethanol-treated animals; by day 32 the animals treated prenatally with ethanol also showed increased levels of long chain n-6 compounds. Behavioral development was retarded by ethanol, but there was no effect of the dietary oils. These results support the hypothesis that effects of ethanol on the developing brain may be modified by the availability of an exogenous supply of long chain fatty acids.
Lipids 24, 989–997 (1989).

During the last decade a series of clinical and epidemiological studies has provided support for the hypothesis that the consumption of ethanol during pregnancy is associated with adverse effects on fetal development (1). In humans a characteristic constellation of defects has been identified and denoted the "fetal alcohol syndrome" (FAS), which includes pre- or postnatal growth retardation, anomalies of facial structure, and central nervous system (CNS) dysfunction. In the absence of the complete syndrome, the presence of some components, such as general growth retardation frequently accompanied by microcephaly and effects on the CNS, has been termed "fetal alcohol effects" (FAE). Research conducted using rodents confirms the effects of ethanol on CNS development, which include lowered brain weight, as well as specific neuroanatomical and neurochemical changes, and which are accompanied by effects on behavioral development (2). This study addresses the effects of ethanol on the developing mouse brain, particularly on the fatty acid composition of the membrane phospholipids, and the influence of dietary supplementation with n-3 fatty acids on developmental outcome.

Because of its physicochemical nature, it is thought that ethanol exerts many of its pharmacological effects through interactions with the lipid constituents of neuronal membranes (3,4). These changes in the brains of adult animals include modifications in the fluidity and fatty acid composition of synaptosomal membranes (5–10). A consistent effect of ethanol on extraneuronal membranes is a decrease in levels of arachidonic acid (AA, 20:4 n-6) (4). There is some evidence in the brain of decreases in AA as well as in docosahexaenoic acid (DHA, 22:6n-3) (5,7,10). This may be the result of the effects of ethanol on essential fatty acid (EFA) metabolism. The metabolism of n-6 and n-3 fatty acids is shown in Figure 1 (adapted from 11). There are data which indicate that ethanol inhibits both the Δ-6 and Δ-5 desaturation of the EFA's (12,13), which may account for the decreased levels of AA in ethanol-exposed animals.

Since the polyunsaturated fatty acids (PUFA), particularly AA and DHA, account for a large proportion of the fatty acids in the structural lipids of the grey matter of the mammalian nervous system (14), the modification by ethanol of the availability of PUFA from dietary precursors may be particularly salient if it occurs during the period which encompasses brain development. The long chain PUFA accrue in the developing brain during the prenatal and suckling periods (15,16). Not only is the developing brain able to incorporate maternally derived PUFA, but it also has the capacity to convert linoleic and α-linolenic acids to their long chain derivatives, with the n-3 having a competitive advantage over the n-6 pathway (17–19). Because of the effects of ethanol in blocking the biosynthesis of the long chain derivatives of the PUFA, it is conceivable that, despite an adequate supply of

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Abbreviations: AA, arachidonic acid; ANOVA, analysis of variance; BAC, blood alcohol concentration; CNS, central nervous system; DHA, docosahexaenoic acid; EDC, ethanol-derived calories; EFA, essential fatty acid; FAE, fetal alcohol effects; FAS, fetal alcohol syndrome; GLC, gas liquid chromatography; GLM, general linear model; PC, phosphatidlycholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PUFA, polyunsaturated fatty acids; SAS, Statistical Analysis Systems; TLC, thin layer chromatography.

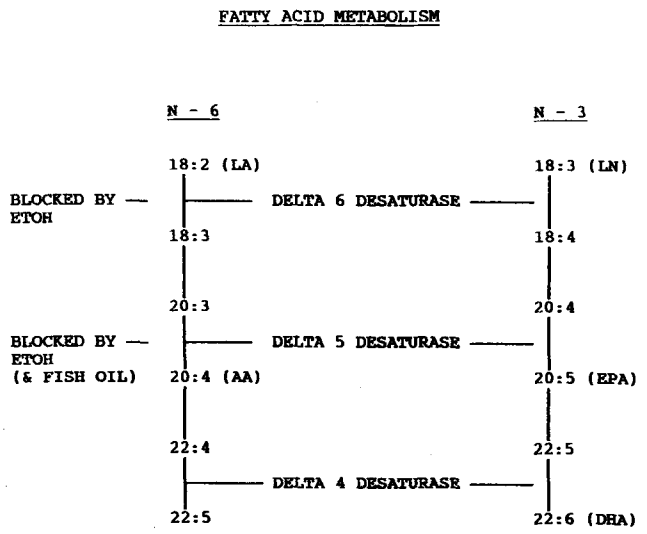

FIG. 1. Metabolism of n-6 and n-3 fatty acids.

dietary precursors, the brains of animals exposed to ethanol during prenatal development may suffer from a functional long chain PUFA deficiency. This would be reflected in the fatty acid composition of the membrane phospholipids and might also be related to effects on neurobehavioral development (20). The present study addressed this by using a factorial design to examine whether the provision to ethanol exposed animals of an exogenous supply of preformed, long chain n-3 fatty acids would ameliorate effects on both brain and behavioral development.

METHODS

Subjects. B6D2F1 parental mice were obtained from Charles River Breeding Laboratories (St. Constant, Quebec). This F1 hybrid cross has been used previously in our laboratory in studies which showed retardation of brain and behavioral development due to prenatal ethanol exposure (21-23). Three to five animals were housed together in groups of the same sex, with free access to laboratory chow (Purina Mouse Chow #5001) and tap water until mating commenced (at eight months). They were maintained under a reversed 12 hour dark:light cycle (dark 0900hr — 2100hr), at 22 ± 1°C, in standard opaque plastic mouse cages (29 × 18 × 13 cm) with Betta-Chip hardwood bedding and several sheets of toilet tissue for nesting material.

Experimental Design. The experiment was a 2 × 2 factorial design. Two levels (0 or 20%) of ethanol-derived calories (EDC) were provided in liquid diets which were especially formulated by BioServ (ref #2187), with 20% of the calories provided by protein (casein) and 40% by carbohydrate (maltose-dextrin). In the case of 0% EDC, ethanol was substituted isocalorically by maltose-dextrin. A further 20% of the dietary calories were supplied by either safflower oil or by 10% safflower oil combined with 10% Polepa (Efamol Research Institute, Kentville, Nova Scotia). The n-6 and n-3 fatty acid composition of the oils is shown in Table 1.

TABLE 1

Selected Fatty Acid % Composition of Dietary Oils

	Safflower	Polepa/Safflower
14:0	0.10	6.05
16:0	5.10	10.20
16:1	0.10	6.00
18:0	0.00	1.45
18:1n-9	76.40	42.80
18:2n-6	16.50	8.75
18:3n-6	0.10	0.05
20:4n-6	0.00	0.00
18:3n-3	0.05	0.28
20:5n-3	0.00	11.15
22:5n-3	0.00	1.05
22:6n-3	0.12	2.76
Total n-6	16.60	8.80
Total n-3	0.17	15.24

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Pregnant females were matched by body weight and then randomly assigned to groups, and equivalence of caloric intake across groups was assured by feeding all animals an average daily amount per g body weight. This had been calculated previously on each day of pregnancy from a group of pilot animals receiving ethanol and Polepa; the daily amount averaged to 0.6 kcal/g/day. A fifth group, which was allowed unrestricted access to lab chow and water, served as a control for the possible effects of slight undernutrition resulting from pair-feeding the liquid diet; it served the additional purpose of providing a normal reference group with respect to brain fatty acid composition. The five groups were designated as follows: n refers to the number of litters tested on day 32, a smaller n on day 22 reflects that some litter sizes did not allow excess pups for culling. The groups are: i) Ethanol/Polepa (E/P) n = 8; ii) Ethanol/Safflower (E/S) n = 8; iii) Maltose-Dextrin/Polepa (MD/P) n = 8; iv) Maltose-Dextrin/Safflower (MD/S) n = 8; and v) Lab Chow (LC) n = 6.

Procedure. Animals were mated between 0900 and 1600 hr. Any female with a subsequent vaginal plug was weighed, randomly assigned to a group, and housed separately. The day of conception was denoted as day 0, and all timing, both pre- and postnatal, was determined from this point as "days post-conception". Feeding of the liquid diets commenced on day 0, with all groups being fed their respective maltose-dextrin control diets until day 5. The ethanol treatment was implemented gradually, with animals receiving 5% EDC on day 5, 10% EDC on day 6, 20% EDC from days 7-16 and 10% EDC on day 17. During this time the pregnant females were weighed every second day and their food intake adjusted accordingly. At 1300 hr on day 11, 100 μl of blood were withdrawn from the tip of the tail in a heparinised capillary tube, centrifuged, and the plasma frozen for subsequent determination of the blood alcohol concentration (BAC). Control animals were subjected to the same procedure, but their blood was discarded. On day 18 the animals were returned to lab chow and tap water and checked every 24 hours for birth, which generally occurred on day 19. On day 22 mother and pups were weighed and litters culled randomly to six pups; the brains of the culled pups

were removed, weighed and, within one minute of weighing, frozen at −80°C for subsequent biochemical analysis. On day 25 the cages were cleaned, but the litters were otherwise undisturbed until day 32, when the behavioral development of two pups of each sex from each litter was assessed using a battery of tests of sensory and motor development. This has been described previously in detail (21). Briefly, each pup was tested on twelve tests measuring such developmental landmarks as righting, cliff aversion, grasping, climbing, eye-opening, visual placing, and auditory startle. The mean score for all tests was entered into a regression equation, derived previously from the scores of untreated B6D2F2 offspring, which predicted age from behavioral score. This procedure allowed quantitative assessment of treatment effects in days. All testing was done with the experimenter blind to the treatment condition of the animal. The animals were weighed and the brains removed from one animal of each sex from each litter, weighed and frozen as before. The brains included the olfactory bulbs anteriorly and 2 mm below the medulla posteriorly.

Biochemical analyses. BAC. These were determined using a micro-adaptation of the ultraviolet NAD/NADH determination method described in the Sigma technical bulletin #332-UV, with standards and reagents obtained from Sigma. The samples were analyzed concurrently and compared against a standard curve. All assays were run in duplicate.

Brain Lipids. The homogenized brains were extracted using a modified Bligh and Dyer method (24), utilizing chloroform/methanol (1:1, v/v), with 0.02% BHT (w/v). After separation and drying under nitrogen, the total lipids were fractionated via one-dimensional thin layer chromatography (TLC), using silica gel plates (Analtech GF, Analtech, Newark, DE) and a chloroform/methanol/acetic acid/water (50/30/4/2) solvent system. The fatty acids in the resulting phospholipid fractions were methylated with 14% Boron trifluoride in methanol, and analyzed on a gas chromatograph (Perkin Elmer 8420, Norwalk, CT) equipped with a flame ionization detector and a 15 m × 0.32 m (i.d.) capillary column (Supelco Wax 10, Bellefonte, PA) The temperature program for the gas liquid chromatography (GLC) run consisted of 2 minutes at 160°C, followed by a 2°C/minute increase to 190°C. After 10 minutes at 190°C, the temperature was increased 5°C/minute to 220°C, followed by 2 minutes at 220°C.

Statistical analyses. The data were analyzed using the general linear model (GLM) provided by the Statistical Analysis System (SAS) to do analysis of variance (ANOVA). Data on day 32 were analyzed initially by sex, and in the absence of a sex by treatment interaction, further analyses were conducted on the data collapsed across sex. The litter-mean score was used as the unit of analysis, and α was set at 0.05. The experimental hypotheses were addressed by the following linear comparisons: i) Main effect of ethanol, E/P + E/S vs MD/P + MD/S; ii) Main effect of EFA, E/P + MD/P vs E/S + MD/S; iii) Ethanol by EFA interaction, E/P + MD/S vs E/S + MD/P; iv) Nutritional effect, MD/P + MD/S vs LC.

RESULTS

Maternal weight gain and BAC. As shown in Table 2, ethanol-exposed animals gained less weight between days 0 and 17 of pregnancy than maltose-dextrin controls, $F(1,34) = 22.75$, $p < 0.0001$, whereas those supplemented with Polepa gained more weight than those fed safflower oil only, $F(1,34) = 7.31$, $p < 0.02$. The differences in maternal body weight on day 17 were no longer apparent after birth. There was no evidence of either an interaction or a nutritional effect. In order to reduce the variability in the data, the BAC were covaried for food-intake during the four hours immediately preceding the blood sampling. There was no difference in BAC between either of the two ethanol-exposed groups, (mean mg% ± SEM: E/P = 42.2 ± 15.6; E/S = 48.6 ± 16.7). Litter size did not differ among the groups.

Pup body and brain weight and behavioral development. As shown in Table 2, there was a significant nutritional effect only on day 22 pup body weight, $F(1,33) = 7.79$, $p < 0.01$, with the pair-fed controls being lighter than those on lab chow. On day 32 none of the effects on body weight was significant. Figure 2 shows a similar nutritional effect on day 22 brain weight, $F(1,29) = 4.62$, $p < 0.05$. Despite the apparent trend in these data towards an increased brain weight in the E/P relative to the E/S group, this was not significant. However, as seen in Figure 3, the data on day 32 brain weight showed a similar pattern of results, and these were supported by a significant interaction, $F(1,33) = 4.20$, $p < 0.05$, which was due to an increased brain weight in the E/P relative to the E/S group, accompanied by a decreased brain weight in the MD/P relative to the MD/S group. It should be noted that, because these data were not normally distributed, a log transformation was used in this analysis; analysis of the untransformed data was similar in outcome, $0.06 > p > 0.05$. The ethanol treatment retarded behavioral development by 0.4 days, $F(1,33) = 4.45$, $p < 0.05$, but none of the other effects was significant. Analysis of the individual behavioral categories (data not shown) supported the average measure in consistently showing effects of ethanol but none of oil.

Brain phospholipid analysis. The selected fatty acid composition of the brain phosphatidylcholine (PC) and phosphatidylethanolamine (PE) fractions from day 22 and 32 animals are shown in Tables 3, 4, 5 and 6. These two fractions constitute the greater proportion of the brain phospholipids, with the PC fraction being the larger prior to weaning (25). When effects were seen in the phosphatidylinositol (PI) and phosphatidylserine (PS) fraction (data not shown), these were generally similar to those in the PE fraction.

At both ages, 22:6n-3 (DHA) comprised a large proportion of the fatty acids in the PE, PI and PS fraction, exceeding that of 20:4n-6 (AA). By comparison, the overall content of both these fatty acids was lower in the PC fraction, and the AA content was relatively higher than DHA. Regardless of diet, the PC levels of 18:2n-6, 20:3n-6, 20:4n-6 and 22:4n-6 were consistently higher in 32, as compared with 22, day old pups. A similar developmental increase was seen in 22:6n-3.

TABLE 2

Effect of Ethanol and Dietary Supplementation With n-3 Fatty Acids on Maternal Weight Gain, Pup Body Weight and Pup Behavioral Development in B6D2F$_2$ Mice[a]

	ETOH/ Polepa n=8	ETOH/ Saff n=8	MD/ Polepa n=8	MD/ Saff n=8	Lab Chow n=6	Effect[c]
Maternal weight gain (g) Day 0–17	11.12 (0.78)	9.08 (0.76)	14.60 (0.55)	12.65 (0.86)	14.55[b] (0.15)	ETOH[d] OIL[f]
Pup body weight (g) Day 22	1.88 (0.09)	1.83 (0.06)	1.91 (0.10)	1.91 (0.11)	2.27 (0.11)	NUT[e]
Day 32	7.97 (0.15)	7.71 (0.23)	8.05 (0.22)	7.91 (0.23)	7.99 (0.22)	
Pup Developmental Age (days)	30.9 (0.18)	30.9 (0.21)	31.3 (0.19)	31.3 (0.17)	31.7 (0.13)	ETOH*

[a]Data are presented as means with [SEM], n = number of litters tested on day 32 post-conception.
[b]n = 2.
[c]ETOH = Main effect of ethanol; OIL = Main effect of oil; INT = Ethanol by oil interaction; NUT = Nutritional effect.
[d]$p<0.0001$.
[e]$p<0.01$.
[f]$p<0.05$.

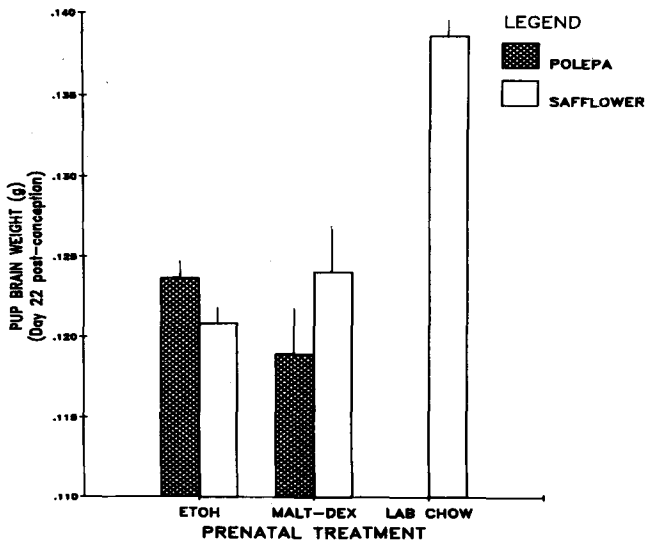

FIG. 2. Effect of prenatal ethanol and dietary supplementation with n-3 fatty acids on brain weight of B6D2F$_2$ mice on day 22 post-conception. Nutrition, $p < 0.05$.

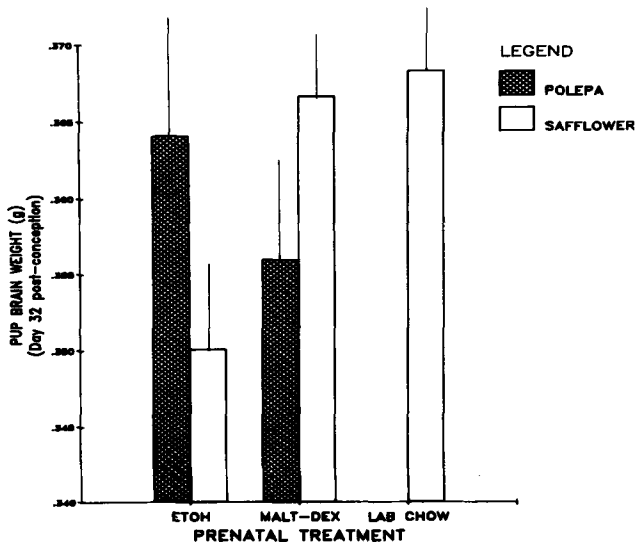

FIG. 3. Effect of prenatal ethanol and dietary supplementation with n-3 fatty acids on brain weight of B6D2F$_2$ mice on day 32 post-conception. Ethanol by Oil Interaction, $p < 0.05$.

The day 22 data are shown in Tables 3 and 4. A summary of the effects on the long chain PUFA's is shown in Figure 4. This represents the long chain n-3:n-6 ratio in the PC fraction, i.e., (20:5n-3 + 22:5n-3 + 22:6n-3)/20:4n-6 + 22:4n-6 + 22:5n-6). This was increased by Polepa, F (1,29) = 98.78, $p < 0.0001$, and ethanol, F (1,29) = 19.94, $p < 0.0001$; and, in addition, there was a significant interaction, F (1,29) = 10.30, $p < 0.005$, with the brains of animals treated with Polepa and ethanol showing a greater incorporation of the n-3 fatty acids than the maltose dextrin controls.

Analysis of the individual fatty acids shown in Table 3 indicates that these effects were true of 22:5n-3 and 22:6n-3, with the interaction term being marginal in the latter. The effect of Polepa on long chain PUFA's was similar in the PE fraction, but a marginal effect of ethanol was seen on 22:6n-3 only, and despite a trend towards the interaction seen in the PC fraction, this was not statistically significant. Ethanol decreased PC levels of 12:0, 14:0, 16:0 and increased levels of 18:0 and 18:1. The PE data supported the decreased levels of 12:0 and 16:0, but not the increase in 18:0. There was a marginal effect of ethanol in increasing PC levels of 18:2n-6. Polepa increased PC levels of 18:2n-6; it also increased 20:3n-6, while decreasing levels of 20:4n-6 (PC and PE). Following from this, the ratio of 20:3n-6 to 20:4n-6 (data not shown) was also increased by Polepa [PC: F(1,29) = 131.37, $p < 0.0001$; PE: F(1,29) = 18.36,

TABLE 3

Selected Fatty Acid Composition on Day 22 Post-Conception of Brain Phosphatidylcholines of B6D2F$_2$ Pups of B6D2F$_1$ Dams Fed Ethanol and Either Fish (Polepa) or Safflower Oils During Gestation[a]

Fatty Acid	ETOH/ Polepa n=6	ETOH/ Saff n=8	MD/ Polepa n=8	MD/ Saff n=8	Lab Chow n=4	Effect[b]
12:0	3.18 (0.70)	3.07 (0.49)	3.85 (0.48)	4.53 (0.24)	4.52 (0.30)	ETOH[e]
14:0	0.14 (0.02)	0.15 (0.02)	0.21 (0.03)	0.22 (0.04)	0.17 (0.01)	ETOH[e]
16:0	47.99 (0.83)	49.24 (0.56)	49.75 (0.39)	50.52 (0.56)	50.67 (0.66)	ETOH[e]
18:0	7.31 (0.54)	6.52 (0.35)	5.64 (0.28)	5.71 (0.28)	5.83 (0.28)	ETOH[d] ETOH[e]
18:1n-9	18.72 (0.51)	18.34 (0.36)	18.49 (0.49)	16.86 (0.19)	16.87 (0.41)	OIL[e] MDP ETOH[f]
18:2n-6	1.19 (0.04)	0.98 (0.03)	1.14 (0.03)	0.92 (0.02)	1.11 (0.02)	OIL[c] MDS[d]
18:3n-6	0.14 (0.009)	0.12 (0.006)	0.14 (0.009)	0.12 (0.007)	0.13 (0.009)	OIL[e]
20:3n-9	0.32 (0.003)	0.26 (0.009)	0.31 (0.02)	0.24 (0.01)	0.25 (0.003)	OIL[c] MDP[e]
20:3n-6	0.51 (0.008)	0.31 (0.014)	0.46 (0.02)	0.29 (0.008)	0.38 (0.005)	ETOH[e] OIL[c] MDP/S[d]
20:4n-6	4.04 (0.17)	4.98 (0.13)	4.08 (0.23)	4.62 (0.15)	4.80 (0.24)	OIL[d] MDP[e]
20:5n-3	0.11 (0.009)	0.005 (0.005)	0.10 (0.013)	0.006 (0.006)	0.01 (0.012)	OIL[c] MDP[c]
22:4n-6	0.28 (0.03)	0.53 (0.04)	0.25 (0.02)	0.47 (0.05)	0.43 (0.04)	OIL[c] MDP[d]
22:5n-6	0.06 (0.02)	0.45 (0.06)	0.08 (0.03)	0.41 (0.05)	0.14 (0.02)	OIL[c] MDS[d] ETOH[d]
22:5n-3	0.29 (0.02)	0.11 (0.007)	0.20 (0.02)	0.10 (0.007)	0.12 (0.009)	OIL[c] INT[e] MDP[d]
22:6n-3	3.95 (0.34)	2.62 (0.18)	2.64 (0.19)	2.16 (0.30)	2.38 (0.24)	ETOH[d] OIL[d] INT[f]

[a]Data are presented as mean % composition with (SEM), n = number of litters.
[b]ETOH = Main effect of ethanol; OIL = Main effect of oil; INT = Ethanol by oil interaction; MDP = MD/Polepa vs lab chow; MDS = MD/Safflower vs lab chow.
[c]$p<0.0001$.
[d]$p<0.01$.
[e]$p<0.05$.
[f]$0.1>p>0.05$.

$p < 0.0002$], but was unaffected by ethanol. An additional effect of Polepa was to increase levels of 20:3n-9 (PC and PE).

The day 32 data are shown in Tables 5 and 6. Ethanol effects were evident in the PC fraction only. There was a remaining marginal increase in 22:6n-3 and, in addition, levels of 20:3n-6 and 22:4n-6 at this age were increased by ethanol. The only consistent dietary effects seen on day 32 were an increase in 22:5n-6 in the safflower oil groups, and an increase in the PC 20:3n-6 to 20:4n-6 ratio by Polepa, $F(1,33) = 6.16$, $p < 0.02$.

DISCUSSION

These results replicate previous work in the rat showing that long chain PUFA, 20:4n-6 and 22:6n-3, accrue rapidly in the developing brain and that the brain lipid composition in the offspring will reflect large alterations of the maternal diet (25–32). On day 22 post-

TABLE 4

Selected Fatty Acid Composition on Day 22 Post-Conception of Brain Phosphatidylethanolamines of B6D2F$_2$ Pups of B6D2F$_1$ Dams Fed Ethanol and Either Fish (Polepa) or Safflower Oils During Gestation[a]

Fatty Acid	ETOH/ Polepa n=6	ETOH/ Saff n=8	MD/ Polepa n=8	MD/ Saff n=8	Lab Chow n=4	Effect[b]
12:0	0.21 (0.13)	0.46 (0.08)	0.87 (0.20)	0.48 (0.12)	0.36 (0.12)	ETOH[e] INT[e] MDP[e]
14:0	3.10 (1.29)	3.83 (0.69)	3.43 (0.66)	4.89 (0.39)	5.36 (0.22)	
16:0	10.15 (0.99)	9.76 (0.55)	12.17 (0.83)	11.76 (0.69)	10.54 (0.57)	ETOH[e]
18:0	26.33 (3.26)	24.84 (2.65)	28.51 (2.72)	22.65 (0.66)	21.22 (0.29)	MDP[e]
18:1n-9	7.20 (0.30)	6.53 (0.27)	7.68 (0.44)	7.03 (0.42)	6.33 (0.10)	
18:2n-6	0.72 (0.10)	0.61 (0.09)	0.71 (0.09)	0.53 (0.11)	0.48 (0.03)	OIL[e] MDP[d]
20:3n-9	0.73 (0.06)	0.57 (0.03)	0.71 (0.04)	0.62 (0.06)	0.45 (0.02)	MDS[e] OIL[c]
20:3n-6	0.63 (0.03)	0.32 (0.02)	0.60 (0.04)	0.33 (0.02)	0.50 (0.06)	MDS[d] OIL[e]
20:4n-6	14.42 (1.97)	15.80 (0.95)	12.54 (1.43)	17.22 (0.45)	16.98 (0.51)	MDP[e] OIL[e]
20:5n-3	0.41 (0.09)	0.05 (0.02)	0.30 (0.06)	0.07 (0.05)	0.06 (0.04)	MDP[e] OIL[c]
22:4n-6	1.77 (0.15)	3.79 (0.11)	1.76 (0.24)	3.72 (0.25)	3.93 (0.09)	MDP[c] OIL[c]
22:5n-6	0.32 (0.02)	3.42 (0.18)	0.49 (0.26)	3.71 (0.26)	1.04 (0.05)	MDS[c] OIL[c]
22:5n-3	1.55 (0.06)	0.52 (0.02)	1.33 (0.13)	0.54 (0.06)	0.66 (0.02)	MDP[c] ETOH[f]
22:6n-3	23.08 (1.97)	18.44 (0.57)	20.38 (1.24)	16.14 (1.31)	22.05 (0.79)	OIL[e] MDS[d]

[a]Data are presented as mean % composition with (SEM). [b]ETOH = Main effect of ethanol; OIL = Main effect of oil; INT = Ethanol by oil interaction; MDP = MD/Polepa vs lab chow; MDS = MD/Safflower vs lab chow.
[c]$p<0.0001$.
[d]$p<0.01$.
[e]$p<0.05$.
[f]$p = 0.51$.

conception, the brains of animals supplemented with fish oil concentrate during gestation showed higher levels of n-3 and lower levels of n-6 fatty acids than those fed safflower oil. However, by day 32, two weeks after the animals had been returned to lab-chow feeding, most of these effects were no longer apparent, except for a decreased level of 22:5n-6. Previous work on recovery from the effects of EFA deficiency also reports lingering effects on 22:5n-6 (25). The increased ratio of 20:3n-6 to 20:4n-6 in the fish oil groups supports previous findings showing that long chain n-3 fatty acids block Δ-5 desaturase (33). The observed increase in 20:3n-9 by fish oil may be explained by the higher 18:1n-9 to 18:2n-6 ratio in the fish/safflower oil mixture compared with safflower oil alone, resulting in less competition in the former with respect to access to Δ-6 desaturase by 18:1n-9.

More importantly, these results provide support for the hypothesis that ethanol interacts with the fatty acid composition of the diet to affect the developing brain. When an exogenous supply of n-3 long chain fatty acids was supplied in excess to animals being treated with moderate amounts of ethanol during gestation, the PC fraction of the brains of the pups on day

TABLE 5

Selected Fatty Acid Composition on Day 32 Post-Conception of Brain Phosphatidylcholines of B6D2F$_2$ Pups of B6D2F$_1$ Dams Fed Ethanol and Either Fish (Polepa) or Safflower Oils During Gestation[a]

Fatty Acid	ETOH/ Polepa n=8	ETOH/ Saff n=8	MD/ Polepa n=8	MD/ Saff n=8	Lab Chow n=6	Effect[b]
12:0	2.15 (1.03)	1.67 (0.44)	2.26 (0.12)	2.74 (0.76)	2.00 (0.23)	
14:0	0.57 (0.33)	0.23 (0.07)	0.31 (0.09)	0.54 (0.29)	0.25 (0.11)	
16:0	45.47 (1.60)	48.70 (1.28)	50.51 (0.17)	48.94 (1.34)	49.82 (1.15)	ETOH[d] INT[e]
18:0	9.38 (0.34)	8.70 (0.31)	8.42 (0.47)	8.16 (0.45)	8.28 (0.48)	ETOH[e]
18:1n-9	18.08 (0.86)	17.22 (0.47)	16.86 (0.33)	16.74 (0.65)	16.79 (0.44)	
18:2n-6	2.02 (0.16)	1.70 (0.11)	1.61 (0.06)	1.81 (0.12)	1.64 (0.12)	INT[d]
20:3n-6	0.74 (0.07)	0.57 (0.04)	0.56 (0.02)	0.47 (0.07)	0.53 (0.02)	ETOH[c] OIL[d]
20:4n-6	7.97 (0.55)	7.55 (0.49)	6.97 (0.21)	7.37 (0.44)	7.39 (0.41)	
22:4n-6	0.66 (0.04)	0.67 (0.08)	0.54 (0.03)	0.49 (0.08)	0.58 (0.06)	ETOH[d]
22:5n-6	0.04 (0.02)	0.26 (0.06)	0.13 (0.03)	0.11 (0.03)	0.21 (0.09)	OIL[d] INT[d]
22:5n-3	0.17 (0.04)	0.23 (0.06)	0.16 (0.04)	0.16 (0.05)	0.10 (0.04)	
22:6n-3	4.54 (0.42)	4.06 (0.28)	3.73 (0.19)	3.76 (0.28)	3.99 (0.32)	ETOH[e]

[a]Data are presented as mean % composition with (SEM), n = number of litters.
[b]ETOH = Main effect of ethanol; OIL = Main effect of oil; INT = Ethanol by oil interaction.
[c]$p<0.01$.
[d]$p<0.05$.
[e]$0.1>p>0.05$.

22 post-conception indicated an increased incorporation of these dietary compounds relative to maltose-dextrin controls. On day 32, two weeks following the cessation of both treatments and reinstatement of feeding with lab chow, ethanol-treated animals continued to show increased levels not only of long chain n-3, but also of n-6 fatty acids. It should be noted that at both ages these effects were seen mainly in the PC fraction. These data could be interpreted as an indication of an increased demand for pre-formed long chain PUFA's by ethanol-treated animals; the increased accumulation of both n-3 and n-6 compounds postnatally, when *de novo* synthesis was able to proceed unhindered, may be an indication of some sort of continuing adaptive or "rebound" effect. Previous reports of changes in the synaptic plasma membranes of the brains of guinea pigs after three weeks of feeding an ethanol liquid diet also showed an effect of ethanol in increasing PE levels of 22:4n-6 and 22:6n-3 (34). Such changes may be related to recently reported effects of prenatal ethanol exposure on neuronal membrane order (35). There were also effects of ethanol on the composition of the saturated fat in the developing brain, reducing levels of 16:0 and increasing those of 18:0. As the fatty acid levels are reported as a percentage of the total fatty acids, the changes in the saturated fats may merely be the converse of the observed increases in the PUFA. Although the inhibition of Δ-6 desaturase by ethanol was slightly supported by the trend towards an increase in 18:2n-6 levels in the ethanol treated animals on day 22, this was not accompanied by a reduction of 20:4n-6, which would be the expected consequence of Δ-5 desaturase blockade. This anomaly may be related to the differential sensitivity of the two enzymes, as well as the possibility that maternal stores of AA may have been offsetting treatment effects. The relatively high levels of n-3 PUFA in the safflower oil groups would support the latter process.

TABLE 6

Selected Fatty Acid Composition on Day 32 Post-Conception of Brain Phosphatidylethanolamines of B6D2F$_2$ Pups of B6D2F$_1$ Dams Fed Ethanol and Either Fish (Polepa) or Safflower Oils During Gestation[a]

Fatty Acid	ETOH/ Polepa n=8	ETOH/ Saff n=8	MD/ Polepa n=8	MD/ Saff n=8	Lab Chow n=6	Effect[b]
14:0	2.64 (0.64)	2.57 (0.69)	1.74 (0.76)	2.80 (0.73)	1.87 (0.87)	
16:0	6.91 (0.53)	6.54 (0.72)	5.48 (0.70)	6.26 (0.60)	5.06 (0.55)	
18:0	29.5 (3.01)	32.21 (2.51)	33.03 (2.97)	30.81 (2.40)	31.43 (3.53)	
18:1n-9	8.46 (0.78)	7.34 (0.40)	7.91 (0.48)	7.98 (0.65)	7.48 (0.38)	
18:2n-6	1.06 (0.23)	0.77 (0.08)	0.72 (0.04)	0.87 (0.22)	0.63 (0.06)	
20:3n-6	0.68 (0.03)	0.64 (0.02)	0.77 (0.05)	1.16 (0.58)	0.72 (0.03)	
20:4n-6	15.91 (0.83)	16.38 (0.91)	15.42 (1.24)	15.44 (1.03)	16.44 (1.71)	
20:5n-3	0.27 (0.19)	0.23 (0.21)	0.22 (0.14)	0.51 (0.50)	0.04 (0.03)	
22:4n-6	3.28 (0.38)	3.64 (0.26)	3.59 (0.19)	3.63 (0.28)	4.11 (0.22)	
22:5n-6	0.57 (0.11)	1.13 (0.12)	0.66 (0.09)	1.22 (0.09)	0.90 (0.14)	OIL[c]
22:5n-3	0.74 (0.15)	0.50 (0.17)	0.65 (0.15)	0.88 (0.32)	0.51 (0.05)	
22:6n-3	21.27 (1.42)	20.91 (1.20)	23.62 (1.64)	20.98 (0.80)	23.81 (0.96)	

[a]Data are presented as mean % composition with (SEM), n = number of litters.
[b]OIL = Main effect of oil.
[c]$p<0.0001$.

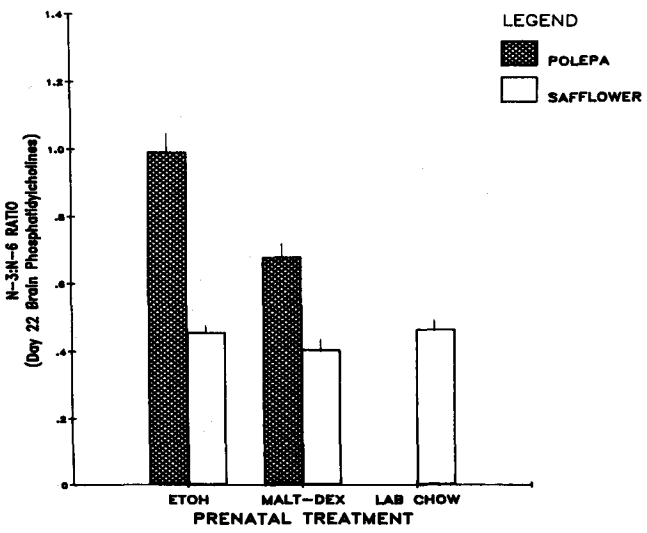

FIG. 4. Effect of prenatal ethanol and dietary supplementation with n-3 fatty acids on the long chain n-3:n-6 ratio in the phosphatidylcholine fraction of B6D2F$_2$ mouse brain on day 22 post-conception. Ethanol by Oil interaction, $p < 0.05$.

Supplementation with n-3 increased brain weight in ethanol-treated animals, but decreased it in maltose-dextrin controls. The calculated levels of n-3 fatty acids in this study were 3% of the dietary energy, which is high relative to the normal dietary range. It is possible that these levels were appropriate for what amounted to a therapeutic intervention in animals with an increased demand, viz., the ethanol-treated groups, but were in fact inappropriately high for normal demands. A further dose-response study of the effects of n-3 fatty acids on brain development should resolve this issue. In view of the effects seen on the brain, it was disappointing to find that treatment with fish oil did not appear to have any effect on behavioral development. Because the n-3 fatty acids have been implicated in effects on retinal development (36), we were particularly interested in the outcome of those tests which assessed visual function, viz., eye-opening and visual placing, but again found effects only of ethanol.

In summary, these results demonstrate that prenatal ethanol treatment interacts with long chain n-3 fatty acid dietary supplementation to affect brain weight and the fatty acid composition of the PC fraction of

the developing mouse brain. Further work should address the dose-response relationship between ethanol and n-3 supplementation, as well as the relationship between the timing of the dietary intervention and effects on development in ethanol-exposed animals. The outcome of such studies may support the possible therapeutic potential of dietary enrichment with long chain PUFA in combating some of the adverse effects of ethanol on brain development.

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Lipids

Notices
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IBC Conference on PAF and PAF Antagonists: Research Developments and Therapeutic Applications. Sheraton New Orleans Hotel, New Orleans, Louisiana, December 11-12, 1989. The Conference will cover cellular and metabolic aspects of PAF action as well as recent developments towards therapeutic applications of PAF antagonists. For further information contact Laurie Henry at IBC USA Conferences, Inc., Charles River Court, 8 Pleasant Street, Building D, South Natick, MA 01760. Phone (508) 650-4700; Fax (508) 653-1627.

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Guinea Pig Epidermis Generates Putative Anti-Inflammatory Metabolites from Fish Oil Polyunsaturated Fatty Acids

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Clinical studies have indicated that dietary fish oil may have therapeutic value in the treatment of psoriasis, a hyperproliferative, inflammatory skin disorder characterized by elevated LTB_4. To evolve a possible mechanism for these beneficial effects, we determined the metabolic fate of fish oil derived n-3 fatty acids in the skin. Specifically, we incubated guinea pig epidermal enzyme preparations with [^{3}H]eicosapentaenoic acid (20:5n-3) and [^{14}C]docosahexaenoic acid (22:6n-3). Analyses of the radiometabolites revealed the transformation of these n-3 fatty acids into n-6 lipoxygenase (arachidonate 15-lipoxygenase) products: 15-hydroxyeicosapentaenoic acid (15-HEPE) and 17-hydroxydocosahexaenoic acid (17-HDHE), respectively. Since 15-lipoxygenase products have been suggested as possible endogenous inhibitors of 5-lipoxygenase (an enzyme which catalyzes the formation of LTB_4) we tested the ability of 15-HEPE and 17-HDHE *in vitro* to inhibit the activity of the 5-lipoxygenase. Incubations of these metabolites with enzyme preparations from rat basophilic leukemia (RBL-1) cells demonstrated that 15-HEPE (IC_{50} = 28 μM) and 17-HDHE (IC_{50} = 25 μM) are respectively potent inhibitors of RBL-I-5-lipoxygenase. The inhibitory potential of these fish oil metabolites provides a possible mechanism by which fish oil might act to decrease local cutaneous levels of LTB_4, and thereby alleviate psoriatic symptoms.
Lipids 24, 998–1003 (1989).

There are reports that dietary fish oil may be beneficial, at least in part, in the treatment of psoriasis (1–3), a hyperproliferative, inflammatory skin disorder. Although the exact mechanism by which fish oil functions to ameliorate the cutaneous lesions of psoriasis has not been elucidated, there is some indication that fish oil may act by decreasing local epidermal levels of leukotriene B_4 (LTB_4), a 5-lipoxygenase product which is elevated in psoriatic epidermis (4–6). The notion that a regulation of LTB_4 synthesis may be involved is based upon increasing evidence that LTB_4 is a powerful proinflammatory agent with activities which include chemotaxis of neutrophils, augmentation of neutrophil adherence to endothelial cells and enhanced expression of C3b receptors on neutrophils (7). Leukotriene B_4 may also have a role in modulating epidermal proliferation based on its ability to stimulate DNA synthesis in keratinocytes, a primary epidermal cell type (8). *In vivo*, applications of LTB_4 to normal skin result in the induction of psoriatic-type lesions with both neutrophil infiltration (9,10) and epidermal hyperproliferation (11,12). Administration of 5-lipoxygenase inhibitors, topical or systemic, have been reported to improve the psoriatic lesions (13,14). Thus, if constituents of fish oil are transformed in the epidermis into metabolites which inhibit 5-lipoxygenase activity and LTB_4 synthesis, this may explain, at least in part, how fish oil alleviates psoriatic symptoms.

The epidermis is capable of converting plant-derived n-6 fatty acids into 15-lipoxygenase products (n-6 hydroxy fatty acids) (15,16). These hydroxy fatty acids have been shown to inhibit LTB_4 synthesis (16–18). The most abundant 15-lipoxygenase products in guinea pig epidermis are 13-hydroxyoctadecadienoic acid (13-HODE), a product of linoleic acid (18:2n-6), and 15-hydroxyeicosatetraenoic acid (15-HETE), a product of arachidonic acid (20:4n-6) (19). However, unlike the n-6 fatty acids, the metabolic fate of fish oil-derived fatty acids in the epidermis is unknown. Fish oils contain low levels of n-6 fatty acids but are rich in the n-3 polyunsaturated fatty acids (PUFAs) — eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3) (20). We reasoned that it is possible that 20:5n-3 and 22:6n-3, like the n-6 fatty acids, are transformed in the epidermis into 15-lipoxygenase products, and that these products could inhibit local LTB_4 synthesis. To investigate this hypothesis, we examined the metabolic fates of 20:5n-3 and 22:6n-3 by incubating them with guinea pig epidermal preparations. The metabolites were identified and then tested for their abilities to inhibit the biosynthesis of LTB_4 generated by RBL-I (rat basophilic leukemia) cells.

MATERIALS AND METHODS

[1-^{14}C]18:2n-6 (Spec. act. = 55.6 mCi/mmol), [1-^{14}C]20:4n-6 (Spec. act. = 54.9 mCi/mmol), [1-^{14}C]22:6n-3 (Spec. act. = 56.9 mCi/mmol) and [^{3}H]20:5n-3 (Spec. act. = 79.0 Ci/mmol) were purchased from DuPont, NEN Products Division (Boston, MA). Unlabelled fatty acids were purchased from NuChek Prep (Elysian, MN). 13-HODE, 15-HETrE, 15-HETE, 12-HEPE and 15-HEPE were purchased from Cayman Chemical (Ann Arbor, MI). Reduced glutathione (GSH) was purchased from Boehringer Mannheim (Indianapolis, IN), $CaCl_2$ was purchased from JT Baker Chemical Co. (Phillipsburg, PA) and *N,O-bis*(trimethylsilyl)trifluorocetamide (BSTFA) was purchased from Supelco, Inc. (Bellfonte, PA). All solvents were of HPLC quality and were purchased from Fisher Scientific Co (Fair Lawn, NJ).

Preparation of epidermal homogenates and incubations with 20:5n-3 and 22:6n-3. Incubations with guinea pig epidermal homogenates were conducted to determine the metabolic fate of 20:5n-3 and 22:6n-3 in the epidermis. Hair from the dorsum of male Hartley guinea pigs (400–450 g; Simonsen) was removed by shaving

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Abbreviations: PUFA, polyunsaturated fatty acid; LTB_4, leukotriene B_4; HODE, hydroxyoctadecadienoic acid; HETrE, hydroxyeicosatrienoic acid; HETE, hydroxyeicosatetraenoic acid; HEPE, hydroxyeicosapentaenoic acid; HDHE, hydroxydocosahexaenoic acid; GC/MS, gas chromatography/mass spectrometry; RP-HPLC, reverse phase-high performance liquid chromatography; GSH, reduced glutathione; BSTFA, *N,O-bis*(trimethylsilyl) trifluoroacetaminde

and depilation with sodium thioglycollate (Nair, Carter-Wallace, Inc., New York, NY) followed by rinsing with deionized water. The animals were sacrificed by cervical dislocation and the skin was rapidly removed. Superficial 0.2 mm slices were then removed by keratome (Storz, St. Louis, MO); histologic evaluation showed these keratome slices to be >85% epidermis. These epidermal slices were promptly placed in ice-cold buffer (50 mM phosphate, pH 7.4, 1 mM EDTA) and were homogenized with a Polytron (Brinkmann Instruments, Westbury, NY). This crude epidermal homogenate was centrifuged at 10,000 g for 20 min at 4°C to obtain a supernatant containing a mixture of particulate and cytosolic (incl. lipoxygenase) enzymes. The 10,000 g supernatant was used for incubations. A typical incubation consisted of 10 mg protein diluted in 2 mls buffer with GSH (1.0 mM). After pre-incubation at 37°C for 10 min, the fatty acid substrate was added (20 μM, 0.2 μCi/incubation) and the mixture incubated at 37°C for 20 min. The incubation was terminated by placement of the tube in ice and immediate acidification to pH 3.0. The incubation products were extracted with $CHCl_3$/MeOH (2:1, v/v).

Generation and identification of epidermal lipoxygenase metabolites from 20:5n-3 and 22:6n-3. The extracted radiometabolites of incubations of [^{3}H]20:5n-3 and [^{14}C]22:6n-3 with the epidermal 10,000 g supernatant were analyzed by chromatographic co-migration with radiolabelled hydroxy fatty acid standards. Soybean lipoxidase was used to convert 20:5n-3 and 22:6n-3 to the reference 15-lipoxygenase products, 15-HEPE and 17-HDHE, respectively, as previously reported (21). Similarly, human platelet preparations were used to convert 20:5n-3 and 22:6n-3 to the reference 12-lipoxygenase products, 12-HEPE (22,23); 14-HDHE and 11-HDHE (24), respectively. Identities of these reference metabolites were confirmed by GC/mass spectrometry. Analysis of each of the epidermal radiometabolites was performed by using two reverse phase-high performance liquid chromatography (RP-HPLC) systems with a Beckman 5um Ultrasphere ODS column (25 cm × 4-6 mm id), Beckman model 100A/100A pumps, and a Radiomatic HS Flo-one on-line radioactive flow detector. RP-HPLC system I utilized a solvent-system of methanol and H_2O (acidified to pH 3.0 with acetic acid) (25). This system was run at a flow rate of 1.0 ml/min in a stepwise gradient with 74% methanol from 0-55 min. For additional confirmation, the metabolites were chromatographed on RP-HPLC system II utilizing a solvent system of acetonitrile and water (0.02% H_3PO_3) modified from Van Rollins *et al.* (26). This second system was run at a flow rate at 20.0 ml/min at 50% acetonitrile from 0-30 min and 100% acetonitrile from 30-45 min. Co-migration of the metabolites with authentic samples in both systems was considered a base for purity.

For additional confirmation of identity, the epidermal metabolite of 20:5n-3 was subjected to gas chromatography/mass spectrometry (GC/MS). Specifically, that fraction of the eluate corresponding with 15-HEPE was collected by a Pharmacia FMC 100 fraction collector (Pharmacia Biochemicals, Piscataway, NJ) and treated with fresh methanolic ethereal diazomethane and BSTFA to form the fatty acid methyl ester, trimethylsilyl ether derivative (27). Authentic 15-HEPE was similarly derivatized. These derivatives were analyzed by GC/MS using an HP5790 gas chromatograph (Hewlett-Packard, Palo Alto, CA) equipped with a 15 m DB-1 fused silica column (J & W Scientific, Rancho Cordova, CA), interfaced with a VG ZAB-HS-ZF mass spectrometer (VG Analytical, Wythenshawe, England), as previously described (16). The oven was operated with a thermal gradient beginning at 220°C increasing 4°C; the MS was in electron impact (EI) mode at 70 mV. The fragment ions from the derivatized epidermal metabolite corresponds with those of the derivatized authentic 15-HEPE and those reported by Mitchell *et al.* (29) and Takenaga *et al.* (23). The prominent ions (*M/Z*) and mode of origin of the derivatized epidermal metabolite were: 404(M), 389(M-15, loss of CH_3), 373 (M-31, loss of OCH_3), 335(M-69, loss of C_5H_9, indicating an n-3 fatty acid), 245(335-90, loss of TMSOH), and 173 ($C_5H_9CHOTMS$, indicating hydroxylation at the n-6 position). The inability to obtain authentic sample for 17-HDHE for comparison prevented us from obtaining a satisfactory GC/MS profile of the epidermal metabolite of 22:6n-3 (17-HDHE). Identity of the epidermal metabolite of 22:6n-3 was therefore limited to chromatography on two RP-HPLC systems and on UV spectra.

Generation of 15-HEPE and 17-HDHE by soybean lipoxidase. Samples of 15-HEPE and 17-HDHE were synthesized by incubating the precursor fatty acids (20:5n-3 and 22:6n-3) with soybean lipoxidase (23) (Sigma Chemical Co., St. Louis, MO), followed by reduction of the intermediate hydroperoxides with triphenylphosphine and purifying the resultant hydroxy fatty acids by HPLC (21). The identities of these two soybean lipoxidase hydroxy fatty acids were confirmed by RP-HPLC and GC/MS, as previously published. Quantification was achieved by integrated optical density at 237 nm against an authentic standard of 15-hydroxyeicosatrienoic acid (15-HETrE). The HPLC and GC/MS characterized metabolites of soybean lipoxidase served as authentic references for the generated epidermal lipoxygenase products.

Effects of generated epidermal 15-HEPE and 17-HDHE on the activity of TBL-1 5-lipoxygenase pathway. The finding that the epidermal lipoxygenase products of 20:5n-3 and 22:6n-3 are 15-hydroxyeicosapentaenoic acid (15-HEPE) and 17-hydroxydocosahexaenoic acid (17-HDHE) led us to investigate whether these hydroxy fatty acids could modulate the synthesis of LTB_4 (a 5-lipoxygenase product of 20:4n-6). To evaluate the effects of epidermal 15-hydroxyeicosapentaenoic acid (15-HEPE) and 17-hydroxydocosahexaenoic acid (17-HDHE) on the biosynthesis of leukotriene B_4 (LTB_4) we used the *in vitro* model of RBL-1 cell homogenate (an effective system for evaluating the effects of chemical agents on 5-lipoxygenase pathway (16). Specifically, RBL-1 cells were placed in buffer (50 mM phosphate, pH 7.4, 1 mM EDTA) at a concentration of 3×10^7 cells/ml and then homogenized by sonication. This crude homogenate was centrifuged at 10,000 g to obtain the supernatant which contained the 5-lipoxygenase activity. The supernatant enzyme preparations were pre-incubated at 37°C for 10 min with 2 mM $CaCl_2$ and varying concentrations (0-50 μM) of either 15-HEPE

or 17-HDHE. The reaction was initiated by the addition of [^{14}C]20:4n-6 (20 μM; 0.2 μCi) to each respective incubation mixture. After 20 min at 37°C, the incubations were terminated by placement of tubes in ice, followed by acidification to pH 3.0. The incubation products were extracted with $CHCl_3$/MeOH (2:1, v/v). Conversion of 20:4n-6 into 5-lipoxygenase products (mainly 5-HETE and LTB_4) by RBL-1 supernatant was determined by a reverse phase-HPLC system using a solvent system of methanol/water (0.08% HOAc, pH 6.2) modified from Henke *et al.* (28). This system was run at a constant flow rate of 1.4 ml/min with an increasing gradient of methanol as follows: 55% methanol from 0–20 min, 66% from 20–40 min, 77% from 40–60 min, and 100% from 60–75 min. Similarly, we tested the effects of the generated epidermal metabolites of n-6 fatty acids, such as the 15-lipoxygenase products of arachidonic acid, (15-HETE) and linoleic acid, 13-hydroxyoctadecadienoic acid (13-HODE) in order to compare their inhibitory potentials with those of the n-3 fatty acids.

RESULTS

Identification of epidermal lipoxygenase products of 20:5n-3 and 22:6n-3. Analysis of the radiometabolites of [^{3}H]20:5n-3 (20 μM) and [^{14}C]22:6n-3 (20 μM) from incubations with the epidermal 10,000 g supernatant showed conversion predominantly into the 15-lipoxygenase products: [^{3}H]15-HEPE and [^{14}C]17-HDHE, respectively. Using two RP-HPLC systems, the identities of the epidermal metabolites of [^{3}H]20:5n-3 and [^{14}C]22:6n-3 were shown to have chromatographic profiles similar to the radiolabelled hydroxy fatty acid standards of the soybean lipoxidase products of 20:5n-3 and 22:6n-3. A typical profile of the separation of the epidermal lipoxygenase products of 20:5n-3 and 22:6n-3 in the solvent system I is shown in Figure 1A. This Figure shows that the major epidermal metabolite of 20:5n-3 co-chromatographs with the 15-lipoxygenase product, 15-HEPE when using the methanol-based RP-HPLC system described above. Figure 1B similarly shows that the major epidermal metabolite of [^{14}C]22:6n-3 co-chromatographs with the 15-lipoxygenase product, 17-HDHE. Co-migration of these two epidermal hydroxy metabolites with soybean lipoxidase products was similarly established using a second solvent system II —acetonitrile-based RP-HPLC system. Additionally, the identity of the epidermal metabolite of 20:5n-3, and not 22:6n-3, was confirmed by comparing the mass spectrometry profiles from the fatty acid methyl ester/trimethylsilyl (TMS) ether derivatives of this metabolite and authentic 15-HEPE as described under Methods.

Comparative metabolic transformations of n-3 and n-6 polyunsaturated fatty acid into monohydroxy acids by guinea pig epidermal homogenates. In these experiments, the incubations of the two n-3 PUFAs 22:6n-3 and 20:5n-3 with epidermal homogenates at a similar concentration (20 μM) and time (20 min) revealed that 30.4 ± 1.0% of 22:6n-3 was converted into 17-HDHE, whereas only 17.8 ± 1.4% of 20:5n-3 was converted into 15-HEPE (Fig. 2). On the other hand, similar incubations with 18:2n-6 and 20:4n-6 revealed that the two n-6 PUFAs were less readily converted to their corresponding 15-lipoxygenase products when compared to the n-3 fatty acids. For instance, 15.7 ± 0.5% of 20:4n-6 was converted into 15-HETE, whereas only 4.0 ± of 18:2n-6 was converted into 13-HODE.

Inhibition of RBL-1 cell 5-lipoxygenase activity by guinea pig generated 15-HEPE and 17-HDHE. Incubations of RBL-1 cell homogenates with generated guinea pig 15-HEPE and 17-HDHE revealed that both monohydroxy fatty acids are potent inhibitors of 20:4n-6 transformation into LTB_4 and 5-HETE, the two major metabolites of 20:4n-6 by RBL-1 homogenate (Fig. 3). The inhibitory effects are dose dependent (0–50 μM). Since the major metabolites of 20:4n-6 by the RBL-1 homogenate are LTB_4 and 5-HETE, the inhibitory effects of both 15-HEPE and 17-HDHE are on the common 5-lipoxygenase pathway. The determined IC_{50} of 15-HEPE and 17-HDHE were 28 μM and 25 μM, respectively. For comparison, the determined IC_{50} of 15-HETE (the epidermal 15-lipoxygenase product of 20:4n-6) and the IC_{50} of 13-HODE (the 15-lipoxygenase product of linoleic acid) were 37 μM and >50 μM, respectively. Taken together, the ability of the epidermal 15-lipoxygenase products to inhibit 5-lipoxygenase follows the order 17-HDHE≥15-HEPE≥-HETE>>13-HODE.

DISCUSSION

Fish oil has been reported to be beneficial in the treatment of psoriasis (1–3), a hyperproliferative, inflammatory skin disorder which is associated with elevated levels of LTB_4, a 5-lipoxygenase product (4–6). To elucidate a possible mechanism of these beneficial effects we investigated whether the major PUFA constituents of fish oil, 20:5n-3 and 22:6n-3, can be transformed by epidermal preparations into metabolites which inhibit LTB_4 synthesis. Our data indicate: i) that both 20:5n-3 and 22:6n-3 are readily converted by epidermal 15-lipoxygenase into 15-HEPE and 17-HDHE, and ii) that both 15-HEPE and 17-HDHE are potent inhibitors of RBL-1 5-lipoxygenase activity. Specifically, incubations of guinea pig epidermal enzyme preparations with [^{3}H]20:5n-3 and [^{14}C]22:6n-3 resulted in the formation of radiometabolites which were chromatographically similar to the 15-lipoxygenase products, 15-HEPE and 17-HDHE, respectively (Fig. 1). Similar incubations with 18:2n-6 and 20:4n-6 (16) showed that these PUFAs are also converted to their corresponding 15-lipoxygenase products — 13-HODE and 15-HETE, respectively. A comparison of the relative utilization of the above four n-3 and n-6 polyunsaturated fatty acids at the same concentration and time showed preferential metabolism of the fatty acid substrates by the guinea pig epidermal 15-lipoxygenase as follows: 22:6n-3>20:5n-3>20:4n-6>18:2n-6 (Fig. 2). The finding that guinea pig epidermal homogenates convert n-3 fatty acids to 15-lipoxygenase products is consistent with the ability of human epidermal homogenates to transform 20:5n-3 to 15-HEPE (30). These results add to other similarities which exist in polyunsaturated fatty acid metabolism in the epidermis of these species (31,32).

Since 20:5n-3 and 22:6n-3 are readily converted to epidermal 15-lipoxygenase products, and since 15-

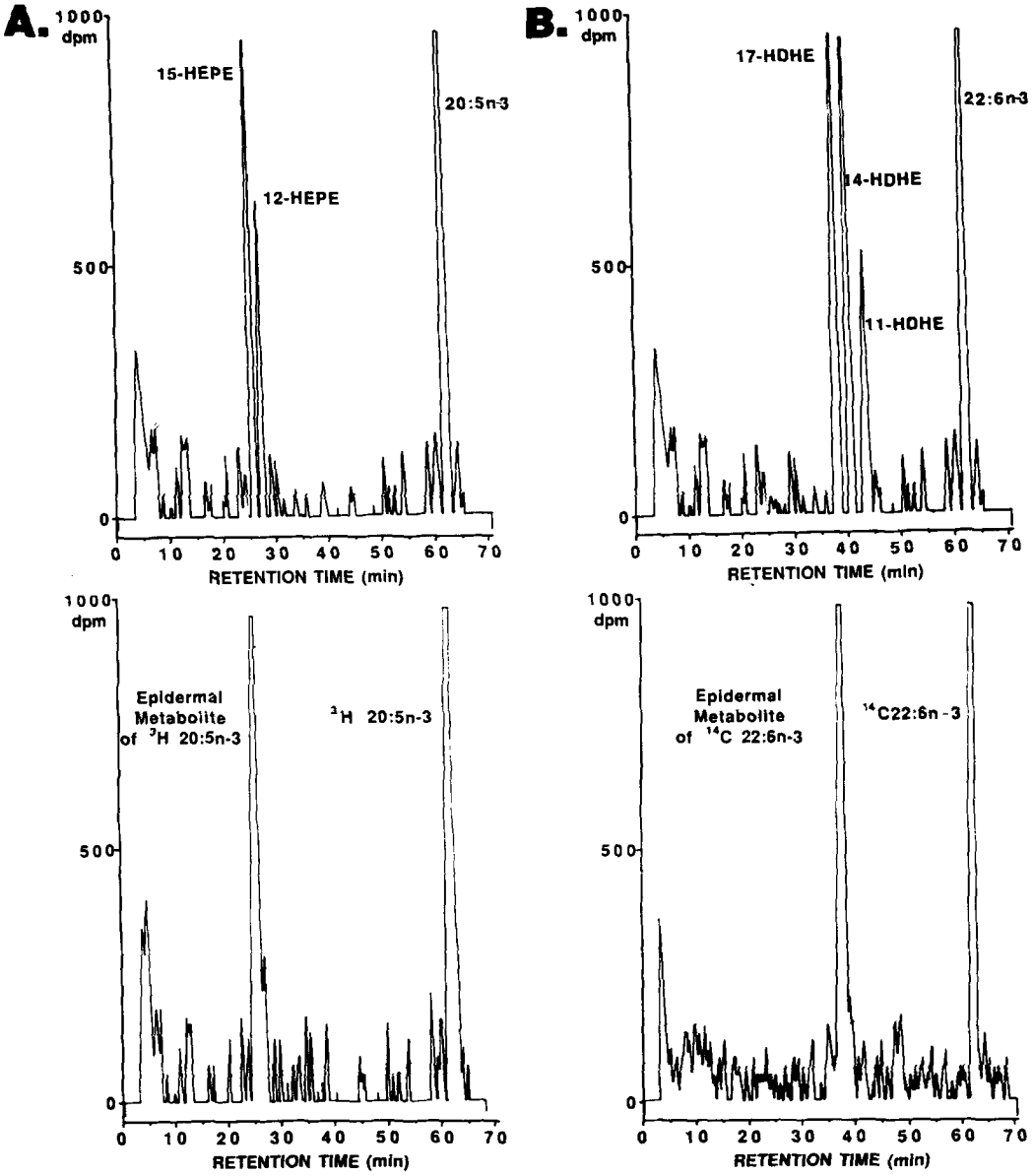

FIG. 1. RP-HPLC radiochromatograms of lipoxygenase products of n-3 fatty acids using solvent system I of 74% MeOH in H_2O acidified to pH 3.0 with HOAc, as described in the text. A) Above: Reference lipoxygenase metabolites of [^{3}H]20:5n-3 from soybean lipoxidase (15-HEPE, TR = 25.8) and human platelets (12-HEPE, RT = 28.0). Below: Lipids extracted from incubations of [^{3}H]20:5n-3 (20 μM) with guinea pig epidermal homogenates (RT = 25.6). B) Above: Reference lipoxygenase metabolites of [^{14}C]22:6n-3 from soybean lipoxidase (17-HDHE, RT = 38.3) and human platelets (14-HDHE and 11-HDHE, RT = 41.0 and 43.9). Below: Lipids extracted from incubations of [^{14}C]22:6n-3 (20 μM) with guinea pig epidermal homogenates (RT = 38.3).

lipoxygenase products have been reported to inhibit LTB_4 synthesis (17,18), we tested the ability of 15-HEPE and 17-HDHE to inhibit 5-lipoxygenase activity using an *in vitro* model of RBL-1 5-lipoxygenase. Our data demonstrated that 15-HEPE and 17-HDHE are both potent inhibitors of the RBL-1 5-lipoxygenase (*Fig. 3*). 15-HEPE inhibited 5-lipoxygenase with an IC_{50} of 28 μM, whereas 17-HDHE inhibited with an IC_{50} of 25 μM. The n-6 fatty acid derived 15-lipoxygenase products 15-HETE and 13-HODE were less potent inhibitors; 15-HETE inhibited with an IC_{50} of 37 μM while 13-HODE inhibited with an IC_{50} of >50 μM. The inhibition data for the 5-lipoxygenase in our RBL-1 homogenate system shows qualitative agreement with similar studies with intact neutrophils in which 15-HETE and 13-HODE inhibited 5-lipoxygenase with IC_{50} of 8 μM and 32 μM, respectively (18). Therefore, our results support the hypothesis that 20:5n-3 and 22:6n-3, the major constituents of fish oil, are converted by the epidermis to 15-lipoxygenase products (15-HEPE and 17-HDHE), and that these epidermal metabolites may increase the overall levels of en-

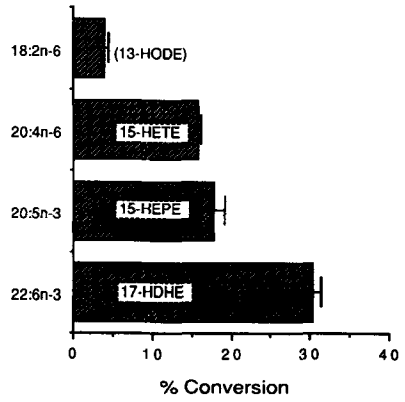

FIG. 2. Comparative metabolic transformations of n-3 and n-6 fatty acids (20 μM) into 15-lipoxygenase products by guinea pig epidermal homogenates. Details of respective incubations are contained in the text. Each value represents the mean ± SEM of three separate experiments.

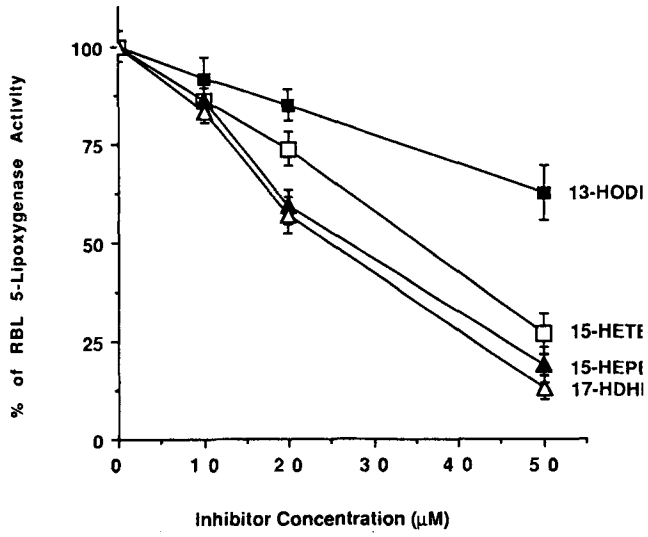

FIG. 3. Inhibitory effects of: 13-HODE (■), 15-HETE (□), 15-HEPE (▲), and 17-HDHE (△) on the activity of 5-lipoxygenase from RBL-1 cell homogenates. Each point represents the mean ± SEM of three experiments. Approximate IC_{50} values in μM for 13-HODE, 15-HETE, 15-HEPE, and 17-HDHE are >50, 37, 28 and 25, respectively.

dogenous inhibitors of 5-lipoxygenase in the skin. Such a possibility was recently demonstrated in the epidermis of guinea pigs fed fish oil rich in 20:5n-3 and 22:6n-3 PUFAs (33). This implies that dietary fish oil may lead to a decrease in local LTB_4 synthesis by neutrophils which are known to infiltrate the epidermis in lesions of psoriasis.

Interestingly, there is precedence to support the above hypothesis with fish oil. In humans, dietary vegetable oils rich in γ-linolenic acid (18:3n-6) have been reported to alleviate the inflammatory skin lesions associated with atopic eczema (34,35), a condition which is also characterized by elevated lesional LTB_4 (36). In guinea pigs, dietary supplementation with 18:3n-6 results in significantly elevated epidermal levels of 18:3n-6, its elongase product dihomo-γ-linolenic acid (20:3n-6), and the latter's 15-lipoxygenase product 15-hydroxyeicosatrienoic acid (15-HETrE). Since 15-HETrE is a potent inhibitor of 5-lipoxygenase activity (16,17), it has been suggested that the beneficial effects of oils rich in 18:3n-6 on atopic eczema may result from an inhibition of lesional LTB_4 synthesis (16, 37).

The synthesis of 15-lipoxygenase products *in vivo* depends on the incorporation of the dietary fatty acids into the epidermal phospholipids and their subsequent release prior to transformation into hydroxy fatty acids by the epidermal 15-lipoxygenase. Neutrophils and platelets, for instance, readily incorporate 20:5n-3 and 22:6n-3 into phospholipids. However, when these cells are stimulated by appropriate agonists, there is negligible release of 22:6n-3 when compared to 20:5n-3 (38). Similarly, epidermis may favor the release of 20:5n-3 over 22:6n-3. Recent data from our laboratory does indicate that although both 20:5n-3 and 22:6n-3 are incorporated into the epidermal phospholipids of guinea pigs fed fish oil diets, the *in vivo* epidermal level of 15-HEPE is markedly greater than that of 17-HDHE (33), suggesting a possible impaired hydrolysis and release of free 22:6n-3 from the epidermal phospholipids. This possibility may explain why clinical improvement of psoriatic patients whose diets were supplemented with fish oil correlated with patients with elevated epidermal ratios of 20:5n-3/22:6n-3 (1).

The conversion of n-3 and n-6 polyunsaturated fatty acids contained in certain dietary oils to 15-lipoxygenase products which can inhibit LTB_4 synthesis provides an attractive mechanism by which these oils may exert beneficial effects on inflammatory, hyperproliferative skin disorders. It is, however, premature to suggest that the beneficial effects of fish oil on psoriasis is limited to or dependent upon this mechanism alone. In fact, 20:5n-3 has been reported to directly inhibit the conversion of 20:4n-6 to LTB_4 in neutrophils (39,40), presumably acting as a substrate competitor. Nonetheless, our studies strongly suggest that the role of epidermal 15-lipoxygenase in generating local putative antiinflammatory hydroxy acids may prove to be more important in modulating local generation of LTB_4 by neutrophils which infiltrate the epidermis. This possibility underscores the need for further investigation into the role of fish oil-derived 15-lipoxygenase products in other hyperproliferative and inflammatory skin disorders.

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Inhibitory Effect of Stearidonic Acid (18:4 n-3) on Platelet Aggregation and Arachidonate Oxygenation

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The effect of stearidonic acid (18:4n-3) present in fish and some plant oils, such as black currant seed oil, was studied on human platelets. When added to platelets simultaneously with collagen, arachidonic acid or endoperoxide mimetic U46619, 18:4n-3 appeared as a weak inhibitor of platelet aggregation. In addition, 18:4n-3 did not alter the metabolism of exogenous arachidonic acid. In contrast, when preincubated with platelets after precoating onto albumin, 18:4n-3 inhibited platelet aggregation induced by thrombin, collagen, arachidonic acid or U46619, and was as potent as eicosapentaenoic acid (20:5n-3) tested under similar conditions. Stearidonic acid also altered the endogenous arachidonate oxygenation stimulated by low doses of thrombin, but to a significantly lesser extent than did 20:5n-3. It seems therefore that, in addition to competing with endogenous arachidonate metabolism, 18:4n-3 may affect platelet aggregation by another mechanism.
Lipids 24, 1004–1007 (1989).

Observations in Greenland Eskimos (1,2), who live on a marine diet, as well as studies with normal human volunteers on a sea fish or fish oil diet (3–6), suggest that n-3 fatty acid-rich diets may prevent atherosclerosis and thrombosis. Numerous studies have pointed out the potential inhibitory effect of α-linolenic (18:3n-3), eicosapentaenoic (20:5n-3) and docosahexaenoic (22:6n-3) acids on platelet aggregation (7–11). Several hypotheses have been proposed to explain the mechanism by which these acids decrease platelet function. It has been suggested that 20:5n-3 competes with arachidonic acid (20:4n-6) for its liberation from membrane phospholipids and its oxygenation by platelet cyclooxygenase (3,11–14). On the other hand, the strong inhibitory effect of 22:6n-3 has been related to phospholipid modification itself and to a possible interaction between esterified 22:6n-3 and the cyclooxygenase/lipoxygenase synthase complex (4). However, seafood is not only rich in 20:5n-3 and 22:6n-3 but also contains minor percentages of other n-3 polyunsaturated fatty acids like stearidonic acid (18:4n-3). Another important source of 18:4n-3 is the seed oil of plants like black currant (15). Stearidonic acid is the product of 18:3n-3 desaturation by Δ6-desaturase which is considered a limiting step in the desaturation/elongation process in animal fatty acid metabolism (16). The metabolism of 18:4n-3 in platelets is still not well understood. Weiner and Sprecher (17) previously observed that 18:4n-3 is poorly incorporated into platelet phospholipids and that it is rapidly chain elongated to 20:4n-3 and subsequently incorporated into phospholipids.

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Abbreviations: CPD, citrate-phosphate-dextrose buffer; THB, Tyrode HEPES buffer; U46619, 9-methano analogue of PGH_2; Tx, thromboxane.

In the present study, the effect of 18:4n-3 on platelet function was investigated both when added exogenously and after preincubation with platelets as albumin complex. Similar studies were done with 20:5n-3 as a reference.

MATERIALS AND METHODS

Materials. Human albumin, thrombin, and unlabelled arachidonic, eicosapentaenoic and linoleic acids were obtained from Sigma-France (L'Isle d'Abeau). Stearidonic acid was provided by the Nestle Research Center (CRN) (Lausanne, Switzerland). [1-^{14}C]Arachidonic acid was purchased from CEA (Gif/Yvette, France). The 9-methano analogue of PGH_2 was a generous gift from Dr. J.E. Pike (the Upjohn Co., Kalamazoo, MI), and collagen was obtained from Horm (Munich, FRG). Silica gel plates were purchased from Merck (Darmstadt, FRG), and nucleosil C_{18} 5 μm from Macherey-Nagel (Düren, FRG). Other reagents were furnished by Prolabo (Paris, France), and organic solvents by SDS (Peypin, France).

Platelet preparation. Venous blood was collected, using 14% CPD (pH 5.6, 0.327% citric acid, 2.63% sodium citrate, 0.249% monosodic phosphate, 2.32% dextrose) as an anticoagulant, from human volunteers who had not taken any drug for at least 10 days. Platelets were isolated from their plasma as described previously (18). To study the effect of exogenous 18:4n-3 on platelet function, platelets were simply resuspended into a tyrode HEPES buffer solution (THB) (0.3×10^9 platelets/ml). When the study concerned platelets preincubated with 18:4n-3 or 20:5n-3, platelets were resuspended into a THB solution containing albumin preloaded with 18:4n-3 or 20:5n-3. This THB solution was prepared with 50 μM free fatty acids human albumin (3.5 g/l), 50 μM 18:2n-6, 5 μM 20:4n-6 and 5 μM 18:4n-3 or 20:5n-3 (the molecular ratio: polyunsaturated fatty acid/albumin was then 1, 0.1 and 0.1, respectively, with 18:2n-6, 20:4n-6, and 18:4n-3 or 20:5n-3), and was kept 16 hr at 37°C under nitrogen before the addition of platelets. The presence of 18:2n-6 and 20:4n-6 simulates the physiological situation better (19). Platelets were incubated in this THB solution for 2 hr at 37°C under gentle shaking. The incubate was then acidified to pH 6.4 with citric acid, centrifuged at 700 g for 12 min, and platelets were resuspended (0.3×10^9 platelets/ml) into THB containing 0.1% gelatin to help keep platelets viable after the second washing. Control platelets were obtained after incubation with THB containing only albumin, 18:2n-6 and 20:4n-6.

Platelet aggregation. Platelet aggregation was performed according to the method of Born (20). Platelet aggregability was tested with collagen, arachidonic acid, analogue of PGH_2 (U46619) and thrombin as inducers. Aggregating agents were used at concentra-

tions inducing about 60% aggregation of control platelets after 4 min, in order to account for either a decreased or an increased aggregation of treated platelets. Concentrations of inducers differed slightly (collagen 1-2.5 μg/ml, arachidonic acid 1-2.5 μM, U46619 30-100 ng/ml and thrombin 15-30 mU/ml) depending on platelet sensitivity observed from the various batches. When 18:4n-3 was tested exogenously without preincubation, it was added in ethanol (final concentration 0.5%) simultaneously to the aggregating agent.

Metabolism of arachidonic acid. The oxygenation of exogenous 20:4n-6 through the cyclooxygenase and lipoxygenase pathways was studied in the presence or absence of 18:4n-3. Platelet suspension, 0.4 ml, was incubated with 10 μM [1-^{14}C]20:4n-6 and 0, 5, or 10 μM unlabelled 18:4n-3 for 4 min at 37°C. Incubations were terminated by the addition of three volumes of ethanol, and total lipids were extracted twice with chloroform in the presence of butylated hydroxytoluene (5×10^{-5}M) as an antioxidant. The lipids were applied to thin-layer plates (TLC, Silica Gel G) to separate successively monohydroxy derivatives of 20:4n-6 (1st elution with hexane/diethyl ether/acetic acid, 60:40:1, v/v/v) and prostanoids from total phospholipids (2nd elution with diethyl ether/methanol/acetic acid, 90:1:2, v/v/v). A quantitative radiochromatogram was run after each elution. Total radioactivity was considered as representing the initial amount of labelled 20:4n-6. The integrated peaks, then calculated as percentage of total radioactivity, could be quantified in nmol of products (21).

Control platelets and platelets preincubated with 18:4n-3 or 20:5n-3 were stimulated by 25 mU/ml thrombin for 4 min at 37°C. Incubations were terminated by acidification to pH 3, and 20:4n-6 derivatives were extracted with 10 volumes of diethyl ether containing [1-^{14}C]15-OH-5, 8,11,13-20:4 (soybean lipoxygenase product of 20:4n-6) as an internal standard. The extract was subjected to TLC to separate total monohydroxy derivatives of 20:4n-6 from the other lipids. These derivatives were then subjected to reverse-phase HPLC to determine 12-OH-5,8,10,14-eicosatetraenoic acid (12-HETE), the normal end-product of the platelet lipoxygenase pathway, and 12-OH-5,8,10-heptadecatrienoic acid (HHT), a product of the cyclooxygenase pathway. Measurements were done as described previously (22).

Statistics. The paired Student's t-test was used to compare the various groups of results.

RESULTS

Effects of 18:4n-3 added during platelet activation. To seek for a possible effect of 18:4n-3 on platelet activity, we first tested platelet aggregation in the presence of different concentrations of 18:4n-3 (0.5, 1, 2 and 4 μM). Stearidonic acid was administered in ethanol simultaneously with the aggregating agent (collagen, 20:4n-6 or U46619). Platelet aggregation tended to decrease in the presence of 18:4n-3 (Table 1). Two μM 18:4n-3 induced a significant inhibition of collagen- or 20:4n-6-induced aggregation. U46619-induced aggregation was not significantly altered by 18:4n-3.

The metabolism of exogenous 20:4n-6 (10 μM) was also tested in the presence of 5 or 10 μM 18:4n-3. The formation of both cyclooxygenase (HHT, T × B_2) and lipoxygenase (HETE) products was not changed by 18:4n-3 (Table 2).

Platelet activities after preincubation with 18:4n-3 or 20:5n-3. Platelets were preincubated in the presence of 18:4n-3 or 20:5n-3 precoated onto albumin at molecular ratio: fatty acid/albumin of 0.1. These incubations were done in presence of 18:2n-6 and 20:4n-6 also precoated onto albumin at molecular ratios of 1 and 0.1, respectively, for simulating their normal concentrations in the plasma unesterified fatty acid pool.

Platelet aggregation was tested in the presence of collagen, 20:4n-6, U46619 or thrombin. Independently of the aggregating agent used, platelets preincubated with 18:4n-3 or 20:5n-3 were significantly less sensitive than control platelets (Table 3).

HHT and HETE, produced from endogenous 20:4n-6, were measured after 4 min stimulation of platelets by 25 mU/ml thrombin at 37°C. A significant inhibition of HHT and HETE synthesis was observed in platelets preincubated with 18:4n-3 as well as in those pretreated with 20:5n-3 (Table 4). In seven experiments, samples of platelets have been treated parallel with 18:4n-3 or 20:5n-3. The paired t-test comparison of both groups revealed that 18:4n-3 was less potent in inhibiting thrombin-induced oxygenation of endogenous 20:4n-6 than was 20:5n-3.

TABLE 1

Effects of 18:4n-3 Added Simultaneously to Collagen, 20:4n-6 or U46619 on Platelet Aggregation[a]

Aggregation by[b]		Concentrations of 18:4n-3 (μM)			
		0.5	1	2	4
Collagen	A	53 ± 12	60 ± 9	55 ± 12	50 ± 2
	B	45 ± 17	49 ± 17	35 ± 11*	39 ± 17
		n=3	n=3	n=3	n=3
20:4n-6	A	53 ± 7	53 ± 5	52 ± 7	61 ± 3
	B	46 ± 8	44 ± 11	39 ± 11*	50 ± 11
		n=6	n=6	n=7	n=5
U46619	A	62 ± 3	59 ± 1	54 ± 5	53 ± 9
	B	59 ± 3	54 ± 2	48 ± 6	31 ± 12
		n=3	n=3	n=4	n=4

[a]Aggregations were performed with 0.3×10^9 platelets/ml. Results represent the mean ± S.D. of percentage aggregations obtained 4 min after the addition of the aggregating agent.
[b]A: control platelets; B: platelets + 18:4n-3; B vs A: * = $p<0.05$.

DISCUSSION

In the present work, the effects of 18:4n-3 upon platelet aggregation and 20:4n-6 oxygenation were investigated *in vitro*. When added to platelet suspensions, simultaneously with aggregating agents involving 20:4n-6 metabolism, 18:4n-3 weakly inhibited the aggregation. Moreover, 18:4n-3 did not alter the metabolism of exogenous 20:4n-6. These first results suggested that 18:4n-3 may inhibit platelet aggregation without altering platelet cyclooxygenase and lipoxygenase activities.

In the second part of the study, we simulated a supplemented diet by preincubating platelets with very

TABLE 2

Effect of 18:4n-3 on the Oxygenated Metabolism of Exogenous 20:4n-6 (10 μM)[a]

Metabolites[b]	Control	18:4n-3 5 μM	18:4n-3 10 μM
HHT	2.4 ± 1.0	2.2 ± 1.4	2.6 ± 1.4
T × B_2	1.3 ± 0.9	1.3 ± 0.5	1.5 ± 0.9
HETE	1.4 ± 0.5	1.8 ± 0.6	1.7 ± 0.7
PL	1.8 ± 0.2	1.7 ± 0.2	1.6 ± 0.2

[a]Results in nmol/10^9 platelets represent the mean ± S.D. (n=3) of 20:4n-6 metabolites after 4 min incubation.
[b]PL: amount of exogenous 20:4n-6 incorporated into phospholipids.

TABLE 3

Platelet Aggregation After Preincubation with 18:4n-3 or 20:5n-3[a]

Aggregation by[b]	A	B	C
Collagen n=8	58 ± 9	43 ± 20*	48 ± 13***
20:4n-6 n=5	57 ± 5	50 ± 7*	48 ± 4*
U46619 n=7	64 ± 10	49 ± 16*	43 ± 11***
Thrombin n=8	65 ± 6	57 ± 9**	58 ± 9*

[a]Aggregations were performed with 0.3 × 10^9 platelets/ml. Results represent the mean ± S.D. of percentage aggregations 4 min after the addition of the aggregating agent (collagen, 20:4n-6, U46619 or thrombin).
[b]A: control platelets; B and C: platelets preincubated with 18:4n-3 and 20:5n-3, respectively; B vs A and C vs A: * = $p<0.05$, ** = $p<0.02$, *** = $p<0.01$.

low concentrations of fatty acids precoated onto albumin (molecular ratio fatty acid/albumin = 0.1) in the presence of 18:2n-6 and 20:4n-6, the main fatty acids of the plasma unesterified fatty acid pool. Hajarine and Lagarde (19) have studied the incorporation of different polyenoic acids into human platelet lipid stores under various conditions, and found a decrease of fatty acid uptake in the presence of 18:2n-6 and 20:4n-6. The presence of 18:2n-6, 20:4n-6, or both in the incubating medium did not substantially modify platelet aggregation (data not shown). The preincubation with 18:4n-3 induced a significant inhibition of platelet aggregability regardless of which inducer was used. Likewise, platelets preincubated with 20:5n-3 in the same conditions were also significantly less sensitive to the four aggregating agents tested than were control platelets. This is in agreement with previous work reporting that platelet aggregability was significantly decreased in platelets preloaded with 20:5n-3 (3,11,13). In the present study we succeeded in obtaining similar results by using a low ratio fatty acid/albumin [ratio of 0.1 rather than 0.5 (11)].

We observed an inhibition of HHT and HETE production in both 18:4n-3 and 20:5n-3 preincubated platelets when they were stimulated by low thrombin concentration (25 mU/ml). We did not measure T × A_2, but the cyclooxygenase activity is generally well reflected in HHT formation. Our results concerning 20:5n-3-treated platelets are in accordance with the hypothesis previously proposed to explain the mechanism by which 20:5n-3 decreases platelet functions. Several authors have proposed that 20:5n-3 might inhibit the aggregation in reducing both the stimulated 20:4n-6 liberation from phospholipids and the formation of T × A_2 via a competitive inhibition of 20:4n-6 cyclooxygenation (3,5,11,23). As 18:4n-3 inhibited both platelet aggregation and the endogenous 20:4n-6 metabolism, we propose that 18:4n-3 acts similarly. However, 18:4n-3 appeared less potent than 20:5n-3 in inhibiting HHT/HETE formation. In spite of this, 18:4n-3 was as potent as 20:5n-3 in inhibiting platelet aggregation. Therefore, an additional mechanism parallel to competing with 20:4n-6 metabolism may explain the decrease of 18:4n-3-treated platelets' sensibility to aggregating agents.

We conclude that 18:4n-3 and 20:5n-3 alter both platelet aggregation and the oxygenated metabolism of platelet endogenous 20:4n-6. Although the acids appear equipotent for inhibiting the aggregation, 18:4n-3 is less potent than 20:5n-3 in inhibiting the conversion of endogenous 20:4n-6 into both cyclooxygenase and lipoxygenase products. This suggests that, in addition to competing with 20:4n-6 metabolism, 18:4n-3 may inhibit platelet aggregation by another mechanism, which is now under investigation.

ACKNOWLEDGMENTS

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TABLE 4

Effects of 18:4n-3 or 20:5n-3 Preincubation on Endogenous 20:4n-6 Oxygenated Metabolism

	A_1		B	A_2		C	
HHT	301 ± 116	n=13	230 ± 111*	308 ± 145	n=8	189 ± 93***	(a)
HETE	109 ± 62	n=13	78 ± 44***	117 ± 96	n=8	62 ± 60**	(b)

Results in pmol/10^9 platelets represent the mean ± S.D. of HHT and HETE, as indexes of cyclooxygenase and lipoxygenase activities, respectively, after 4 min stimulation by 25 mU/ml thrombin. A_1 and A_2: control platelets; B and C: platelets preincubated with 18:4n-3 and 20:5n-3, respectively; B vs A_1 and C vs A_2: * = $p<0.05$, ** = $p<0.02$, *** = $p<0.01$; C vs B: (a) = $p<0.05$ (n=7), (b) = $p<0.02$ (n=7).

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X-Ray Diffraction Study of Sodium Soaps of Monounsaturated and Polyunsaturated Fatty Acids

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X-Ray powder diffraction patterns of the sodium soaps of 14 monounsaturated and polyunsaturated fatty acids were obtained at room temperature. The patterns of the soaps of 9,12-*trans,trans*-octadecadienoic acid, 11,14-*cis,cis*-eicosadienoic acid 11,14,17-all *cis*-eicosatrienoic acid and 5 monounsaturated fatty acids were typical of the crystalline lamellar phase. The patterns of the soaps of 9,12-*cis,cis*-octadecadienoic, all *cis*-9,12,15-octadecatrienoic, all *cis*-8,11,14-eicostrienoic, all *cis*-5,8,11,14-eicosatetraenoic, all *cis*-5,8,11,14,17-eicosapentaenoic and all *cis*-4,7,10,13,16,19-docosahexaenoic acids were indicative of the less ordered forms reported for sodium oleate at elevated temperature. The diffraction data from the less ordered soaps are consistent with the melted form of the hydrocarbon chains of the unsaturated acids at room temperature.
Lipids 24, 1008–1013 (1989).

There are few published reports of x-ray diffraction studies of unsaturated fatty acids and their derivatives. The disorder and oxidative instability of these compounds have presumably hindered structural investigations. The study of the polymorphism of sodium oleate as a function of temperature (1) and the single crystal study of linoleic acid at low temperature (2) are the principal sources of information about the structure of unsaturated fatty acids.

The importance of the long chain unsaturated fatty acids in biological systems is well known. Their presence can alter fluidity in membranes (3), and both energy calculations (4) and NMR (5) have been utilized to predict their conformation.

Another approach to the study of the unsaturated fatty acid conformations is through stabilizing them as soaps. Soaps crystallize in phases that are dominated by the strong ionic forces of the cation-carboxylate interaction. A second interaction that stabilizes the lattice is that of the van der Waals forces involved in hydrocarbon chain-chain packing and the resultant energy minima. The magnitude of the chain-chain lattice stabilization energy is small compared with the ionic energy term. A comparison of the ionic forces with the chain packing forces is illustrated by the melting points of three compounds with similar packing and order of the hydrocarbon chains, but with varying polarity in the head group.

These melting points Methyl Palmitate, 30.4°C; Palmitic Acid, 63°C; Sodium Palmitate, 270°C show the relatively small effect of the hydrocarbon packing interactions dominating methyl palmitate relative to the hydrogen bonding of palmitic acid or the ionic forces of sodium palmitate.

Because of the dominance of the cation-carboxylate interaction in soaps, it is possible to form solid or waxy phases of soaps of fatty acids at room temperature that would solidify as the undissociated acid only at very low temperatures. Because of numerous "kinks" in the hydrocarbon chain, fatty acids with multiple *cis* double bonds melt at very low temperatures, and studies of their chain-chain packing in the solid state require low-temperature techniques. The formation of soaps of these acids, however, generates higher melting phases that can allow measurement of hydrocarbon chain dimensions by x-ray diffraction. We have studied the sodium soaps of 14 unsaturated fatty acids and present here the results of room temperature x-ray diffraction measurements of their long and short spacings.

MATERIALS AND METHODS

Materials were obtained from Nuchek Prep, Elysian, MN (methyl pentadecanoate, elaidic acid, linoelaidic acid, and the sodium salts of α-linolenic—erucic, homo-γ-linolenic, 11,14-eicosadienoic, 11,14,17-eicosatrienoic, arachidonic and docosahexaenoic acids), Sigma Chemical Co., St. Louis, MO (sodium linoleate, *cis*-vaccenic acid, 11-*cis*-eicosenoic acid, oleic acid, and sodium eicosapentaenoate) and Alltech Associates, Arlington Heights, IL (palmitoleic acid). With the exception of sodium eicosapentaenoate, purity claims of 99% for these materials were confirmed by gas chromatography of the methyl esters made from the acids and the soaps. The purity claim for sodium eicosapentaenoate was 90% and found to be >95% by GC.

Sodium soaps of the acids were prepared by dissolving 50–100 mg of the individual fatty acid in methanol followed by the addition of a molar equivalent of sodium hydroxide in a 0.1N aqueous solution. An additional volume of distilled water was added, and the resulting solution was frozen in a tared vessel and dried under vacuum (freeze drier, Hull corporation, Hatboro, PA) for 48–72 hr. Recovery (95 ± 5% of theoretical anhydrous mass) indicated complete removal of solvent, but the variability in the recovered mass does not exclude the possible formation of hemi-, mono-, or dihydrates. Portions of each of the soaps were converted to the methyl esters of the fatty acids by reaction with BF_3 in methanol. These esters were analyzed by gas chromatography (30 M Durabond 225 capillary column). In addition, known masses of the methyl esters of acids with 3 or more double bonds

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Abbreviations: Palmitoleic acid, *cis*-9-hexadecenoic acid; oleic acid, *cis*-9-octadecenoic acid; *cis*-vaccenic acid, *cis*-11-octadecenoic acid; linoleic acid, *cis,cis*-9,12-octadecadienoic acid; α-linolenic acid, *cis,cis,cis*-9,12,15-octadecatrienoic acid; homo-γ-linolenic acid, 8,11,14-eicosatrienoic acid; elaidic acid, *trans*-9-octadecenoic acid; linoelaidic acid, *trans,trans*-9,12-octadecadienoic acid; arachidonic acid, *cis,cis,cis,cis*-5,8,11,14-eicosatetraenoic acid; erucic acid, *cis*-13-docosenoic; eicosapentaenoic acid, EPA, *cis,cis,cis,cis,cis*-5,8,11,14,17-eicosapentaenoic acid; docosahexaenoic acid, DHA, *cis,cis,cis,cis,cis,cis*-4,7,10,13,16,19-docosahexaenoic acid.

were combined with a known mass of methyl pentadecanoate. The absence of oxidative deterioration of the unsaturated fatty acids was established in each case by GC analysis of this combination. Based on the pentadecanoate mass, the unsaturated acid mass based on GC response in all cases was within 4% of theory, thus establishing that oxidative degradation had not taken place. This analysis was simultaneous with the x-ray diffraction analysis for each sample. The sample analyzed by x-ray diffraction, less than 1 mg in a capillary, was of insufficient mass to allow analysis after x-ray exposure.

X-ray diffraction patterns were obtained on flat film at room temperature with nickel-filtered copper radiation (40 kV, 6mA, rotating anode generator). The sample-to-film distance was 20 cm for the measurement of long spacings, and the exposure time was 60 min. Short spacings were measured for 30 min with film to specimen distance of 5 cm. The focal spot as 0.2 × 2.0 mm, with pinhole diameters of 0.0025 and 0.001 mm for the 20 and 5 cm exposures, respectively.

An estimate of the presence of undissociated fatty acid in sodium linoelaidate was made from the relative absorbances of the carbonyl (1700 cm^{-1}) and carboxylate ion (1560 cm^{-1}) groups. This analysis indicated the presence of less than 10% undissociated acid in the sample.

The conformations of DHA were generated from the values reported by Applegate and Glomset (4) with CHEM-X, developed and distributed by Chemical Design Ltd., Oxford, England.

RESULTS

The spacings of the principal diffraction lines are presented in Table 1. The patterns from the soaps of the monounsaturated acids, the diunsaturated acids (except for linoleic) and 11,14,17-eicosatrienoic acid indicated a high level of order. An example of the diffraction from the crystalline soaps is shown in Figure 1, in which the long and short spacing patterns from the soap of 11,14-eicosadienoic acid are given.

The remainder of the soaps that we studied were less ordered, as shown by the broader diffraction lines and only two principal short spacings in the 4.6 and 2.9 A regions. As discussed below, these soaps were in a "waxy" phase. Except for the soaps of DHA and homo-γ-linolenic acid, the patterns from the waxy soaps included a weak line with a spacing somewhat longer than that of an intense and broad long spacing diffraction. An example of the waxy phase diffraction is given in Figure 2, the diffraction pattern from sodium EPA.

The dominant long spacings of the polyunsaturated compounds decreased with the increasing number of double bonds for a given length of hydrocarbon chain. For the eighteen carbon *cis* Δ 9 series, the spacing of 45 A for oleate decreases to 39-43 A for linoleate, and 39-41 A for α-linolenate. The *cis* Δ 11 twenty carbon series intense long spacings (described above) were 49.3, 49.0, 42-46, 39 and 37-41 A for 1, 2, 3, 4 or 5 double bonds, respectively.

A double set of long spacings was observed in the diffraction patterns from sodium linoelaidate. Each set was indicative of a lamellar crystalline phase with ratios of d-spacings of 1:2, 1:3, 1:4.

DISCUSSION

The diffraction patterns from the crystalline sodium soaps of the unsaturated fatty acids are similar to that reported for crystalline sodium oleate at temperatures below 55°C (1). The long spacings are consistent with the distance separating planes of polar regions in a lamellar structure. These spacings are a function of twice the length of the fatty acid's hydrocarbon chain with an allowance for the tilt of the chain's axis and a contribution of approximately 8 A from the sodium and carboxylate ions [estimated from sodium stearate (6)].

The "doublet" of spacings observed for crystalline sodium linoelaidate may have resulted from the presence of two solid phases in the sample. The GC and IR analyses supported the purity of the compound and the absence of undissociated acid. The long spacings of 48 and 51-52A (based on the second to fourth orders) are similar to those reported for β-monoclinic and α-sodium stearate (6), which would presumably have long spacings similar to the all *trans* conformation of sodium linoelaidate.

The relationship of the diffraction patterns to the array and conformation of molecules in the less ordered soap phases is not well understood. We can, however, interpret the data in light of the studies of the polymorphism of sodium oleate and sodium palmitate as a function of temperature (1,7), the relationship of long spacing to the number of double bonds in a fatty acid, and predictions of the expected dimensions of the hydrocarbon chains.

The less ordered phases of the sodium soaps include phases described as waxy polymorphs. The waxy phases of sodium palmitate found between 117 and 208°C yielded short spacings similar to those observed for the polyunsaturated waxy phases with broad lines in the 4.6-4.8 A and 2.8-2.9 A regions (7). The pattern of the long spacings of the waxy phases of the polyunsaturated soaps that we studied at room temperature is similar to that found for sodium oleate at temperatures of 55-119°C (phases II and II) (1). This pattern includes a long spacing that is somewhat greater than that of the bilayer distance of the crystalline lamellar structure as well as a spacing that is somewhat less than that of the crystalline phase. The intensity of the latter (shorter) of these diffraction lines is much stronger than the former for the polyunsaturated soaps (Table 1).

The pattern for sodium homo-γ-linolenate is suggestive of that of the face centered cubic phase of sodium oleate at 144-175°C (1). The diffraction from sodium DHA suggests that of the sodium oleate "ribbon" phase reported at 175-243°C (1).

The diffraction patterns from all of the less ordered soaps included an intense long spacing with a broad range of several angstroms. Although we cannot unequivocally determine the relationship of this intense long spacing to the molecular arrangement in the waxy soap, there are indications that this spacing does reflect the dimension of the fatty acid chains separat-

TABLE 1

Spacings of Sodium Soaps of Unsaturated Fatty Acids[a]

Fatty acid carbon atoms configuration	Phase	Long spacings (A)	Short spacings (A)
Palmitoleic	C	37.6 S	4.7 S
16:1		19.2 W	3.0 M
cis		13.0 W	
Oleic	C	45.0 S	4.7 W
18:1		23.0 W	4.6 W
cis		15.2 M	4.5 W
		11.4 W	2.9 W
Elaidic	C	49.8 S	4.6 M
18:1		25.3 S	4.0 M
trans		16.7 M	
		12.7 W	
		10.2 M	
cis-Vaccenic			
18:1	C	44.1 S	4.8 W
cis		22.2 W	4.4 M
		14.7	4.2 M
		8.8 W	3.9 S
			3.7 S
			3.0 S
Linoleic	W	39–43 S	4.5 S
18:2		22.1 W	
all-*cis*		20.3 W	
		14.6 W	2.9 M
		13.6 W	
		10.8 W	
		8.6 W	
Linoelaidic	C	48.0 S (56 S)	4.6 M
18:2		24.0 S (26 S)	4.4 W
all-*trans*		16.0 S (17 S)	
		12.0 W (13 S)	
α-Linolenic	W	51.4 W	4.6 S
18:3		38.6–41.1 S	
all-*cis*		26.8 W	
		21.0 M	2.9 W
		19.9 M	
		14.1 M	
		13.3 W	
		10.5 W	
		8.3 W	
11-Eicosenoic	C	49.3 S	4.6 W
20:1		24.2 M	4.4 W
cis		16.5 M	4.2 M
		12.4 W	3.9 W
		9.9 W	3.6 W
			3.0 W
			2.7 W
			2.6 VW
			2.5 VW
			2.3 VW
			2.3 VW
			2.2 VW
11,14-Eicosadienoic	C	49.0 S	6.7 W
20:2		23.8 M	5.1 M
all-*cis*		15.6 M	4.8 M
		9.4 W	4.6 M
			4.3 M
			4.1 M
			3.9 W
			3.3 W
			3.1 W
			2.9 W
			2.8 W

TABLE 1 (Cont.)

Spacings of Sodium Soaps of Unsaturated Fatty Acids[a]

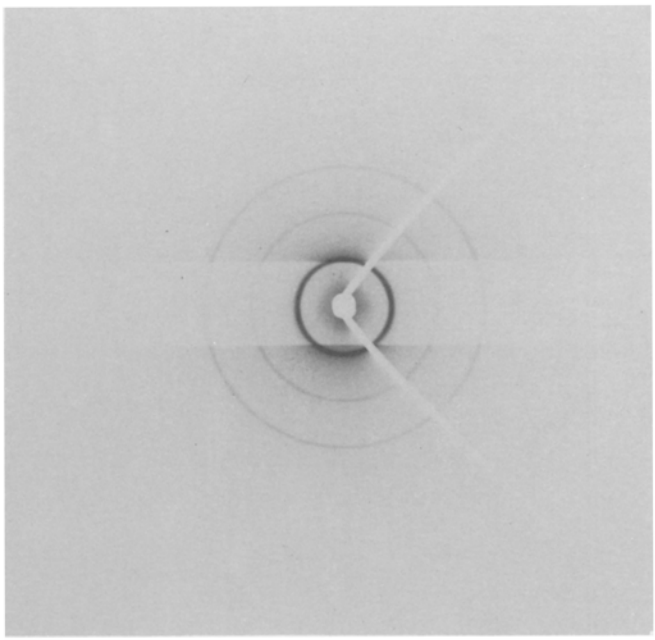
Fatty acid carbon atoms configuration	Phase	Long spacings (A)	Short spacings (A)
Homo-γ-linolenic	F	44.1 W	4.2-4.9 M
20:3		39.6 M	
all-*cis*		36.3 S	2.9 W
		21.3 W	
		18.2 W	
		13.8 W	
		12.4 W	
11,14,17-Eicosatrienoic	C	56.1 W	6.7 W
20:3		42.3-45.7 S	4.7 W
all-*cis*		30.9 W	4.5 M
		22.9 W	4.1 S
		15.5 W	3.8 W
		11.4 W	3.6 W
		9.1 W	3.2 W
			3.0 W
			2.9 W
			2.7 W
			2.5 W
			2.4 W
			2.3 W
Arachidonic	W	50.6 W	4.5 S
20:4		39.1 S	
all-*cis*		22.1 W	2.9 M
		19.6 W	
		14.3 W	
Eicosapentaenoic	W	49.0 W	4.5 S
20:5		37.2-41.1 S	
all-*cis*		22.1 W	
		19.3 W	2.9 W
		14.3 W	
		13.3 W	
Docosahexaenoic	R	28.6-32.5 S	4.0-5.1 S
22:6		17.2 W	2.9 M
all-*cis*		14.5 W	
		11.0 W	

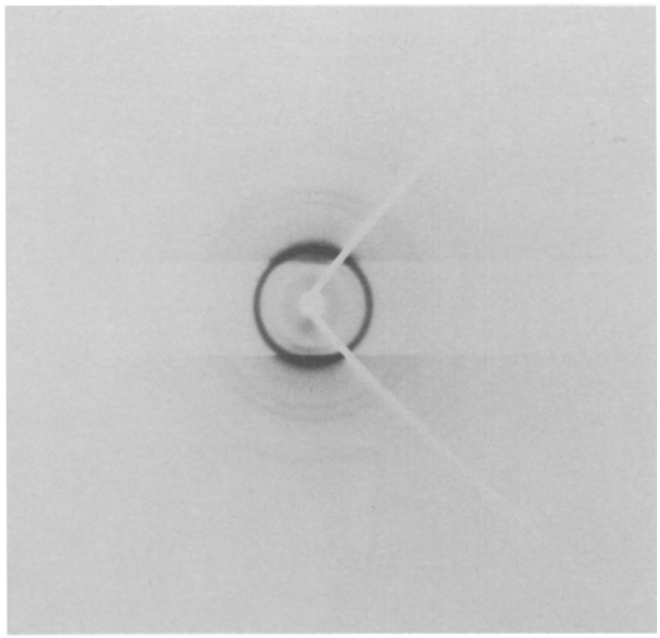
[a]S,M,W, indicate visual estimation of strong, medium, or weak lines, respectively. C,W, refer to crystalline and waxy, respectively. F and R indicate similarity to face centered cubic and "ribbon" phases described in Reference 1.

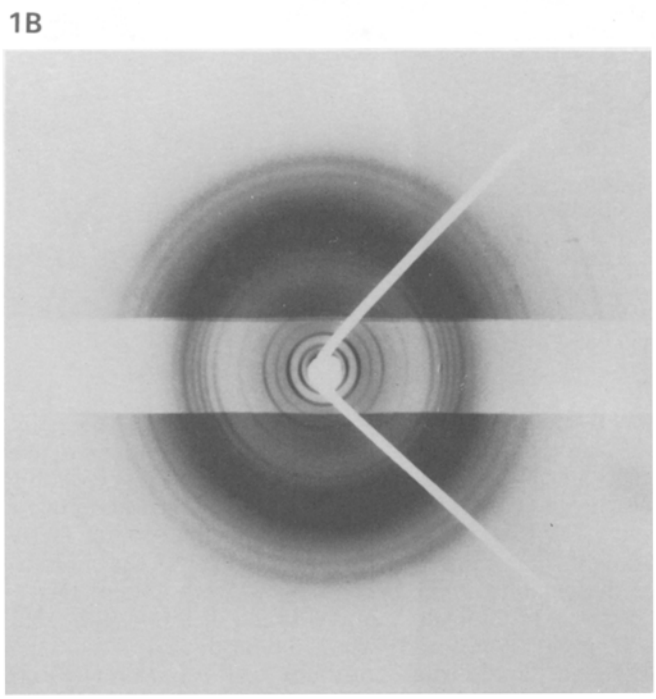
ing the polar regions. First, the decrease in the long spacing with temperature for the waxy phase of sodium oleate (1) is consistent with a melting of the hydrocarbon chains to increase the number of bent molecules separating the polar region planes. Second, the long spacing decreases with an increase in the number of double bonds, as seen in the 20-carbon fatty acid series we report here. In terms of the disruption of a crystalline array of hydrocarbon chains, this effect of an increase in double bonds might be considered to be equivalent to an increase in temperature in terms of introducing disorder into the hydrocarbon region. A third indication of the long spacing reflecting the chain dimensions is seen in the broad width of the most intense long spacing. This width, 3-6 Å, suggests a range of distances separating the polar regions of the waxy phases, consistent with multiple and varied fatty acid conformations in the hydrocarbon layers of the structure. Finally, the strong intensity of this line is consistent with diffraction from planes highly populated with the sodium atoms of the soap.

The dimensions of the intense long spacing are also consistent with the dimensions that are probable for the bent conformers of the polyunsaturated fatty acids. For example, the long spacing for sodium DHA (28.6-32.5 Å) can be considered in light of the molecular dimensions of the probable conformations of DHA. The bent, or "hairpin" conformation of DHA (4) is 13.9 Å (Fig. 3), compared with 22.6 and 22.7 Å for the extended "angle iron" and helix conformation (4) (Fig. 3). The long spacing of sodium DHA (minus 8 A for the contribution of the ionic region) is 20-25 Å, somewhat less than the 27.8 Å for twice the long dimension of the hairpin conformer. The long spacing of sodium DHA could reflect the dimension of a bilayer of hairpin conformers with a tilt of the chains or intercalation of the chains from the separate halves of the bilayer.

These conjectural dimensions of "melted" hydrocarbon chains from the long spacing are consistent with the formation of bent conformers that have been suggested in other studies. A bent conformation of DHA was proposed to explain the slow lipase-







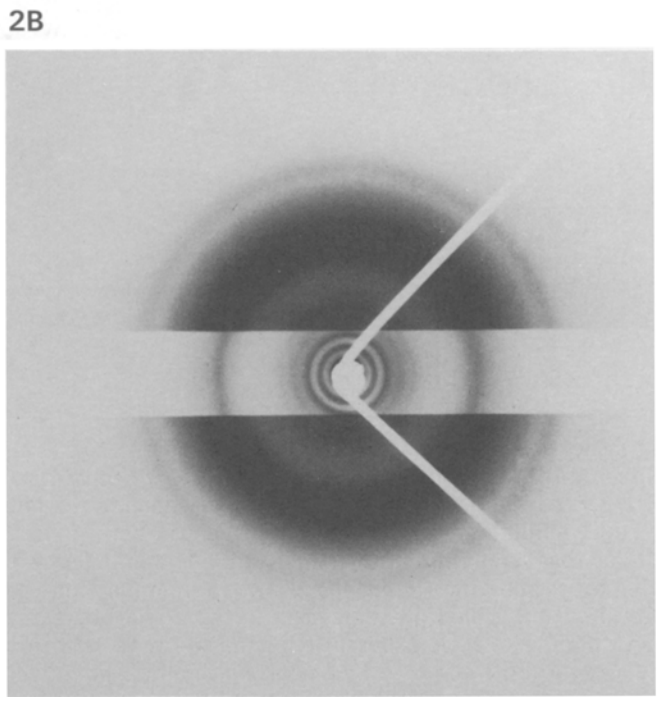


FIG. 1. X-Ray diffraction patterns of the sodium salt of 11,14-eicosadienoic acid. Sample to film distance was 20 cm (a) and 5 cm (b).

FIG. 2. X-Ray diffraction patterns of sodium eicosapentaenoate. Sample to film distance was 20 cm (a) and 5 cm (b).

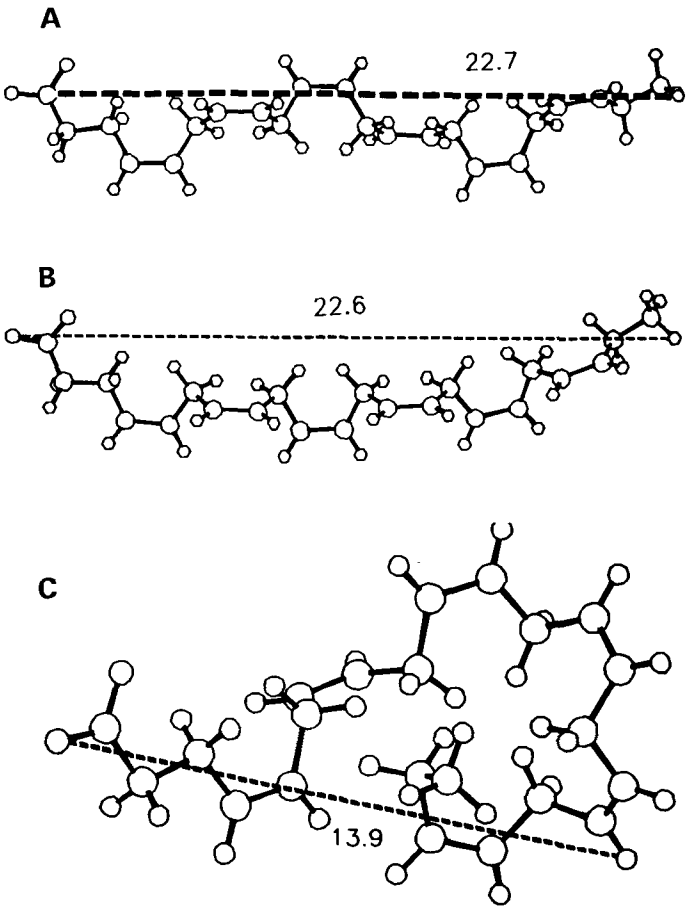


FIG. 3. Three low energy conformations of docosahexaenoic acid as calculated by Applegate and Glomset (4). (a) The "helix" is shown with a maximum molecular distance of 22.7 Å from carboxylate oxygen to a hydrogen atom of the terminal methyl group. (b) The "angle iron" with a distance between the same atoms is 22.6 Å. (c) The longest dimension of the "hairpin" conformer is 13.9 Å from the carboxylate oxygen to the hydrogen on carbon atom 17 of the molecule.

catalyzed hydrolysis of triglycerides containing DHA through steric hindrance of the ester group by the terminal part of the DHA chain (8). The bent conformation is also implied in the results of a study of 1-palmitoyl-2-docosahexaenoyl lecithin (5) by NMR. As part of phospholipids, bent polyunsaturated fatty acid conformations would be expected to reduce cell membrane thickness compared with fatty acids with greater proportions of extended fatty acid conformations.

The diffraction data presented here are consistent with an increase in disorder in hydrocarbon chains with increasing unsaturation. Even in a system stabilized by the ionic forces of a sodium soap, the *cis* double bonds greatly hinder the packing of hydrocarbon chains in an ordered array.

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Influence of Testosterone Administration on the Biosynthesis of Unsaturated Fatty Acids in Male and Female Rats

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The *in vivo* effect of testosterone administration to male or female rats on the biosynthesis of unsaturated fatty acids of liver was studied. Twenty-four hours after injection of testosterone (260 μg/kg), Δ9 desaturase activity increased significantly, whereas the activities of Δ5 and Δ6 desaturases were strongly depressed. These effects were more pronounced in female than in male animals. The fatty acid composition of plasma and liver (homogenates, crude microsomes and cytosol) showed differences between the sexes. In males, the percentage of palmitic acid and the 18:1/18:0 ratio were higher whereas the 20:4(n-6)/18:2(n-6) ratio was lower than in female rats. The administration of testosterone significantly modified the fatty acid pattern in all fractions studied. Analytical data correlated with alterations in the fatty acid desaturase activities caused by the hormone. It is suggested that the mechanism by which testosterone exerts its effect on unsaturated fatty acid biosynthesis is different than that previously demonstrated by glucocorticoid action. The effects produced by testosterone may be of biological significance in atherosclerosis pathogenesis.
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In many industrialized countries the combined incidence of mortality in all age groups as a consequence of coronary atherosclerosis is several-fold higher in males than in females (1). The molecular mechanisms underlying this sex difference are at present poorly understood, although considerable emphasis has been placed on the role that sex steroids may play in modifying serum lipoprotein profiles (2–8).

Polyunsaturated essential fatty acids and their metabolites, particularly arachidonic acid, are required as constituents of cellular membranes and as precursors of eicosanoids (9). It has also been reported that they are involved in the reduction of atherosclerosis risk (10). Kingsbury *et al.* (10) reported that low linoleic and arachidonic acid concentrations, associated with high levels of monoenoic acids (palmitoleic and oleic), correlate with the incidence of atherosclerosis in humans.

The formation of arachidonic acid from linoleic acid in mammals requires the function of two desaturases and one elongase. Linoleic acid is converted to 18:3 (γ-linolenic acid) by Δ6 desaturase (11–13), 18:3 is rapidly elongated to 20:3(n-6) acid, and the eicosatrienoic acid is subsequently desaturated by Δ5 desaturase to arachidonic acid (11,14). Liver microsomal Δ5 and Δ6 desaturases are the principal regulatory enzymes in the biosynthesis of polyunsaturated fatty acids (12,15,16). Δ9 Desaturase is not involved in the biosynthesis of arachidonic acid, but acts by inserting a double bond in saturated fatty acids (11,17) to produce oleic and palmitoleic acids.

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Abbreviations used: GLC, gas-liquid chromatography; Me, extracted microsomes; Sp, supernatant soluble fractions; Te, testosterone-injected.

It is generally understood that increased testosterone production is responsible for the incidence of increased atherosclerosis in men. Since polyunsaturated fatty acid metabolism appears to have a role in the pathogenesis of this disease (10,18–21), we investigated the *in vivo* effect of the sexual steroid hormone testosterone in Δ9, Δ6 and Δ5 rat liver microsomal desaturase activities. The correlations of the desaturase activities with the fatty acid composition of plasma and different subcellular fractions of liver and the possible existence of a testosterone-induced factor that modulated desaturase activities were also tested.

MATERIALS AND METHODS

Chemicals. [1-^{14}C]Palmitic acid (58.7 mCi/mmol, 98% radiochemically pure) was provided by Commissariat a l'Energie Atomique (France). [1-^{14}C]Linoleic acid (52.6 mCi/mmol, 98% radiochemically pure) and [1-^{14}C]eicosa-8,11,14-trienoic acid (54.9 mCi/mmol, 99% radiochemically pure) were purchased from New England Nuclear Corp. (Boston, MA). Unlabeled palmitic, linoleic and eicosatrienoic acids were supplied by Nu-Chek Prep, Elysian, MN. NADH, ATP, CoASH and testosterone were obtained from Sigma Chemical Company (St. Louis, MO). All other chemicals used were of analytical grade.

Treatment of animals. Adult Wistar rats weighing 220 ± 20 g were used. Two rats per cage were placed in a room thermostatically controlled at 22 ± 2°C. Food (Cargill type "C," Argentina) and water were available *ad libitum*. Cargill standard pelleted diet, type "C," consisted of (as calories) 56.7% carbohydrates, 10.4% lipids and 32.9% protein, vitamins and minerals. The fatty acid relative percentages of this diet were 21.4 palmitic, 2.1 palmitoleic, 8.2 stearic, 24.9 oleic, 37.7 linoleic and 0.2 arachidonic. Different groups of animals (male and female rats) were injected intraperitoneally with 260 μg/kg testosterone. The group used as control was injected with the vehicle in which the hormone was dissolved (20 mM ethanol). The rats were killed by decapitation 24 hr after the injection; blood was allowed to drain and was collected in separate tubes for testosterone assay and analytical determinations. In order to avoid individual differences among the animals, all the rats were fasted for 24 hr, refed for 2 hr and then killed 12 hr after the end of the refeeding period.

Testosterone assay in rat plasma. The concentration of the hormone was determined by radioimmunoassay using an International-CIS (solid phase) kit from CIS, Gif-Sur-Yvette, France.

Isolation of subcellular fractions. Liver (four rats per group, processed individually) was rapidly excised, rinsed, weighed and homogenized in ice cold homogenizing solution (3 ml per gram of tissue) (22). Crude microsomal fractions were separated by differential centrifugation at 110,000 × *g* as described previously (22). The supernatant fraction from the ultracentrifugation was used as

cytosol, and the microsomal pellets were resuspended in cold homogenizing solution to a final protein concentration of 50 mg/ml.

Protein content in the different fractions was determined by the micromethod of Lowry *et al.* (23), using bovine serum albumin as standard.

Microsome extraction. In some experiments, extracted microsomes (Me) were obtained following the procedure previously described (24). Briefly, crude microsomes from control or testosterone-treated rats in the proportion of 5 mg protein per 3 ml of standard extraction solution (24) were shaken at 0-4°C under air for 15 min. This suspension was then centrifuged at 110,000 × *g* at 1-2°C for 60 min in a L-2 Spinco ultracentrifuge. Two fractions were obtained—one corresponded to the extracted microsomes (Me) and the other to the supernatant soluble fractions (Sp).

Fatty acid desaturase activity assay. Desaturation of fatty acids was measured by estimation of the percentage conversion of [1-^{14}C]palmitic to palmitoleic acid, [1-^{14}C]linoleic acid to γ-linolenic acid and [1-^{14}C]eicosa-8,11,14-trienoic acid to arachidonic acid. Five mg of microsomal protein were incubated at 37°C for 10 min in a shaker with 5 nmol labeled acid and 95 nmol unlabeled acid in the same solution used for the extraction of crude microsomes. The only difference was that in the desaturase activity assay, 3.5 mM ATP, 0.2 mM CoASH and 1.5 mM NADH were added. Under these experimental conditions, the desaturase enzymes were completely saturated with the corresponding substrates (25). In some experiments, cytosolic fractions (0.6 mg protein) or 110,000 × *g* supernatant soluble fractions (0.6 mg protein) were added to the incubation medium. In all cases the final volume of incubation was 1.5 ml. The tubes were placed in a metabolic shaker (70 strokes per min) at 37°C, and the reaction was started by addition of crude or extracted microsomal suspension. The incubation was carried out for 10 min and the reaction was stopped by addition of 2 ml of 10% KOH in ethanol plus 500 μl methanol to facilitate the subsequent extraction step. The fatty acids were recovered after saponification of the incubation mixture at 85°C under nitrogen for 45 min, acidification with HCl and extraction with petroleum ether (b.p. 30-40°C). They were esterified with methanolic 3N HCl (at 68°C under nitrogen for 3 hr), extracted from the reaction mixture and stored in the dark under nitrogen at −20°C.

Radioactivity of the recovered methyl esters was determined in a Beckman liquid scintillation counter (model LS-3133 P) with 96% efficiency for ^{14}C. Distribution of radioactivity among the fatty acid methyl esters was measured by gas-liquid radiochromatography using an Acromat CG-100 equipped with a Packard proportional counter and a Honeywell recorder. Percentage conversion was calculated from the radioactivity distribution between substrate and product measured directly on the radiochromatogram (11). The column was packed with 10% SP-2330 on Chromosorb WAW-DMCS (100-200 mesh) (Supelco Inc., Bellefonte, PA).

Fatty acid composition. Different fatty acid fractions from plasma and liver (homogenates, crude microsomes and cytosol) were methylated as described above and analyzed by gas-liquid chromatography in a Hewlett-Packard 5840 A apparatus equipped with a 5840 A terminal computer integrator system. The column was packed as described above and the temperature of the oven was programmed from 140 to 220°C at 3°C/min after a 1-min initial hold. The fatty acid methyl esters were identified by comparison of their relative retention times with authentic standards and the mass distribution was calculated by gas liquid chromatography (GLC) in the presence of an internal standard of eicosa-11-monoenoic acid.

Student's *t*-test was used for the statistical data analysis.

RESULTS

Testosterone levels in rat plasma. Table 1 shows that the administration of testosterone to male or female rats (260 μg/kg) produced, after 24 hr, a significant increase in the plasma testosterone levels. This increase was higher in female animals than in males.

Effect of in vivo *administration of testosterone on liver microsomal desaturase activities.* Figure 1 shows the results obtained for liver microsomal Δ9 desaturase

TABLE 1

Plasma Testosterone Levels in Male and Female Rats

Rats	Testosterone (ng/ml)
Males	4.1 ± 0.1[a]
Females	0.2 ± 0.03
Males injected with testosterone	7.7 ± 0.2
Females injected with testosterone	5.5 ± 0.2

Testosterone was determined as described in the Materials and Methods section.

[a]Results are the mean of three rats (each analysis performed in triplicate) ± 1 SEM.

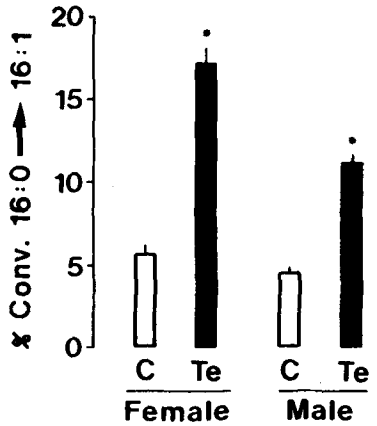

FIG. 1. Effect of testosterone, administered to normal rats, on the liver microsomal Δ9 desaturase activity. Five mg of microsomal protein from control (C) (open bar) or testosterone-injected (Te) (solid bar) rats were incubated at 37°C for 10 min in a shaker with 100 nmol of a mixture of labeled and unlabeled palmitic acid (0.25 μCi/tube). Details of incubation conditions are described in the Materials and Methods section. Results are given as percent conversion of [1-^{14}C]palmitic acid to [1-^{14}C]palmitoleic acid and they are the mean of four rats (each analysis performed in duplicate) ± SEM. *Statistically significantly different from the respective control $P < 0.01$.

activity 24 hr after administering the rats a single dose of testosterone. The hormone produced a marked increase in the conversion of palmitic to palmitoleic acid in both male and female rats. The effect on Δ5 and Δ6 desaturase activities was different, since testosterone caused a significant inhibition in both enzymes reflected by a decrease in the conversion of linoleic acid to γ-linolenic acid (Fig. 2) and eicosatrienoic acid to arachidonic acid (Fig. 3). The depression observed in the activities of Δ6 and Δ5 desaturases of liver microsomal fraction from female rats was more evident than in male rats.

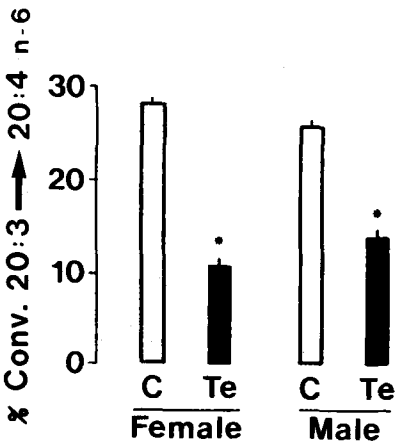

FIG. 2. Effect of testosterone, administered to normal rats, on the liver microsomal Δ5 desaturase activity. Five mg of microsomal protein from control (C) (open bar) or testosterone-injected (Te) (solid bar) rats were incubated at 37°C for 10 min in a shaker with 100 nmol of a mixture of labeled and unlabeled eicosatrienoic acid (0.25 μCi/tube). Details of incubation conditions are described in the Materials and Methods section. Results are given as percent conversion of [1-^{14}C]eicosa-8,11,14-trienoic acid to [1-^{14}C]arachidonic acid and they are the mean of four rats (each analysis performed in duplicate) ± SEM. *Statistically significantly different from the respective control $P < 0.01$.

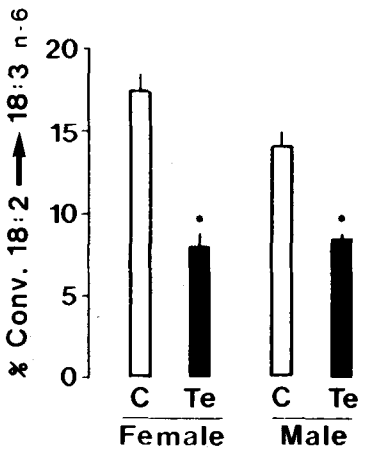

FIG. 3. Effect of testosterone, administered to normal rats, on the liver microsomal Δ6 desaturase activity. Five mg of microsomal protein from control (C) (open bar) or testosterone-injected (Te) (solid bar) rats were incubated at 37°C for 10 min in a shaker with 100 nmol of a mixture of labeled and unlabeled linoleic acid (0.25 μCi/tube). Details of incubation conditions are described in the Materials and Methods section. Results are given as percent conversion of [-^{14}C]-linoleic acid to γ-[1-^{14}C]linolenic acid and they are the mean of four rats (each analysis performed in duplicate) ± SEM. *Statistically significantly different from the respective control $P < 0.01$.

Effect of testosterone administration to male or female rats on the fatty acid composition of plasma and different liver cellular fractions. From the analysis of fatty acid composition of plasma and liver (homogenates, crude microsomes and cytosol) obtained from female (Table 2) and male (Table 3) untreated animals, it can be seen that in males the percentage of palmitic acid and the 18:1/18:0 ratio were higher, whereas the 20:4(n-6)/18:2(n-6) ratio was lower than in female rats. These changes were more pronounced in plasma than in the different liver fractions studied. The administration of testosterone to rats (Tables 2 and 3) significantly modified the fatty acid pattern in all fractions studied. The most important changes observed were: an increase in palmitic, oleic, linoleic and eicosatrienoic acids and a decrease in arachidonic and docosahexaenoic acids. The effect of the hormone was more pronounced in plasma and in the liver microsomal fraction.

The 18:1/18:0 ratio, which indirectly indicates the activity of Δ9 desaturase, increased under testosterone treatment in plasma and all liver fractions studied. However, the 20:4(n-6)/18:2(n-6) ratio was decreased by the hormonal treatment, a finding that is consistent with an inhibition of Δ5 and Δ6 desaturase activities.

Table 3 shows the percent distribution of fatty acids from plasma and liver fractions obtained from male control and testosterone-injected rats. In general, the hormone caused changes similar to those observed in female animals. However, they were less pronounced.

Effect of supernatant soluble fractions and cellular cytosolic fractions on Δ5 desaturase activity from hormonal-treated and control rats. Results of the conversion of eicosatrienoic to arachidonic acid of crude and extracted microsomes with or without the addition of soluble fractions (Sp or cytosol) from control or testosterone-injected animals are shown in Table 4. Liver Δ5 desaturase activity was strongly depressed by extraction of microsomes with a low ionic strength solution, both in control or in hormonal-treated rats. The lowest values corresponded to extracted microsomes from testosterone-injected animals (Me_{Te}). The addition of 0.6 mg protein of soluble fraction obtained from control livers (Sp_C) to extracted microsomes of untreated animals (Me_C) restored approximately 90% of Δ5 desaturase activity. A similar effect was observed with added soluble protein fractions obtained from testosterone-treated rats (Sp_{Te}) or cytosolic fractions from control ($Cytosol_C$) or testosterone-injected animals ($Cytosol_{Te}$). When extracted microsomes from hormonal-treated rats (Me_{Te}) were incubated in the presence of soluble fractions obtained from control or testosterone-injected animals (Sp or Cytosol), the Δ5 desaturase activity increased up to the values observed with whole microsomes from treated animals (M_{Te}). Moreover, the conversion of eicosatrienoic to arachidonic acid in liver microsomes from testosterone-injected rats was significantly depressed (compared to control animals) even after the addition of the different soluble fractions tested.

TABLE 2

Effect of Testosterone Treatment on Fatty Acid Composition of Plasma and Hepatic Subcellular Fractions from Female Rats

Fatty acids[b]	Plasma		Liver					
			Homogenate		Microsome		Cytosol	
	C	Te	C	Te	C	Te	C	Te
14:0	0.3 ± 0.1[a]	0.3 ± —	0.1 ± 0.03	0.1 ± —	0.1 ± —	0.2 ± 0.03	0.3 ± 0.1	0.5 ± 0.1
16:0	14.5 ± 0.1	21.4 ± 0.3[e]	14.0 ± 0.3	16.4 ± 0.3[d]	13.2 ± 0.1	15.4 ± 0.2[e]	19.1 ± 0.2	23.0 ± 0.1[e]
16:1	1.8 ± 0.2	2.0 ± 0.2	1.4 ± 0.03	1.7 ± 0.1	0.7 ± 0.1	0.6 ± 0.1	2.6 ± 0.2	3.1 ± 0.2
18:0	14.8 ± 0.2	9.1 ± 0.2[e]	22.2 ± 0.3	21.0 ± 0.2[c]	28.0 ± 0.2	24.6 ± 0.2[e]	15.3 ± 0.1	12.1 ± 0.2[e]
18:1n-9	10.5 ± 0.3	14.9 ± 0.2[e]	10.0 ± 0.2	12.5 ± 0.1[e]	7.9 ± 0.1	10.3 ± 0.3[d]	17.9 ± 0.1	19.2 ± 0.2[d]
18:2n-6	14.8 ± 0.1	21.0 ± 0.4[e]	11.0 ± 0.3	12.3 ± 0.5	8.1 ± 0.1	10.8 ± 0.2[e]	14.7 ± 0.2	14.3 ± 0.5
20:3n-6	0.5 ± 0.1	1.4 ± 0.2[c]	0.8 ± 0.1	1.0 ± 0.3	0.7 ± 0.1	2.5 ± 0.2[d]	0.6 ± 0.1	1.0 ± 0.2
20:4n-6	26.8 ± 0.2	18.0 ± 0.4[e]	21.7 ± 0.2	19.5 ± 0.2[d]	26.8 ± 0.2	21.4 ± 0.4[e]	19.6 ± 0.2	16.5 ± 0.2[e]
20:5n-3	0.6 ± 0.2	0.9 ± 0.3	3.0 ± 0.3	2.7 ± 0.3	0.4 ± 0.1	0.5 ± 0.2	1.0 ± 0.2	0.8 ± 0.3
22:2n-6	1.4 ± 0.2	1.0 ± 0.2	tr —	0.1 ± 0.03	0.7 ± 0.3	0.8 ± 0.3	0.6 ± 0.1	0.8 ± 0.2
22:3n-3	1.5 ± 0.2	0.9 ± 0.2	1.4 ± 0.2	1.1 ± 0.2	1.0 ± 0.1	1.1 ± 0.1	0.3 ± 0.1	0.7 ± 0.2
22:4n-6	1.6 ± 0.3	1.1 ± 0.2	1.5 ± 0.3	1.2 ± 0.2	1.3 ± 0.1	1.3 ± 0.3	0.8 ± 0.2	0.7 ± 0.2
22:5n-6	2.4 ± 0.2	1.3 ± 0.2	1.4 ± 0.3	1.2 ± 0.2	1.2 ± 0.1	1.5 ± 0.3	0.7 ± 0.2	0.9 ± 0.2
22:5n-3	2.5 ± 0.4	2.0 ± 0.2	2.0 ± 0.3	2.2 ± 0.1	1.1 ± 0.1	1.7 ± 0.3	0.8 ± 0.1	1.5 ± 0.3
22:6n-3	6.0 ± 0.1	4.7 ± 0.1[e]	8.6 ± 0.2	7.0 ± 0.1[d]	8.8 ± 0.2	7.3 ± 0.2[d]	5.7 ± 0.1	4.9 ± 0.2

[a] Data are expressed as percent weight of total fatty acids and are given as means of three individual determinations ± SEM. Analysis of the samples was done as described under Chromatographic measurements. C, samples obtained from control animals; Te, from testosterone-treated rats.

[b] Numbers of carbons:number of double bonds; n, the biological series of the acid.

[c] Statistically significant difference from control $P < 0.02$ (student's *t*-test).

[d] Different from control $P < 0.01$.

[e] Different from control $P < 0.001$.

TABLE 3

Effect of Testosterone Treatment on Fatty Acid Composition of Plasma and Hepatic Subcellular Fractions from Male Rats

Fatty acids[b]	Plasma		Liver					
			Homogenate		Microsome		Cytosol	
	C	Te	C	Te	C	Te	C	Te
14:0	0.2 ± 0.05[a]	0.2 ± 0.03	0.1 ± —	0.2 ± 0.03	0.1 ± —	0.1 ± —	0.6 ± 0.1	0.4 ± 0.1
16:0	19.3 ± 0.2	22.3 ± 0.3[c]	15.0 ± 0.3	16.4 ± 0.2[c]	15.9 ± 0.3	17.3 ± 0.2[c]	19.7 ± 0.3	21.5 ± 0.2[d]
16:1	1.4 ± 0.1	1.1 ± 0.2	1.7 ± 0.3	1.5 ± 0.3	0.7 ± 0.1	0.6 ± 0.1	1.3 ± 0.2	1.8 ± 0.1
18:0	11.8 ± 0.3	9.5 ± 0.2[d]	22.0 ± 0.3	20.1 ± 0.2[d]	24.0 ± 0.2	21.8 ± 0.2[d]	15.9 ± 0.3	10.8 ± 0.2[e]
18:1n-9	13.2 ± 0.2	16.0 ± 0.2[e]	11.2 ± 0.1	12.0 ± 0.2[c]	8.0 ± 0.2	10.1 ± 0.1[e]	11.8 ± 0.2	15.7 ± 0.2[e]
18:2n-6	19.6 ± 0.3	21.4 ± 0.3[c]	11.4 ± 0.2	12.8 ± 0.2[d]	10.5 ± 0.3	13.4 ± 0.2[d]	17.2 ± 0.3	24.4 ± 0.2[e]
20:3n-6	0.4 ± 0.1	0.4 ± 0.1	0.8 ± 0.2	0.9 ± 0.1	0.9 ± 0.1	1.3 ± 0.1	0.7 ± 0.1	0.9 ± 0.2
20:4n-6	23.9 ± 0.3	20.0 ± 0.2[c]	20.3 ± 0.3	18.1 ± 0.1[d]	25.6 ± 0.3	22.0 ± 0.2[e]	20.4 ± 0.3	15.3 ± 0.2[e]
20:5n-3	0.5 ± 0.1	0.2 ± —	2.5 ± 0.1	2.6 ± 0.2	0.3 ± 0.1	0.6 ± 0.1	1.1 ± 0.4	1.0 ± 0.3
22:2n-6	1.1 ± 0.3	1.5 ± 0.1	0.2 ± 0.03	0.3 ± —	1.7 ± 0.2	1.9 ± 0.1	0.5 ± 0.1	0.7 ± 0.1
22:3n-3	0.4 ± —	0.5 ± 0.1	1.5 ± 0.3	1.7 ± 0.1	1.6 ± 0.1	1.4 ± 0.1	1.0 ± 0.1	0.9 ± 0.2
22:4n-6	1.7 ± 0.2	1.7 ± 0.3	1.1 ± 0.1	1.2 ± 0.1	0.9 ± 0.1	1.3 ± 0.2	0.6 ± 0.1	0.5 ± 0.1
22:5n-6	1.2 ± 0.1	0.7 ± 0.2	2.0 ± 0.2	2.2 ± 0.2	0.7 ± 0.1	1.0 ± 0.2	0.2 ± —	0.4 ± 0.1
22:5n-3	1.1 ± 0.3	1.0 ± 0.2	3.3 ± 0.4	4.0 ± 0.3	1.1 ± 0.2	1.2 ± 0.3	1.0 ± 0.2	0.7 ± 0.1
22:6n-3	4.2 ± 0.1	3.5 ± 0.1[d]	6.9 ± 0.3	6.0 ± 0.3	8.0 ± 0.2	6.0 ± 0.2[d]	8.0 ± 0.3	5.0 ± 0.1[e]

[a] Data are expressed as percent weight of total fatty acids and are given as means of three individual determinations ± SEM. Analysis of the samples was done as described under Chromatographic measurements. C, samples obtained from control animals; Te, from testosterone-treated rats.

[b] Number of carbons:number of double bonds; n, the biological series of the acid.

[c] Statistically significant difference from control $P < 0.02$ (Student's *t*-test).

[d] Different from control $P < 0.01$.

[e] Different from control $P < 0.001$.

TABLE 4

Effect of the Addition of Soluble Fractions from Control or Testosterone-Injected Rats to Extracted Microsomes on the Δ5 Desaturase Activity

	% Conversion 20:3→20:4(n-6)
Whole microsomes from control rats (M_C)	31.7 ± 1.4
Extracted microsomes from control rats (Me_C)	19.0 ± 0.8
Me_C + Sp_C	28.0 ± 0.3
Me_C + Sp_{Te}	28.8 ± 0.8
Me_C + $Cytolsol_C$	29.1 ± 0.5
Me_C + $Cytosol_{Te}$	30.1 ± 0.9
Whole microsomes from testosterone-treated rats (M_{Te})	12.1 ± 0.6
Extracted microsomes from testosterone-treated rats (Me_{Te})	7.5 ± 0.3
Me_{Te} + SP_C	12.2 ± 0.5
Me_{Te} + Sp_{Te}	10.9 ± 0.7
Me_{Te} + $Cytosol_C$	11.9 ± 0.4
Me_{Te} + $Cytosol_{Te}$	11.5 ± 0.5

Five mg of liver extracted microsomes (Me) were incubated at 37°C for 10 min in a shaker with 100 nmol of a mixture of labeled and unlabeled eicosatrienoic acid (0.25 μCi/tube). In some cases 0.6 mg protein/tube of 110,000 g supernatant soluble fractions from control ($Cytosol_C$) or testosterone-treated rats ($Cytosol_{Te}$) or soluble fractions obtained after washing control or testosterone crude microsomal suspensions (Sp_C or Sp_{Te}, respectively) were added to the incubation medium. In all the cases the final volume of incubation was 1.5 ml. Results are expressed as percent conversion of [1-^{14}C]eicosa-8,11,14-trienoic acid to [1-^{14}C]arachidonic acid and they are the mean of three determinations ± SEM.

DISCUSSION

Several studies have revealed sex-mediated differences in lipid metabolism. It has been reported that the synthesis of triacylglycerol from exogenous fatty acids is increased in livers from females when compared to males (26,27). In addition, differences in esterification and oxidation of unsaturated fatty acids in liver and the content of fatty acid binding protein have been related to sex (27-32). As previously reported (33-37) and as shown in the present paper, the sex of the animal also affects the fatty acid composition of plasma and of different cellular fractions of rat liver (Tables 2 and 3). Females had a higher level of stearic acid than males, whereas males had more palmitic acid than females. Female rats also exhibited higher values of polyunsaturated fatty acids than male rats. In addition, it was previously demonstrated that females are generally less susceptible than male animals to essential fatty acid deficiency (38-45) and they retain, under this condition, higher levels of 20:4(n-6) (arachidonic acid) in tissues (37,39-41). These differences between sexes may be due, in males, to the action of testosterone, which caused a depression of liver Δ5 and Δ6 desaturase activities. Consequently, the synthesis of arachidonic acid was decreased, leading to an accumulation of its principal precursor, linoleic acid. This assumption was corroborated by the decreased liver microsomal Δ6 and Δ5 desaturase activities after testosterone treatment (Figs. 2 and 3). Moreover, when the hormone was administered to female rats, these effects were more pronounced than those observed in males.

Results obtained in the present study also indicate that Δ9 desaturase activity increased after testosterone injection (Fig. 1). This finding is consistent with an increase in the monoenoic/saturated fatty acid ratio observed in males as compared to females. This was consistently observed in the fatty acid composition after hormonal treatment of all liver fractions studied. Several authors have shown that an increase in Δ9 desaturase activity is closely related to an enhancement of fatty acid synthetase activity (46-48). The large amount of palmitic acid observed in plasma and liver fractions after testosterone injection could be due to a stimulation of fatty acid synthetase activity produced by the hormone.

It was already known that a factor present in the cell cytosolic fraction that binds to microsomal surface is required to achieve fully active desaturation reaction *in vitro* (24). This factor, which has no measurable desaturase activity per se, is loosely bound to the microsomes since it could be separated by washing once with a low ionic strength solution (24). The presence of the aforementioned factor was also evidenced in these experiments since washed rat liver microsomes lost approximately 40% of their Δ5 desaturation activity. Previous studies from this laboratory provided evidence on the existence of another soluble protein induced by dexamethasone that modulates fatty acid desaturase activities (49,50). This factor, leading to a decrease in the activity of Δ5 and Δ6 desaturases (49) and to an increase in Δ9 desaturase (50), was also loosely bound to microsomes since it was present in a soluble fraction obtained after washing crude microsomes from hormonal-treated rats with a low ionic strength solution (49,50). Additional experiments have been done to investigate the possibility that the modification of the desaturase activities evoked by testosterone could be produced through the induction of a soluble regulatory factor. The results obtained in these experiments demonstrated that testosterone directly modified the fatty acid composition of liver microsomal membranes and the activity of the different

desaturase enzymes (Figs. 1-3). However, these hormonal effects were not mediated by a soluble factor induced by testosterone treatment (Table 4). This fact implies that the mechanism by which testosterone exerted its effect on unsaturated fatty acid biosynthesis is different than that already demonstrated by the action of glucocorticoids (49-51), since the testosterone-induced defect would be in the microsomal residue.

In conclusion, it is reported that testosterone modifies the fatty acid composition of plasma and liver and that it is involved in unsaturated fatty acid biosynthesis through the modulation of liver microsomal desaturases. Several reports support the suggestion that changes in polyunsaturated fatty acid biosynthesis are related to the occurrence of atherosclerosis and occur before clinical manifestation of the disease (10,18-21). An increase in Δ9 desaturase activity and a decrease in Δ5 and Δ6 desaturases after testosterone treatment would lead to an increase in monoenoic acids and a decrease in tetraenoic acid concentration in plasma lipids. These changes evoked by the hormone, together with the differences observed between male and female animals, should be of biological significance in the pathogenesis of atherosclerosis.

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Regulation of Squalene Synthetase and Squalene Epoxidase Activities in *Saccharomyces cerevisiae*

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Squalene synthetase (EC 2.5.1.21) and squalene epoxidase (EC 1.14 99.7) activities have been measured in cell-free extracts of wild type yeast grown in aerobic and semi-anaerobic conditions as well as in sterol-auxotrophic mutant strains grown aerobically. The results show that both enzymes are induced resulting in an almost two- to five-fold increase in enzymatic activities in mutant strains containing limited sterol amounts and are repressed in the wild type strain cultured in anaerobiosis in excess of sterol. The results show also that squalene epoxidase is repressed by lanosterol, and that the mevalonic acid pool may regulate squalene synthetase levels.

The large change in the activities of the two enzymes, depending on the sterol needs of the cells, as well as their low specific activities in comparison with those of the enzymes involved in the early stages of sterol synthesis strongly suggests that squalene synthetase and squalene epoxidase are of importance in regulating the amount of sterol synthesized by yeast.
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The sterol pathway provides eukaryotic cells with the sterol molecules they need for membrane build-up. In addition it is the source of other isoprenoids involved in cell physiology, i.e., isopentenyl groups of ser and tyr tRNAs, the side chain of ubiquinone and dolichol, a sugar carrier in the glycosylation pathway.

In animal cells the regulation of the cholesterol pathway resides primarily at the level of HMG-CoA reductase: high levels of serum sterols repress enzyme activity both by reducing enzyme synthesis and by shortening the half-life of the protein (1). In contrast to the situation in animal cells, much less is known regarding the regulation of sterol synthesis in yeast. Several reports also point to a major role for HMG-CoA reductase, especially since two different genes, HMG 1 and HMG 2, have been isolated in the *S. cerevisiae* genome (2).

Our group and others (3–6) could not detect modifications in HMG-CoA reductase specific activity linked to the cellular requirement for sterol; however, changes in acetoacetyl-CoA thiolase and HMG-CoA synthase activities were observed. These activities are repressed under conditions of excess sterol in anaerobiosis; conversely, they are induced under sterol starvation conditions which can be attained in ergosterol auxotrophic mutants (3).

Branched pathways are generally regulated at one of the first steps of the overall pathway and at the branch point where biosynthetic precursors are diverted to one or the other specific route. Farnesyl pyrophosphate is the major branch point in polyisoprene biosynthesis. Squalene synthetase (EC 2.5.1.21), which catalyzes the condensation of two molecules of farnesyl pyrophosphate into squalene, is the first enzyme specific of the sterol branch. Indeed, in *Tetrahymena pyriformis* it has been shown that the activity of squalene synthetase is repressed by exogenous cholesterol, leading to arrest of tetrahymanol biosynthesis and to cholesterol incorporation into membrane systems (7).

Squalene epoxidase (EC 1.14 99.7) catalyzes the next step in sterol biosynthesis; it is the first reaction involving molecular oxygen. During anaerobic growth, yeast cells do not produce ergosterol, but they do accumulate large quantities of squalene (8). After exposure to oxygen, the squalene pool is rapidly converted to sterols (9). Little is known about the regulation of squalene epoxidase in yeast, which is particularly due to difficulties in measuring specific activity *in vitro.* In cell-free extracts, squalene, the substrate of the enzyme, is poorly converted into its reaction product. Recently, we have overcome this difficulty by using farnesyl pyrophosphate as substrate (10), which is possible because the specific activity of squalene synthetase in a cell-free supernatant is 5 times higher than the specific activity of squalene epoxidase. With this methodology, we obtained a specific activity of 0.10 nmol farnesyl pyrophosphate converted/min/mg 12000 g protein at 30°C which is very similar to that reported by Jahnke and Klein (11) with endogenously synthesized squalene as substrate. This specific activity is one of the lowest in the first part of the pathway leading to the triterpene lanosterol. For example, the specific activities of squalene synthetase, HMG-CoA reductase, HMG-CoA synthase and acetoacetyl-CoA thiolase are 0.25, 2.75, 22 and 140 nmol product formed/min/mg 12000 g protein respectively (3,10). It is therefore reasonable to postulate that squalene epoxidase be a rate limiting step in ergosterol biosynthesis in yeast.

In the present study we measured the specific activities of squalene synthetase and squalene epoxidase in cell-free supernatants of yeast cells as a function of sterol content. The results clearly show that the two enzyme activities are regulated by the sterol need of the cells.

EXPERIMENTAL

Strains and culture conditions. The strains used were derived from *Saccharomyces cerevisiae* FL100 (A.T.C.C. 28383) haploid, a mating type, or from an isogenic strain FL200 (A.T.C.C. 32119), haploid, α mating type. The sterol auxotrophic mutant strains have been previously described (3–5).

The complete medium consisted of (per liter): yeast extract (Biokar), 10 g; peptone pepsique de viande (Biokar), 10 g; and glucose, 20 g. Ergosterol and oleic acid were supplied by dilution of stock solutions in a mixture of Tergitol NP40/ethanol (1:1, v/v).

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Abbreviations: DTT, dithiothreitol; erg, mutant gene involved in ergosterol biosynthesis; FAD, flavin adenine dinucleotide; HMG1, HMG2, genes; HMG-CoA, hydroxy methyl glutaryl coenzyme A; LDL, low-density lipoprotein; TLC, thin-layer chromatography; tRNA, transfer ribonucleic acid

Aerobic growth was carried out in Erlenmeyer flasks at 28°C with continuous shaking. Semi-anaerobic growth was started by inoculating yeast cells in 1 liter volumetric flasks almost completely filled with the growth medium and covered with liquid paraffin (2–3 cm).

Enzyme preparation. Cells were harvested at late logarithmic growth phase and washed with ice-cold 0.05 M potassium phosphate buffer pH 7.4. The cells were disrupted in the same buffer containing 0.5 mM dithiothreitol as previously described (3). A 12000 g supernatant was used as a crude enzyme preparation. Protein was determined by the biuret procedure using crystalline bovine serum albumin as a standard.

Chemicals. [^{14}C]Farnesyl pyrophosphate (4 mCi/mmol) was synthesized according to the Popjak method using [2-^{14}C](R) mevalonate (1.6 mCi/mmol; 12). Squalene-2,3-epoxide was synthesized as described by Van Tamelen and Curphey (13).

Squalene synthetase assay. The standard assay mixture contained, in a total volume of 0.25 ml, 50 mM phosphate buffer pH 7.4, 0.5 mM DTT, 4 mM KF, 10 mM $MgCl_2$, 1 mM NADPH, 12 mM glucose-6-phosphate, 2 units glucose-6-phosphate dehydrogenase, and 20 μM [^{14}C]farnesyl pyrophosphate. After 10 min of preincubation at 30°C, the assay was initiated by addition of crude enzyme: 20–80 μg for the wild type strain and 10–40 μg for the mutant strains. The standard incubation period was 30 min at 30°C and incubations were stopped by addition of 1 ml methanolic KOH (15%, w/v). Squalene, 2,3-oxidosqualene, lanosterol, and ergosterol were added as carriers and the mixture was heated at 60°C for 30 min in order to precipitate proteins. Non-saponifiable products were extracted with hexane. After concentration under nitrogen, the residue was dissolved in a known volume of hexane (200 μl). An aliquot (10 μl) was counted for radioactivity by liquid scintillation. The remainder was applied to silica gel G plates and developed with cyclohexane/ethyl acetate (9:1, v/v) as solvent. Distribution of the radioactivity incorporated was then determined using a Berthold 2382 TLC Linear Analyzer. Nanomoles of reaction products were calculated from liquid scintillation data. Squalene synthetase activity was determined as the sum of radioactivity in the squalene, squalene epoxide and lanosterol fractions and expressed in nanomoles of farnesyl pyrophosphate converted per min per mg of protein. In the range of protein concentration used, the enzymatic reaction was shown to be linear over 40 min at 30°C, and the major reaction product was squalene. Trace amounts of 2,3-oxidosqualene and lanosterol are only observed at high protein concentrations.

Squalene epoxidase assay. The standard assay mixture contained, in a total volume of 0.25 ml, 50 mM phosphate buffer pH 7.4, 0.5 mM DTT, 4 mM KF, 10 mM $MgCl_2$, 5 mM NADPH, 25 mM glucose-6-phosphate, 0.04 mM FAD, 2 units glucose-6-phosphate dehydrogenase, and 20 μM [^{14}C] farnesyl pyrophosphate. After 10 min of preincubation at 30°C, the assay was initiated by addition of crude enzyme: 250–750 μg for the wild type strain and 65–125 μg for the mutant strains. The reaction was stopped after 30 min incubation at 30°C, by addition of 1 ml of methanolic KOH (15%, w/v). Non-saponifiable lipids were extracted and quantified as described above (squalene synthetase assay). Squalene epoxidase activity was determined as the sum of radioactivity in the squalene epoxide and lanosterol fractions and was expressed in nanomoles of farnesyl pyrophosphate converted per min per mg of protein. In the range of protein concentration used, the reaction rate was constant over 45 min.

RESULTS

Two possibilities exist to manipulate sterol levels in yeast. The first one involves growing wild type strains anaerobically. Because yeast cannot synthesize the sterol ring in the absence of molecular oxygen, it becomes permeable to exogenously supplied sterols (14). Depletion or enrichment with sterols can then be achieved under these conditions. The second possibility involves the use of sterol auxotrophic mutant strains which incorporate exogenously provided sterols in aerobic cultures. These mutants also lend themselves to the study of possible regulatory effects of precursor molecules of ergosterol accumulating as a result of the enzymatic lesion.

Under standard aerobic conditions at 28°C, sterol auxotrophic mutants show optimal growth at an exogenous ergosterol concentration of 2 mg/l: the ergosterol content is about 0.3% of dry cell weight. With or without sterol supplementation, the wild type strain shows a stable sterol content of about 0.8% (3).

We have shown that the low ergosterol content in auxotrophic mutant strains correlates with the induction of the activities of enzymes such as acetoacetyl-CoA thiolase and HMG-CoA synthase (3). Therefore, we measured, under the same conditions, squalene synthetase and squalene epoxidase activities *in vitro*. Enzyme activities were measured in 12000 g cell-free supernatant as described in the experimental section. The results (Table 1) show that squalene synthetase specific activity is induced 1.5- to 5-fold in sterol auxotrophic mutant strains with the exception of *erg 12* (blocked in mevalonate kinase) and *erg 18* and *erg 19* (blocked in squalene epoxidase and mevalonate pyrophosphate decarboxylase, respectively). In the last two strains the activity is similar to that of the wild type strain. In contrast, the *erg 12* mutant strain shows a 25% decrease of the squalene synthetase activity in comparison with wild type strain.

The fact that squalene synthetase is most strongly enhanced (4–5 fold) in mutant strains, *erg 10* and *erg 11*, blocked before mevalonic acid synthesis and lowered in *erg 12* mutant strain defective in mevalonate kinase suggested that the mevalonic acid pool may influence squalene synthetase specific activity. To test this hypothesis, we measured squalene synthetase activity in a cell-free extract of *erg 12 pAO1* strain bearing the mevalonate kinase gene on a multicopy vector, leading to a 15-fold increase of mevalonate kinase specific activity in comparison with the wild type strain (A. Oulmouden and F. Karst unpublished data). Table 1 (bottom) shows that in this strain, with wild type phenotype, the specific activity of squalene synthetase is 2.5-fold higher than in the parent strain *erg 12* due to plasmid introduction. Since it can be assumed that high mevalonate kinase activity decreases the mevalonate intracellular pool, the induction of squalene synthetase in *erg 12 pAO1* could directly be related to a lowering of intracellular mevalonate concentration.

TABLE 1

Squalene Synthetase and Squalene Epoxidase Specific Activities in Wild Type and Mutant Strains

Strains[a]	Enzyme lesion	Squalene synthetase[b]	Squalene epoxidase[b]
WT	—	.47 ± .02(8)	.10 ± .02(8)
erg 10	Acetoacetyl-CoA thiolase	2.25 ± .01(3)	.41 ± .05(3)
erg 11	HMG-CoA synthase	1.97 ± .01(3)	.46 ± .04(3)
erg 12	Mevalonate kinase	.36 ± .04(2)	.24 ± .02(2)
erg 8	Mevalonate phosphate kinase	1.11 ± .02(3)	.48 ± .06(3)
erg 19	Mevalonate pyrophosphate decarboxylase	.44 ± .09(3)	.43 ± .08(2)
erg 9	Squalene synthetase	0	Nonmeasurable
erg 18	Squalene epoxidase	.44 ± .05(3)	0
erg 7	Oxidosqualene-lanosterol cyclase	.60 ± .01(2)	.54 ± .03(3)
erg 16	Lanosterol-14 demethylase	.76 ± .02(3)	.03 ± .01(3)
erg 12 pAO1	—	.89 ± .04(3)	.21 ± .02(3)

[a]Wild type strain and ergosterol auxotrophic mutants were grown in a complete medium supplemented with ergosterol 2 mg/l at 28°C with shaking. Cells were harvested during late exponential growth phase and the enzyme assays were performed using 12000 g proteins as described in the experimental section.

[b]The results are expressed in nanomoles of farnesyl pyrophosphate converted/min/mg protein ± S.D. (number of independent experiments).

Squalene epoxidase activity is raised 2- to 5-fold in all mutant strains, except in *erg 16* (blocked in lanosterol-C14 demethylase), which by contrast shows a 4-fold drop in squalene epoxidase activity as compared to the wild type strain. The highest squalene epoxidase specific activity is observed with mutant strain *erg 7* blocked in oxidosqualene-lanosterol cyclase (5-fold enhancement).

To study the influence of excess sterol on enzyme activities, we grew the wild type strain in anaerobiosis. Under these conditions wild type yeast cells require an exogenous supply of sterol and unsaturated fatty acid (14). The results (Table 2) show that squalene synthetase activity is not modified in comparison with aerobic cultures if the ergosterol supplement is 2 mg/l. By contrast, with a high ergosterol supplementation (50 mg/l), squalene synthetase activity is lowered two fold. The same anaerobic growth conditions always repress squalene epoxidase activity independently of the exogenous ergosterol supply. A 6–10-fold lowering is observed in comparison with a corresponding aerobic culture.

TABLE 2

Squalene Synthetase and Squalene Epoxidase Specific Activities in Wild Type Strain Grown Anaerobically: Effect of Ergosterol Supplementation

Culture medium[a]	Squalene synthetase[b]	Squalene epoxidase[b]
Ergosterol 2 mg/l	.42 ± 0.04(2)	.017 ± 0.005(2)
Ergosterol 50 mg/l	.23 ± 0.03(2)	.010 ± 0.002(2)

[a]The wild type strain FL100 was grown anaerobically for 72 hr at 28°C, in a complete medium supplemented with oleic acid (50 mg/l) and ergosterol as indicated.

[b]Specific activities are expressed in nanomoles farnesyl pyrophosphate converted/min/mg protein ± S.D. (number of independent experiments).

DISCUSSION

Measurement of squalene synthetase and squalene epoxidase activities shows firstly that these two enzymes are regulated by the ergosterol level of the cells. Indeed, sterol auxotrophic mutants with a low sterol level are generally depressed in comparison to the wild type strain. In anaerobic cultures with an excess of sterol, the enzyme activities are lower than in corresponding aerobic cultures.

The second observation is that squalene epoxidase activity is sharply decreased in anaerobic cultures. The results we described in a previous publication (10) showed that squalene epoxidase synthesis is dependent on molecular oxygen. However, with high ergosterol supplementation, the enzyme activity is further lowered (Table 2), showing that the enzyme is also controlled by ergosterol in anaerobiosis.

Pinto *et al.* (15) have shown that the amount of squalene accumulated in anaerobically-grown yeast cells is sharply decreased at high ergosterol supplementation in comparison to a low ergosterol supply. The two-fold lowering of squalene synthetase activity observed in the presence of high ergosterol supplementation (Table 2) might, in part, account for this result.

Our results also show that the intracellular metabolic pools of ergosterol precursors may regulate the enzyme levels, although squalene synthetase and squalene epoxidase have different sensitivities to these intermediary metabolites.

The results show that a high induction (5-fold) of squalene synthetase is observed in mutants, *erg 10* and *erg 11,* blocked before mevalonic acid synthesis. In contrast, *erg 12* which cannot metabolize mevalonic acid due to inactivation of mevalonate kinase shows reduced

activity in comparison to the wild type strain. The observation that mutant strain *erg 12* bearing the mevalonate kinase gene on a multicopy plasmid, which leads to overexpression of the kinase, recovers induced squalene synthetase activity strongly supports the hypothesis that the mevalonic acid pool may regulate squalene synthetase activity. The significance of such a regulation could be to protect the cell from excess ergosterol owing to enlargement of the mevalonate pool. We have previously also reported that HMG-CoA reductase is regulated by mevalonic acid (3).

The other important observation is that lanosterol represses squalene epoxidase. In fact in *erg 16* mutant strain blocked in lanosterol C-14 demethylase, the activity is lowered 3-fold in comparison with wild type strain and 19-fold in comparison with the most strongly induced strain (*erg 7*). The repression of squalene epoxidase by lanosterol could account for the accumulation of squalene observed in mutant strain *erg 16* and in *por* mutant strains blocked in porphyrin synthesis (16). This regulation could be crucial for the cellular physiology since lanosterol does not fulfill all the functions of demethylsterols (17). It has been shown that lanosterol neither modifies the glucose efflux from lecithin liposomes (18) nor alters the fluidity of liposomes (19). The highest specific activity of squalene epoxidase is observed in *erg 7* blocked in oxidosqualene-lanosterol cyclase. This high epoxidase activity could explain the formation of dioxidosqualene observed in *erg 7* mutant strains as well as in wild type strains blocked by cyclase inhibitors (20).

These results show that several regulatory mechanisms may exist in the ergosterol pathway in yeast in addition to those involving the end products. Moreover, the results obtained here and those previously reported (3) suggest that the regulatory mechanisms of the overall ergosterol pathway in yeast are different from those described in mammalian cells. In fibroblasts, the HMG-CoA reductase is the key enzyme in the regulation of cholesterol synthesis. Squalene synthetase appears to be under the same control, i.e., LDL-cholesterol suppression, but squalene synthetase specific activity is about ten times higher than is reductase activity (21). Faust *et al.* (21) therefore suggested that squalene synthetase would never be limiting in the cholesterol pathway, but that the physiological interest of its repression is to allow farnesyl pyrophosphate molecules synthesized in scarce amounts under conditions of excess cholesterol to be directed towards dolichol and ubiquinone. In yeast, HMG-CoA reductase activity does not change significantly with the sterol level of the cells. Moreover, the reductase specific activity is about 2- and 10-fold higher than those of squalene synthetase and squalene epoxidase respectively. Therefore, the latter enzymes with their low specific activities, could be the main regulation sites of ergosterol biosynthesis in yeast.

The changes in the specific activities of squalene synthetase and squalene epoxidase are likely due to a transcriptional control of protein synthesis. Indeed we have shown that lanosterol and ergosterol do not inhibit these enzyme activities *in vitro* (10). Moreover the addition of 105000 g supernatant to washed microsomes does not modify the specific activities of these enzymes (10). Therefore we assume that soluble cytopolasmic activators, such as SPF (22), are not involved in these enzymatic activities. However we cannot rule out the possibility of a control of enzyme activities by membrane-bound effectors.

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Phorbol Myristate Enhanced Specific Incorporation of Arachidonic Acid into Phospholipids Through Lysophospholipid Acyltransferase in Cultured Smooth Muscle Cells

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The effect of stimulation of phospholipase with phorbol 12-myristate 13-acetate and lipopolysaccharide on 1-acylglycerophospholipid acyltransferase was studied in cultured rabbit aorta smooth muscle cells. The acyltransferase in smooth muscle cells without stimulation was active on a wide range of unsaturated fatty acids and was not arachidonic acid specific. Upon increase in phospholipase activity, acyltransferase activity only with arachidonic acid as substrate increased in a time-dependent fashion. Apparent acyltransferase activity was increased most upon increase in phospholipase activity when lysophosphatidylcholine was used as acceptor. These results suggest that arachidonic acid specific acyltransferase was induced in smooth muscle cells by increase in phospholipase activity. The role of this acyltransferase is postulated to be the specific incorporation of endogenously released arachidonic acid.
Lipids 24, 1024-1027 (1989).

The biologically active pool of arachidonic acid (AA) bound to phospholipids, especially phosphatidylcholine, is reported to decrease in atherosclerotic lesions (1,2). Depletion of AA has been suggested to be involved in the development of atheromatous lesions (3-6). In this work we tried to clarify the mechanism of the decrease in AA. Polyunsaturated fatty acids (PUFA), including AA, are incorporated into phospholipids through lysophospholipid acyltransferase (Lands' pathway; [7]), and AA is thought to be incorporated into phospholipids mainly by this pathway (8). The AA content of phospholipids is partly maintained by the balance between phospholipase (deacylation) and lysophospholipid acyltransferase (reacylation) activities. Phospholipase activities are increased in atheromatous lesions (9), but it is unknown whether there is any relationship between phospholipase and acyltransferase activities. Since phorbol 12-myristate 13-acetate (TPA) is known to stimulate phospholipase activities (10-13), we studied the effect of stimulation of phospholipase by TPA on lysophospholipid acyltransferase activities in cultured smooth muscle cells (SMC) of rabbit aorta.

MATERIALS AND METHODS

Chemicals. Phorbol 12-myristate 13-acetate (TPA), lipopolysaccharide (LPS), adenosine triphosphate (ATP) and coenzyme A (CoA-SH) were purchased from Sigma Chemical Co. (St. Louis, MO). Free fatty acids were from Nu-Chek Prep (Elysian, MN), and labeled free fatty acids were from New England Nuclear (Boston, MA). Lysophospholipids were purchased from Serdary Research Laboratories (Ontario, Canada).

Culture of SMC from media in rabbit aorta. SMC from the aorta of Japanese white rabbits *(Oryctolagus cuniculus var. domesticus)* were cultured as described previously (14). The culture medium was Dulbecco's modified Eagle's medium (DME) containing 10% fetal bovine serum (FBS) (lot 27N3541, Grand Island Biological Co., Grand Island, NY). SMC were used at the third passage.

Preparation of enzyme solutions. Confluent SMC in culture dishes (Corning, NY, 35 × 10 mm) were treated with the indicated concentration of TPA in 2 ml of culture medium and incubated for 1-36 hr at 37°C under 5% CO_2 in air. The dishes were washed five times with 2 ml of phosphate buffered saline (pH 7.4) and SMC were collected with a rubber policeman. SMC from five dishes were combined and homogenized in 1 ml 0.25M sucrose-5 mM Tris-HCl-buffer (pH 7.4) by 10 strokes of a teflon-glass homogenizer. The homogenate was centrifuged at 800 × g for 5 min, and the supernatant was used as enzyme solution for assay of acyltransferase activities.

Assay of lysophospholipid acyltransferase activities. Acyltransferase was assayed as described previously (8,15,16). The reaction mixture contained 20 mM ATP, 200 μM CoA-SH, 50 mM $MgCl_2$, 150 mM Tris-HCl-buffer (pH 7.4), 0.2% bovine serum albumin (fatty acid free), 250 μM lysophospholipid, 200 μM free fatty acids (FFA), 0.2 μCi/tube [1-^{14}C]FFA and 15-30 μg protein/tube of enzyme solution in 0.25 ml. After incubation at 37°C for 60 min, phospholipids were separated by thin layer chromatography on silica gel G (Merck, Darmstadt) with chloroform/methanol/acetic acid/water (100:60:16:8, v/v/v/v) as solvent, and the radioactivities of phospholipid fractions were counted. Phospholipid synthesis was proportional to the protein amount of the enzyme solution with up to 100 μg of protein and to the incubation time for up to 90 min.

Assay of arachidonic acid content in phosphatidylcholine (PC) of SMC. AA content was assayed by the method of Morisaki *et al.* (14). In brief, lipids were extracted by the method of Folch *et al.* (17), and then PC was separated as previously described. Hydrolysis of PC and methylation of the fatty acids of SMC were achieved by incubating the extracts with 1 ml of 0.6 N NaOH in 100% methanol for 30 min at room temperature. After incubation, the mixture was neutralized with 6 N HCl and the methylated fatty acids were extracted with 2 ml of hexane. The fatty acid composition was analyzed by gas chromatography in a GC 7-A model apparatus (Shimadzu Co., Kyoto, Japan).

Assay of prostaglandin E_2 (PGE_2) content of culture medium. PGE_2 in culture medium was estimated using radioimmunoassay (PGE_2 [^{125}I] Kit, New England Nuclear.

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Abbreviations: AA, arachidonic acid; PUFA, polyunsaturated fatty acid; TPA, phorbol 12-myristate 13-acetate; SMC, smooth muscle cells; LPS, lipopolysaccharide; DME, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; LysoPC, lysophosphatidylcholine; LysoPE, lysophosphatidylethanolamine; LysoPI, lysophosphatidylinositol; LysoPS, lysophosphatidylserine.

TABLE 1

Effects of TPA on AA Content of Phosphatidylcholine (PC) in SMC and Prostaglandin E_2 (PGE_2) Content of Culture Medium

	SMC incubated		
	Without TPA (n = 4)	With TPA (n = 4)	Difference
AA content of PC (%)	8.2 ± 1.9	5.0 ± 0.9	$p < 0.05$
PGE_2 content of culture medium (ng/ml)	46 ± 4	375 ± 22	$p < 0.001$

Confluent SMC in dishes (35 mm) were incubated in 1 ml of culture medium containing 10^{-8} M TPA for 24 hr. AA content of PC in SMC and PGE_2 content of culture medium were assayed as described in the Materials and Methods section. Values are mean ± SD.

Measurement of protein. Protein was measured by the method of Lowry *et al.* (18).

Statistics. Statistical analysis was performed using either two way analysis of variance (ANOVA) or Student's *t*-test.

RESULTS

Effects of TPA on arachidonyl-CoA acyltransferase activities as a function of incubation time. As can be seen in Figure 1, acyltransferase activities with AA and lysophosphatidylcholine (lysoPC) as substrates increased steeply between 12 and 24 hr after the start of incubation with 10^{-8} M TPA, reached a plateau at 24 hr (328% of the control), and remained at this level at least until 36 hr (Fig. 1). This increase in activities with 10^{-8} M TPA at 24 and 36 hr was highly significant ($p < 0.01$, ANOVA). Acyltransferase activities of 244, 328 and 98% of the control were observed with concentrations of 10^{-9} M, 10^{-8} M and 10^{-7} M TPA at 24 hr, respectively. Therefore, in the following experiments an incubation time of 24 hr and TPA concentration of 10^{-8} M TPA were used.

Effects of TPA on AA content of PC in SMC and PGE_2 content of culture medium. As can be seen in Table 1, PGE_2 is reported to be one of the metabolites of AA in phospholipid and to be released into culture medium (19). When SMC were incubated with 10^{-8} M TPA for 24 hr, the AA content in SMC significantly ($p < 0.05$) decreased from 8.2 ± 1.9 (n = 4) to 5.0 ± 0.9% (n = 4) in fatty acids of PC and the content of PGE_2 in culture medium significantly ($p < 0.001$) increased from 46 ± 4 to 375 ± 22 ng/ml. In place of AA, the content of linoleic and oleic acid was slightly increased.

Fatty acid specificity of lysophosphatidylcholine (lysoPC) acyltransferase activities induced by TPA or LPS in SMC. LysoPC acyltransferase activities in the control culture were highest with linolenic acid as substrate. The activities with linoleic acid, eicosatrienoic acid and eicosapentaenoic acid were similar, but lower than that with linolenic acid (Table 2). The lysoPC acyltransferase activity with AA was about one-third of that with linolenic acid. The activities with saturated fatty acid (palmitic acid) and docosahexaenoic acid were almost zero.

In the presence of 10^{-8} M TPA, only lysoPC acyltransferase activity with AA ($p < 0.001$) increased three-fold. The activities with other fatty acids were similar to those in the absence of TPA.

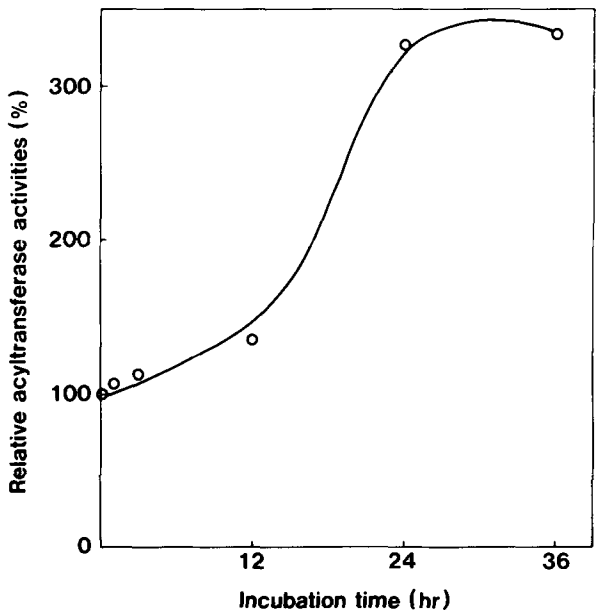

FIG. 1. Effects of TPA on arachidonyl-CoA acyltransferase activities as a function of incubation time. SMC were incubated with 10^{-8} M TPA in 2 ml of culture medium for 0–36 hr. Arachidonyl-CoA:lysoPC acyltransferase activities in SMC were assayed as described in the Materials and Methods section. Data are averages for values in two separate cultures. Two values at each period did not differ more than 10% from each other.

When 10^{-9} or 10^{-8} M TPA was directly added to the acyltransferase assay system, the acyltransferase activity with TPA was 109% or 78% compared to controls, respectively.

Another phospholipase stimulator, LPS (20,21) was also used in the same system. In the presence of 1.0 μg/ml LPS, lysoPC acyltransferase activity was significantly ($p < 0.001$) increased to 217% of the control when AA was used as substrate. When oleic acid, linoleic acid and eicosapentaenoic acid were used as substrate, apparent lysoPC acyltransferase activities in the presence of LPS was 97%, 98% and 106% of the control, respectively.

Lysophospholipid specificity of arachidonyl-CoA acyltransferase activities induced by TPA in SMC. Measured arachidonyl-CoA acyltransferase activities of control cultures using various lysophospholipids declined in the order lysophosphatidylinositol (lysoPI) > lysophosphatidylcholine (lysoPC) ≒ lysophosphatidylethanolamine (lysoPE) > lysophosphatidylserine (lysoPS) (Table 3).

TABLE 2

Fatty Acid Specificity of LysoPC Acyltransferase Activities Induced by TPA in SMC

Treatment	Fatty acids					
	18:1 (n-9)	18:2 (n-6)	18:3 (n-3)	20:3 (n-6)	20:4 (n-6)	20:5 (n-3)
	nmol/mg protein/60 min					
Control	24.2 ± 1.5 (n = 4)	48.8 ± 3.6 (n = 4)	55.6 ± 1.1 (n = 4)	42.5 ± 3.0 (n = 4)	18.5 ± 1.8 (n = 4)	37.8 ± 6.0 (n = 4)
TPA	23.6 ± 1.8 (n = 6)	51.2 ± 4.2 (n = 6)	40.2 ± 9.4 (n = 4)	44.2 ± 3.8 (n = 4)	55.0 ± 7.6 (n = 10)	45.6 ± 6.7 (n = 6)
% Change	(98)	(105)	(62.3)	(104)	(297)	(121)
Difference	NS	NS	NS	NS	$p < 0.001$	NS

Confluent SMC were incubated with 10^{-8} M TPA for 24 hr. LysoPC acyltransferase activities were assayed with various fatty acids as substrate as described in the Materials and Methods section. Values are mean ± SD. Numbers in parentheses show relative acyltransferase activities with TPA as percentages of those without TPA. NS: not significant.

TABLE 3

Lysophospholipid Specificity of Arachidonyl-CoA Acyltransferase Activities Induced by TPA in SMC

Treatment	Lysophospholipid			
	LysoPC	LysoPE	LysoPI	LysoPS
	nmol/mg protein/60 min			
	(n = 4)	(n = 3)	(n = 4)	(n = 3)
Control	20.0 ± 0.9	19.6 ± 1.7	34.6 ± 0.2	3.23 ± 0.04
TPA	52.8 ± 7.9	22.3 ± 4.3	56.3 ± 1.0	3.51 ± 0.86
% Change	(264)	(113)	(163)	(108)
Difference	$p < 0.001$	NS	$p < 0.001$	NS

Confluent SMC were incubated with 10^{-8} M TPA for 24 hr. Arachidonyl-CoA acyltransferase activities were assayed with various lysophospholipids as substrates as described in the Materials and Methods section. Values are mean ± SD. Numbers in parentheses show relative acyltransferase activities with TPA as percentages of those without TPA. NS: not significant.

TPA significantly ($p < 0.001$) increased the acyltransferase activity with lysoPC as substrate, namely 2.6-fold as compared with the control. TPA increased acyltransferase with lysoPI 1.6-fold, but had no appreciable effect on the activities with lysoPE or lysoPS as substrate.

Fatty acid specificity for lysoPC acyltransferase activities induced by AA in SMC. In the presence of 90 μM AA, lysoPC acyltransferase activities increased slightly with various fatty acids, but the relative increase of lysoPC acyltransferase activities was similar with various fatty acids (Table 4).

DISCUSSION

There are reports that AA is mainly incorporated into phospholipids by lysophospholipid acyltransferase (Lands' pathway [7]). In atheromatous lesions, the AA content of phospholipids is decreased and phospholipase A_2 activities are increased (9). TPA and LPS are known to increase phospholipase activities (10-13,20,21). In cultured SMC, TPA increased prostaglandin E_2 synthesis 8.1-fold over controls during an incubation period of 24 hr. It also decreased the AA content of SMC to 61% of that in control SMC (Table 1). These results indicate that TPA increased phospholipase activities in cultured SMC. In the present work, we studied the changes of lysoPC acyltransferase activities in SMC when phospholipase activities were stimulated by TPA or LPS. Results showed that TPA induced arachidonic acid specific acyltransferase in SMC, and that the induced enzyme activity also had a high specificity for lysoPC. The largest increases of acyltransferase activity by TPA in SMC were seen when AA and lysoPC were used as substrates.

When AA was added directly to the culture media, arachidonyl-CoA acyltransferase activities increased slightly, but arachidonic acid specific activity did not (TAble 4). Thus, the substrate of AA specific acyltransferase *in vivo* may be endogenous AA released by phospholipases from membrane phospholipids. Direct addition of 10^{-9}-10^{-8} M TPA to the acyltransferase assay system did not affect the enzyme activities, indicating that the increase in acyltransferase activities by TPA is not caused by a direct effect of TPA on the enzyme. Thus AA specific acyltransferase may be induced by increase of phospholipase activities (13).

As we used an acyl-CoA generating system in the acyltransferase assay, we examined the effect of TPA on acyl-CoA synthetase activities. Results showed that TPA

TABLE 4

Fatty Acid Specificity for LysoPC Acyltransferase Activities Induced by AA in SMC

Treatment	Fatty acids					
	18:1 (n-9)	18:2 (n-6)	18:3 (n-3)	20:3 (n-6)	20:4 (n-6)	20:5 (n-3)
	nmol/mg protein/60 min					
Control	24.2	48.8	55.6	42.5	20.0	41.8
AA	29.9	69.6	72.2	56.2	24.2	58.9
% Change	(123)	(142)	(129)	(132)	(121)	(141)

Confluent SMC were incubated in 2 ml of culture medium containing with 90 μM AA for 24 hr. LysoPC acyltransferase activities were assayed with various fatty acids as substrate as described in the Materials and Methods section. Numbers in parentheses show relative acyltransferase activities with AA as percentages of those without AA. Data are averages for values in two separate cultures. Two values at each condition did not differ more than 10% from each other.

had no effect on acyl-CoA synthetase activities (22) (control, 61.9 nmol/mg protein/10 min; TPA 10^{-8} M, 59.8 nmol/mg protein/10 min), indicating that the increase in acyltransferase activities was not due to increased acyl-CoA synthesis.

The above results were obtained from SMC from aortic media. In atherosclerosis, a major site of the lesion is the intima of the aorta, and one of the proliferating cell types in intima are smooth muscle cells. Therefore, we examined the changes of the acyltransferase activities in SMC from aortic intima. Our preliminary experiments showed that TPA did not significantly change arachidonyl-CoA acyltransferase activities in intimal SMC of atheromatous lesions [control: 38.1 ± 4.1, 10^{-8} M TPA: 37.1 ± 8.1 (nmol/mg protein/60 min, mean ± SD)]. Thus, AA specific acyltransferase may not be induced in atheromatous lesions. The characterization of this enzyme is now in progress.

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Turnover and Fate of Plasma Free Fatty Acids in Briefly-Fasted Lymphoma-Bearing Mice

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Body fat loss during tumor growth may be due to increased mobilization of adipose triglycerides. Earlier work from this laboratory suggested that (i) lymphoma-bearing AKR mice have a circulating lipid mobilizing factor (LMF) which caused body fat loss during cancer growth; that (ii) fatty acids (FA) mobilized in these tumor-bearing (TB) mice were not oxidized to CO_2 as in starved mice that lose their body fat; and that (iii) instead, the mobilized FA were sequestered by the lymphoma. We tested these hypotheses by injecting [1-^{14}C]palmitate-albumin into lymphoma-bearing and control mice. We measured turnover of plasma FFA for 24 hr and predicted the cumulative conversion of tracer into breath $^{14}CO_2$ (at 85 min) in the TB mice. Plasma FFA were mobilized more slowly in briefly fasted tumor-bearing mice than in controls with the same plasma FFA pool sizes. The fractional catabolic rate (FCR) (min^{-1}) of plasma FFA turnover in both groups decreased during the night when the mice ate: postabsorptive controls, 1.07 (±5.6%); fed controls, 0.25 (±13%); postabsorptive TB, 0.53 (±4.6%); fed TB, 0.29 (±7.3%). Virtually all of the plasma FFA irreversible disposal in TB mice was accounted for as breath $^{14}CO_2$ (30 to 40% I.D.), not as tumor lipids (1.1 ± 0.22% I.D.). Thus, FFA oxidation to CO_2 is the major fate of plasma FFA turnover in TB mice, and sequestration of FFA (palmitate) by tumor cells is a quantitatively minor process. The putative circulating LMF did not cause increased FFA mobilization in these lymphoma-bearing mice in the post-absorptive state.
***Lipids 24,* 1028–1034 (1989).**

Lymphoma-bearing AKR mice, like many other cancer-bearing rodents, are known to lose their body fat reserves as the cancer grows (1), a dramatic aspect of a more general wasting, i.e., cancer cachexia. Considerable evidence has been presented to support the hypothesis that this loss of fat is due, at least in part, to increased mobilization of adipose tissue triglyceride fatty acids (TGFA) in response to the effects of circulating proteins that act on adipose tissue. One such agent, cachectin/tumor necrosis factor (TNF), is produced by macrophages when they become activated during the immune response (2). Another such protein is thought to be produced by the cancer cells themselves, and it has been suggested that increased fat mobilization could provide essential fatty acids (FA) that are required by the tumor cells for their growth especially if, as suggested by Kitada *et al.*, oxidation of fatty acids is spared in tumor-bearing (TB) animals [(1) and R. Kannan, M. Gan-Elepano, and N. Baker, manuscript in preparation]. As yet, no purified lipid-mobilizing protein derived from cancer cells has been described, to our knowledge, and studies of fat mobilization in mice bearing different types of cancers are often difficult to reconcile with each other.

For example, Kitada *et al.* have suggested (1) that fat pads of SL-3 lymphoma-bearing AKR mice mobilize their fatty acids at an increased rate under the influence of a cancer cell-derived (1,3,4) lipid mobilizing factor (LMF). They also concluded that very little of the FFA mobilized from the labeled adipose tissue of the tumor-bearing mice was oxidized to CO_2. They contrasted this finding with the response to fasting in mice without tumors; in the latter case FFA mobilized from adipose tissue were obviously being oxidized to CO_2. Kitada *et al.* also suggested that the major metabolic fate of mobilized FFA in TB mice was sequestration by the cancer cells (1).

This interpretation of their data contrasts markedly with the findings of Baker *et al.* in Ehrlich ascites tumor-bearing (EATB) mice (5–7). The reported that only about 1% of I.V.-injected labeled plasma FFA is sequestered by well-developed ascites tumors (5,6); moreover, they found that plasma FFA were being mobilized (5) and oxidized to CO_2 (7) at approximately the same rates in EATB and control mice.

The principal investigators of the two laboratories in which these discrepant findings were made have collaborated here to re-examine the fate of plasma FFA in lymphoma-bearing AKR mice as measured by techniques similar to those used to study the fate of plasma FFA in EATB mice, namely, I.V. tracer injection of [1-^{14}C]-palmitate complexed to mouse serum albumin using multicompartmental analysis to measure the turnover of plasma FFA, in the postabsorptive state. We extended the experiment from a brief 30 min (5) to a prolonged 5-hr period to obtain a more reliable estimate of the plasma FFA irreversible disposal rate, which theoretically (8) should closely approximate that of plasma FFA oxidation to CO_2, the major metabolic end-product of FFA metabolism. By estimating these rates in TB and control mice and by comparing the predicted (and also, in separate mice, the observed) cumulative $^{14}CO_2$ output with the incorporation of [^{14}C]FFA into tumor tissue [^{14}C]TL 90 min after tracer injection into TB mice, we could test our hypotheses.

In developing our hypotheses, we assumed, as in earlier studies of EATB mice (5–7), that if adipose tissue TGFA is being mobilized at an increased rate in response to a circulating lipolytic factor, and if this factor is active in briefly fasted or post-absorptive TB mice, the increased mobilization would manifest itself by an increase in the

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[1]This work is dedicated to the memory of Dr. James F. Mead who died in December 1987 without reading this version of the manuscript. The senior author assumes sole responsibility for the interpretation and manner of presentation of the data.

Abbreviations: Compt., compartment; EATB, Ehrlich ascites tumor-bearing; FCR, fractional catabolic rate; FFA, free fatty acids; TGFA, triglyceride fatty acids; I.D., injected dose; LMF, lipid mobilizing factor; SAAM/CONSAM, Simulation Analysis and Modeling/Conversational SAAM; TB, tumor-bearing; TL, total lipids; TNF, tumor necrosis factor.

flux of plasma FFA. We also assumed, based on a large body of published work from many laboratories, including ours (N.B.), that when adipose tissue TGFA is mobilized at an increased rate, as in fasting, both essential and non-essential FA in adipose TGFA are similarly affected, and the manifestations of the increased mobilization again will be evidenced to the same degree whether one uses an essential or a non-essential FFA as tracer for the plasma FFA compartment.

MATERIALS AND METHODS

Seven-week-old male AKR/J mice (Jackson Laboratory, Bar Harbor, ME) were maintained on Purina Lab Chow and water *ad libitum*. The mice were divided into three groups: controls fed *ad libitum*, lymphoma-bearing mice fed *ad libitum*, and pair-fed controls (i.e., each control mouse paired with an experimental animal and fed, the following day, the amount of food consumed by a corresponding tumor-bearing mouse).

One group of tumor-bearing mice was inoculated i.p. with 1×10^6 freshly harvested SL-3 tumor cells. SL-3 is an *in vitro* cell line of T lymphoma cells derived from a spontaneous lymphoma of an AKR mouse (9). The cells were injected in 0.2 ml Delbucco's modified Eagle's medium or Krebs 0.1M potassium phosphate buffer pH 7.4. The tumors usually developed in about 2 wks and were detected by a palpable abdominal mass near the inoculation site and by an increase in body weight compared to the pair-fed control. Experimental animals were studied when the difference in body weight was 5 to 10 g, usually within a week of initial detection.

[1-^{14}C]Palmitic acid (58 mCi/mmol) was purchased from ICN Chemical Co. (Irvine, CA). Fatty acid-mouse serum albumin complexes were prepared as described previously (6). The dose, 1.0 μCi in a volume of 0.20 ml mouse serum, was injected I.V. into a tail vein of the unanesthetized mouse.

Blood was obtained from an opthalmic venous sinus in heparinized capillaries. Initially, unanesthetized mice were used for studies of 30 min duration. When an unexpected rise in the plasma radioactivity curve occurred between 15 and 30 min, the experiments were repeated in that interval (10 min to 30 min) and extended to 300 min in the briefly fasted state. This allowed the secondary peak to be confirmed and its magnitude defined. Mice used for this second series of experiments were lightly anesthetized with diethyl ether for several seconds during each of the momentary periods required to obtain blood samples from the orbital venous sinus. The data between 10 and 30 min were unaffected by this very brief exposure to anesthesia. Only two samples per mouse were taken prior to a third, terminal sample. The capillaries were kept in ice and then centrifuged at 1,000 rpm for 1 min prior to separation of plasma from red blood cells.

For the first 10 min of the experiment, total ^{14}C radioactivity as measured by direct liquid scintillation of 10 μL aliquots of plasma was assumed to approximate the [^{14}C]FFA remaining in plasma (6,10; confirmed by separate comparison of directly assayed plasma ^{14}C vs extracted plasma FFA-^{14}C at 10 min). Thereafter, plasma FFA were extracted before being counted. Extraction and storage of plasma FFA in Dole's extraction solution (11), further extraction of FFA by the method of Hagenfeldt (12), and assaying of radioactivity were carried out as described previously (8). Since FFA recoveries average at least 95%, no corrections were made for incomplete recoveries. Plasma FFA concentrations were determined according to the method of Hron and Menahan (13) as in our earlier study (8). Incorporation of labeled FFA into TL of solid tissue samples (whole thymus glands and tumor masses dissected from the peritoneal cavity) was measured after first obtaining the tissue wet weights by extracting the tissues using the Folch procedure (14). [^{14}C]TL was determined by liquid scintillation counting.

Breath $^{14}CO_2$ and total rates of CO_2 excretion were measured by the techniques described earlier (8), namely, by trapping expired CO_2 in Hyamine hydroxide solution, titrating to a phenolphthalein end point to measure production rates (15), and measuring radioactivity by liquid scintillation counting.

Multicompartmental analyses were carried out using the SAAM (16) and CONSAM programs (17) with a VAX 11-780 computer. These programs use an iterative process to calculate a set of fractional rate constants (±S.E.) that describes the assumed model and gives a least squares best fit to the data. The logic and assumptions used in developing the models have been presented in an earlier study (8). Part of the control data (up to 300 min) has been previously published to validate the model and experimental approach (8).

The present analysis of plasma FFA flux in tumor-bearing and control mice is based upon the measured plasma FFA concentrations (pool sizes calculated assuming a plasma volume of 5.0% of body wt) and the plasma FFA FCR, calculated by analysis of plasma [^{14}C]FFA disappearance measured at intervals over a 24-hr period. The mice were briefly fasted 4 hr prior to tracer injection and remained in the post-absorptive state for an additional 5 hr after tracer injection. They were allowed to eat food *ad libitum* after that until the terminal sample at 24 hr. Plasma FFA levels were not measured in the fed state but were assumed to fall a minimum of 50% (compared to fasted levels) in both groups, as they do both in TB (lymphoma or EAT) and control mice after they are fed a single test meal [(5), and R. Kannan, M. Gan-Elepano, and N. Baker, manuscript in preparation].

Disappearance of injected tracer from the plasma reflects both mixing-exchange processes and the replacement of the tracee, which is assumed to be in an approximate steady state (see below). The present experiments were designed to estimate this replacement rate in the briefly fasted state.

We used Model 2, as described (8), to calculate the plasma FFA fractional catabolic rate (FCR). The FCR is equivalent to the fraction of plasma FFA (Compt. 1) that leaves irreversibly by entering an oxidative intermediate compartment (Compt. 10) before the FFA-C is oxidized to breath $^{14}CO_2$. The fractional rate constant (min^{-1}) describing this flow of FFA-C from Compt. 1 to Compt. 10 is L(10,1). If a circulating metabolite is kept in a steady state by replacement with new molecules entering the compartment that is being sampled, in this case the plasma FFA compartment, then its FCR is also mathematically equal to the injected dose (normalized to 1.0) divided by the area under the plasma curve which describes the fraction of the dose remaining as a function

of time (18,19). Therefore, secondary recycling peaks, if they are relatively small so that they do not increase the area under the curve appreciably, can be ignored, as we have done here, without affecting the FCR. This has been validated (8).

We assume a near-steady state for plasma FFA during the briefly fasted state of the tracer study, based upon the observation that plasma FFA concentrations in a separate group of briefly fasted animals did not change significantly during a 5-hr experimental period (unpublished observations). All fractional rate constants were assumed to remain constant during any given dietary state, but L(10,1) was allowed to change after the animals were given food, for it is known that rates of plasma FFA mobilization and oxidation decrease when fasted animals are fed. The approximate average value of L(10,1) during the fed state was estimated for the time period 300–1440 min following tracer injection by means of SAAM/CONSAM using the "time change" convention (16,17), whereby the value of L(10,1) is allowed to assume some new fixed value that will give the least squares best fit to the observed data at the beginning and end of the interval.

The cumulative per cent dose excreted as breath $^{14}CO_2$ was predicted at 85 min based upon the above model using CONSAM. A separate group of TB mice was studied to measure the cumulative excretion of breath $^{14}CO_2$ during that period to verify the order of magnitude of this major metabolic pathway (8). These analyses of the plasma FFA turnover and oxidative rates were then used along with the directly measured incorporation at 90 min of tracer into the TL of the major tumor mass and of the thymus gland in the tumor-bearing mice to test our hypotheses based on Kitada *et al.* (1).

The fractional rate constants describing exchanges among plasma FFA, extra-plasma FFA and FA-ester compartments (8) are given in the Appendix. In the present analysis artificially large weights were needed for the data points at 120 and 300 min in order to obtain a satisfactory fit to the terminal slope (TB mice only). When the observed FSD's were used to weigh these two values, the calculated terminal slope in the postabsorptive state, prior to the time change in the value of L(10,1), was greater than in the present analysis. Rate constants for both analyses are included in the Appendix.

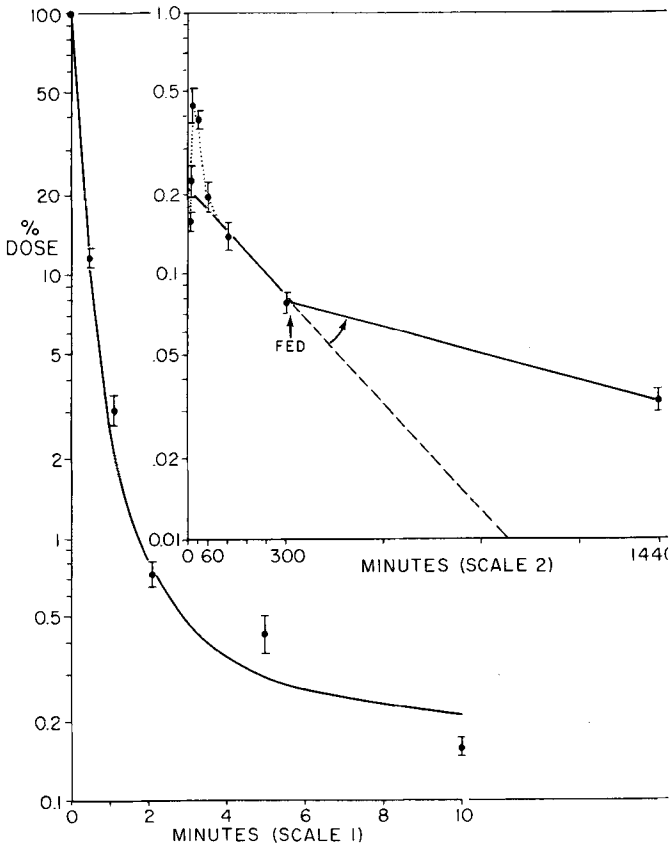

FIG. 1. Plasma disappearance of I.V.-injected [1-^{14}C]palmitate complexed to mouse serum (albumin) in control mice. Ordinate, logarithmic scale, is percent of injected dose remaining in the plasma [means of 10 mice (±S.E., vertical bars)] at the times indicated. Note the use of two scales: scale 1, 0 to 10 min; scale 2, 10 min to 1440 min. The animals were fasted for 4 hr before injection and not allowed access to food (Purina Lab Chow) during the experiment, except for mice used to obtain datum at 1440 min. The latter were given food after the 300-min plasma sample was obtained. The broken line after 300 min is the predicted fall in radioactivity if the FCR of plasma FFA, equal to L(10,1), were to remain constant after feeding. The solid curve is the least squares best fit of the model to the data calculated using CONSAM. Secondary recycling between 10 and 60 min, shown by a dotted line drawn by eye, was ignored, but the data were not excluded during the fitting process.

RESULTS

The disappearance of [1-^{14}C]palmitate from the plasma of the two control groups, the data for which were combined because they did not differ, and from that of the TB mice, respectively, are shown in Figures 1 and 2. If the mobilization of FFA is increased in lymphoma-bearing mice, this disappearance would be expected to be faster; instead it was significantly slower, as indicated by the differences in FCR (see below). After eating, there appeared to be less of an inhibitory effect on FFA mobilization in the TB mice, but this could not be determined with confidence because of the need to use arbitrary weights to fit the data for the TB mice between t = 120 and 300 min (see Appendix).

The FFA pools sizes were not significantly different among all groups of mice; however, as noted earlier (8), FFA concentrations in separate subgroups of both control and TB mice that were used for serial blood sampling without anesthesia tended to be 33% greater than in mice that were either sampled without having had serial blood samples drawn or that were lightly anesthetized with ethyl ether for several seconds just before drawing the blood samples, a process that also usually takes only several seconds. For example, the FFA pool sizes for unanesthetized mice subject to serial blood sampling (3 samples per mouse including the terminal sample) were as follows: Combined controls, 20 ± 2.0 (n = 17) vs TB mice, 22 ± 2.7 (n = 7) μg-atoms FFA-C/30 g body wt (mean ±S.E.). The lower values shown in Table 1—16 ± 1.8 and 15 ± 1.2 units/30 g body wt—were obtained for controls and lymphoma-bearing mice, respectively, that had either been lightly anesthetized during blood sampling or had only one terminal blood sample removed.

We have used the mean plasma FFA pool sizes shown in Table 1 to calculate the rates of plasma FFA turnover [plasma FFA pool size × L(10,1), where L(10,1) is the

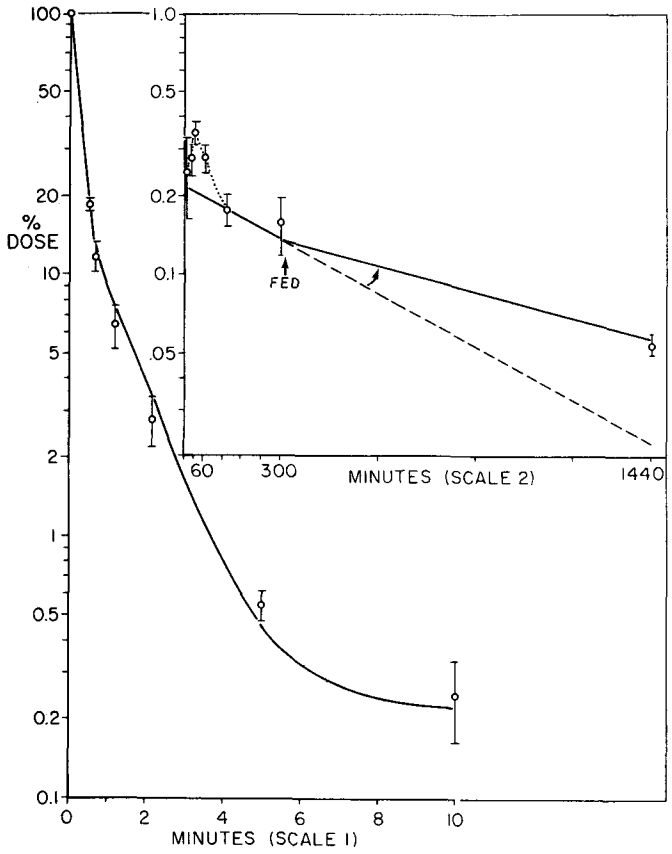

FIG. 2. Plasma disappearance of I.V.-injected [1-^{14}C]palmitate complexed to mouse serum (albumin) in lymphoma-bearing mice. The means of 5 values (±S.E., vertical bars) are plotted at each time point. (See legend to Figure 1 for further details.) Note the secondary rise in radioactivity peaking at about 30 min. (See insert, scale 2, in which the first point of the insert corresponds to the last point at 10 min of the early part of the curve.) This phenomenon, termed "delayed recycling," was previously described in the control mice (8) that were used in this study. The extent of this recycling appears to be similar in both groups. The significance of delayed recycling has been discussed earlier (8).

fractional rate of plasma FFA oxidation to breath CO_2]. Units of μg-atoms FA-C are used for convenience when estimating rates of FA oxidation to CO_2. We assumed an average of 17 μg-atoms C/μmole FFA and a plasma volume of 1.5 ml/30 g body wt; thus, dividing the mean control values for plasma FFA pool size shown in Table 1 —15.8 ± 1.8 units/30 g body wt—by (17 × 1.5) gives the observed mean plasma FFA concentrations in more conventional units: 0.62 ± 0.070 μmoles/ml. Similarly, the corresponding mean plasma FFA concentration for the TB mice with plasma FFA pool sizes of 15.3 ± 1.2 units (Table 1) was 0.60 ± 0.078 μmoles/ml.

In the postabsorptive state the TB mice mobilized their plasma FFA at a statistically significant lower rate, about half that of the combined controls [8 (±4.8%) vs 17 (±5.9%), respectively; units as in Table 1]. Using the higher plasma FFA pool sizes observed for the TB and control groups that were unanesthetized when serial blood samples were drawn (see above), the corresponding plasma FFA mobilization rates for lymphoma-bearing and control mice were 12 (±5.3%) and 21 (±5.9%) units, respectively, $p < 0.01$. In the fed state, the turnover rates (fluxes) in TB and control mice appeared to be comparable (approximately 2.2 and 2.0 units, respectively), and both reduced markedly compared to the postabsorptive or briefly fasted state. Inhibition of FFA flux by eating, a well-known regulatory process that we did not plan to study in our original experimental design, may have been greater in the controls than in TB mice, but this could not be established with any validity due to the data limitations and arbitrary weighting used. We obtained no evidence that adipose tissue TGFA are being mobilized to plasma FFA faster in SL-3 lymphoma-bearing mice than in controls, at least not in the briefly fasted state.

At least 96% of the FFA removed irreversibly from the circulation was accounted for as expired CO_2, as shown in Table 2. Moreover, only about 3 to 4% of the irreversibly disposed plasma FFA was sequestered by the tumor (i.e., 1.1% of the dose found in tumor lipids compared to at least 30% converted to breath CO_2, as shown in Table 2). The mice used to determine the uptake by the tumor tissue 90 min after tracer palmitate injection were typical with respect to growth patterns and food intake. Body weights (mean ±S.E., n) of controls fed *ad libitum* were 28.6 ± 0.91 g, n = 8 at the time the pair-fed control group was established (1 wk after tumor inoculation). Two wks later, at the time of the tracer study, the 8 *ad libitum* fed controls weighed 31.2 ± 0.89 g. The corresponding weights for the pair-fed controls initially were 27.6 ± 0.60 g, n = 6, not significantly different from the controls at that time. However, at the time of the tracer study their body weights had decreased significantly to 25.8 ± 0.77 g. Eight of the 8 controls fed *ad libitum* gained weight, while 6 of 6 of the pair-fed controls lost weight. The TB mice weighed initially (one week after tumor inoculation) 28.4 ± 0.56 g (n = 6), the same as the controls at that time. Their body weights increased significantly to 32.4 ± 1.5 g, a mean of 6.6 g greater than that of the pair-fed controls. The mean food intake in the controls fed *ad libitum* was 4.7 ± 0.21 g/day during the several days preceding the tracer study, whereas that of the TB mice and their pair-fed controls fell to 3.5 ± 0.19 g/day after 2 wk of tumor growth and paired feeding and to 1.8 ± 0.28 g during the several days preceding the tracer experiment, approximately 3 wks after tumor inoculation.

Although the specific activity of lipids (% dose per g wet wt of tissue) in the thymus gland was greater than that found in the tumor tissue of lymphoma-bearing mice, the total mass of thymus tissue was small compared to that of the tumor mass in the peritoneal cavity, and only a negligible fraction of the plasma FFA disposal was due to sequestration by the thymus in our TB mice. The thymus gland was not significantly enlarged at late stages of tumor growth using the present model in which SL-3 lymphoma cells were injected i.p. This contrasts with the massive enlargement of the thymus, with marked tumor infiltration in both the spleen and liver, found after SL-3 viruses are injected into neo-natal AKR mice. Kitada *et al.* used the latter method to induce SL-3 lymphomas in their earlier study of LMF and its effects on adipose TGFA mobilization (1).

DISCUSSION

We hypothesized that, if LMF is active in briefly fasted TB mice, we should be able to observe an increased

TABLE 1

Plasma FFA Pool Sizes and Turnover Rates in Briefly Fasted and Fed Lymphoma-Bearing and Control Mice

	Plasma FFA		
	Pool size	FCR[a]	Mobilization rate
Group	[μg-atoms FA-C per 30 g body wt ± % S.E. (n)]	[min^{-1} ± %S.E.]	[μg-atoms FA-C/min per 30 g body wt (±%S.E.)]
Controls			
Briefly fasted	16 ± 1.8% (14)	1.07 ± 5.6%	17 ± 5.9%
Fed	8.0[b]	0.25 ± 13%	2.0[b]
Lymphoma			
Briefly fasted	15 ± 1.2% (12)[c]	0.53 ± 4.6%[d]	8.0 ± 4.8%[d]
Fed	7.5[b]	0.29 ± 7.3%	2.2[b]

[a] FCR values = L(10,1), using model 2 (8); other rate constants are given in the Appendix. The control values are based on the combined pair-fed and *ad libitum*-fed control data shown in Figure 1; the lymphoma values are based on the data shown in Figure 2.

[b] Maximum estimate (see text). Plasma FFA concentrations were not measured after the mice were given food. Separate studies in 6 lymphoma-bearing mice and 6 pair-fed control AKR mice showed the same fall in plasma FFA levels in response to a single 250-mg test meal in both groups (Kannan, R., M. Gan-Elepano and N. Baker, manuscript in preparation).

[c] n.s., lymphoma-bearing vs controls.

[d] $p < 0.01$, lymphoma-bearing vs controls. The %S.E. of the plasma FFA mobilization rates was estimated as the square root of the sum of the squares of the %S.E. values for the corresponding plasma FFA pool size and FCR, the product of which is the mobilization rate.

TABLE 2

Incorporation of [1-^{14}C]Palmitate-Albumin into [^{14}C]TL of Lymphoma and Thymus Tissues and into Breath $^{14}CO_2$ 85–90 Min After I.V. Tracer Injection in Lymphoma-Bearing Mice

Per cent dose [Mean ±S.E. (n = 6)] incorporated:

	Time (min)	Per g wet wt	Per total tissue mass
Lymphoma-[^{14}C]TL[a]	90	0.16 ± 0.025	1.1 ± 0.22[b]
Thymus-[^{14}C]TL[c]	90	0.67 ± 0.12	0.077 ± 0.013
		Calc.[d] % I.D.	Obs.[e] % I.D.
Breath $^{14}CO_2$	85	30	40 ± 2.9

Per cent of plasma FFA irreversible disposal (FCR) to "end products"[f]:

Lymphoma-TL	90	3.5
Thymus-TL	90	0.25
Breath CO_2	85	96

[a] Sampled from the solid tumor mass found in peritoneal cavity.

[b] Calculated by assuming that the mean difference in body weights (6.7 ± 1.2 g) between 6 pair-fed controls and the corresponding lymphoma-bearing mice equals the tumor mass at the time of the tracer study. Thus, 0.16%/g (±16% S.E.) × 6.7 g (± 18% S.E.) = 1.1% I.D. (± 20% S.E.).

[c] The mean (± S.E.) thymus weight was 87 ± 16 mg (n = 6) and did not differ significantly from those of the controls, either pair-fed [80 ± 15 mg (n = 5)] or fed *ad libitum* [100 ± 16 mg (n = 7)]. In a separate study of SL-3 TB mice inoculated neonatally with SL-3 virus, the mean thymus weight was 720 ± 94 mg (n = 10).

[d] Calculated from the data of Figure 2 using Model 2 (8).

[e] Mean ± S.E. (n = 6) based on a separate group of 6 lymphoma-bearing mice.

[f] Calculated on the assumption that the estimated percentage of injected dose incorporated into the total lipids of the total tumor mass and into breath CO_2 in 85 to 90 min represents the radioactivity that has been irreversibly converted to "end products." Using the lower of the two estimated values for conversion to breath CO_2, namely 30%, to maximize the estimated percent converted to tumor lipids, the total % dose converted to end products was 31.18%. The remaining 69% of the dose at about 90 min is distributed in exchangeable FA pools that recycle back to plasma FFA (8). Therefore, the % of plasma FFA irreversible disposal to CO_2 is 30%/31.18% = 96%, etc.

mobilization of adipose tissue TGFA in lymphoma-bearing mice by measuring the replacement of plasma FFA using one of the major FFA components, palmitate, as tracer. Our study offers no support for this or for other hypotheses that were based on Kitada *et al.* (1). There was no evidence that adipose tissue TGFA of the TB mice was being lost due to increased FFA mobilization or that the FFA mobilized were being selectively sequestered by the tumor cells rather than oxidized to CO_2. In this respect the mice with solid SL-3 lymphomas appeared to behave very much like EATB mice (5–7). There was an indication that at the advanced stages of tumor growth, when the present TB mice were studied, the rate of FFA oxidation to CO_2 was lower than normal, consistent with depressed FA oxidation in livers of these mice (20) and with both the generally decreased metabolic rates and the decreased respiratory quotients seen in similarly anorexic mice bearing methylcholanthrene-induced sarcomas (21). However, two other observations indicate that in the briefly fasted state, plasma FFA turnover and oxidation may be of approximately equal magnitude in control and TB mice: the plasma FFA pool sizes of the two groups did not differ and the percent of the injected FFA converted in 85 min to its major end-product, breath $^{14}CO_2$, was approximately the same as that reported earlier (8) for the control mice (about 40% of the injected dose in each case). None of these observations is consistent with there being a faster rate of mobilization in the lymphoma-bearing mice.

There appeared to be a very marked decrease in the plasma FFA FCR [L(10,1)] in the control mice when they were allowed to eat compared to the rate estimated in the briefly fasted state. This change in rate, when expressed as the percentage of the rate in the briefly fasted state, seemed less striking in the TB mice. Further work is required to study this phenomenon for several reasons. First, inferences regarding the effects of eating on plasma FFA mobilization are based, to a very large extent, on a single datum (the mean of 6 animals per group) taken at 24 hr after injection and 19 hr after the mice were given access to food. Second, the times at which the mice ate were not determined. Third, arbitrary assumptions with respect to weighting of the data were made to fit some critical points. Experiments specifically designed to study this aspect of FFA regulation are needed to quantify the extent to which food is able to suppress FFA mobilization and oxidation to CO_2 at night in TB and in control mice. Obviously, defective regulation of this process could result in the loss of body fat.

Although regulation of FFA mobilization by dietary glucose has been previously tested in EATB mice (5), where no differences between TB and control mice were found, it deserves re-examination. Estimates of the plasma FFA FCR in those studies were based upon experiments of only 30 min duration (5); such estimates are invalid unless $^{14}CO_2$ data are included and the FCR estimated from the plasma FFA oxidation rate rather than from the area under the plasma [^{14}C]FFA disappearance curve (8). This criticism applies to (5) where the reported plasma FFA FCR would be a measure, for the most part, of the FFA and FA-ester exchange rates and, only to a minor extent, the actual rate of plasma FFA irreversible disposal. Second, the experiments that were the main focus in the work of Kitada *et al.* (1) showed that circulating LMF produced its effect in mice that had been fasted and then refed. There was no claim that LMF was active in the fasted state. Its mechanism of action could well be to antagonize the effects of insulin and insulin-like hormones released when food is eaten. If so, major differences in plasma FFA flux and oxidation in TB mice, which we assume to have circulating LMF (1), might only appear in the fully fed or fasted-refed states.

An enigma remains. If LMF is produced by cancer cells (1,3,4,22–25); if other lipid mobilizing agents, such as TNF/cachectin, are produced by the immune system (2,26,27); and if all of these are circulating in the blood of TB mice, why did we fail to observe an increase in the mobilization of plasma FFA in our studies? In reviewing the literature, we fail to see any evidence that LMF or any other agent has had an effect on the adipose tissue of TB mice. The *in vivo* LMF assays carried out by Kitada *et al.* were done using control mice, i.e., animals without tumors. One might hypothesize that adipose tissue of TB mice becomes resistant (refractory) to the effects of a putative LMF or that there are circulating antagonistic hormones or other agents in the TB mice that are not present in control mice. It is also possible, as mentioned above, that LMF can only be demonstrated *in vivo* in fed or in fasted-refed animals, not in the fasted state that we used to establish a steady, basal state in our experiments.

There are additional important differences between our studies and the earlier work of Kitada *et al.* (1), especially in the experimental approaches used. Their tracer was an essential FA, [^{14}C]linoleate, whereas ours was a non-essential FA, [^{14}C]palmitate. We established earlier that the uptake of labeled palmitate by EAT cells is similar to that of labeled linoleate *in vivo*, and we have confirmed this in unpublished studies using lymphomas in AKR mice (R. Kannan, M. Gan-Elepano, and N. Baker, manuscript in preparation). We have also shown that labeled palmitate and labeled linoleate are both oxidized to similar extents in EATB and control mice, with linoleate being burned at a somewhat faster rate (7,28). Therefore our choice of labeled palmitate as tracer is unlikely to account for the differences noted. Their tracer was administered in the form of a transplanted fat pad, labeled in the TGFA moiety; ours was given in the form of the FFA-albumin complex, I.V. Theoretically, the two techniques should yield the same results if appropriately analyzed by quantitative techniques; however, Kitada *et al.* did not calculate adipose TGFA or plasma FFA fluxes. Their tumor was induced by virus injection into neo-natal mice. The tumor developed in different anatomical locations than those induced by i.p. inoculation of SL-3 lymphoma cells (see Results).

Some of these differences in experimental design could have contributed to our inability to obtain evidence of increased FFA mobilization in our TB mice. However, one observation of Kitada *et al.* seems to require modification. Their assertion that the tumor can sequester most of the FFA mobilized from adipose tissue when it loses its TGFA during tumor growth seems highly unlikely. In examining their data on the uptake of label (from TGFA-labeled adipose tissue) by liver that had been invaded by cancer cells (and other tissues when data are available), only about 7% of the mobilized FA appears to have been sequestered by the tumor tissue, mainly in the liver. This is not inconsistent with the present

findings, especially considering the variations in methodology listed above. Although FFA uptake by the tumor is small compared to the rate of FFA oxidation to expired CO_2, this degree of sequestration may be important for the growth of the tumor, especially in the case of an essential FA, as suggested by Kitada *et al.* (1). Earlier work implicating the importance to the tumor of essential FA supplied by the host is cited by Kitada *et al.*, and other relevant work has appeared more recently (e.g., 29–33). We are currently studying the transport of essential FA from the host to tumor cells under varying nutritional states employing techniques similar to those used here and in our earlier studies (5–8).

ACKNOWLEDGMENTS

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APPENDIX

Fractional rate constants [min^{-1}, mean (± %SE)] obtained by CONSAM in fitting the data shown in Figures 1 and 2 to Model 2 (8):

	Combined controls	Tumor-bearing[a]	
		A	B
L(1,2)	0.80 (±46%)	1.8 (±17%)	1.34 (±22%
L(3,1)	2.5 (±5.0%)	2.1 (±4.1%)	2.0 (±8.4%)
L(1,3)	0.011 (±7.6%)	0.0073 (±11%)	0.012 (±14%)
L(10,1[b] (fasted)	1.07 (±5.6%)	0.53 (±4.6%)	0.73 (±20%)
L(10,1)[c] (fed)	0.25 (±13%)	0.29 (±7.3%)	0.16 (±44%)

[a] The two solutions for TB mice are based on the same set of data shown in Figure 2; however in A artificially high weights at 120 and 300 min were introduced to improve the fit to the apparent terminal slope, whereas in B, the weights at these times, like those at all other times, were based on the observed S.E.'s. The predicted cumulative values for $^{14}CO_2$ (or sum of all ^{14}C-labeled end-products) at t = 85 min predicted by solutions A and B, respectively were 30% and 43%, differences consistent with the more steeply falling slope calculated by the parameters of solution B.

[b] L(10,1) is the only irreversible outlet from plasma FFA. It flows by way of a brief delay to the bicarbonate system and then to expired CO_2.

[c] A time change (TC) was introduced for L(10,1) at 301 min when mice were allowed to eat *ad libitum*. Another experimental design is required to quantify accurately the change in this and other rate constants in fed vs fasted states. We assumed for simplication that only L(10,1) changed following feeding and that fractional exchange rates were unaffected.

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Preferential Redistribution of Lipoprotein-Unassociated ApoA-IV to an HDL Subpopulation with a High Degree of LCAT Modification

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The relationship between LCAT mediated HDL modification and the redistribution of lipoprotein-unassociated apoA-IV to HDL was investigated *in vitro*. Immunoaffinity-isolated rat lipoprotein-unassociated apoA-IV was added to apoB-, apoE-, apoA-IV depleted, [^{3}H]cholesterol labelled rat plasma and incubated at 37°C. The addition of lipoprotein-unassociated apoA-IV resulted in a modest (10%) but significant reduction in the rate of cholesterol esterification. Incubations conducted in the presence of active LCAT led to a time-dependent increase in the amount of the ^{3}H label retained by an anti-apoA-IV immunoaffinity column. Lipoproteins retained by the anti-apoA-IV immunoaffinity column had experienced a greater conversion of [^{3}H]cholesterol to [^{3}H]cholesteryl esters (48% esterification at 30 min) than the unretained lipoproteins (19% esterification at 30 min). These data suggest that during the course of LCAT-induced cholesterol esterification, lipoprotein-unassociated apoA-IV transfers to a subpopulation of HDL which has been modified by LCAT to a greater extent than the remaining HDL. Further analysis of the data demonstrates that 48% cholesterol esterification is sufficient to allow apoA-IV to be accommodated on the surface of an HDL particle.
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ApoA-IV was first described in rats by Swaney *et al.* (1) in 1974. At present, no specific physiological role for apoA-IV has been conclusively demonstrated. DeLamatre *et al.* (2) demonstrated that in rats, the relative amount of apoA-IV associated with HDL could be influenced *in vitro* by the action of LCAT, a finding which has since been confirmed in human plasma (3,4). DeLamatre *et al.* (2) speculated that apoA-IV becomes associated with HDL following LCAT-induced reduction of lipoprotein surface components relative to core components. It was argued that loss of surface phospholipids and unesterified cholesterol, and expansion of the core by cholesteryl esters would provide sufficient space between the phospholipid head groups to accommodate apoA-IV. However, time course studies (5) indicated that the degree of cholesterol esterification required for complete redistribution of lipoprotein-unassociated apoA-IV to HDL would not increase the available surface area on HDL sufficiently to accommodate apoA-IV. This led to the speculation that either apoA-IV displaces other apolipoproteins as it associates with the HDL or that it associates with a subpopulation of HDL that has undergone a greater degree of LCAT modification than the bulk of the HDL. In this report, we have examined more closely the interaction of lipoprotein-unassociated apoA-IV with LCAT-modified rat HDL.

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Abbreviations: HDL, high density lipoproteins; EDTA, ethylenediaminetetraacetic acid; DTNB, 5,5′-dithio*bis*(2-nitrobenzoic acid); apo, apolipoprotein; PBS, phosphate buffered saline; LCAT, lecithin:cholesterol acyltransferase.

METHODS AND MATERIALS

Plasma and lipoprotein preparations. Blood was obtained from male rats (300-350 g) after an overnight fast. Blood was collected into tubes containing disodium EDTA (1 mg/ml) and immediately centrifuged (1500 × *g*) at 4°C. Following centrifugation, DTNB was added to plasma at a final concentration of 1.4 mM to inhibit LCAT. Lipoprotein-deficient plasma (d > 1.21 g/ml) was isolated by ultracentrifugation in a Beckman SW-40 rotor.

Preparation of immunoaffinity columns. Rat apoA-IV was isolated from a d < 1.21 g/ml lipoprotein fraction using a combination of gel filtration chromatography and heparin-affinity chromatography (6). Antisera to rat apoA-IV were produced in goats as previously described (7). The antisera was monospecific as judged by immunoblots against both total lipoproteins and plasma following SDS-PAGE.

A rat HDL-Sepharose column was produced by coupling 100 mg of rat HDL to 20 ml of CNBr-activated Sepharose 4B (Pharmacia; Piscataway, NJ) according to manufacturer's instructions. Goat anti-rat apoA-IV antibodies were isolated as follows. One hundred ml of goat anti-rat apoA-IV antisera were passed through a 1.6 × 10 cm column containing rat HDL-Sepharose at a flow rate of 1 ml/min. The column was washed with PBS until the absorbance at 280 nm was less than 0.02. Bound IgG was recovered from the column by elution with 3 M NaSCN, pH 7.0. The eluent was immediately passed through a Sephadex G-25 column coupled in series to remove NaSCN. The affinity-isolated goat anti-rat apoA-IV was coupled to CNBr-activated Sepharose 4B at a concentration of 5 mg protein per ml bed volume.

Isolation of lipoprotein-unassociated apoA-IV. Nonsaturating amounts of rat-lipoprotein-deficient plasma was passed through a 1.6 × 10 cm column containing anti-rat apoA-IV-Sepharose at a flow rate of 1 ml/min. The column was washed with PBS until the absorbance at 280 nm was less than 0.02. The bound apoA-IV was eluted with 3.0 M NaSCN, pH 7.0 and then quickly desalted on a Sephadex G-25 column run in series. Nondenaturing gradient gel electrophoresis of the eluted material revealed the presence of lipoprotein-unassociated apoA-IV (protein migrating below albumin) as well as other higher molceular weight material including HDL-associated apoA-IV, apoA-IV aggregates, and albumin. The apoA-IV preparation was concentrated under pressure (Amicon PM10 membrane filter) and applied to a 0.9 × 100 cm Biogel A-.5m gel filtration column. The column was eluted with PBS containing 0.01% EDTA and 0.01% NaN_3 at a flow rate of 5.0 ml/hr. Fractions containing protein were subjected to nondenaturing gradient gel electrophoresis. Fractions containing lipoprotein-unassociated apoA-IV were combined and again concentrated under pressure.

The final lipoprotein-unassociated apoA-IV preparation appeared as two bands on nondenaturing gradient gel electrophoresis. The major band (approximately 90%)

migrated below albumin and reacted strongly to anti-rat apoA-IV following electrophoretic transfer to charge-modified nylon membranes (8). A minor band appeared with an apparent diameter of 7.6 nm and was presumed to be an apoA-IV dimer (9). In all preparations, albumin contamination was minor.

Preparation of [^{3}H]cholesterol-labelled apoB-, apoE- and apoA-IV-depleted plasma. Two ml of rat plasma containing 1.4 mM DTNB were applied to a 1.5 × 10 cm column of heparin-Sepharose (Pharmacia; Piscataway, NJ) to remove apoB and apoE. The eluent was directed onto a second 1.6 × 15 cm column containing anti-rat apoA-IV-Sepharose. Both columns were maintained at 4°C and were operated at a flow rate of 0.5 ml/min. The non-retained eluent was concentrated back to its original volume (2.0 ml). As determined by electroimmunoassay, greater than 95% of apoB, apoE and apoA-IV was removed by this procedure.

The apoB-, apoE-, apoA-IV-depleted plasma was labelled with [^{3}H]cholesterol essentially as described by Stokke and Norum (10). Two to ten μCi of [7(N)-^{3}H]cholesterol was evaporated to dryness under N_2 and then redissolved in 100 μl of acetone. The [^{3}H]cholesterol was added dropwise with constant stirring under N_2 to 1.0 ml of 5% BSA in 100 mM phosphate buffer, pH 7.4. The BSA/[^{3}H]cholesterol solution was continuously stirred under N_2 until no acetone could be detected. To each 1.0 ml of the apoB-, apoE-, apoA-IV-depleted plasma, 330 μl of the BSA/[^{3}H]cholesterol was added and then incubated for 2 hr at 37°C. The final product had a specific activity of 2,000 to 10,000 dpm/μl plasma.

Experimental design. The [^{3}H]cholesterol-labelled apoB-, apoE-, apoA-IV-depleted plasma was divided in half. Lipoprotein-unassociated apoA-IV was added to one sample (plasma with A-IV) to provide a final concentration of 100 μg protein/ml plasma while the second sample (plasma without A-IV) received an equivalent volume of PBS. Endogenous LCAT was activated by the addition of 100 μl 0.1 M β-mercaptoethanol to each 900 μl plasma preparation (containing the equivalent of 500 μl of original plasma). Samples were incubated for 0, 30, 60 and 90 min at 37°C.

At the end of each incubation period, a 20 μl aliquot was removed from both the plasma with A-IV sample and the plasma without A-IV sample for determination of the extent of cholesterol esterification. A second 100 μl aliquot was removed and applied to the top of a disposable column packed with anti-rat apoA-IV-Sepharose (0.5 ml packed bed volume). Preliminary experiments had shown that under these conditions, all the apoA-IV in the 100 μl aliquot of the plasma with A-IV sample was removed by this column. The column was washed with 2.5 ml of PBS, and the non-retained material kept for analysis. The anti-rat apoA-IV-Sepharose (containing the retained material) was then quantitatively removed from the column and suspended in an equal volume of methanol. An aliquot was assayed for ^{3}H. The remainder of the fraction was subsequently extracted for lipids.

Analytical methods. Lipids were extracted by the method of Bligh and Dyer (11). Separation of [^{3}H]cholesterol into unesterified cholesterol and cholesteryl esters was accomplished by thin layer chromatography (Baker-flex silica gel 1B2) in a solvent system comprised of hexane/diethyl ether/acetic acid (85:15:2, v/v/v). Unesterified cholesterol and cholesteryl esters were visualized by exposure to iodine; the appropriate areas were cut from the TLC plate and assayed for ^{3}H.

Apoproteins were assayed by the method of Laurell (12) as previously modified (13). Total and free cholesterol were assayed enzymatically (14). Data are presented as mean ± SEM. Significant effects due to time of incubation and presence of lipoprotein-unassociated apoA-IV were analyzed by two-way analysis of variance employing a randomized complete block design. When significant effects were indicated, multiple paired t-tests were used to identify significant differences between treatments at each incubation time point.

RESULTS

The rate of cholesterol esterification was estimated by following the conversion of [^{3}H]cholesterol to [^{3}H]cholesteryl esters. Addition of lipoprotein-unassociated apoA-IV to apoB-, apoE-, apoA-IV-depleted plasma was associated with a small decrease in the rate of cholesterol esterification (Fig. 1). This decrease was statistically significant ($P < 0.05$ by paired t-test) at 60 and 90 min. When the extent of cholesterol esterification was examined during each 30 min period, it was found that the greatest reduction of LCAT activity occurred between 30 and 60 min (Fig. 1, inset).

As judged by the amount of label retained by the anti-rat apoA-IV-Sepharose column, little [^{3}H]cholesterol was specifically associated with apoA-IV before activation of LCAT (Fig. 2). After reversal of the LCAT inhibition by the addition of β-mercaptoethanol, a time-dependent increase in the amount of [^{3}H]cholesterol (and [^{3}H]-

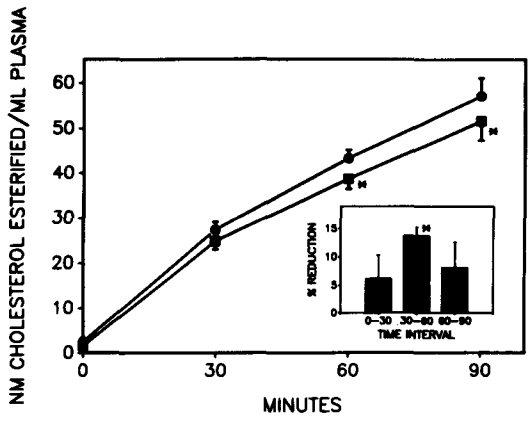

FIG. 1. Reduction in cholesterol esterification due to the addition of lipoprotein-unassociated apoA-IV. DTNB-treated plasma depleted of apoB, apoE and apoA-IV and labelled with [^{3}H]cholesterol was prepared as described in the text. Half of the plasma received 100 μg lipoprotein-unassociated apoA-IV per ml plasma (plasma with A-IV, squares) while the other half received an equal volume of PBS (plasma without A-IV, circles). Plasma LCAT was activated by the addition of β-mercaptoethanol and incubated at 37°C for the times indicated. The addition of lipoprotein-unassociated apoA-IV resulted in a small but statistically significant (asterisks: $P < 0.05$ by paired t-test) reduction in the extent of cholesterol esterification at 60 and 90 minutes. Inset: Percent reduction in the rate of cholesterol esterification due to the addition of lipoprotein-unassociated apoA-IV. The greatest reduction in cholesterol esterification occurred between 30 and 60 minutes. Asterisk indicates value is significantly different from zero. Data are Mean ± SEM of four experiments.

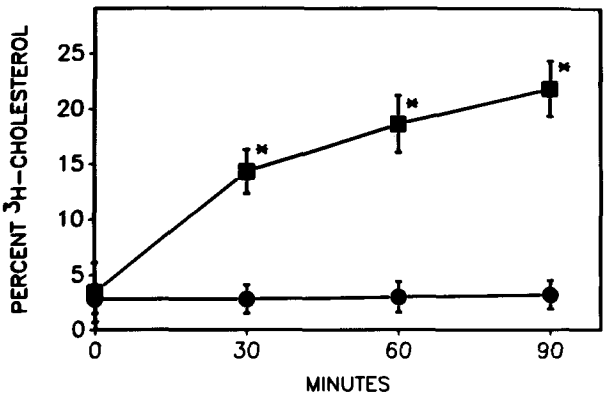

FIG. 2. Retention of [^{3}H]cholesterol (and [^{3}H]cholesteryl esters) by anti-apoA-IV-Sepharose following incubation of LCAT-activated plasma. Following activation of LCAT and incubation at 37°C for the times indicated, samples of both plasma with A-IV (squares) and plasma without A-IV (circles) were applied to anti-rat apoA-IV Sepharose columns. Following thorough washing of the columns, the column matrix was quantitatively removed and an aliquot assayed for ^{3}H. With increasing length of incubation, progressively more plasma ^{3}H became associated with the immunoaffinity column in those samples to which lipoprotein-unassociated apoA-IV had been added. Data are Mean ± SEM of three experiments. Asterisks indicate significant differences ($P < 0.05$ by paired t-test) between plasma with A-IV and plasma without A-IV.

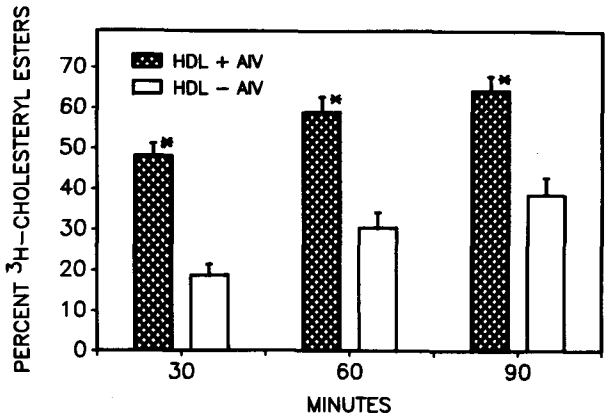

FIG. 3. Degree of LCAT modification in HDL with apoA-IV (HDL + AIV) and HDL without apoA-IV (HDL − AIV). Following incubation for the times indicated, aliquots of the plasma with A-IV sample were applied to the anti-rat apoA-IV Sepharose columns. Following thorough washing of the columns, the column matrix was removed and extracted for lipids. The lipid extracts were subjected to thin layer chromatography for neutral lipids. Regions corresponding to unesterified cholesterol and cholesteryl esters were visualized with iodine, removed, and assayed for ^{3}H. Data for HDL + AIV represent analysis of material bound to the immunoaffinity column. Data for HDL-AIV were determined from the non-retained immunoaffinity column fraction. Data are corrected for non-specific binding as determined from parallel columns to which equivalent aliquots of the plasma without A-IV sample had been applied. Data are Mean ± SEM of three experiments. Asterisks indicate significant differences ($P < 0.05$ by paired t-test) between HDL with apoA-IV and HDL without apoA-IV.

cholesteryl esters) retained by the anti-apoA-IV column was noted when plasma with A-IV was applied. At the earliest time point measured (30 min), lipoproteins retained by the anti-apoA-IV column (apoA-IV-associated lipoproteins) had undergone a 48% conversion of cholesterol to cholesteryl esters (Fig. 3). This contrasts with a 19% conversion in the non-retained fraction (non-apoA-IV-associated lipoproteins). In both fractions, the degree of cholesterol esterification increased with time, reaching 64% in the apoA-IV-associated lipoproteins and 39% in the non-apoA-IV-associated lipoproteins. The distribution of plasma [^{3}H]cholesteryl esters between apoA-IV-associated and non-apoA-IV-associated lipoproteins changed only slightly with time. ApoA-IV-associated [^{3}H]cholesteryl esters account for 26.1 ± 1.9%, 27.9 ± 0.6%, and 29.8 ± 1.6% of total plasma [^{3}H]cholesteryl esters at 30, 60 and 90 min, respectively.

DISCUSSION

ApoA-IV has been shown to activate LCAT under specific conditions (15,16). It was therefore surprising to find that the addition of lipoprotein-unassociated apoA-IV to apoB-, apoE-, and apoA-IV-depleted plasma resulted in a modest but significant reduction in the rate of cholesterol esterification. This reduction in cholesterol esterification could have resulted from interactions between LCAT and apoA-IV in either its lipoprotein-associated or lipoprotein-unassociated form. That lipoprotein-unassociated apoA-IV can cause a reduction in LCAT activity has precedence in the observations of Jonas *et al.* (17); addition of excess lipoprotein-unassociated apoA-I to apoA-I/phosphatidylcholine/cholesterol complexes decreased the rate of LCAT-dependent cholesterol esterification due to non-productive binding of LCAT to the lipoprotein-unassociated apolipoprotein. However, such inhibition was achieved at only relatively high concentrations of lipoprotein-unassociated apoA-I, and thus may not have physiological relevance. In the present study, a significant decrease in the rate of cholesterol esterification occurred only between 30 and 60 min. Based upon previously published reports (5) and the data presented in Figure 2, it would be expected that by 30 min, a substantial proportion of apoA-IV would be associated with the HDL. This would suggest that HDL-associated apoA-IV and not lipoprotein-unassociated apoA-IV was primarily responsible for the observed modest reduction in cholesterol esterification.

This reduction in the rate of cholesterol esterification appears to be contradictory to previously published reports regarding apoA-IV's ability to activate LCAT. However, in both these studies, apoA-IV was less effective than apoA-I in activating LCAT. The reduction in cholesterol esterification rate seen with apoA-IV could have been a result of a competition between HDL-associated apoA-I and HDL-associated apoA-IV for binding to LCAT, with the apoA-IV-LCAT complex producing a lower rate of cholesterol esterification.

The above data support the hypothesis that during LCAT catalyzed cholesterol esterification in rat plasma, lipoprotein-unassociated apoA-IV preferentially associates with a subpopulation of HDL which has undergone a substantially greater degree of modification than the bulk of the HDL. To assess whether the approximately 50% cholesterol esterification found at the 30 min point is sufficient to fully accommodate apoA-IV on the HDL surface, we relied upon the equations provided by Shen *et al.* (18). The relationships between the particle radius

and composition of the surface hydrophilic lipoprotein shell are given by the following equations:

$$68.5n_{pl} + 39.1n_c = 4\pi(r - 20.2)^2 \quad [1]$$

$$15.6n_{aa} + 62.7n_{pl} = 4\pi r^2 \quad [2]$$

where n_{pl}, n_c and n_{aa} are the number of phospholipid (PL), unesterified cholesterol (UC) and amino acids (AA) per particle. From the composition data provided by Swaney *et al.* (19) for rat HDL, we can derive molar ratios for HDL surface components of 3:1 for PL:UC and 8.6:1 for AA:PL. We have previously shown that approximately 80 percent of rat apoA-I resides on HDL displaying a median diameter of 100 Å on nondenaturing gradient gels (8). Using this diameter, the molar ratio of PL:UC and equation 1, we calculated that each HDL should contain 46 molecules of unesterified cholesterol and 137 molecules of phospholipid. From the ratio of AA:PL, we calculated that each HDL should also contain 1,178 amino acid residues. Assuming an average amino acid molecular weight of 100 (the value used in the derivation of the above equations), then the aggregate protein component should have a molecular weight of 117,800. This value agrees quite well with aggregate protein molecular weight of 110,000 for control rat HDL as determined by chemical crosslinking (19).

From the number of phospholipids per HDL, the HDL diameter, and equation 2, we determined that the maximum number of amino acid residues that can be accommodated on the surface of the HDL is 1463 or 285 amino acids more than is calculated to actually occupy the surface. Again, assuming an amino acid molecular weight of 100, this excess surface capacity is insufficient to accommodate apoA-IV (MW, 46,000).

We next examined the case where 50% of the unesterified cholesterol has undergone esterification by LCAT. From data provided by Shen *et al.* (18), the following assumptions were made: 1) the lipoprotein hydrophobic core has a radius of r − 20.5 Å, where 20.5 Å is the approximate width of the hydrophilic shell and 2) each additional cholesteryl ester molecule increases the core volume by 1068 Å^3. Additionally, we assumed that the width of the hydrophilic shell did not change following cholesterol esterification. Based on these assumptions, the addition of 23 cholesteryl ester molecules to the core would increase the core radius from 29.5 Å to 31.6 Å and increase the HDL radius to 52 Å. Taking into account the reduction in surface phospholipids and the increase in HDL diameter, we can use equation 2 to calculate a new maximum of 1,720 amino acid residues per particle. The excess capacity of 542 amino acids is more than sufficient to accommodate apoA-IV on the surface of the HDL.

The above calculations predict that apoA-IV-containing HDL would have an average diameter of 104 Å. This agrees remarkably well with measured median diameter of 104 Å for rat plasma apoA-IV-containing HDL as determined by non-denaturing gradient gel-immunoblot analysis (8). The above calculations also suggest that rat HDL may be relatively deficient in surface components. When similar calculations are applied to compositional data for human HDL_2 (18) and dog HDL_2 (20), the discrepancy between the maximum possible number of amino acid residues on the surface and the actual number of amino acid residues is less than 75 residues. This relative deficiency in surface components in normal rat HDL may explain why lipoprotein-unassociated apoA-IV readily associates with HDL following brief (2 hr) incubation at 37°C. In human plasma, incubation at 37°C for 6 hr results in only a 25% transfer of lipoprotein-unassociated apoA-IV to the HDL (4).

In summary, our data suggests that the expansion of the hydrophobic core and the reduction in surface phospholipids due to 50% cholesterol esterification of a rat HDL are sufficient to increase the surface area between phospholipids to accommodate apoA-IV without loss of additional HDL apolipoproteins. Once associated with HDL, apoA-IV may compete with apoA-I for binding by LCAT leading to reduction in the rate of cholesterol esterification. Such a mechanism may serve to limit the extent to which a given HDL particle is modified by LCAT.

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Increased Liver Lipase Activity in Rats with Essential Fatty Acid Deficiency

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Liver lipase activity was measured in EFA-deficient rats (long-term) and in control rats and rats fed an EFA-deficient diet for two weeks (short-term). Liver lipase activity was significantly enhanced by EFA deficiency, both in long-term and short-term experiments. The enhanced liver lipase activity could be normalized by feeding these rats normal laboratory chow for 14 days. Since during EFA deficiency prostaglandin synthesis is impaired, the possible involvement of prostaglandins in the observed changes in liver lipase activity during EFA deficiency was studied. Administration of the prostaglandin synthesis inhibitor indomethacin (5 mg/kg body weight, i.p.) to normally fed rats for two days led to an increase of liver lipase activity. Prostaglandin E_2 was found to inhibit the secretion of liver lipase activity by freshly isolated parenchymal liver cells *in vitro*. These results indicate that the increase in liver lipase activity during EFA deficiency may be due to an impairment of the prostaglandin synthesis.
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Animals fed a diet without essential fatty acids (EFA) develop a syndrome known as EFA deficiency (1,2). Biochemically, alterations in the fatty acid composition of tissue lipids (3), development of a fatty liver (4) and changes in the levels of the serum lipoproteins (5) are found. The most remarkable effect is the reduction in the concentration of circulating triglycerides, unesterified cholesterol and phospholipids (5). Especially the proportion of triglycerides in very low density lipoprotein (VLDL) (6) and the proportion of unesterified cholesterol in high density lipoprotein (HDL) are decreased during EFA deficiency (7). De Pury and Collins (8) suggested that the increase in lipase activity released into the bloodstream after injection of heparin, which was considered to represent lipoprotein lipase activity, is responsible for the reduction in the level of VLDL triglycerides. Later it became clear that, besides lipoprotein lipase, a lipase derived from the liver (liver lipase) is also released into the bloodstream after injection of heparin (9). This liver lipase contributes largely to the overall postheparin lipolytic activity in the rat (9,10), and the enzyme is believed to play a role in the metabolism of HDL cholesterol (unesterified) (11,12) and possibly in the metabolism of triglycerides in intermediate density lipoproteins (IDL) (13,14), especially in the postprandial phase (15). It may well be that at least part of the effect of EFA deficiency on postheparin lipase activity is due to changes in the liver enzyme. Therefore, we studied the effect of dietary EFA deficiency on liver lipase activity in the liver and in the blood plasma after injection of heparin. Since prostaglandin synthesis is impaired in EFA-deficient animals (16), we studied, in addition, whether prostaglandins are involved in the regulation of liver lipase activity.

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Abbreviations: EFA, essential fatty acids; HDL, high density lipoprotein; IDL, intermediate density lipoprotein; VLDL, very low density lipoprotein.

MATERIALS AND METHODS

Animals. EFA-deficient male Wistar rats of about 200 g were obtained from Unilever Research, Vlaardingen, The Netherlands. The EFA deficiency was induced by feeding these rats an EFA-deficient diet from weanling. The same EFA-deficient diet was used in the experiments. They showed all the signs of EFA deficiency (1-5). The rats had free access to food and water, unless noted otherwise. They were housed under controlled conditions; temperature 20-22°C, lights on between 7.00 and 19.00 hr.

Experimental design. The EFA-deficient rats were divided into two groups: (i) One group maintained the EFA-deficient diet (Hope Farms, Woerden, The Netherlands), composition by percentage of weight: Casein, 20; glucose, 65; α_2-cellulose, 4.0; hydrogenated coconut oil, 4.0; dried yeast, 1.0; $CaCO_3$, 1.0; $CaHPO_4$, 1.6; KCl, 1.0; NaCl, 0.5; MgO, 0.3; DL-methionine, 0.2 and vitamins, 1.4; (ii) The other group was fed laboratory chow for two weeks. Short-term EFA deficiency was attained with normal male Wistar rats of the same weight. They were also divided into two groups: One group was fed laboratory chow and the other group received the EFA-deficient diet for two weeks. In addition, two groups of male Wistar rats were fed a diet enriched with safflower or hydrogenated coconut oil for two weeks. In addition one group of normally fed male Wistar rats was treated with indomethacin (5 mg/kg body weight, i.p.) for two days; the control group received saline instead of indomethacin.

Secretion experiments. Parenchymal cells were isolated from rat liver by collagenase treatment, as described previously (17). Experiments to study the secretion of lipase activity *in vitro* were carried out as reported elsewhere (17), with or without the addition of prostaglandin $F_{2\alpha}$ or E_2 (Unilever Research, Vlaardingen, The Netherlands).

Biochemical measurements. Liver lipase activity was determined in tissue extracts of the liver, exactly as described before (18) and in postheparin blood plasma obtained 5 min after injection of 200 units of heparin/kg body weight, i.v. (Thromboliquine, Organon, Oss, The Netherlands), collected in ice-cooled tubes. Lipase activity was estimated in the presence of 1 M NaCl, using the method as described by Huttunen *et al.* (19). To determine the lipase activity that is not readily released from the liver by heparin, the livers of heparin-injected animals were rinsed by a 5 min perfusion (12 ml/min) through the portal vein with a modified Tyrode buffer, pH 7.4 (20). Then tissue extracts were prepared and lipase activity was estimated (18). Protein was determined with the biuret method. Cholesterol and triglycerides were estimated enzymatically, using kits from Boehringer Mannheim (West Germany).

Statistical procedures. All values are given as mean ± S.D. Statistical significance of differences between groups was determined with the unpaired Student's t-test.

TABLE 1

Effect of Long-Term EFA Deficiency on Serum Triglycerides, Liver Weight, Liver Protein Content and Relative Liver Weight in Fed and Overnight Fasted Animals[a]

Animals	n	Feeding condition	Serum triglycerides (mM)	Body weight (g)	Liver weight (g)	Liver protein content (mg/g wet weight)	Relative liver weight (g/100 g body weight)
Control	4	Fed	1.96 ± 0.035	288 ± 37	11.7 ± 0.8	181 ± 2	4.08 ± 0.33
EFA-deficient	4	Fed	1.50 ± 0.28[b]	278 ± 39	14.2 ± 1.7[b]	159 ± 5[b]	5.14 ± 0.30[b]
Control	4	Fasted	0.77 ± 0.02	247 ± 10	6.9 ± 0.1	210 ± 11	2.78 ± 0.07
EFA-deficient	4	Fasted	0.78 ± 0.30	294 ± 57	8.8 ± 1.1[b]	200 ± 10	3.04 ± 0.25

[a]The results represent the mean value ± S.D.; n = number of rats.

[b]$p < 0.01$ vs the control group.

TABLE 2

Effect of Long-Term EFA Deficiency on the Total and the Heparin Releasable Liver Lipase Activity

Animals	n	Feeding condition	Liver lipase activity[a] (mU/total liver)	Liver lipase activity[a] (mU/ml postheparin plasma)
Control	4	Fed	6955 ± 930	465 ± 37
EFA-deficient	4	Fed	14036 ± 1770[b]	800 ± 98[b]
Control	4	Fasted	4950 ± 780	410 ± 37
EFA-deficient	4	Fasted	6960 ± 1240[c]	530 ± 17[b]

[a]Liver lipase activity was measured in the liver and in postheparin plasma of fed and fasted EFA-deficient and control rats. Data are presented in mean value ± S.D.

[b]$p < 0.01$.

[c]$p < 0.025$, compared to the corresponding control group.

TABLE 3

Influence of Long-Term EFA Deficiency on the Total and Non-Releasable Liver Lipase Activity

Animals	n	Feeding condition	Liver lipase activity[a] (mU/mg protein) Total activity	Liver lipase activity[a] (mU/mg protein) Non-releasable activity
Control	4	Fed	3.39 ± 0.08	0.21 ± 0.02
EFA-deficient	4	Fed	6.13 ± 0.55[b]	0.45 ± 0.14[b]
Control	4	Fasted	2.53 ± 0.23	0.24 ± 0.03
EFA-deficient	4	Fasted	4.01 ± 0.73[b]	0.88 ± 0.60[c]

[a]Liver lipase activity was measured in the livers of rats before or after injection with heparin. Values represent the mean ± S.D.

[b]$p < 0.01$, compared to the corresponding control group.

[c]$p < 0.05$, compared to the corresponding control group.

RESULTS

Long-term EFA deficiency. Table 1 shows the effect of long-term EFA deficiency on serum triglycerides, relative liver weight and protein content of the liver, in fed and overnight fasted rats. In fed EFA-deficient rats the serum triglycerides were lower than in control animals. After an overnight fast, no difference between controls or EFA-deficient animals was found. The absolute and relative liver weight was higher in EFA deficiency—this was more pronounced in the fed than in the fasted state. Besides the higher liver weight, there was a 10% lower protein content in the liver in the EFA-deficient rats.

The effect of long-term EFA deficiency on liver lipase activity is shown in Table 2. To obtain a measure for the total amount of enzyme activity and the heparin-releasable part thereof, liver lipase was measured in liver and plasma after heparin injection. The lipase activity in the liver was twice as high in the EFA-deficient rats as in control rats. In postheparin plasma, liver lipase activity was increased by 60%. The difference in liver lipase activity was most pronounced in the EFA-deficient rats in the fed state. The lipase activity which remained in the liver after heparin administration was considered as the non-(readily)-releasable lipase activity. Especially in the fasted EFA-deficient rats, this non-releasable lipase activity was much higher than in control animals (Table 3)—about 20% of the lipase activity in the fasted EFA-deficient rats is non-releasable compared to 10% in the fasted control rats.

Short-term EFA deficiency. Short-term effects of dietary EFA's were studied by feeding control rats an EFA-deficient diet for 14 days, and by feeding EFA-deficient animals a diet of normal laboratory chow for the same period. The results of these experiments are shown in Table 4. An EFA-deficient diet in control rats led to a significant increase in lipase activity, while, on the other hand, liver lipase activity was normalized in EFA-deficient rats fed laboratory chow for 14 days. Because the EFA-deficient diet contains more than 70 energy percent carbohydrates, which may exaggerate the effects of EFA deficiency, we also studied the effect of short-term EFA deficiency with diets enriched with safflower oil and hydrogenated coconut oil. Lipid enrichment with hydrogenated coconut oil led to a further stimulation of liver lipase activity—a level similar in rats fed the carbohydrate-rich EFA-deficient diet. (Hydrogenated coconut oil 13379 ± 1364mU/total liver, safflower oil 10847 ± 1528 mU total liver, $p < 0.01$, n = 6.)

Involvement of prostaglandins. To investigate the possible involvement of prostaglandins in the enhancement of liver lipase activity in the EFA-deficient rats, we measured the lipase activity in rats treated with indomethacin for two days. In these rats the total and

non-releasable liver lipase activity was significantly increased (Table 5).

The apparent involvement of prostaglandins in the regulation of liver lipase activity was also studied *in vitro*. Prostaglandin E_2 was found to inhibit the secretion of liver lipase activity by 25% (Table 6), while prostaglandin $F_{2\alpha}$ was without effect.

TABLE 4

Short-Term Effect of the EFA-Deficient Diet on Liver Lipase Activity

Animals	n	Diet	Liver lipase activity[a] (mU/total liver)	(mU/ml postheparin plasma)
Control	4	Chow	6955 ± 930	465 ± 37
Control	4	EFA-deficient	8500 ± 807[b]	585 ± 79[b]
EFA-deficient	4	EFA-deficient	14036 ± 1770	800 ± 98
EFA-deficient	4	Chow	7400 ± 800[c]	502 ± 53[c]

[a]EFA-deficient and control rats were fed with an EFA-deficient diet or laboratory chow for 14 days. Liver lipase activity was measured in fed animals, in the liver and in the plasma after injection of heparin. Results are expressed as the mean ± S.D.

[b]$p < 0.05$, compared to the corresponding control group.

[c]$p < 0.01$, compared to the corresponding control group.

TABLE 5

Effect of Indomethacin on Liver Lipase Activity

Treatment	n	Liver lipase activity[a] (mU/liver before heparin)	(mU/liver after heparin)
None	4	6940 ± 694	398 ± 40
Indomethacin	4	8350 ± 577[b]	549 ± 35[b]

[a]Chow fed rats were treated with indomethacin (5 mg/kg body weight, i.p.) for two days and liver lipase activity was measured in the liver before or after injection with heparin. Results represent the mean value ± S.D.

[b]$p < 0.05$.

TABLE 6

Effect of Prostaglandins on the Secretion of Liver Lipase Activity

Additions	n	Liver lipase activity[a] (mU/mg cell protein)
Control (0.1% ethanol)	4	2.21 ± 0.12
Prostaglandin $F_{2\alpha}$ (2 μg/ml)	3	2.09 ± 0.14
Prostaglandin E_2 (0.4 μg/ml)	4	1.74 ± 0.05[b]

[a]Freshly isolated parenchymal liver cells were incubated in the presence of solvent (ethanol), prostaglandin E_2 or prostaglandin $F_{2\alpha}$, during 4 hr. This is the activity after the incubation, cells and medium were separated by centrifugation and liver lipase activity was measured in the medium. The letter "n" denotes number of experiments. Results are expressed as the mean value ± S.D.

[b]$p < 0.01$.

DISCUSSION

We have investigated the effect of dietary EFA's on liver lipase activity. It was found that this activity is remarkably higher during EFA deficiency both in postheparin plasma and the liver. Relatively short periods of EFA deprivation were sufficient to cause a significant increase in liver lipase activity (Table 4). The increase in liver lipase activity was also found when rats were fed an EFA-deficient diet enriched with hydrogenated coconut oil. The activity did not reach the values found in EFA-deficient animals presumably because a real EFA-deficient state is not reached in a relatively short period, as EFA's may still be mobilized from the fat stores. On the other hand, the addition of EFA's to the diet rapidly led to normalization of the lipase activity in EFA-deficient animals. This clearly indicates that the EFA deficiency is the primary cause of the high liver lipase activity and that this is not due to other factors, such as breeding conditions or the strain of the rats or the high amount of glucose in the EFA-deficient diet.

The relation between changes in serum lipid levels and liver lipase activity is not unambiguously clear. The enzyme may be of major importance for the cholesterol homeostasis, and play an important role in the catabolism of HDL phospholipids (11,21) and the uptake of HDL cholesterol (unesterified) by the liver (11,12). Therefore the increase in liver lipase activity in EFA-deficient rats may be responsible for the decrease of HDL cholesterol and the observed changes in HDL subpopulations in EFA-deficient rats (7,22). Moreover, the increase in liver lipase activity may also lead to an enhanced uptake of HDL cholesterol which may contribute to the observed increase in liver cholesterol in these rats (5). A role for the enzyme in the metabolism of triglycerides has also been proposed (13–15). Therefore the observed changes in plasma lipids during EFA deficiency could be explained by the increase in liver lipase activity.

One consequence of EFA deficiency is impairment of prostaglandin synthesis (16). An acute inhibition of prostaglandin synthesis by dietary means is difficult to obtain. Therefore prostaglandin synthesis was inhibited by the administration of indomethacin. Already within two days the liver lipase activity was significantly increased, indicating that the lack of prostaglandins may be responsible for the enhancement of the enzyme activity in the EFA-deficient animals. That the prostaglandins indeed affect the liver lipase activity can be seen in Table 6 which shows that the synthesis or secretion by the parenchymal liver cells is inhibited by prostaglandin E_2. A possible role of prostaglandins in the regulation of liver lipase is of interest. Prostaglandins are synthesized in the endothelium and may affect metabolism in the parenchymal cells. For example, prostaglandins of the E series, synthesized in the liver endothelium, stimulate glycogenolysis (23–26). Liver lipase, which is presumably active at the vascular endothelium of the liver (18,27) in the hydrolysis of lipoprotein phospholipids (11,20), is synthesized in the parenchymal liver cells (17). During phospholipid hydrolysis, EFA's are liberated and these fatty acids may serve as the precursor for prostaglandin synthesis in the endothelium. These prostaglandins affect, as shown in this study, liver lipase activity. In this way the prostaglandins may play a communicative role

between the site of action of liver lipase and the site where the enzyme is synthesized and transported to the endothelium. Such a role of the prostaglandins would be of interest in view of the coordinate regulation of liver lipase activity at the endothelium and parenchymal cells, as was found to occur during hypercorticoism in the rat (28). In what respects prostaglandin E_2 inhibits the secretion of liver lipase activity is not known, but can only be speculated on. We recently showed that the synthesis of liver lipase is suppressed after (nor)-epinephrine administration (29), a condition with a high endogenous free calcium level. Therefore it is tempting to speculate that the effect of prostaglandin E_2 on the synthesis of the enzyme may also be mediated by changes in the endogenous calcium concentration. It cannot be excluded, however, that the prostaglandins or EFA's are (also) operative in the regulation of the binding of the lipase to the endothelium, or in the transport of the lipase from the parenchymal cells to the endothelium.

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Ascorbic Acid, Glutathione and Synthetic Antioxidants Prevent the Oxidation of Vitamin E in Platelets

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An earlier report from this laboratory showed that tocopherol in human platelets is oxidized when the platelets are incubated *in vitro* in Tyrode medium with arachidonate (or other oxidants). Arachidonate is a more potent oxidizing agent in 50 mM potassium phosphate buffer at pH 7.4 with 0.1 mM ethylenediaminetetraacetic acid (EDTA) than in Tyrode medium. Forty to fifty percent of total platelet tocopherol was oxidized upon incubation with 40–50 μM arachidonate in the phosphate-buffered medium. The tocopherol oxidation took place within 15 min after the addition of arachidonate. Preincubation of platelets with ascorbate blocked the oxidation of tocopherol. This is one of the first direct *in vitro* demonstrations of the vitamin E-sparing action of vitamin C in media containing biological cellular material. Other compounds which blocked the oxidation of platelet tocopherol were ascorbyl palmitate, propyl gallate, butylated hydroxytoluene, hydroquinone and glutathione. If ascorbate or glutathione was added after the tocopherol was oxidized to the quinone there was no reversal of the oxidation.

Lipids 24, 1043–1047 (1989).

Many experimental studies support the antioxidant theory of action of vitamin E (tocopherol) (1). During this protective antioxidant action tocopherol is expected to be oxidized to the quinone and/or higher molecular weight products. However, the concentrations of oxidation products, such as the quinone, are quite low in normal samples of mammalian tissues (2). This could be due to the regeneration of tocopherol by other antioxidant nutrients such as vitamin C. As early as 1941, Golumbic and Mattill (3) had shown that ascorbic acid enhanced the antioxidant activity of vitamin E in lard and hydrogenated cottonseed oil even though ascorbic acid itself is a poor antioxidant. Later, Tappel (4) proposed that vitamin C plays an important role in the regeneration of tocopherol from tocopheroxyl radical. Such regeneration of tocopherol from the chromanoxyl radical has been demonstrated to occur in chemical media by Niki *et al.* (5) and Packer *et al.* (6). Nutritional studies in experimental animals also suggest that dietary vitamin C stabilizes tissue tocopherol concentration (7–9). A study of blood levels of vitamins C and E in elderly human subjects by Jacob *et al.* (10) showed that vitamin C spares vitamin E. Nevertheless, experimental support for the regeneration of tocopherols by vitamin C has come mostly from studies in chemical solutions or media containing liposomes (see review by McCay [11]). The *in vitro* incubation of human blood platelets with a variety of oxidants has been used in our laboratory as a model system for the study of oxidation of tocopherols (12). Using this model, the current report shows that vitamin C blocks the oxidation of membrane tocopherol in human platelets.

The biological interactions between vitamin E and synthetic antioxidants are well known. Some of the commercially available antioxidants, such as *N,N*-diphenyl-*p*-phenylenediamine and ethoxyquin, have been reported to have vitamin E activity in the chick (13). Antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are widely used as food and feed antioxidants. These compounds exhibit vitamin E activity when administered in high doses (14). Draper (15) reviewed the nutritional interrelationships between vitamin E and synthetic antioxidants and concluded that some synthetic antioxidants are unable to substitute for vitamin E due to inefficient absorption, transport, translocation at cell boundaries or binding to active sites. Further work on the interaction between vitamin E and commercial antioxidants is needed to clarify the mechanism of interaction between vitamin E and synthetic antioxidants. This report provides experimental evidence showing that commercial antioxidants block the oxidation of tocopherol in human platelets under *in vitro* conditions.

MATERIALS AND METHODS

Human subjects. Human male subjects within the ages of 25 and 55 years were selected for the study. These healthy volunteers fasted for 12 hr prior to a morning drawing of venous blood from the arm. None of the subjects were on any weight-reducing or other dietary regimens or were taking high doses of vitamins or minerals.

Chemicals. All chemicals used were of reagent grade purity from standard sources. Solvents used for chromatography were high performance liquid chromatography (HPLC) grade from Burdick and Jackson Laboratories (Muskegon, MI). α-Tocopherol, γ-tocopherol and tocopherolquinone were from Kodak Laboratory Chemicals (Rochester, NY). Absolute ethanol was obtained from Midwest Solvents Company (Pekin, IL) and was redistilled prior to use. Sodium salts of the fatty acids were obtained from Nuchek Prep, Inc. (Elysian, MN).

Platelet incubations. All operations with platelets were done at room temperature (20–22°C). Blood was drawn into tubes containing ethylenediaminetetraacetic acid (EDTA) as anticoagulant and mixed gently using a hematologic tipper. The platelet-rich plasma samples were obtained by centrifugation at 200 × *g* for 15 min. The platelets from the platelet-rich plasma were sedimented by centrifugation for 10 min at 1200 × *g* and washed twice with phosphate-buffered saline containing 4 mM EDTA. After washing, the platelets were suspended in 50 mM potassium phosphate buffer with 0.1 mM EDTA at pH 7.4, and assayed for total protein using a modified Lowry technique which employs sodium dodecyl sulfate for solubilizing proteins (16). All incubations were performed

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Abbreviations: BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; EDTA, ethylenediaminetetraacetic acid; HPLC, high performance liquid chromatography.

at room temperature (20-22°C) for 120 min in the same medium. The final protein concentration was kept at 500 μg per ml. The total reaction volume ranged from 1 to 3 ml. The sodium arachidonate was always kept under argon after the sealed vial was broken and used for only a week after opening. The sodium salts were dissolved in water which was exhaustively deaerated with argon, and this solution was added to start the reaction. During the incubation, all tubes were vortexed very briefly four or five times. After incubation for 120 min, the tubes were centrifuged at 20,000 × *g* for 15 min. The supernatant was removed, and the tocopherol and tocopherolquinone in the platelet pellet determined by liquid chromatography.

Water soluble compounds such as glutathione and ascorbic acid were dissolved in water and the pH of the solutions adjusted to 7.4 before addition to the incubation medium. Ascorbyl palmitate and BHT were added as ethanolic solutions.

Liquid chromatography of tocopherols. The method for the liquid chromatography of tocopherol has been developed in this laboratory over the past several years. Briefly, 2 ml ethanol containing 0.025% BHT and 0.1 ml of 30% ascorbic acid were pipetted into the tubes containing samples for tocopherol analysis. The mixture was saponified at 60°C for 30 min after the addition of 1 ml of 10% potassium hydroxide solution. The tubes were cooled, and 2 ml of water was added followed by 3 ml of hexane containing 0.025% BHT. Tocopherol was extracted into the hexane phase by vortexing for one minute. The hexane phase was separated and a known volume was evaporated and the residue redissolved in methanol. The tocopherols were separated by reverse phase liquid chromatography (Zorbax-C18 column, mobile phase, methanol/water, 98:2, v/v). Tocopherol was detected by its native fluorescence using excitation and emission wavelengths of 295 and 340 nm, respectively. Details of the instrumentation have been reported (12).

Liquid chromatographic conditions for analysis of tocopherolquinone. The procedure for determination of tocopherolquinone has been reported previously (17). The platelet samples were saponified as described above and the tocopherolquinone extracted with hexane. Known volumes of the hexane layer were pipetted into tubes and the solvent evaporated off under nitrogen using an N-evap. The residue was redissolved in the mobile phase (methanol/water, 98:2, v/v) and injected on the reverse phase column (Zorbax C-18). The tocopherolquinone was detected by its absorption at 265 nm.

All experiments were repeated using platelets from a different human volunteer. The experimental points in each of the figures represent the mean of data from three or four separate incubation tubes. The coefficients of variation of the various data points were less than 5% in all cases.

RESULTS

The earlier report from this laboratory showed that incubation of human platelets in Tyrode medium in the presence of polyunsaturated fatty acids, diamide or superoxide resulted in the oxidation of tocopherol (12). The concentration of arachidonate needed to oxidize 50% of the total platelet tocopherol was 3-5 × 10^{-4} M. A few experiments were conducted to simplify the medium in addition to enhancing the extent of oxidation. It was found that an incubation medium containing 50 mM potassium phosphate buffer at pH 7.4 and 0.1 mM EDTA was suitable for all incubations. Platelets were isolated from the blood of one donor and then incubated with varying concentrations of arachidonate. The decline in α-tocopherol and increase in tocopherolquinone concentrations were monitored as described under Methods. The data in Figure 1 show that the concentration of arachidonate that oxidized 50% of the total tocopherol was roughly 47 μM which was considerably less than the 300-500 μM required in the earlier study (12). In this experiment, as well as in all others, α-tocopherol levels in incubated control platelets were determined. In a typical experiment, α-tocopherol levels in control platelets were unchanged for at least 60 min and even at 120 min only 6% of the original α-tocopherol was oxidized. The amounts of α-tocopherol in platelets shown in Figure 1 were obtained after correcting for this minor loss of tocopherol during incubations without arachidonate.

Even though the incubation times in this and earlier studies have been kept at 120 min, it was of interest to examine whether the oxidation of tocopherol induced by arachidonate was relatively fast. Therefore, platelet samples were incubated with 0.5 mM arachidonate, the higher arachidonate concentration being used to ensure enhanced oxidizing power. The incubations were stopped at 15, 30, 60 and 120 min by centrifugation and removal of the incubation medium containing arachidonate. All samples were then analyzed for α-tocopherol and tocopherolquinone. The results (Fig. 2) illustrate that the arachidonate-induced oxidation of tocopherol is relatively fast and that most of the oxidation takes place within the first 15 min.

The effect of ascorbate, glutathione and the synthetic antioxidant butylated hydroxytoluene upon arachidonate-induced oxidation of platelet tocopherol was then

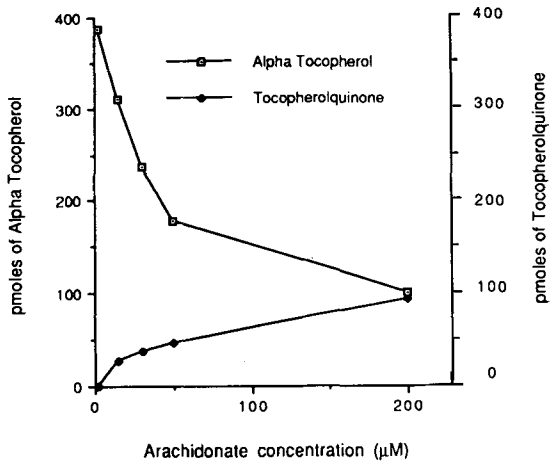

FIG. 1. The effect of arachidonate concentration in the incubation medium upon oxidation of α-tocopherol and production of tocopherolquinone in human platelet samples. Platelets (900 μg protein) were incubated at room temperature (20-22°C) for 120 min after the addition of arachidonate (see Methods for details). The variabilities of the means (coefficient of variation less than 5%) are too small to be visible as error bars on this and other figures in this report.

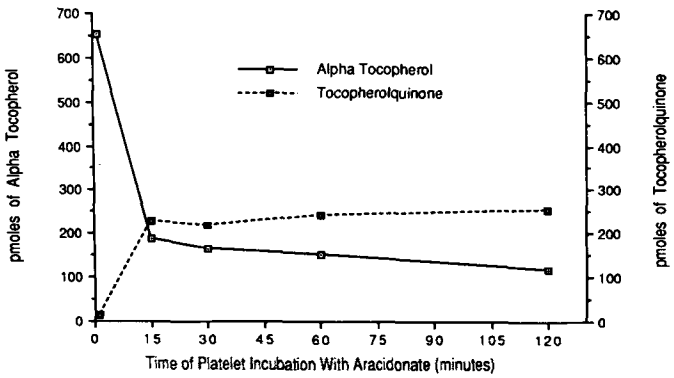

FIG. 2. The effect of time of incubation upon oxidation of α-tocopherol in human platelets induced by arachidonate. Each incubation tube containing 750 μg of platelet protein was incubated with 0.5 mM arachidonate at room temperature (20–22°C). (See legend for Fig. 1.)

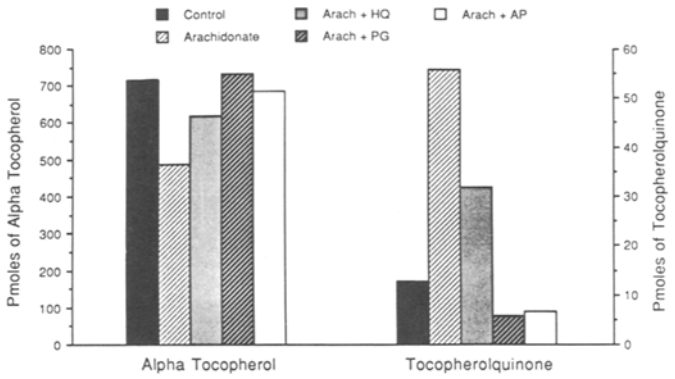

FIG. 4. The effect of synthetic antioxidants hydroquinone, propyl gallate and ascorbyl palmitate upon the oxidation of platelet tocopherol. Platelets (1000 μg protein) were preincubated for 5 min with 0.25 mM hydroquinone, 0.25 mM propyl gallate or 0.02 mM ascorbyl palmitate before the addition of 25 μM arachidonate. (See legend for Fig. 1.)

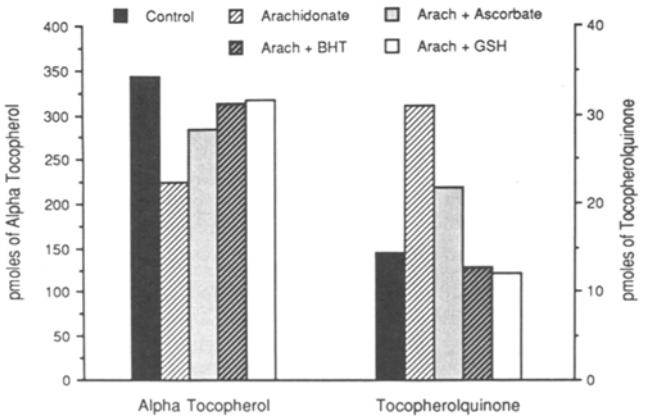

FIG. 3. The effect of addition of ascorbate, glutathione and butylated hydroxytoluene upon arachidonate-induced oxidation of tocopherol in human platelets. Platelet samples (625 μg protein in each) were preincubated for 5 min with 0.142 mM ascorbate, 2.5 mM glutathione or 0.227 mM butylated hydroxytoluene before the addition of 25 μM arachidonate. (See legend for Fig. 1.)

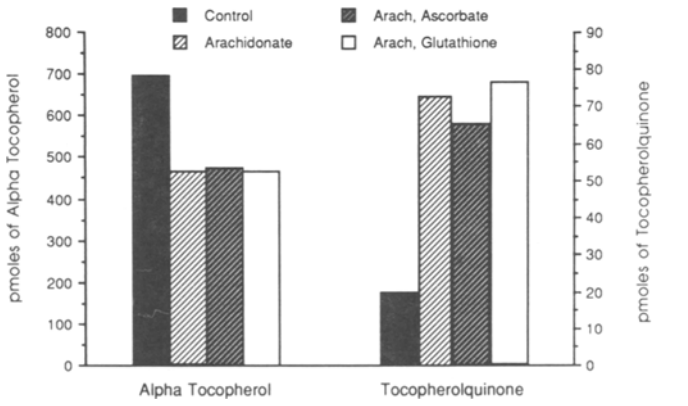

FIG. 5. The lack of effect of the addition of ascorbate or glutathione after oxidation of platelet tocopherol by arachidonate. The platelets (832.5 μg protein) were first oxidized by incubation with 25 μM arachidonate. The incubation medium was removed by centrifugation and the oxidized platelets were then incubated with 0.142 mM ascorbate or 2.5 mM glutathione, and the tocopherol and tocopherolquinone in the platelets were determined. (See legend for Fig. 1.)

investigated. Platelets were isolated and preincubated at room temperature (20–22°C) for 5 min with 0.142 mM ascorbate, 2.5 mM glutathione or 0.227 mM BHT. After adding sodium arachidonate (final concentration 25 μM) the incubation and analysis were conducted as before. The results are shown in Figure 3. All three compounds blocked the oxidation of α-tocopherol and production of tocopherolquinone to various extents. The ascorbate concentrations used correspond to human plasma levels at the higher end of the normal range. It was necessary to add 2.5 mM glutathione in order to demonstrate inhibitions of vitamin E oxidations induced by arachidonate. It is noteworthy that glutathione and ascorbate, which are water-soluble antioxidants, were capable of blocking the oxidation of tocopherol which is present in the lipid portion of the platelet membrane.

The next experiment tested the effectiveness of three other commercial antioxidants. The platelet samples were preincubated with 0.25 mM hydroquinone, 0.25 mM propyl gallate or 0.02 mM ascorbyl palmitate, and the tocopherol oxidation induced by 25 μM arachidonate was followed as before. The results in Figure 4 show that these antioxidants are very effective in blocking tocopherol oxidation induced by arachidonate. Ascorbyl palmitate was much more potent than ascorbate.

Since it has been suggested that some of the endogenous biological antioxidants such as glutathione and ascorbate can regenerate tocopherol, another experiment was conducted to see the effect of adding these compounds after the tocopherol in the platelets had been oxidized. Platelets were incubated with 25 μM arachidonate and the medium containing arachidonate was removed by centrifugation. The sedimented platelets were then resuspended in the original medium to which either 0.142 mM ascorbate or 2.5 mM glutathione was added. Incubations, separation of platelets by centrifugation and analyses for tocopherol and tocopherolquinone were performed as before. The results (Fig. 5) show that oxidation of

tocopherol induced by arachidonate is not reversible if ascorbate or glutathione are added after the tocopherol had been oxidized to the quinone.

DISCUSSION

Experimental observations in chemical media strongly suggest that the tocopheroxyl radical can be regenerated by other reducing compounds such as ascorbate and glutathione (4-6). It has been proposed that peroxy radicals are scavenged by a series of non-enzymatic reactions which involve sequential hydrogen transfer as follows: NADH → RSH → Ascorbate → vitamin E (18). NADH and NADPH act as electron donors, and oxygen or oxidized cytochrome C functions as the final electron sink. Our results support this scheme since the oxidative elimination of tocopherol can be blocked by ascorbate and glutathione. Even though the hydrophobic tocopherol molecule is expected to be in the lipid bilayer of the membrane it is possible that the phenolic hydroxyl group is oriented towards the hydrophilic environment where the tocopheroxyl radical can be converted back (repaired) to tocopherol by ascorbate and glutathione. As expected, hydrophobic antioxidants (such as ascorbyl palmitate) were found to be more efficient in blocking the oxidation of tocopherol than water soluble compounds (Fig. 4).

Platelet aggregation induced *in vitro* by arachidonic acid is inhibited by vitamin E (19-22). A number of studies also show that arachidonic acid metabolism in platelets is modulated by vitamin E. Mower and Steiner (23) found that the *in vitro* addition of α-tocopherol reduced the activity of cyclooxygenase in platelets. Correspondingly, vitamin E deficiency has been shown to stimulate the formation of cyclooxygenase products *in vivo* (24). Gwebu *et al.* (25) showed that vitamin E inhibited platelet lipoxygenase *in vitro*, and Reddanna *et al.* (26) reported that purified 5-lipoxygenase from potato tubers was strongly inhibited by vitamin E and its analogs. Douglas *et al.* (27) found that vitamin E inhibits phospholipase A2 under both *in vitro* and *in vivo* conditions. These are a few selected examples of reports which indicate that vitamin E modulates arachidonate metabolism in platelets at various levels. The findings have at times been contradictory. For example, Agradi *et al.* (28) found that vitamin E has limited effects on metabolism of arachidonate in platelets. The observation of Srivastava (29) that both vitamin E and vitamin C induced an increase in arachidonate metabolites *in vitro* is of special interest. The data suggest that both vitamins act as scavengers of lipid peroxides which inhibit enzymes like cyclooxygenase. The interactions between vitamin E and the various antioxidants during such scavenging activity is the subject of this paper.

The model system in this study involved the *in vitro* incubation of platelets with arachidonate resulting in the oxidation of platelet membrane tocopherol. In another experimental model which involved the incubation of isolated hepatocytes in the presence or absence of calcium, the tocopherol in the cells was depleted in the absence of calcium in the medium (30). Our observation from platelets is somewhat different from this. It was necessary to incubate the platelets in a calcium-free medium for at least 2 hr to observe a decrease (6%) in the tocopherol content (see Results). The arachidonate-induced oxidation of platelet tocopherol occurred over and above the small amount of spontaneous loss of platelet tocopherol due to incubations alone.

The interaction between vitamin E and ascorbic acid has been of great interest from nutritional and biochemical considerations. The data in Figure 3 show that ascorbate blocked the oxidation of platelet tocopherol. To the best of our knowledge this is one of the first such demonstrations of an interaction between vitamins C and E under *in vitro* conditions utilizing biological cellular material. The data in Figure 5 show that the oxidation induced by arachidonate is such that neither glutathione nor ascorbate could regenerate the tocopherol after the oxidation had progressed to the stage of quinone and other products. Presumably the oxidation would have been reversible if it (oxidation) was arrested at the level of the tocopheroxyl radical intermediate before the quinone or polymeric products were formed. The high reactivity of the tocopheroxyl radical would make it difficult to stop the reaction at the radical stage.

The effects of synthetic antioxidants BHT, hydroquinone and propyl gallate in blocking arachidonate-induced oxidation of platelet tocopherol were very similar to that of ascorbate and glutathione. Ascorbyl palmitate was the most potent of all the compounds tested. Cort (31) studied the comparative effectiveness of antioxidants in stabilizing vegetable oils and found that ascorbyl palmitate was better than BHT. Various commercial antioxidants are also known to spare vitamin E under different conditions. The data presented in this report confirm this biological interaction in an *in vitro* system. Perhaps the most interesting observation is that both water-soluble and lipid-soluble antioxidants (both endogenous and synthetic) are effective in blocking the arachidonate-induced oxidation of platelet tocopherol.

Details of the mechanism by which ascorbate, glutathione and the antioxidants prevent the oxidation of vitamin E in platelets is unknown. Nevertheless, some speculations could be offered. It is well known that the incubation of platelets with arachidonate results in conversion of arachidonate to oxidized products (32,33). Endoperoxides and hydroperoxides are intermediates in the biosynthesis of these products and it is possible that the peroxides may be the active agents for tocopherol oxidation. On the other hand, oxidizing agents derived from peroxidation of arachidonate may have been formed in the medium during the incubation. The experiments described in this report do not discriminate between these two ways of production of the oxidants. In fact, one cannot rule out the possibility that an oxidative process independent of peroxides could have been initiated in the platelet membrane by arachidonate. If intracellular peroxides are involved in inducing tocopherol oxidation, then it could be assumed that ascorbate, glutathione and the antioxidants could react with the peroxy radical and/or regenerate the tocopherol within the platelet. However if peroxides are being produced and destroyed in the medium, the tocopherol in the platelet membrane would not be oxidized at all, and then there would be no need for regeneration of the tocopherol. The oxidation of tocopherol may involve a quinone hemiacetal, tocopherone as an intermediate as proposed by Goodhue and Risley (34) and Durckheimer and Cohen (35). If such unstable intermediates were indeed formed in our experiments the

would not have survived the extraction and sample treatment procedures employed during analysis. Further work is needed to understand the details of how the antioxidants (especially the water-soluble compounds) prevent the oxidation of tocopherol in the human blood platelet membrane.

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Apparent Lack of Effect of Obesity on the Soluble Phosphatidic Acid Phosphatase Activity in Human Adipose Tissue

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In view of previous reports that the activity of the Mg^{++}-dependent phosphatidic acid phosphatase in adipose tissues of rat and mouse is elevated in obesity, we attempted to assay this activity in biopsies of human omental adipose tissue obtained from normal-weight and morbidly obese subjects in connection with operations.

The major portion of the phosphatidic acid phosphatase activity was found in the cytosol, and the small amount found in the microsomal fraction was too low for accurate measurement. It was not possible to assay the activity in the crude cytosol. After precipitation with ammonium sulfate, however, the enzyme activity was linear with both the incubation time and the concentration of enzyme. It was not possible to obtain substrate saturation of the enzyme under the conditions employed.

When assayed in the presence of a high concentration of substrate (0.6 mmol/l) the activity obtained in normal-weight patients, 7.8 ± 2.4 nmol/mg protein/min (n = 10), was not significantly different from that in morbidly obese patients, 5.6 ± 0.8 nmol/mg protein/min (n = 10). There was no relation between the size of adipose cells and phosphatidic acid phosphatase activity. Furthermore, there was no apparent relation between phosphatidic acid phosphatase activity in omental adipose tissue and that in the liver.

The findings suggest that the increased biosynthesis of triglycerides in human obesity is not associated with an increased capacity of the soluble phosphatidic acid phosphatase in adipose tissue.
Lipids 24, 1048–1052 (1989).

Evidence has been presented that phosphatidic acid phosphatase is a rate-limiting enzyme in the biosynthesis of triglycerides in the liver. The *in vitro* activity of this enzyme is lowest of all enzymes in the pathway. The activity varies in parallel with dietary and hormonal induction of increased or decreased triglyceride synthesis (1). Whether or not phosphatidic acid phosphatase is of regulatory importance also in adipose tissue is controversial. It has been reported that the specific activity of the microsomal phosphatidic acid phosphatase in adipocytes is 20-fold greater than in the liver, indicating that the phosphatase has a great reserve capacity and may not be rate-limiting (1,2). Furthermore, in a fat cell model, the 3T3-L1 cells phosphatidic acid phosphatase was the only activity of those involved in triglyceride synthesis that did not increase significantly during adipocyte differentiation (3,4). On the other hand, there are several reports demonstrating that the activity of phosphatidic acid phosphatase in fat varies under different conditions in a way that is consistent with a regulatory role for this enzyme. Thus, the Mg^{++}-dependent soluble phosphatidic acid phosphatase activity was found to be higher in adipose tissue from obese rats and mice than in the corresponding control animals (5,6). Starvation, as well as treatment with various lipolytic factors such as epinephrine and cyclic AMP seems to reduce the activity of this enzyme *in vitro* (7–9).

Phosphatidic acid phosphatase is present in both the cytosolic and the microsomal fraction. In the presence of optimal concentrations of Mg^{++}, the major part of the activity is found in the cytosol (5,9,10). According to some investigations (9), the microsomal enzyme has properties different from that of the soluble enzyme and responds differently to different hormonal stimuli. However, the relative role of the soluble and particulate enzyme is not known.

To our knowledge, phosphatidic acid phosphatase has not previously been assayed in human adipose tissue. In the present work we have attempted to assay this activity in omental adipose tissue obtained from patients in connection with operations. In view of the previous reports (5,6) that the activity is higher in adipose tissue from obese rodents than in control animals, we have also assayed this activity in adipose tissue from morbidly obese human subjects.

MATERIALS AND METHODS

Subjects. The study comprised ten normal-weight patients with uncomplicated gallstone disease or ulcus duodeni, admitted for operation. Six were males and four were females. Eight obese females and one obese male admitted for gastric operation for obesity were also studied, together with one obese female who was admitted for elective cholecystectomy (clinical data are given in Table 1). One normal-weight male had slight hypertriglyceridemia and hypercholesterolemia, otherwise all patients tested were normolipidemic. None of the patients had been treated with drugs affecting lipid metabolism for at least two months prior to operation. No clinical or laboratory evidence of diabetes mellitus, kidney, liver or thyroid disease or ethanol overconsumption was present. All the obese patients were weight stable at the time of the study and were thought to be in a steady-state.

Experimental procedure. The patients were hospitalized in the surgical ward, where laboratory tests and a clinical examination were performed. They were fed the regular hospital diet for 2–3 days. All operations were performed between 8 and 9 a.m. after a 12 hr fast to avoid a possible diurnal variation of enzyme activity (12). Anaesthesia was induced by thipentotal and continued with nitrous oxide, diazepam and fentanyl. After opening the abdomen, a 4–10 g omental biopsy was taken. This biopsy was immediately frozen in liquid nitrogen. In some cases a 1–3 g liver biopsy was obtained from the left lobe of the liver (13). In a few cases a 2–4 g subcutaneous biopsy was also taken.

The ethical aspects of this study were approved by the Ethical Committee at the Karolinska Institute. Informed consent to perform the biopsies was obtained from each patient before operation.

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TABLE 1

Clinical Data and Soluble Phosphatidic Acid Phosphatase Activity in Normal Weight and Obese Subjects[a]

Patient number	Sex	Age in years	Body weight		Phosphatidic acid phosphatase activity nmol/mg protein/min	Size of adipose cells[b] $mm^3 \times 10^6$
			kg	Relative %		
Normal weight						
1. L.F.	M	44	78	90	3.1	580
2. E.T.	F	52	59	87	27.4	—
3. B.S.	M	56	71	90	5.9	643
3. B.S.	M	56	71	90	5.9	643
4. N.A.	M	52	77	99	5.0	839
5. A.B.	F	78	62	91	8.3	511
6. B.B.	M	48	72	96	4.3	282
7. A.A.	M	70	82	108	1.2	758
8. B.S.	F	33	69	93	5.5	430
9. A.K.	M	74	57	64	12.0	—
10. M.A.	F	49	—	—	5.5	1172
Obese						
1. M.H.	F	34	113	166	3.1	695
2. A-K E.	F	30	130	188	4.3	1185
3. S.J.	F	55	115	183	1.6	1194
4. A.M.	F	31	127	176	4.6	878
5. E.L.	F	33	122	194	7.3	1278
6. M.V.	F	40	83	134	9.9	—
7. R.Q.	F	29	145	238	4.7	1252
8. M.J.	F	45	143	228	6.1	826
9. H.N.	M	22	165	181	9.0	1351
10. A.N.	F	62	96	168	5.1	810
Normal weight (n = 9)		56 ± 5	70 ± 3	91 ± 4	7.8 ± 2.4	652 ± 97
Obese (n = 10)		38 ± 4	124 ± 8	187 ± 9	5.6 ± 0.8	1052 ± 82

[a] Individual values and mean ± S.E.M.

[b] Reference 11.

Preparation of phosphatidic acid phosphatase. The frozen fat (or isolated cells) was pulverized in a mortar at 4°C within 1 hr after the operation. When needed, liquid frozen nitrogen was added to keep the material in the frozen state during the pulverization. The fine dry powder obtained was carefully mixed with the least possible amount of 50 mM Tris-Cl buffer, pH 7.4 at 4°C. The porridge-like mixture obtained was centrifuged at 10,000 × *g* for 10 min, and the supernatant was recentrifuged at 100,000 × *g* to obtain a microsomal and a cytosolic fraction. The cytosol was treated with 0.23 g ammonium sulfate per ml solution for 10 min at 4°C. By this procedure, more than 80% of the soluble phosphatidic acid phosphatase activity was found to be precipitated (14). The mixture was then centrifuged at 10,000 × *g* for 10 min and the precipitate was washed gently with 50 mM Tris-Cl, pH 7.4 containing 0.23 M. sucrose, dissolved in 0.3 M sucrose and dialyzed overnight against 0.3 M sucrose containing 0.5 mM dithiothreitol (DTT). Part of the dialyzed material was incubated immediately, and part was frozen and stored at −20°C. This storage did not significantly affect the activity of the enzyme during the time of the study, provided thawing and freezing of the preparation was avoided.

In two cases, free fat cells were isolated from adipose tissue according to the method of Rodbell (15) as modified by Smith, Sjöström and Björntorp (16). The size of adipocytes was measured as described by Hirsch and Gallian (17). Phosphatidic acid phosphatase was prepared from these cells by the same method as above.

Preparation of membrane bound and free ^{14}C-labelled phosphatidic acid. Membrane-bound phosphatidic acid used in some experiments was prepared as described by Lamb and Fallon (14) by incubation of 1-^{14}C-labelled palmitate (obtained from Radiochemical Centre, Amersham, England) with rat liver microsomes in the presence of ATP, CoA, *sn*-glycerol-3-phosphate, albumin and NaF. The microsomal solution contained about 2 mg of protein per ml. This preparation was frozen and stored at −20°C prior to use. Before incubation, the thawed suspension was heated at 85°C for 5 min in order to remove intrinsic microsomal activity. More than 95% of the radioactivity in the final preparation was obtained in the phosphatidic acid fraction and less than 1% in the diglyceride fraction as judged by thin-layer chromatography of an extract of the microsomal suspension. Assuming that no significant dilution with endogenous phosphatidic acid took place, the specific radioactivity of the membrane-

bound phosphatidate was found to be 4.2×10^3 cpm per nmol.

For preparation of labelled soluble phosphatidic acid, membrane-bound phosphatidic acid was first prepared as above, using 1-^{14}C-palmitate of maximal specific activity (Radiochemical Centre, Amersham, England). The microsomal phosphatidic acid obtained was then extracted and isolated by preparative thin-layer chromatography. The material was finally diluted with dipalmitoyl phosphatidic acid (obtained from Sigma) to a specific radioactivity of 2×10^3 cpm per nmol.

Incubation procedure. The standard incubation mixture contained phosphatidic acid phosphatase (100-300 μg protein), membrane-bound phosphatidate (39 nmol, 0.18×10^6 cpm) or sodium salt of free phosphatidic acid (500 nmol, 1×10^6 cpm), Tris-maleate, 60 μmol, pH 6.9, and $MgCl_2$, 1.6 μmol, in a total volume of 0.8 ml. In some experiments, the incubation mixture was preincubated for 5 min (prior to addition of substrate) with 10 I.U. of alkaline phosphatase (isolated from E. Coli, Sigma), dissolved in 240 μl of a solution containing 5 μmol of imidazol buffer, pH 7.4, and 0.7 μmol DTT. In the corresponding control experiments, the preincubation was performed only with imidazol buffer and DTT. In some experiments ATP was added to a final concentration of 2 mmol/l. Under these conditions, the concentration of $MgCl_2$ was 4 mmol/l. All incubations were performed at 37°C for 15 min and terminated by addition of 4 ml of chloroform/methanol (2:1, v/v).

Analysis of incubation mixture. The chloroform phase obtained in the extraction of the incubation mixture with 4 ml of chloroform/methanol (2:1, v/v) was evaporated under nitrogen and the residue subjected to thin-layer chromatography using diisopropylene ether/acetic acid (24:1, v/v) as solvent. The solvent was allowed to move only 10 cm. The chromatoplate was then dried in air for 30 min and immediately subjected to a second chromatography, using hexane/diethyl ether/acetic acid (80:20:1, v/v/v) as solvent. The conversion of labelled phosphatidic acid into diglyceride was measured by scanning the chromatoplates with a radioscanner (Berthold, Wildbad, West Germany). The conversion in each experiment with ATP and alkaline phosphatase was compared to the conversion in the corresponding control experiment. Protein was determined according to the Lowry procedure (18).

RESULTS

The present mode of preparation of adipose homogenate was found to be more simple and to yield more reproducible results than did homogenization in the unfrozen state. In comparative experiments, there was no evidence that the initial freezing of the adipose tissue decreased the enzyme activity. The maximum inter-assay difference obtained with separate soluble enzyme preparations from the same biopsy was less than 15% with the method used.

When assayed as described in Materials and Methods, more than 80% of the phosphatidic acid phosphatase activity was found in the cytosol, and less than 20% in the microsomal fraction. In most cases, the amount of activity in the microsomal fraction was too low to permit an adequate assay and therefore, this activity was not studied further. It should be emphasized that centrifugation for longer time than one hour at 100,000 × *g* did not further increase the yield of the microsomes and the microsomal activity. When using homogenization in the unfrozen state for the preparation of enzyme, the yield was generally lower.

It was not possible to assay phosphatidic acid phosphatase in the crude cytosol under enzymological conditions. In general, the activity was linear with incubation time but not with enzyme protein. Similar results were obtained both with soluble and membrane-bound phosphatidic acid as substrate. The activity was, however, considerably lower in the latter case.

After precipitation with 23% ammonium sulfate according to the procedure described for the hepatic enzyme (14) and incubation with soluble substrate, the activity was linear with concentration of enzyme up to at least 600 μg of protein as well as with incubation time up to 15 min (Fig. 1A and B). The figures shown are each obtained from one specific preparation of cytosol from adipose tissue. At least three additional experiments were made with similar results (linearity with enzyme concentration and time). It was not possible to saturate the enzyme with substrate regardless of whether soluble or membrane-bound phosphatidic acid was used. The use of membrane-bound substrate gave a considerably lower degree of conversion (20–30%). Also with membrane-bound substrate, however, there was linearity with enzyme concentration and time (not shown). In the following experiments, a relatively high amount of soluble phosphatidic acid, 500 nmol, was always added as substrate. With this amount there was a high rate of conversion. If higher concentration of substrate was used, the conversion increased somewhat, but occasional solubility problems occurred. The activity was stimulated by Mg^{++}, and the optimal concentration was 2 mmol/l (Fig. 1C). It should be pointed out that the present assay measures the sum of the Mg^{++}-sensitive and the Mg^{++}-insensitive activity. In view of the very low Mg^{++}-insensitive activity, no attempts were made to assay the two activities separately.

In view of the previous finding that treatment with ATP or alkaline phosphatase affected the activity of the hepatic enzyme (12,18), the ammonium sulfate precipitate of the phosphatidic acid phosphatase isolated from the control patients and the obese patients was assayed after preincubation with alkaline phosphatase (10 U) or ATP (2 mmol/l). Both with ATP and with alkaline phosphatase, there was a small stimulation (20–30%) (results not shown). The degree of stimulation was similar in preparations from both groups of patients. The addition of more alkaline phosphatase did not further stimulate the activity. Addition of 1 mmol/l of ATP had about the same stimulatory effect as addition of 2 mmol/l. Addition of 4 mmol/l of ATP depressed the activity.

The mean activity of the phosphatidic acid phosphatase in the preparations from the control subjects was 7.8 ± 2.4 nmol/mg protein/min (mean ± S.E.M.). This activity was not significantly different from that of the preparations from the obese subjects, 5.6 ± 0.8 nmol/mg protein/min (Table 1). Only one specific subject, E.T., was responsible for the fact that the mean activity was somewhat higher for the control subjects than for the obese subjects. If the result from this subject was deleted, the mean of the phosphatidic acid phosphatase activity from the control subjects, 5.7 + 1.0 nmol/mg protein/min, was almost identical to that obtained for the obese subjects.

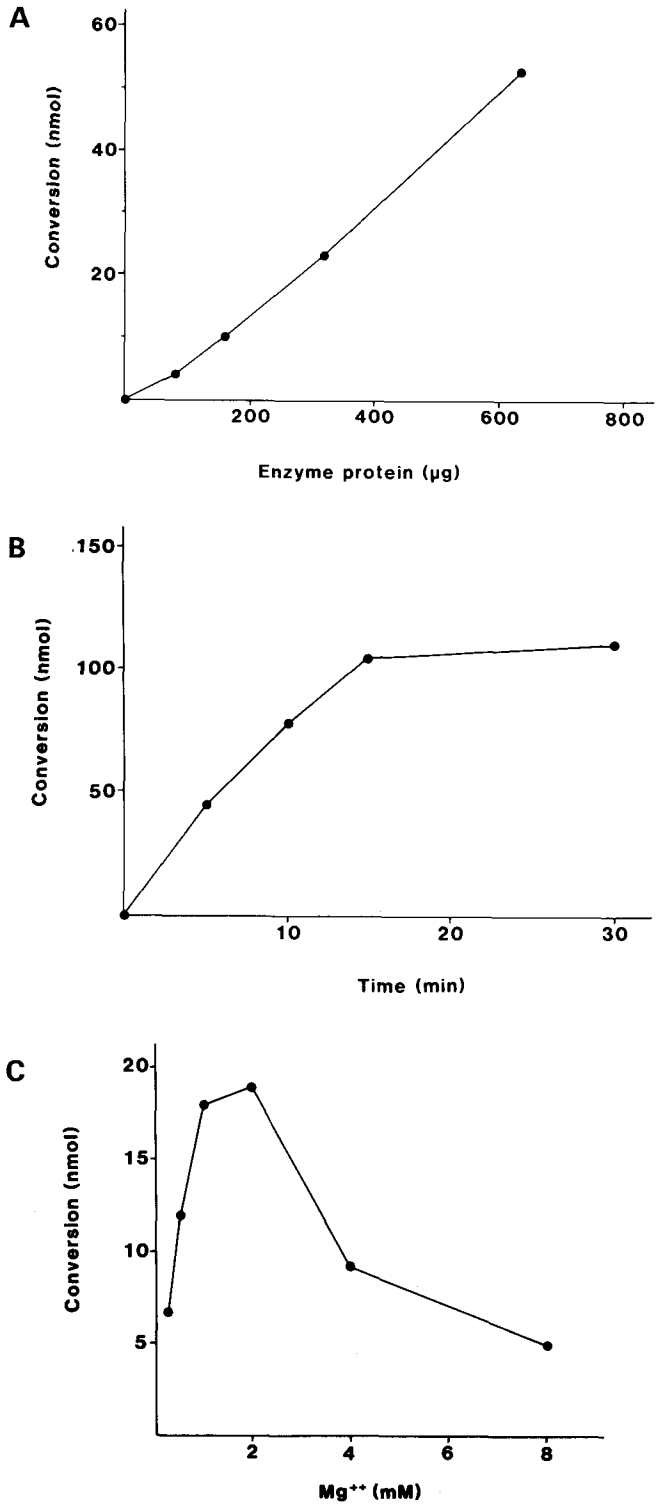

FIG. 1. Effect of enzyme protein (A), time (B) and concentration of Mg^{++} (C) on the activity of phosphatidic acid phosphatase. With the exception of the varied parameter, standard incubation conditions were used.

In most subjects the size of the adipose cells was measured (Table 1). There was no relation between the size of the adipose cells and the phosphatidic acid phosphatase activity. However, the mean of the size of the adipose cells from the obese subjects was significantly higher than that of the adipose cells from the control subjects ($p < 0.05$, Student's t-test).

In the ten obese subjects and in four of the control subjects, the hepatic phosphatidic acid phosphatase was also assayed (13). There was no apparent correlation between the two activities when all the results were taken into consideration (not shown).

For several reasons we used adipose tissue fragments instead of isolated fat cells in order to characterize phosphatidic acid phosphatase. First, the use of isolated fat cells necessitated very large amounts of adipose tissue and, for ethical reasons, it was usually not possible to obtain such large amounts of tissue. Second, the fact that the collagenase procedure used for the isolation of fat cells could alter the properties of the enzyme. It has been previously demonstrated that phosphodiesterase of human adipose is markedly and artificially altered in isolated human adipocytes due to the action of collagenase (11).

However, sufficient adipose tissue was obtained from two subjects to prepare phosphatidic acid phosphatase from a homogenate of free adipose cells. In both cases the activity of the preparation obtained was very similar to that of the corresponding preparation obtained directly from the adipose tissue (differences less than 20%). The lack of difference between specific activities of the two fat preparations may be due to the fact that the contribution of non-fat cells to the total protein content of human adipose tissue is low (20).

In six cases it was also possible to prepare phosphatidic acid phosphatase from subcutaneous fat. The activities of these preparations were lower in general than those obtained in the corresponding omental biopsies (74 ± 9%).

DISCUSSION

The difficulties associated with assay of phosphatidic acid phosphatase are well documented (1,5,21). In the present work both membrane-bound and free phosphatidic acid were tried as a substrate. The latter was preferred due to the higher activity obtained. In some work, chemically defined liposome substrate of equimolar phosphatidate and phosphatidylcholine has been used as a substrate for the enzyme (21). Such a substrate may be more suitable than that used here.

In accordance with previous studies in rat (10), the major part of the phosphatidic acid phosphatase present in human adipose tissue was present in the soluble fraction and was stimulated by Mg^{++}. The activity in the microsomal fraction was too low to permit adequate analysis. The activity in the cytosol was, however, lower than that reported in most studies with rat and mouse. It has been reported that the specific activity of phosphatidic acid phosphatase from adipose tissue of rats is up to 20-fold higher than the corresponding activity in the liver of the same animal (1). The level of activity found in the present work was similar to that found in the human liver, although there was little or no correlation between the two activities within the same individual. The relatively low activity of the phosphatidic acid phosphatase in adipose tissue is consistent with the possibility that this enzyme may be of regulatory importance. However, simultaneous assay of other enzymes involved

in the biosynthesis of triglycerides will be required before more definite conclusions can be drawn.

As in previous studies on the hepatic enzyme (13,19,22), it was not possible to assay the activity in the crude cytosolic fraction, and precipitation with ammonium sulfate was found to be necessary in order to obtain linearity between the rate of conversion and the concentration of enzyme. That the precipitation step may remove factor(s) of regulatory importance for the enzyme cannot be excluded. The lack of reproducibility in the studies with the crude cytosol made it necessary to restrict the study to determine the activity of the ammonium precipitate. Since optimal conditions were used, our studies can only give information of the potential capacity of the phosphatidic acid phosphatase under the different conditions used.

In most of the subjects studied, it was possible to measure the size of the adipocytes. In contrast to some animal studies (23), we did not find any correlation between phosphatidic acid phosphatase activity and the size of the adipocytes.

With the assay conditions used, the soluble phosphatidic acid phosphatase activity in the adipose tissue of the obese subjects was not significantly different from that in the control subjects. Unforunately, it was not possible to assay the activity under saturated conditions. Therefore, it cannot be completely excluded that small differences may have been observed if it had been possible to assay the activity under substrate saturation. It may be argued that there were differences between the two groups of subjects with respect to age and sex. In the limited number of subjects available, however, there were no indications that age and sex had any significant effect on the activity. It may thus be concluded that other factors than the activity of the soluble phosphatidic acid phosphatase are responsible for the increased triglyceride biosynthesis in obesity. This finding does not, however, rule out the possibility that the enzyme may be of regulatory importance under other conditions.

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METHODS

A Method for the Simultaneous Analysis of Unconjugated and Glycine-Conjugated Bile Acids by Capillary Gas-Liquid Chromatography

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A method for the simultaneous analysis of unconjugated and glycine-conjugated bile acids by means of capillary gas-liquid chromatography without need for prior deconjugation is described. The method involves: i) the use of an aluminum-clad fused-silica capillary column coated with a very thin film (0.1 μm) of a highly thermostable bonded and crosslinked methyl polysiloxane, and ii) the analysis of the bile acids as their methyl ester-dimethylethylsilyl ether derivatives. This method, used to separate the major free and glycine-conjugated bile acids from human gall bladder bile, should be applicable for the analysis of other biological fluids.

Lipids 24, 1053–1055 (1989).

Bile acids in biological fluids are not only present in free form, but also as glycine and taurine conjugates (1). Current methods of analysis of conjugated bile acids by gas-liquid chromatography (GC) and GC-liquid-mass spectrometry (GC-MS) require initial hydrolysis (alkaline or enzymatic) and subsequent preparation of suitable derivatives of the resulting free bile acids for their separation and quantitation by GC (2,3). Such two-step methods can be hampered because quantitative information on the conjugates may be lost and because the hydrolysis step, which is hard to control, is often either incomplete or produces side products.

A method recently reported by Street *et al.* (4) for the direct GC analysis of glycine-conjugated bile acids in plasma without need for preliminary hydrolysis to the free acids is indeed useful, but has two major shortcomings, especially when used for routine GC and GC-MS analyses. Hydrogen, which is used as carrier gas, requires a very high flow rate (ca. 20 ml/min), which can be hazardous, and the time needed for a determination (ca. 1 hr) is quite long.

We have therefore explored the use of a recently introduced, highly thermostable, aluminum-clad, flexible fused-silica capillary column for direct analysis of bile acid conjugates. According to the catalog (No. CA189-138) from the manufacturer (Shimadzu Corp, Kyoto, Japan), this column is coated with the very thin film (0.1 μm) having an upper limit of 390°C, which has been reported to be suitable for the separation of high boiling materials such as waxes and triglycerides. This paper describes an improved method for the simultaneous analysis of free and glycine-conjugated bile acids in human bile.

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Abbreviations: CA, 3α,7α,12α-trihydroxy-5β-cholanoic acid; CDCA, 3α,7α-dihydroxy-5β-cholanoic acid; DCA, 3α,12α-dihydroxy-5β-cholanoic acid; DMESI, *N*-dimethylethylsilylimidazole; EEDQ, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; GC, gas-liquid chromatography; GC-MS, gas-liquid chromatography-mass spectrometry; HDCA, 3α,6α-dihyroxy-5β-cholanoic acid; HFIP-TFA, hexafluoroisopropyl ester-trifluoroacetyl; LCA, 3α-hydroxy-5β-cholanoic acid; Me-DMES, methyl ester-dimethylethylsilyl; Me-TFA, methyl ester-trifluoroacetyl; Me-TMS, methyl ester-trimethylsilyl; TLC, thin-layer chromatography; TMS-HT, hexamethyldisilazane and trimethylchlorosilane in anhydrous pyridine solution; UDCA, 3α,7β-dihydroxy-5β-cholanoic acid.

EXPERIMENTAL

Sample and reagents. Lithocholic (3α-hydroxy-5β-cholanoic acid; LCA), deoxycholic (3α,12α-dihydroxy-5β-cholanoic acid DCA), chenodeoxycholic (3α,7α-dihydroxy-5β-cholanoic acid; CDCA), ursodeoxycholic (3α,7β-dihydroxy-5β-cholanoic acid; UDCA) and cholic (3α,7α,12α-trihydroxy-5β-cholanoic acid; CA) acids and cholesterol (cholest-5-en-3β-ol) were obtained from Tokyo Kasei Kogyo Co. (Tokyo, Japan). LCA, DCA, CDCA, UDCA and CA were converted to the corresponding glycine (GLCA, GDCA, GCDCA, GUDCA and GCA, respectively) conjugates by using the peptide coupling reagent, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) according to the method of Tserng *et al.* (5). The purity of the compounds synthesized was checked by thin-layer chromatography (TLC) and proton nuclear magnetic resonance spectroscopy—all compounds showed only a single spot on TLC.

TMS-HT (hexamethyldisilazane and trimethylchlorosilane in anhydrous pyridine), *N*-dimethylethylsilylimidazole (DMESI), hexafluoroisopropanol and trifluoroacetic anhydride were used as GC derivatizing reagents. TMS-HT and DMESI were commercially available from Tokyo Kasei Kogyo Co. All other reagents and solvents were of analytical grade.

Amberlite XAD-2 resin was supplied by Rohm and Haas Co. (Philadelphia, PA), and Sep-Pak SIL cartridges (700 mg) by Waters Associates (Milford, MA).

Human gall bladder bile of a patient with a mixed gallstone was collected at a surgical operation (College of Medicine, Nihon University) and stored at −20°C in glass tubes until analysis.

GC derivatization procedure. The following GC derivatives of free and glycine-conjugated bile acids were evaluated because of their excellent reported GC properties for free bile acid analyses (2,3): methyl ester-trifluoroacetyl (Me-TFA), methyl ester-trimethylsily ether (Me-TMS), methyl ester-dimethylethylsilyl ether (Me-DMES) and hexafluoroisopropyl ester-trifluoroacetyl (HFIP-TFA) derivatives. HFIP-TFA de-

rivatives were prepared in one step by using hexafluoroisopropanol and trifluoroacetic anhydride (6,7). The remaining three derivatives were prepared in two steps: initially, methylation of the carboxyl group of the side chains in free and glycine-conjugated acids was carried out by the acetyl chloride-methanol method (8); the methyl esters thus obtained were then derivatized to their complete Me-TFA, Me-TMS (9) and Me-DMES (9,10) derivatives, using trifluoroacetic anhydride, TMS-HT, and DMESI, respectively. The purity of these derivatives was checked by TLC prior to GC analysis.

Extraction procedure. Free and glycine-conjugated bile acid fractions were extracted according to Goto *et al.* (11) and Street *et al.* (12). Briefly, the procedures were as follows: the bile sample (0.5 ml) was diluted with water (2 ml) and percolated through a column of Amberlite XAD-2 resin (20 g). The column was washed with water (50 ml) and total bile acids were eluted with methanol (50 ml) (11). After evaporation of methanol, the residue was redissolved in ethanol/chloroform/water (20:80:1, v/v/v, 5 ml) and an aliquot (1 ml) of the solution was applied to a Sep-Pak SIL cartridge at 4°C. Free and glycine-conjugated bile acid fractions were eluted with ethanol/chloroform/water/acetic acid (20:80:1:0.02, v/v/v/v, 6 ml) and then with ethanol/chloroform/water/acetic acid (20:80:3:5, v/v/v/v, 10 ml). Taurine-conjugated bile acids were retained on the cartridge with the eluent systems (12). After evaporation of the solvents to dryness, the residue was derivatized as described above, and an aliquot (0.5 μl) of the solution was injected into GC.

GC instrument and column. The apparatus used was a Shimadzu GC-7A gas chromatograph equipped with a flame ionization detector, capillary split injector, and data-processing system (Chromatopac C-R3A). 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 crosslinked methyl polysiloxane (equivalent to OV-101) was installed into the apparatus. The column, HiCap CBPM1, was purchased from Shimadzu Corp. (Kyoto, Japan). An analogous column is also commercially available from Quadrex Corp. (New Haven, CT) and SGE Co. (Ringwood, Victoria, Australia). Helium was used as carrier gas under the following conditions: flow rate, 1.5 ml/min; auxiliary gas, 40 ml/min. The oven and detector temperatures were raised from 200–280°C at 2°C/min for Me-TFA and HFIP-TFA derivatives, from 230–280°C at 2°C/min for Me-TMS ethers, and from 270–310°C at 2°C/min for Me-DMES ethers.

RESULTS AND DISCUSSION

As expected, both free (LCA, DCA, CDCA, UDCA and CA) and glycine-conjugated (GLCA, GDCA, GCDCA, GUDCA and GCA) bile acids were converted easily to their complete Me-TFA, HFIP-TFA, Me-TMS and Me-DMES derivatives. Since taurine-conjugated bile acids are much less volatile and have not been successfully run on direct GC without prior hydrolysis (2,3), they did not interfere with the chromatogram of the derivatized free and glycine-conjugated bile acids. In fact, no peaks corresponding to taurine conjugates could be seen under the various GC conditions examined.

The ten bile acids and their glycine conjugates, which are commonly found in biological materials and frequently used for evaluation of the resolving power of a column, were compared to determine which of the four derivatives (Me-TFA, HFIP-TFA, Me-TMS or Me-DMES) would give the best separation on the HiCap CBPM1 column. Table 1 gives a comparison of the retention times found.

As seen in the Table, the Me-TFA derivatives of the free acids are poorly resolved. The corresponding glycine-conjugated derivatives under various GC conditions showed no peaks at all, probably because of thermal decomposition. The HFIP-TFA derivatives were also considered unsatisfactory; the main peaks of the glycine conjugates were often accompanied by minor peaks. Whether these artifacts are due to faulty derivatization or thermal decomposition was not further investigated.

Although the Me-TMS ether derivatives behaved better and gave generally sharp peaks, resolution was unsatisfactory. CDCA and CA partially overlap, and GCDCA and GCA completely overlap (4). In addition, relatively high concentrations of glycine-conjugated samples were required.

However, the Me-DMES ethers proved to be well-suited for use with this column, giving excellent resolution, short analysis times and high sensitivity without appearance of artifacts. Figure 1 illustrates the clean separation of the 11 compounds (the ten Me-DMES derivatives of the free and glycine-conjugated bile acids plus cholesterol) even at the elevated column temperatures (270–310°C). The Me-DMES ethers emerged in the order LCA < cholesterol < DCA < CDCA < UDCA < CA < GLCA < GDCA < GCDCA < GUDCA < GCA. Analysis was complete within about 22 min. In contrast to the use of hydrogen as carrier gas (4), high flow rates were not needed to obtain good responses and peak shapes. With helium, even at the moderately low flow rate of 1.5 ml/min, the Me-DMES ether derivatives gave excellent resolution and response. Furthermore, a good linear relationship (correlation coefficient, r 0.998) was found between peak area and amount of each glycine conjugate in the examined range

TABLE 1

Retention Times[a] of Unconjugated and Glycine-Conjugated Bile Acid Derivatives on an Aluminum-Clad Fused-Silica Capillary Column, HiCap CBPM1

	Derivative			
Bile acid	Me-TFA	HFIP-TFA	Me-TMS	Me-DMES
LCA	1.10	1.20	0.89	0.74
DCA	1.00 (1.7 min)	1.00 (4.9 min)	1.00 (8.4 min)	1.00 (7.8 min)
CDCA	1.10	1.20	1.05	1.07
UDCA	1.10	1.36	1.13	1.15
CA	1.00	1.05	1.07	1.32
GLCA		2.83	2.35	2.12
GDCA		2.51	2.48	2.48
GCDCA		2.71	2.56	2.61
GUDCA		2.95	2.68	2.72
GCA		2.51	2.56	2.92

[a]Expressed relative to DCA derivatives.

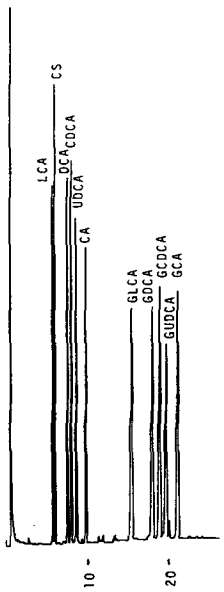

FIG. 1. Capillary gas chromatogram of the Me-DMES derivatives of a standard mixture of unconjugated and glycine-conjugated bile acid on HiCap CBPM1 capillary column. Conditions for GC and abbreviations for compounds are described in the text.

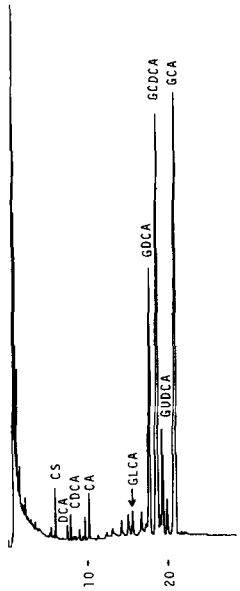

FIG. 2. Capillary gas chromatogram of the Me-DMES ether derivatives of unconjugated and glycine-conjugated bile acids extracted from gall bladder bile of a patient with a mixed gallstone. GC conditions and peaks identification are the same as in Figure 1. Peaks are tentatively identified from their retention times.

of 20–80 ng when hyodeoxycholic acid (3α,6α-dihydroxy-5β-cholanoic acid; HDCA) was used as an internal standard. Although HDCA (13) was used as an internal standard in this study, other compounds, such as 5α-cholestane, 5β-cholanoic acid, 23-nordeoxycholic acid (2) or 7β,12β-dihydroxy-5β-cholanoic acid (14) seem to be more suitable for the quantitative determination of each bile acid in biological samples, because HDCA may be naturally present in small amounts. The detection limit of glycine-conjugated bile acids was in the range of 3–7 ng with a signal to noise ratio of 5 to 1.

Consequently, the Me-DMES ether derivatives were chosen for trial on the HiCap CBPM1 column for simultaneous analysis of free and glycine-conjugated bile acid mixtures extracted from a biological fluid. Figure 2 shows a typical chromatogram obtained from an extract of the gall bladder bile of a patient with a mixed gallstone, first processed through Amberlite XAD-2 (11) and Sep-Pak SIL (12); taurine-conjugated bile acid fraction was removed in the extraction process as described above. Each bile acid was cleanly separated from other signals and was readily identified by comparison with a reference sample.

We report here this improved and direct method for the determination of free and glycine-conjugated bile acids in biological materials and suggest that it should be useful for the study of bile acid metabolism. We are now applying the method to the analysis of other biological fluids; those results will be reported elsewhere.

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